Flavor Analysis of Mrps Made from Different Enzymolysis Products by Gas Chromatography-Mass Spectrometry

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Abstract. Beef-bone protein hydrolysates were obtained by Flavourzyme (F), Protamex (P), Flavourzyme followed by Protamex (Fp), Protamex followed by Flavourzyme (Pf), and combination of Flavourzyme and Protamex (DE). Samples without any enzymes served as the control (C). Subsequently, the six hydrolysates were subjected to Maillard reaction for further investigation of the contribution of enzymatic hydrolysis to flavor. SPME-GC-MS (gas chromatograph-mass spectrometer) was applied to analyze the volatile substances and taste components. The results showed that the content of volatile compounds detected by the 6 samples was significantly different, while there was little difference in the type of compounds.

1. Introduction

Seven million tons of beef were produced in China in 2016 (data from Animal husbandry information network of China). Cattle slaughter in China accounts for 17.4% of the world's cattle slaughter, which ranks first in the world. Most of these cattle carcasses are deboned in plants, and the meat is sold as packaged beef. During this process, up to 6%-12% (based on carcass weight) of the bones was discarded [1]. On the other hand, China imports a large amount of beef from other countries, such as New Zealand, and most of these beef are transported in the form of frozen dyads and quarters [2]. These frozen carcass will be cut by chainsaw before selling, concomitantly producing a certain proportion of minced beef-bone (MBB). Theses processes could result in approximately 80 thousand tons of bone and minced beef-bone as inedible by-products, and leads to enormous economic losses. For that reason, industries have begun to develop various technologies to make use of this waste, mainly in the form of value-added products, while simultaneously reducing the cost derived from its disposal.

After hydrolysis, bones could be developed into value-added products [3]. For example, Lafarga & Hayes; Lieske & Konrad; Zhan, Tian, Zhang, & Wang found that hydrolyzed protein was an important flavoring agent [4], which could produce MRPs with meat-like flavor [5]. Madruga, Elmore, and Oruna-Concha, et al. [6] reported that by controlling the DH, different constituents of these precursors could generate different flavors. The different compositions of these precursors, which can be derived by controlling the DH, generate a significant difference in flavor because of the different levels of volatiles formed under the thermal reaction conditions. Similar results were also obtained by Song, Tan [7] and Madruga, & Mottram [8].
In this paper, five different enzymatic hydrolysis methods were used to hydrolyze beef bone and meat, and the enzymatic hydrolysate were used as the base material to make MRPs. the volatile compounds of the MRPs were determined by SPME-GC-MS.

2. Materials and methods

2.1. Materials and chemicals
Minced beef-bone (MBB) was obtained from Guayue Food Co., Ltd (Tianjin, China), which contains a notable amount of muscle, connective tissue, bone and fat, and therefore represents a valuable source of proteins, containing approximately 13.7%-32.9% moisture, 16.7%-24.9% protein (collagen), 8.2%-26.8% fat, and 35.8%-48.1% ash. The raw materials were stored at -18°C till required. Food-grade Flavourzyme (500 LAPU/g) and Protamex (1.5AU/g) were purchased from Novozymes Biological Technology Co., Ltd in China (Beijing, China). L-cysteine, glycine, alanine and VB1 were purchased from Jizhou Biotechnology Co., Ltd (Hebei Province, China). Xylose and glucose were purchased from Shandong Xiwang Sugar Co., Ltd (Shandong Province, China).

2.2. Preparation of hot-pressure protein extractions from minced beef-bone (MBBE)
Frozen MBB was thawed at 4°C for 8 h. Then, it was mixed thoroughly with distilled water at a ratio of 1:4 and extracted at 120°C for 4 h and 0.1 MPa in an autoclave sterilizer (TOMY Co., Ltd., Tokyo, Japan). The resulting supernatant was termed as MBBE and kept at 4°C until further use.

2.3. Preparation of minced beef-bone protein hydrolysates by different enzymatic hydrolysis (MBBPHs)

| Hydrolysates | Conditions                                  |
|--------------|---------------------------------------------|
| C            | -                                           |
| F            | 0.06% Flavourzyme, 50 °C, 4.3 h             |
| P            | 0.03% Protamex, 50 °C, 4.5 h                |
| Fp           | 0.06% Flavourzyme, 50 °C, 4.3 h + 0.03% Protamex, 50 °C, 4.5 h |
| Pf           | 0.03% Protamex, 50 °C, 4.5 h + 0.06% Flavourzyme, 50 °C, 4.3 h |
| DE           | 0.06% Flavourzyme and 0.03% Protamex, 50 °C, 4.5 h |

Note: C: control; F: Flavourzyme; P: Protamex; Fp: Flavourzyme followed by Protamex; Pf: Protamex followed by Flavourzyme; DE: Combination of Flavourzyme and Protamex.

MBBE was used as the substrate for hydrolysis. The preparations of six different MBBPHs are listed in Table 1. All six hydrolysates (termed as C, F, P, Fp, Pf and DE) were prepared at the optimal conditions of the enzymes according to our previous study. After enzyme deactivation at 100°C for 20 min, parts of these six hydrolysates were centrifuged (ST-40r, Thermo Scientific Co., Ltd., Tokyo, Japan) at 5,000 g, 4°C for 20 min. The supernatants were stored at -20°C until being used. The other parts of these six hydrolysates were collected at -20°C to prepare the Maillard reaction products (MRPs).

2.4. Preparation of Maillard Reaction Products (MRPs)
In order to enhance the aroma of MRPs, xylose (1.2%), glucose (1.2%), L-cysteine (0.9%), glucose (0.45%), alanine (0.45%) and VB1 (1.8%) were added into the six MBBPHs (prepared according to 2.3), and the mixtures were transferred to conical flasks (250 mL) and adjusted to pH 5.5 with 1 M acetic acid. Then, they were placed in an autoclave sterilizer (TOMY Co., Ltd., Tokyo, Japan) at 110°C for 60 min. These six Maillard reaction products (MRPs C, MRPs F, MRPs P, MRPs Fp, MRPs Pf and MRPs DE) were immediately cooled in ice water (0°C) and filtered through qualitative filter paper, and the supernatants were stored at -18°C for further analyses.
2.5. Determination of volatile compounds in MRPs

The volatile compounds were analyzed by SPME with a 65 µm PDMS/DVB fiber (Supelco, Bellefonte, PA) according to Zhang [9] with some modification. The MRPs (2mL) were placed into a glass vial (20mL), and then the vial was sealed with a lid and placed in a water bath at 60°C for 20 min. When the process was completed, the glass vial was transferred to a solid-phase microextraction unit (Supelco, America) at 60°C for 30 min to age the SPME fiber in the headspace to allow the equilibration of the volatile compounds. Then, the fiber was desorbed at 200°C for 2 min. Separation of the volatile compounds was actualized on a DB-WAX (30 m × 0.25 mm × 0.25 µm) capillary column. Helium was used as the carrier gas, flowing at 1.0 mL/min. The starting temperature of the chromatographic oven was 40°C, which was held for 3 min, followed by a gradual increase to 120°C at a rate of 5°C/min. Then, the temperature was increased to 200°C at a rate of 10°C/min, and this temperature was held for 13 min. The mass spectrometric detector was operated at an electron voltage of 70eV, and the temperature of the ion source was 200°C. The experimental results were compared with the spectra of unknown volatile compounds using the NIST11 database and quantified by the area normalization method.

2.6. Statistical analysis

The average and standard deviation of all data were calculated using Microsoft Excel 2003 and presented as mean ± standard deviation (SD). The analysis of variance (ANOVA) was performed using the general linear model procedure to determine significant differences among the treatments at P < 0.05 (Statistix 8.1, Tukey HSD).

3. Results and discussion

Table 2. Major volatile compounds identified and content (%) of MRPs

| RT   | Compounds                       | C     | F       | P       | Fp      | Pf      | DE      |
|------|--------------------------------|-------|---------|---------|---------|---------|---------|
| 20.062 | 2-Methyl-3-furanthiol         | 1.10±0.03a | 1.28±0.37a | 1.32±0.01a | ND      | ND      | ND      |
| 20.649 | Octanal                       | 2.32±0.44d | 7.97±0.32b | ND      | 4.90±0.10e | 6.37±0.42bc | 20.45±0.80a |
| 20.981 | Formic acid                   | ND    | 1.50±0.28 | ND      | ND      | ND      | ND      |
| 21.452 | 2-methyl-3-Pentanethiol      | 2.35±0.47c | 7.21±0.23a | 3.97±0.49b | 4.92±0.05b | 8.34±0.25a | ND      |
| 23.281 | Nonanal                       | 7.64±0.07c | 28.10±2.64b | 16.14±1.91c | 26.46±4.60c | 27.45±1.02c | 46.76±0.35c |
| 24.624 | Benzaldehyde                  | 1.77±0.31d | 6.39±0.65c | 6.29±0.20c | 10.01±0.42c | 12.60±0.29f | 16.42±0.50c |
| 25.243 | Nonanal                       | 1.90±0.10a | 7.15±0.60c | 3.06±0.38bc | 6.18±0.25a | 10.15±0.21c | 3.85±0.18c |
| 28.633 | Hexanoic acid                 | 4.68±0.23c | 9.27±0.10c | 9.87±0.42ab | 11.43±0.54c | 7.44±1.32bc | 4.09±0.02c |
| 28.725 | 3-Methyl-2-thiophene carboxaldehyde | ND | ND | ND | 1.05±0.04 | ND | ND |
| 29.36  | 5-Formyl-4-methylthiazole      | 1.05±0.05d | 2.53±0.13b | 1.54±0.05cd | 1.70±0.06e | 2.31±0.08bc | 3.55±0.28a |
| 30.288 | Heptanoic acid                | 1.12±0.10a | 1.02±0.12a | 0.79±0.06a | ND | 0.94±0.01a | ND |
| 31.809 | 1-Dodecanol                   | 1.02±0.66 | ND | ND | ND | ND | ND |
| 32.248 | Octanoic acid                 | 1.67±0.06c | 4.24±0.21ab | 3.26±0.42b | 4.63±0.28ab | 4.64±0.41f | ND |
| 34.752 | Nonanoic acid                 | 1.41±0.13b | 7.25±0.63b | 1.92±0.33bc | ND | 2.98±0.04b | ND |
| 39.289 | 4-methyl-5-Thiazoleethanol    | 70.49±2.18b | 21.12±1.44d | 48.93±0.52b | 23.42±3.39cd | 31.29±3.66d | 20.39±0.71a |

RT = Retention time. 
ND = Not detected.

Values are presented as means ± standard deviation (SD) and bearing different lowercase letters (a, b, c, d and e) were significantly different (p < 0.05).

The volatile compounds of these six Maillard reaction products (MRPs) were detected by SPME-GC-MS, and the analyzed results are shown in Table 2. The six MRPs (C, F, P, Pf, Fp and DE) can be detected, and 13, 13, 12, 10, 11 and 7 compounds were identified, respectively. The results showed that the content of volatile compounds detected by the 6 samples was significantly different, while there was
little difference in the type of compounds, including sulfur-containing compounds, aldehydes and others that had very little contribution to the beef aroma characteristics of these six MRPs.

Meat flavors usually have two types: one is based on the amino acids, peptides, nucleotides and sugars after the Maillard reaction to produce basic meat flavors. The other is the combination of thermal degradation products of lipids and Maillard reaction intermediates, which then produces meaty flavors, including aldehydes, alcohols, esters and ketones. As shown in Table 2, the contents of aldehydes in MRPs C, F, P, Fp, Pf and DE were pretty obvious that the method of hot-pressure extraction with enzymolysis can significantly improve the content of characteristic aroma compounds compared with the MRPs C which was treated by hot-pressure extraction alone. Specifically, the contents of aldehydes in MRPs F, P, Fp, Pf and DE increased by 30.73%, 10.70%, 30.69%, 34.69% and 71.90%, respectively, compared with MRPs C.

It is well known that basic meat flavor is associated with sulfur-containing heterocyclic compounds and their derivatives [10]. Van Boekel [11] reported that the meat-related flavor compounds are mainly sulfur-containing compounds, which are derived from cysteine and ribose. There was no significant difference in the total contents of sulfur-containing compounds of MRPs C, F, P, Fp, Pf and DE from Table 5. The main reason was that cysteine was not detected in the six hydrolysates, and then cysteine, an important precursor of sulfur-containing compounds [12], was added to enhance the meat flavor during the Maillard reaction. Moreover, only liquid was determined at the time of detection and fat and liquid had not been homogenized, while fat was an important precursor of the formation of meat flavor [13], which led to the detection of some flavor substances.

4. Conclusion

Analysis by SPME-GC-MS demonstrated that Maillard reaction of MBB hydrolysates could significantly improve the major volatile compounds in the Maillard reaction products, especially those hydrolyzed by the combination of Flavourzyme and Protamex (DE).

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