The Influence of Different Enzymolysis Methods on the Components of Enzymolysis Products

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Abstract. Beef-bone protein hydrolysates were obtained by different enzymatic hydrolysis schemes, including 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). The degree of hydrolysis (DH), contents of low molecular weight peptides and free amino-acids distribution were evaluated. The results indicated that the applied enzymatic hydrolysis significantly increased the DH, contents of low molecular weight peptides and free amino-acids compared with that of group C.

1. Introduction

Beef is widely consumed and has become increasingly popular in the world because of its nutritional and special flavor qualities. Continuous efforts have been made to improve the functional and nutritional value of by-products. Optimization of the enzymatic hydrolysis of animal raw material is essential for obtaining functional meat protein preparation. Enzymes used to hydrolyze proteins are usually composed of incision enzymes, excision enzymes, and flavoring enzymes [1]. The peptide chain inside the protein can be cut off from the middle by the incision enzyme. At the end of a polypeptide chain can be cut off by excision enzyme so as to release the amino acids. The flavor enzyme could be used to optimize the bitterness of the hydrolysate, and then the hydrolysate without bitter taste and fishy smell but with natural rich flavor will be obtained. Recently, enzymatic hydrolysis was employed to extract proteins and produce peptides [2], which was an effective way to recover proteins from the by-products of animal processing. Linder et al. reported that the utilization of bones mainly focused on the enzymatic extraction of nutrients. Therefore, it is necessary to develop an effective way to take advantage of the beef-bone by-product. Besides, methods for controlling the DH to accomplish moderate enzymatic hydrolysis require further investigation.

The main objective of the present study is to develop a new method for the use of by-product minced beef-bone with different hydrolysis schemes, including incision enzyme (Protamex) and excision enzyme (Flavourzyme). Analyzing the DH, free amino acids and MW distribution of minced
beef-bone protein hydrolysates (MBBPHs) following different treatment schemes, and then a method of enzymatic hydrolysis with abundant components of enzymatic hydrolysates was determined.

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). NaOH, formaldehyde and other chemical reagents were obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). All chemicals used were of analytical grade.

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)

Table 1. Condition of preparation of six different 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. Determination of the degree of hydrolysis (DH)

The DH was defined as the percentage of free amino groups cleaved from the protein and calculated as the ratio between amino nitrogen (AN) and total nitrogen (TN) of MBBE. The content of AN of hydrolysates was determined by potentiometric titration of formaldehyde according to Nilsang, Lertsiri, and Suphantharika [3].

2.5. Free amino acid analysis (FAA)

The content (ug/mL) of free amino acid (FAA) was determined according to Cai [4] with some modifications. A total of 1 mL of the samples and 10 mL of 80% (V/V) sulfo salicylic acid were added
to a centrifuge tube (50 mL) and mixed well. The mixture was centrifuged with a high speed freezing centrifuge (ST-40r, Thermo Scientific Co., Ltd., Shanghai, China) at 4 °C, 10,000 × g for 20 min. The supernatant was collected and centrifuged twice according to the above methods. Then, the solution was filtered through qualitative filter paper (12.5 cm), and the supernatant was stored at 4 °C before injection.

The amino acid compositions for the MBBPHs were measured by an L-8900 Amino Acids Automatic Analyzer (Hitachi LTD, Japan). For chromatographic separation, the sample (20 uL) was injected into the system and analyzed with the detector operating at 570/400 nm. A Na+ ion exchange column was used at a constant oven temperature of 57 °C, while the buffer flow rate was 0.4 mL/min, and the ninhydrin flow rate was 0.35 mL/min.

2.6. Determination of Molecular Weight Distribution (MW)

The method used was according to Lan [5]. The molecular weight of peptides in the sample was determined using a Agilent 1200 HPLC (Agilent, USA). The column used was TSK gel filtration column, 2000 SWXL 300 mm × 7.8 mm (Tosoh Co., Tokyo, Japan), whilst the mobile phase consisting of acetonitrile/water/trifluoroacetic acid (45/55/0.1, v/v/v) was delivered at a flow rate of 0.5 mL/min. The column temperature was 30 °C and 10 uL of sample was injected into the HPLC system. The molecular weight calibration curve was obtained from the following five standards from Sigma: cytochrome C (12,500 Da), aprotinin (6500 Da), Vitamin B12 (1355 Da), Oxidized glutathione (612 Da), and tripeptide GGG (189 Da). The chromatogram was recorded using a UV detector at 220 nm and the data were analysed by gel permeation chromatography (GPC) software.

2.7. 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

3.1. DH and AN of different MBBPHs

![Fig. 1 Changes of DH and AN of different MBBPHs](image)
The changes of DH and AN of six MBBPHs are shown in Fig. 1. The DH values and AN content of the six hydrolysates were significantly different. The results showed that enzymatic hydrolysis could increase the DH of MBBPHs remarkably. The DH values of MBBPHs after enzymatic hydrolysis were 15.96%, 17.67%, 22.31%, 25.02% and 31.41%, respectively (Fig. 1), which were significant higher than the control (8.90%). Meanwhile, Protamex was more efficient in increasing the DH and AN of MBBPHs than Flavourzyme. In addition, it was found that the DH and AN of MBBPHs using Flavourzyme and Protamex (including Fp, Pf and DE) were significantly higher than that of the MBBPHs treated with F or P alone. A possible reason might be that different enzymes have different restriction sites, which catalyze the hydrolysis of proteins into different polypeptide fragments, and more amino acids and polypeptides can be obtained by combining Flavourzyme and Protamex. Moreover, compared with that of Fp and Pf, the DH and AN of DE were significantly higher. From the above results, it is clear that enzymatic hydrolysis exerted a considerable influence on the increase of DH and AN, which provides sufficient precursors for Maillard reaction later. It has the best effect on the formation of flavor precursors by combination of Flavourzyme and Protamex (DE).

3.2. FAA composition of different MBBPHs

| Amino acid | C   | F    | P    | Fp   | Pf   | DE   |
|------------|-----|------|------|------|------|------|
| Asp        | 10.00 ± 0.03 | 15.7 ± 0.01 | 64.5 ± 0.02 | 179 ± 0.03 | 31.41% |
| Thr        | 13.20 ± 0.04 | 56.3 ± 0.01 | 32.3 ± 0.03 | 104 ± 0.01 | 31.41% |
| Ser        | 14.70 ± 0.02 | 92.4 ± 0.03 | 38 ± 0.01 | 141 ± 0.03 | 31.41% |
| Glu        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Pro        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Gly        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Ala        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Val        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Met        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Ile        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Leu        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Tyr        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Phe        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Lys        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| His        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Trp        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Arg        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Cys        | 14.70 ± 0.02 | 56.7 ± 0.01 | 22.1 ± 0.01 | 85 ± 0.01 | 31.41% |
| Total      | 293.39 | 3052.5 | 1987.70 | 2250.70 | 31.41% |
| Fresh AA   | 13.20 | 43.00 | 32.70 | 12.40 | 31.41% |
| Bitter AA  | 120.30 | 1253.90 | 959.60 | 1462.30 | 31.41% |

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 FAA compositions and the corresponding contents of six MBBPHs by different enzymatic hydrolysis are presented in Table 2. It is well known that amino acids are precursors of the Maillard reaction, and the composition and content of the free amino acids play an important role in the flavor properties of MMRs directly and indirectly[6]. As shown in Table 2, enzymatic hydrolysis exerted a considerable influence on the increase of free amino acids. Compared with the total FAA contents of C (259.39 µg/mL), the FAA increased by at least 6.8 times. Asp and Ser are important umami amino acids. Sample DE had a high content (45.4 µg/mL) of umami amino acids compared with the others. Met and cystin, which are kinds of sulfhydryl amino acid, can be degraded by Strecker at the
intermediate stage of the Maillard reaction to produce H$_2$S and ammonia, and provide precursor substances for the formation of a large number of heterocyclic flavor compounds, which produce a strong aroma of beef [7]. Sulfur amino acids are usually added as substrates in the preparation of meat flavor seasoning [8]. From the above results, there was no cysteine detection in all of the MBBPHs, and the contents of methionine in the six MBBPHs (C, F, P, Pf and DE) were 5.80 µg/mL, 176 µg/mL, 142 µg/mL, 243 µg/mL and 197 µg/mL, respectively. It was obvious that Met was present at high proportion in the samples after enzymatic hydrolysis compared with C. Among the 18 analyzed amino acids, Pro, Val, Leu, Tyr, Phe and Lys are bitter amino acids. The highest ratio of bitter amino acids was observed on the samples following Fp treatment.

3.3. Molecular weight distribution of different MBBPHs

| MW(Da) | Samples* | C       | F       | P       | Fp      | Pf      | DE      |
|--------|----------|---------|---------|---------|---------|---------|---------|
| >5000  |          | 34.96±0.03d | 57.81±0.16c | 34.87±0.07b | 42.86±0.06b | 27.02±0.03e | 37.18±0.06c |
| 2000-5000 |        | 0.53±0.01d | 0.00±0.00e | 24.54±0.03c | 23.84±0.01c | 25.86±0.07a | 25.03±0.01b |
| 1000-2000 |        | 0.00±0.00b | 8.54±0.03b | 0.00±0.00b | 0.00±0.00b | 0.00±0.00b | 0.00±0.00b |
| 500-1000 |        | 10.69±0.14a | 0.00±0.00b | 0.00±0.00b | 0.00±0.00b | 0.00±0.00b | 0.05±0.00b |
| 180-500 |          | 4.90±0.30a | 21.75±0.44b | 27.99±0.01ab | 19.97±0.07b | 32.88±0.01ab | 22.28±0.07ab |
| <180   |          | 8.92±0.20e | 11.91±0.07d | 12.60±0.11c | 13.34±0.04b | 13.72±0.16b | 15.46±0.04a |

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).

* Peptides in MBBPHs as percent of total area (%)

The molecular weight distributions of six MBBPHs by different enzymatic hydrolysis are shown in Table 3. It is obvious that some peptides less than 180 Da gradually increased with the increase of degree of hydrolysis (Fig. 1). This finding is in agreement with other studies, which showed that the MW distribution of hydrolyzed proteins was highly related to DH [9]. Ogasawara [10] reported that the main compounds that provided characteristic aroma were Maillard peptides whose MW ranged from 1000 Da to 5000 Da. It was found that the Maillard peptides increased significantly after enzymolysis compared with C (0.53%). Specifically, the content of Maillard peptide in the Pf group was the highest, followed by DE, P, Fp and F. Because these peptides contribute to the mouthful flavor, the content of these peptides will directly affect the flavor of the Maillard reaction products.

4. Conclusion

This study revealed that enzymatic hydrolysis was efficient in increasing the DH, content of AN, total free amino acids and Maillard reaction peptides compared with the MBBPHs without enzymatic hydrolysis. The combination of two enzymes has better effect than single enzymatic hydrolysis, and more flavor precursors can be obtained, which provides abundant substrates for the preparation of natural meat flavoring base materials.

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References

[1] Zhang, C. H. Bone source food processing technology [J]. Science Press, 2015, 29-31.
[2] Morimura, S., Nagata, H., Uemura, Y., Fahmi, A., Shigematsu, T., & Kida, K. Development of an effective process for utilization of collagen from livestock and fish waste [J]. Process Biochemistry, 2002, 37(12), 1403-1412.

[3] Nilsang, S., Lertsiri, S., Suphantharika, M., & Assavanig, A. Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases [J]. Journal of Food Engineering, 2005, 70(4), 571-578.

[4] Cai, G.H. Dried fish process optimization and its quality changes in the course of processing [D]. 2016.

[5] Lan, X., Liu, P., Xia, S., Jia, C., Daniel, M., & Zhang, X., et al. Temperature effect on the non-volatile compounds of maillard reaction products derived from xylose-soybean peptide system: further insights into thermal degradation and cross-linking [J]. Food Chemistry, 2010, 120(4), 967-972.

[6] Cao, C., Xie, J., Hou, L., Zhao, J., Chen, F., & Xiao, Q., et al. Effect of glycine on reaction of cysteine-xylose: insights on initial maillard stage intermediates to develop meat flavor [J]. Food Research International, 2017, 99(1), 444.

[7] Yu, A. N., Tan, Z. W., & Wang, F. S. Mechanism of formation of sulphur aroma compounds from -ascorbic acid and -cysteine during the maillard reaction [J]. Food Chemistry, 2012, 132(3), 1316-1323.

[8] Kamhuey, W., Suraini, A. A., & Suhaila, M. Sensory aroma from maillard reaction of individual and combinations of amino acids with glucose in acidic conditions [J]. International Journal of Food Science & Technology, 2010, 43(9), 1512-1519.

[9] Baek, H. H., & Cadwallader, K. R. Enzymatic hydrolysis of crayfish processing by-products [J]. Journal of Food Science, 2010, 60(5), 929-935.

[10] Ogasawara, M., Yamada, Y., & Egi, M. Taste enhancer from the long-term ripening of miso (soybean paste) [J]. Food Chemistry, 2006, 99(4), 736-741.