Effects of Fatty Acids in Enzymatic Hydrolysate of Chicken Bones on Maillard Reaction

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Received: February 19, 2019 **Accepted: October 16, 2019**

Published: December 31, 2019

Abstract: *This paper attempts to develop a novel model to extract the hydrolysed proteins from chicken bones. Firstly, an effective model was set up to detect the degree of hydrolysis with papain by response surface method. Next, the fatty acids of Maillard reaction products were identified by gas chromatography mass spectrometry (GC-MS). After that, the relationship between the fatty acids of Maillard reaction products and the substrate of Maillard reaction was analysed in details. The main research results are as follows: the top three influencing factors on enzyme hydrolysis degree were time (2.5 h), temperature (63 °C) and bone size (1.9 cm); a total of 19 types of free fatty acids were spotted in enzymatic hydrolysis, including 2.56% of polyunsaturated fatty acids (PUFAs), 54.92% of monounsaturated fatty acids (MUFAs) and 39.63% of saturated fatty acids (SFAs). It is concluded that more MUFAs in meat can enhance the special aroma of chicken in Maillard reaction.*

Keywords: *Chicken bones, Enzymatic hydrolysate, Fatty acids, Maillard reaction.*

Introduction

Proteins are the largest non-water component (19%) in chicken bones. Most of the proteins in chicken bones contain bioactive peptides (e.g. collagen and gelatine) that are beneficial to health [20]. Each year, about 28 million tons of chicken is consumed in China, producing 5.6-14 million tons of chicken bones. In 2012 alone, the global consumption of raw chicken amounted to 83 million tons, creating 16.6-41.5 million tons of chicken bones. Virtually all of the chicken bones are discarded, causing a huge waste of valuable protein sources [1].

In animal processing industries, many approaches have been adopted to extract proteins from chicken bones, ranging from acidic/alkaline hydrolysis to enzymatic hydrolysis [17]. Authors in [2] found that some proteases can break both terminal and nonterminal peptide bonds in a peptide chain. These proteases help to make enzymatic proteolysis mild and controllable, and improve the quality and functional properties of proteins [11, 12]. In [9, 10] authors observed that many small peptides in enzymatic proteolysis have antioxidative effects, due to the scavenging of free radicals and/or inhibition of the production of oxidants and pro-inflammatory cytokines. Khan et al. [5] suggested that peptides can enhance emulsion or foam formation, preventing the foams from coalescence or flocculation, and also promote protein solubility, turning the solution into a gel under proper physical-chemical conditions. Lacou et al. [6] learned that enzymatic hydrolysis can occur under mild conditions (pH 6-8; temperature 30-60 °C), and thus minimize the side reactions.

In recent decades, the Maillard reaction products of chicken bones have been extensively used as flavour enhancers for foods, such as roasted coffees, baked goods, and meat products. The reaction is nonenzymatic, involving reducing sugar and amino acid in peptides or proteins. The reaction products are a mixture of native proteins, peptides and amino acids. The mix proportion depends on the hydrolysis condition and has a great impact on the functional properties of the final products. The Maillard reaction products are hailed for their great contribution to the aroma, texture and taste of foods, and their excellent antioxidant and chemo-preventive effects [4, 8, 14, 19].

Recently, it is discovered that the meat flavour enhanced by Maillard reaction products depends heavily on the precursor substrate and the processing technique [3]. The compounds derived from lipid have been confirmed as the main precursor substrates that bring a unique flavour. It is also found that the protein hydrolysates, consisting of free amino acids, peptides, and reducing sugars, are important flavour agents in Maillard reaction products with meat flavour. Considering the richness in essential amino acids, minerals and vitamins, the protein hydrolysates from pork, beef or lamb by-products have the potential to be flavour enhancers, emulsifiers, and water bonding enhancers or nutrients.

According to [7], the precursor content directly bears on the flavour, under the same degree of hydrolysis. In general, the degree of hydrolysis is negatively correlated with the precursor content. Toldra et al. [18] noted that the adipose tissue around beef bones may hinder the combination of protein and protease, leading to a low degree of hydrolysis. As a result, Song et al. [16] called for better ways to fully utilize beef bone by-products [13-15].

In the light of the above studies, this paper attempts to develop a novel model to extract the hydrolysed proteins from chicken bones. Firstly, an effective model was set up to detect the degree of hydrolysis with papain by response surface method. Next, the fatty acids of Maillard reaction products were identified by gas chromatography mass spectrometry (GC-MS). After that, the relationship between the fatty acids of Maillard reaction products and the substrate of Maillard reaction was analysed in details. Finally, the author disclosed how Maillard reaction is affected by the fatty acids of the reaction products [21].

Materials and methods

Raw materials and reagents

The chicken bones were purchased from a supermarket in Anshan, north-eastern China's Liaoning Province. Papain (600 U/mg) was obtained from Tianjin Zhenruguo Food Industry Co., Ltd. The chemical reagents, namely, concentrated sulfuric acid, methylene chloride, methyl alcohol and n-hexane, were procured from Sinopharm Chemical Reagent Co., Ltd.

Processing of raw materials

Firstly, chicken bones were cleaned of meat, fat and bone marrow, and werecut into cubes with a size length of 2.00 cm. Then, the cubes and lard were mixed in deionized water, forming a colloid. The colloid was hydrolysed for 2.5 h with papain under 65±0.5 ℃ on amagnetic stove. Finally, the hydrolysis products were filtered for further use [10].

Measurement of amino acid content

The amino acid content was measured by ninhydrin colorimetric method, referring to the standard curve of L-isoleucine in Fig. 1.

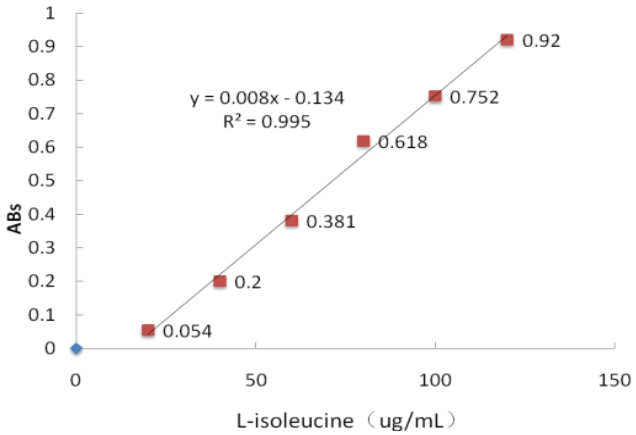

Fig. 1 The standard curve of L-isoleucine

Degree of enzyme hydrolysis

The degree of enzyme hydrolysis (DEH) was calculated by subtracting one from the quotient between the content of amino acids in the initial solution (AN_0) and that in the solution after reaction (AN_1):

$$DEH = (AN_1 / AN_0) - 1.$$

Design of response surface experiment

Drawing on Box-Behnken designs, a three-factor, three-level response surface optimization plan was designed for single-factor tests. Developed on Design-Expert 9.0, the optimization plan was applied to determine the degree of enzyme hydrolysis and the optimal condition for thorough reaction between chicken bone proteins and compound protease. A total of 17 tests were carried out, including 12 factorial tests and 5 repeated tests at the central point.

Preparation of fatty acids

Firstly, dichloromethane-methanol was added into enzymatic hydrolysate of chicken bones to triple the original volume of the hydrolysate, and the lipid was extracted through 30 min digestion. After that, the dichloromethane with lipid extract was further digested 5 times with small aliquots of dichloromethane, and the final organic solution was dried on sodium sulphate, filtered and evaporated until dry under reduced pressure, creating the lipid extract for immediate GC-MS analysis.

Transesterification

Firstly, 15 mL lipid extract was poured into a screw-cap vial and treated with 5 mL sodium methoxide in 2.5 mol/L dry methanol solution. Then, 1 mL dry *n*-hexane was added, and the whole solution was shaken for 15 min at room temperature to wash methanolic residue, which was separated in hexane layer with *n*-hexane (5 × 5 mL). Next, hexane fractions and methanolic residue were evaporated until dryunder reduced pressure for fatty acid methyl ester. After that, 2 mL 1N aqueous hydrogen chloride and peroxide free diethyl ether (5 × 5 mL) were applied to acidify and extractmethanolic residue. Finally, the ethereal solution was dried on sodium sulphate, filtered, and evaporated until dry under reduced pressure for free fatty acid.

Analysis of fatty acids by gas chromatography

Firstly, 1 μL aliquot of chloroform solution (1 mL in total volume) containing fatty acid methyl ester mixture, which was obtained by transesterification of the lipid extract, was injected in the Agilent GCD system. Secondly, an Agilent J & W HP-5 ms polysiloxane MS capillary column (25 m × 0.20 mm × 0.33 μm) was applied for separation under the injector temperature of 250 °C and the detector temperature of 280 °C. During the separation, the injected solution was 0.4 μL in volume, and the mass detector was operated in the electron ionization mode at the electron energy of 70 eV. The column was heated up from the initial temperature of 100 °C at 10 °C/min to 280 °C, and kept under the final temperature for 10 min. During the experiment, the split/splitless ratio was set to 1/20 and Helium was introduced as carrier gas at the flow of 1mL/min. After that, the content of fatty acid methyl ester was retrieved from the G1701BA ChemStation and the literature for artificial spectrum analysis, and the content of free fatty acids was obtained by peak area normalization for quantitative analysis. All samples were injected manually and analysed in triplicate.

Results and discussion

Response surface tests on enzyme hydrolysis conditions

As mentioned above, the three-factor, three-level response surface optimization plan was designed based for Box-Behnken designs on single-factor tests. As shown in Table 1, bone size (A), time (B) and temperature (C) are the top three influencing factors of the response value. The variances of the data in Table 1 were analysed and recorded in Table 2.

Table 1. The optimization plan and the response values

Number	A Bone size, cm	B Time, h	C Temperature, °C	R_1, DEH
1	1.5	2.5	55	0.85
2	2	3	65	0.54
3	1.5	3	60	0.16
4	1.5	2.5	65	0.80
5	1	3	65	0.28
6	1.5	3.5	65	0.06
7	2	2.5	60	0.93
8	1.5	3	60	0.20
9	1.5	3	60	0.11
10	1	3	55	0.53
11	1.5	3	60	0.12
12	2	3	55	0.37
13	2	3.5	60	0.06
14	1	3.5	60	0.07
15	1.5	3	60	0.20
16	1.5	3.5	55	0.39
17	1	2.5	60	0.71

On the basis of the single factor test, a three-factor three-level response surface optimization plan was developed in accordance with the design principle of Box-Behnken design. As is obtained, the three most influencing factors are bone size (A), duration (B) and temperature (C). Please refer to Table 1and Table 2 for the plan, and variance analysis respectively.

Table 2. The variance analysis

Source	*F*-value	*p*-value ($P_{rob} > F$)	Significant
Model	33.92	< 0.0001	significant
A-A	2.49	0.1589	
B-B	201.79	< 0.0001	significant
C-C	5.83	0.0465	significant
AB	3.13	0.12	
AC	9.68	0.0171	significant
BC	4.33	0.759	
A^2	8.3	0.0236	significant
B^2	33.19	0.0007	significant
C^2	29.03	0.001	significant
Residual	4.56E-003		
Lack of fit	4.51	0.0899	not significant

Based on the variance analysis, the test results were fitted with the multivariate quadratic regression equation in the established hydrolysis degree detection model:

$$R_1 = + 33.61588 - 2.86475A - 3.18150B - 0.84103C - 0.23900AB + 0.042000AC - 0.028100BC + 0.37900A^2 + 0.75800B^2 + 7.09000E\text{-}003C^2$$

According to Table 2, the model has a high goodness of fit, small test error and high significance. There was no obvious lack-of-fit despite the significant changes in hydrolysis degree in different conditions, revealing the high reliability of the model. In the model, B, C, AC, A^2, B^2, and C^2 had great weights during the calculation of R_1. Therefore, time, temperature, and bone size are major impactors of enzyme hydrolysis degree.

Next, the *F*-value was employed to reflect the influence degree of each factor. The *F*-value is positively correlated with the importance of the corresponding factor. As shown in Table 2, $F_A = 2.49$, $F_B = 201.79$ and $F_C = 5.83$. Hence, the three factors can be ranked in descending order of impact as time > temperature > bone size.

The response surface of the interaction between temperature and bone size (Fig. 2) was obtained using the regression equation. It can be seen that, when the temperature remained constant, the degree of enzyme hydrolysis increased with bone size.

Prediction and verification of optimal enzyme hydrolysis conditions

The regression equation was solved on Design-Expert 9.0. The optimal conditions for enzyme hydrolysis of chicken bones were determined as the bone size of 1.89 cm, the temperature of 63.22 °C, and the time of 2.51 h. To make the conditions feasible for verification, the three parameters were slightly modified as 1.9 cm, 63 °C and 2.5 h. Three tests were conducted to verify the effectiveness of the modified conditions. Through the tests, the enzyme hydrolysis degree of chicken bones was measured as 0.921, close to the predicted value of 0.934. The relative error was merely 1.39%, indicating the regression equations a reliable tool to analyse and predict the enzyme hydrolysis degree of chicken bones.

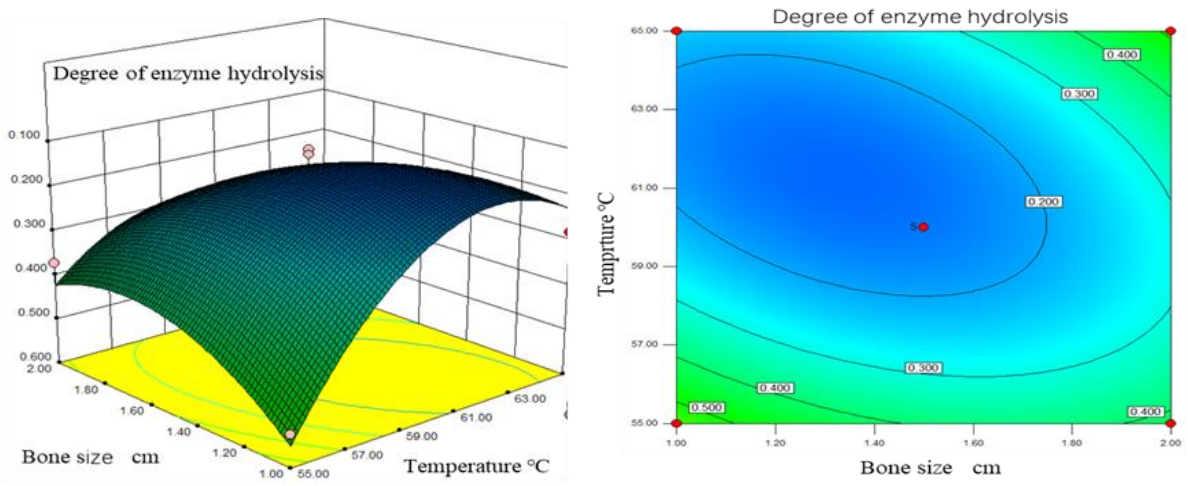

Fig. 2 The effects of bone size and temperature on the degree of enzyme hydrolysis

Analysis of fatty acids in enzymatic hydrolysate

A total of 19 types of free fatty acids were spotted in enzymatic hydrolysis (Fig. 3). Among them, 2.56% were polyunsaturated fatty acids (PUFAs), 54.92% were monounsaturated fatty acids (MUFAs) and 39.63% were saturated fatty acids (SFAs) (Table 3). Eicosatetraenoic acid (1.64%), octadecenoic acid (48.97%), and methyl-heptadecanoic acid (13.7%) were the dominant species in the PUFAs, the MUFAs and the SFAs, respectively. It was deduced that short chain fatty acids might directly contribute to the characteristic flavour of meat, due to their low odour threshold. Meanwhile, the acids with high odour threshold, e.g., octanoic acid and nonanoic acid, may act as precursor substances and have indirect impacts on flavour [13]. Although taking up less than 0.3%, cyclopropaneoctanoic acid may also contribute to the special flavour in the reaction system. Furthermore, it is learned that the MUFAs promote the juiciness, tenderness, and flavour of meat, while intramuscular PUFAssuppress these features due to their poor antioxidative effects. To sum up, more MUFAs in meat can enhance the special aroma of chicken in Maillard reaction.

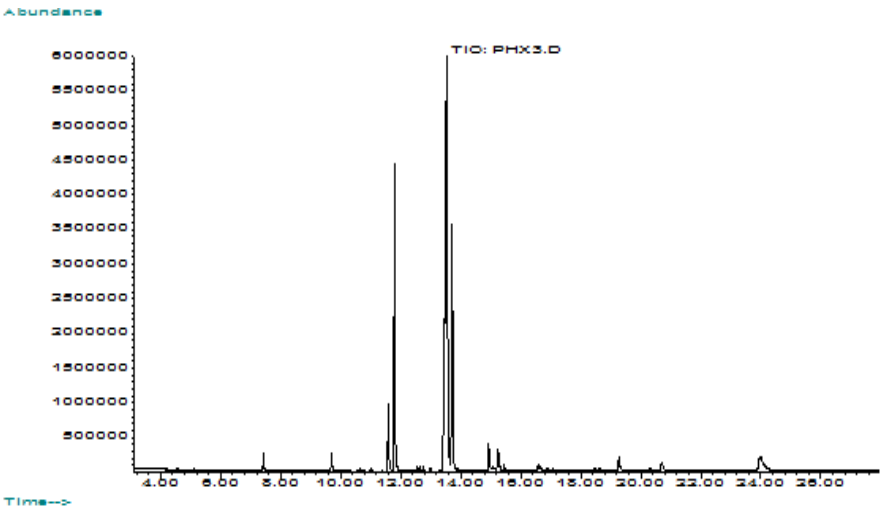

Fig. 3 GC-MS flow diagram of fatty acid ion species inchicken bone hydrolysate

Table 3. GC-MS analysis on free fatty acids in chicken bone hydrolysate

No	Library/ID	Total, %	Molecular formula
1	Tridecanoic acid, 12-methyl-	1.06	$C_{15}H_{30}O_2$
2	11-Hexadecenoic acid	3.69	$C_{17}H_{32}O_2$
3	Hexadecanoic acid	16.66	$C_{17}H_{34}O_2$
4	Cyclopropaneoctanoic acid	0.3	$C_{18}H_{34}O_2$
5	Heptadecanoic acid	0.34	$C_{18}H_{36}O_2$
6	8-Octadecenoic acid	48.97	$C_{19}H_{36}O_2$
7	15-methyl-Heptadecanoic acid	13.7	$C_{19}H_{38}O_2$
8	13,16-Octadecadienoic acid	0.32	$C_{18}H_{32}O_2$
9	5,8-Octadecadienoic acid	0.07	$C_{19}H_{34}O_2$
10	10-Nonadecenoic acid	0.13	$C_{20}H_{38}O_2$
11	5,8,11,14-Eicosatetraenoic acid	1.64	$C_{22}H_{36}O_2$
12	7,10,13-Eicosatrienoic acid	0.36	$C_{21}H_{36}O_2$
13	6,9,12-Octadecatrienoic acid	0.17	$C_{19}H_{32}O_2$
14	11-Eicosenoic acid	1.96	$C_{21}H_{40}O_2$
15	Nonadecanoic acid	0.45	$C_{19}H_{38}O_2$
16	Docosanoic acid	0.23	$C_{23}H_{46}O_2$
17	Tetracosanoic acid	0.14	$C_{25}H_{50}O_2$
18	Cholest-5-en-3-ol (3. beta.)	0.93	$C_{29}H_{48}O_2$
19	Cholesterol	3.42	$C_{27}H_{46}O_2$
	PUFA MUFA SFA	2.56 54.92 39.63	

Conclusions

This paper determines the optimal conditions for enzyme hydrolysis of chicken bones through single-factors tests with Box-Behnken designs and response surface analysis. The top three influencing factors on enzyme hydrolysis degree were identified in descending order as time, temperature and bone size. The optimal conditions were summed up as: the bone size of 1.9 cm, the time of 2.5 h and the temperature of 63 °C. In addition, the regression equation was obtained which is a reliable tool to analyse and predict the enzyme hydrolysis degree of chicken bones. Moreover, a total of 19 types of free fatty acids were spotted in enzymatic hydrolysis, including 2.56% of PUFAs, 54.92% of MUFAs and 39.63% of SFAs. It is concluded that more MUFAs in meat can enhance the special aroma of chicken in Maillard reaction.

Acknowledgment

The present work was supported by the Foundation of Doctoral Scientific Research of Liao Ning Province of China for contract No. 201501200 and the Foundation of National Training Program of Innovation and Entrepreneurship for Undergraduates for No. 201710169011.

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