The Preparation and Identification of Characteristic Flavour Compounds of Maillard Reaction Products of Protein Hydrolysate from Grass Carp (Ctenopharyngodon idella) Bone

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1.Introduction

As a traditional freshwater fish, grass carp (Ctenopharyngodon idella) is one of the major freshwater-cultured fishes [1]. Its production comprises up to 35~40% (3.7 million tons annually) of the total freshwater fish species [2] and it is widely popular with consumers for its palatability, nutritional characteristic, and low price [1, 3]. Therefore, it would not be a surprise that there are various categories of intensive processing products for grass carp in contemporary society, such as fish ball, fish noodle, and fish tofu. With the rapid growth of the fish products industry, the amount of fish bones rich in nutrition, such as protein, fat, and minerals, also increases proportionately. However, in most circumstances, these high-quality resources of nutrition are merely used as animal feed additives and fertilizer or buried directly, which is regarded to be a waste. Therefore, it is of great urgency to develop value-added products from grass carp bone.

The application of enzymolysis technology has attracted considerable interest in the conversion of underutilized resource into a more marketable and functional form, which can promote the physicochemical, functional, and sensory properties of the native protein [4]. Researches showed that...
the grass carp proteins were mainly used to prepare bioactive peptide and functional material in secondary processing [5, 6]. For example, the research of Li et al. [7] revealed that the grass carp protein hydrolysate displayed high antioxidant activities. What is more, Wang et al. [8] claimed that grass carp peptides associated with NO regulation and the rennin-angiotensin system present to have antihypertensive effect. In other researches, fish protein hydrolysate was proved to be of use for flavour enhancer for direct human consumption for the material composition of free amino acids, smaller peptides, salt, and many savory compounds [9]. However, the study of protein hydrolysate from grass carp bone on food flavour agents has not been fully investigated.

The flavour of protein hydrolysate is related to the type of protease and enzymatic condition, among which bitter taste is the major problem affecting the sensory acceptability of protein hydrolysate. Some methods exist to limit the formation of bitter compounds during hydrolysis, such as controlling degree of hydrolysis (DH), choosing some specific enzymes, and masking by cyclodextrin [10]. The choice of protease affects the functional and flavour characteristics of protein hydrolysate. Flavourzyme is one commonly used protease in various commercial proteases to produce low-bitter flavour peptide compounds. It is endo- and exopeptidase enzymes mixture, which can effectively minimize the bitterness produced by the previous hydrolysis in the hydrolyzed peptide product [11]. It can hydrolyze proteins and produce more flavour peptides as well as amino acids for the product, which is more suitable for the hydrolysis of seafood. Also, recent studies show that a higher DH is more conducive to the formation of flavour amino acids and potential flavouring material [11, 12]. Therefore, it is an effective method to produce hydrolysate using flavourzyme under longer enzymatic time from grass carp bone protein (GCBP).

Besides, the Maillard reaction has also been reported to be an effective method to improve the functional properties of protein hydrolysate [13], masks the initial fish off-flavours, and improves the odour characteristics of hydrolysates [14]. Other researches regard that the peptides with smaller molecular weight showed a higher reaction degree to produce more characteristic flavour compounds through Maillard reaction [15]. According to these researches, it can be concluded that enzymolysis of protein and Maillard reaction of protein hydrolysate were two effective methods for preparing flavour compounds. However, which one is beneficial for making full use of grass carp bone is still uncertain.

Therefore, the objective of this study was to demonstrate the use of by-products of the fish industry (fish bone) for the generation of bioactive substances and fish flavour compounds. The content includes the three following contents: (1) to optimize enzymatic parameters of GCBP and Maillard reaction conditions of GCBP hydrolysate, (2) to investigate the bioactivities of GCBP hydrolysate and its Maillard reaction products with the thermal degradation reaction as one positive control, and (3) to analyze and compare the flavour components of the GCBP hydrolysate and its Maillard reaction products with the thermal degradation reaction as one positive control. It is also hoped that the results of the present research will be of great value to the application of grass carp bone and even other fish bones.

2. Materials and Methods

2.1. Materials. Grass carp (Ctenopharyngodon idella) bone was provided by a fish market of Zhenjiang, Jiangsu, China. Flavourzyme (activity of 2.44 × 10⁴ U/mg) was purchased from Novozymes Co. Ltd. (China). Angiotensin-I-converting enzyme (ACE) was extracted from the pig lung according to the reported method (Maruyama et al., 1999). N-[3-(2-Furyl)-acryloyl]-L-phenylalan glycyl glycine (FA PGG), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), and 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich Corp. (USA). All the other chemicals and solvents used in the experiment were of analytical grade.

2.2. Extraction of Fish Bone Protein. In this study, alkali extraction combined with high-temperature treatment was applied to improve protein purity. Briefly, the fresh fish bone with a few of meat (byproduct of fish balls) was washed by using running water and then chopped into pieces (~0.3 cm) homogenized with water (w/v, 1 : 4.5) and the mixture was adjusted to pH 9.0 by adding 2 mol/L NaOH. Afterwards, the mixture was placed in high-pressure cooking machine at 120°C for 60 min. The homogenate was cooled to room temperature and centrifuged for 20 min at 5,000 r/min. The insoluble pellets and grease suspensions were discarded. Soluble protein in the supernatant was frozen-dried to prepare fish bone protein powder. The protein content of the fish bone powder was assayed by the Kjeldahl method (AOAC 991.20) and the value was 92.38% (dry weight).

2.3. Screening of Enzymolysis Conditions of Fish Bone Protein. In order to optimize enzymolysis conditions of fish bone protein for the maximum DH, single factor experimental design was employed. The fish bone powder was mixed with distilled water and then the mixture was hydrolyzed with flavourzyme according to the enzymatic conditions defined by the experimental design with DH as an index. The single factor experiment was conducted under the following conditions: substrate concentration of 20, 30, 40, 50, and 60 g/L; pH value of 6, 6.5, 7, 7.5, and 8; temperature of 40, 45, 50, 55, and 60°C; enzyme-to-substrate ratio of 5%, 5.5%, 6%, 6.5%, 7%, and 7.5%; and enzymolysis time of 5, 6, 7, 8, and 9 h. The reaction was terminated by boiled water bath for 15 min to inactivate the enzyme. Then the sample was centrifuged for 15 min at 4000 r/min after cooling to the room temperature (25 ± 1°C). The supernatant of protein hydrolysate was used for further analysis. All reactions were operated in triplicate.

2.4. Determination of Degree of Hydrolysis. DH is defined as the percentage of the number of peptide bonds broken to the
total number of bonds per unit weight, which was calculated from the ratio of $\alpha$-amino nitrogen to total nitrogen. The amino nitrogen content was determined by a formaldehyde titration method [16]. An equal amount of distilled water was added to 10 mL supernatant of protein hydrolysate (Section 2.3) and the mixture was adjusted to pH 7.0 using 0.1 mol/L NaOH. Then 10 mL of 38% (v/v) formaldehyde solution was added to the mixture and titration was continued to the end point at pH 9.5 with 0.2 mol/L standard NaOH solution. The total nitrogen content was determined by Kjeldahl method (AOAC 991.20). Tests were conducted in triplicate.

2.5. Optimization of Maillard Reaction Condition. Based on the optimal protein enzy molysis condition (Section 2.3), the supernatant of the grass carp bone protein hydrolysate was prepared for Maillard reaction. According to our previous experiment results, the supernatant of protein hydrolysate was mixed with glucose of 4% (w/w), glycine-arginine (ratio of 1:1) mixture of 3% (w/v), vitamin C of 0.15% (w/v), and vitamin B$_1$ of 0.05% (w/v). Then the sample (protein concentration of 1 mg/mL) was put in sealed tube and heated for 1 h at different independent variables ($i$) protein, the protein hydrolysate was directly heated for 1 h at thermal degradation reaction of protein hydrolysate was designed by the Box-Behnken design (Table 1).

In order to optimize the reaction parameters, Box-Behnken design was selected and used to encode the test factors. The actual and coded values for the independent variables are shown in Table 1. The factors with the same coded levels (−1, 0, 1) and corresponding actual values were as follows: initial pH of 6, 7, and 8; reaction temperature of 90, 110, and 130°C; and reaction time of 1, 2, and 3 h. The degree of graft (DG) was an index to evaluate Maillard reaction and it was set as the respond ($Y$). Each run was conducted in triplicate. The quadratic equation was the same as that of equation (2).

The complete quadratic equation reflecting the effect of the three independent variables on the DG was shown as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_iX_i + \sum_{i=1}^{3} \beta_{ii}X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij}X_iX_j,$$  \hspace{1cm} (1)

where $Y$ is the response variable; $\beta_0$ is a constant; $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ are the regression coefficients for intercept, linearity, square, and interaction, respectively; $X_i$ and $X_j$ are the different independent variables ($i \neq j$). To deduce the quadratic equation (1), seventeen factorial points and five replicates of the center points were designed by the Box-Behnken design (Table 1).

In order to evaluate the effects of Maillard reaction, thermal degradation reaction of protein hydrolysate was employed to be a positive control. After hydrolysis of fish bone protein, the protein hydrolysate was directly heated for 1 h at 120°C in oil bath without adding glucose, glycine-arginine mixture, vitamin C, and vitamin B$_1$ under the optimal conditions of Maillard reaction, including temperature, pH, and time. Then the mixture was immediately cooled in ice bath for 30 min to terminate the heat reaction. The thermal degradation products (TDP) were stored at −20°C for further analysis. All trials were performed in triplicate.

2.6. Determination of the DG. The DG value was determined by o-phen thaldialdehyde method [17] with light modifications. The preparation method of o-phen thaldialdehyde reagent was as follows: 40 mg of o-phen thaldialdehyde was dissolved in 1.0 mL of methanol, 25 mL sodium tetraborate (0.1 mol/L), 2.5 mL sodium dodecyl sulfate (20%, w/w), and 100 μL of β-mercaptoethanol. Then the mixture was diluted to a final volume of 50 mL with distilled water, after which two hundred microliter sample solution (protein concentration of 1 mg/mL) was added to 4 mL o-phen thaldialdehyde reagent. The solution was mixed briefly and incubated for 2 min at 37°C. The absorbance was read at 340 nm using a spectrophotometer (Unic 7200, Unocal Corporation, Shanghai, China). Tests were performed in triplicate, and DG was calculated according to the following formula:

$$DG(\%) = \frac{A_0 - A_1}{A_0} \times 100\%,$$  \hspace{1cm} (2)

where $A_0$ was the absorbance of the supernatant of protein hydrolysate at 340 nm; $A_1$ was the absorbance of mixture after Maillard reaction at 340 nm.

2.7. DPPH Radical Scavenging Activity and ACE Inhibitory Activity Assays. The DPPH radical scavenging activity was measured by the method of Nasrollahzadeh et al. [18] with some modifications. 2 mL of DPPH solution (0.1 mol/L, dissolved in methanol) was added to 2 mL of sample (protein concentration of 1 mg/mL). Then the mixture was kept in the dark for 20 min at 37°C, followed by the centrifugation for 10 min at 4000 r/min and the measurement of the absorbance at 517 nm using spectrophotometer (Unic 7200, Unocal Corporation, Shanghai, China). The blank and control were prepared with the distilled water instead of samples and methanol instead of DPPH solution, respectively. All reactions were operated in triplicate. The DPPH radical scavenging activity was calculated by the following formula:

$$\text{DPPH radical scavenging rate (\%) } = \left( 1 - \frac{A_1 - A_2}{A_0} \right) \times 100\%,$$  \hspace{1cm} (3)

where $A_0$ is the absorbance of the blank (methanol instead of the sample), $A_1$ is the absorbance of sample, and $A_2$ is the background absorbance (methanol instead of DPPH).

The ACE activity was measured following the method of Zhou et al. [19] with some modifications. The samples were diluted 100 times with a HEPES buffer (80 mmol/L, pH 8.3, 300 mmol/L NaCl). The reaction mixture contained 50 μL ACE, 100 μL sample, and 50 μL FAPGG (1.0 mmol/L). The control was prepared with HEPES buffer instead of samples. After being incubated for 30 min at 37°C, the mixture was...
measured in the absorption at 340 nm. The ACE inhibitory activity was calculated using the following formula:

$$\text{ACE inhibitory activity (\%)} = 1 - \frac{A_{0} - A_{30}}{A_{30} - A_{0}} \times 100\%,$$

(4)

where $A_{0}$ and $A_{30}$ represent the absorption of sample at 0 min and 30 min. $A_{0}$ and $A_{30}$ represent the absorption of blank at 0 min and 30 min.

2.8. Determination of Amino Acids Composition. The total amino acids profile of grass carp bone protein hydrolysates (GCBPH), thermal degradation products (TDP), and Maillard reaction products (MRP) was determined using an amino acid analyzer (S433D, Sykam, German) after hydrolyzing under vacuum with 6 mol/L HCl at 110°C for 24 h in the presence of 1% phenol (v/v). The free amino acid (FAA) profile was measured directly by the analyzer. The peptide-bound amino acid was calculated by subtracting FAA from total amino acid (TAA) [20]. The results were expressed as mg/g dry weight of products.

2.9. Determination of Volatile Compounds by Dynamic Headspace Gas Chromatography-Mass Spectrometry. The volatile compounds in GCBPH, TDP, and MRP were detected using HP6890/5973 dynamic headspace gas chromatography-mass spectrometry (DHS GC-MS) (Agilent Technologies (China) Co., Ltd.) [21] with some modifications. Each sample was prepared from 4 mL of samples (GCBPH, TDP, and MRP) and 1.0 g of NaCl in a 15 mL vial for the headspace technique. During the headspace collection procedure, samples were magnetically stirred at 600 r/min and equilibrated for 40 min at 60°C after purging the nitrogen. The separation of volatile compounds was achieved on a DB-WAX capillary column (60 mm × 0.25 mm × 0.25 μm). Helium was used as carrier gas at a flow rate of 0.8 mL/min. Oven initial temperature was set at 120°C for 4 min and then programmed to 90°C by a temperature ramp of 5°C/min and lastly increased to 210°C at 10°C/min with a final isothermal period of 10 min. The inlet and interface temperature were both kept at 250°C. A mass spectrometer scanned from 33 to 450 u. The ion source was set at 200°C and spectra were obtained by electron impact (70 eV).

In this experiment, the compound standards of C7∼C30 were selected for qualitative analysis and 2,4,6-trimethyl pyridine (chromatographic purity) was used as internal standard for quantitative analysis. The results were expressed as the relative area percentages being indices of the mass percentages of the compounds.

### Table 1: Box-Behnken design and results of Maillard reaction of protein hydrolysate from grass carp bone.

| Level | $X_1$     | $X_2$     | $X_3$     | $Y$    |
|-------|-----------|-----------|-----------|--------|
| 1     | −1 (6)    | −1 (90)   | 0 (2)     | 36.52  |
| 2     | 1 (8)     | −1 (90)   | 0 (2)     | 41.33  |
| 3     | −1 (6)    | 1 (130)   | 0 (2)     | 50.08  |
| 4     | 1 (8)     | 1 (130)   | 0 (2)     | 53.89  |
| 5     | −1 (6)    | 0 (110)   | −1 (1)    | 47.62  |
| 6     | 1 (8)     | 0 (110)   | −1 (1)    | 53.14  |
| 7     | −1 (6)    | 0 (110)   | 1 (3)     | 39.17  |
| 8     | 1 (8)     | 0 (110)   | 1 (3)     | 39.82  |
| 9     | 0 (7)     | −1 (90)   | −1 (1)    | 56.33  |
| 10    | 0 (7)     | 1 (130)   | −1 (1)    | 60.31  |
| 11    | 0 (7)     | −1 (90)   | 1 (3)     | 42.24  |
| 12    | 0 (7)     | 1 (130)   | 1 (3)     | 66.04  |
| 13    | 0 (7)     | 0 (110)   | 0 (2)     | 68.17  |
| 14    | 0 (7)     | 0 (110)   | 0 (2)     | 69.42  |
| 15    | 0 (7)     | 0 (110)   | 0 (2)     | 67.36  |
| 16    | 0 (7)     | 0 (110)   | 0 (2)     | 64.88  |
| 17    | 0 (7)     | 0 (110)   | 0 (2)     | 66.04  |

$R^2 = 0.9891$

| Term          | Sum of squares | Df | Mean square | F-value | p value   |
|---------------|----------------|----|-------------|---------|-----------|
| Model         | 2013.44        | 9  | 223.72      | 70.68   | <0.0001   |
| $X_1$         | 916.04         | 1  | 916.04      | 289.41  | <0.0001   |
| $X_2$         | 108.89         | 1  | 108.89      | 34.40   | 0.0006    |
| $X_3$         | 6.29           | 1  | 6.29        | 1.99    | 0.2014    |
| $X_1 X_2$     | 0.25           | 1  | 0.25        | 0.079   | 0.7868    |
| $X_1 X_3$     | 5.93           | 1  | 5.93        | 1.87    | 0.2134    |
| $X_2 X_3$     | 42.77          | 1  | 42.77       | 13.51   | 0.0079    |
| $X_1 X_1$     | 1033.00        | 1  | 1033.00     | 326.36  | <0.0001   |
| $X_2 X_2$     | 154.41         | 1  | 154.41      | 48.78   | 0.0002    |
| $X_3 X_3$     | 181.93         | 1  | 181.93      | 57.48   | 0.0001    |
| Residual      | 22.16          | 7  | 3.17        |         |           |
| Lack of fit   | 9.54           | 3  | 3.18        | 1.01    | 0.4763    |
| Pure error    | 12.62          | 4  | 3.15        |         |           |
| Sum           | 2035.59        | 16 |             |         |           |
2.10. Statistical Analysis. The data were expressed as means ± standard differences. Results were statistically analyzed to identify differences by using one-way ANOVA and the Duncan test under the significance level of \( p < 0.05 \) using SPSS 19.0 software (IBM Corporation, NY, USA). Design expert 8.0.5b (Trial Version, State-Ease, Inc, Minneapolis, MN, USA) software package was used to analyze the experimental data of Box-Behnken design.

3. Results

3.1. Single Factor Experimental Results of Enzymolysis of Grass Carp Bone Protein. In order to determine the optimal levels of each variable of enzymolysis conditions for maximum DH, single factor experimental design was employed. Figures 1(a)--1(e) show the DH of grass carp bone protein at different substrate concentration (20–60 g/L), pH value (6.0–8.0), temperature (40–60°C), enzyme-to-substrate ratio (5.5–7.5%), and enzymolysis time (5–9 h). When screening the first parameter, other parameters were set as follows: pH value of 7.0, temperature of 50°C, enzyme-to-substrate ratio, other parameters were set as follows: substrate concentration, other parameters were set as follows: pH value of 7.5, enzymatic temperature (Figure 1(c)). At the temperature of 50°C, the highest DH of 23.54% was obtained. Moreover, enzymatic time of 8h was determined to be the optimal enzymatic time of 8h. Under these conditions, the DH of grass carp bone protein increased gradually with its extension. However, the data of 8h had no significant effect (\( p < 0.05 \)) (Figure 1(d)). In order to reduce the cost, enzymatic time of 8h was selected to optimize the following parameters. As shown in Figure 1(b), the results showed that different pH values influenced (\( p < 0.05 \)) the DH of grass carp bone protein to a great extent and the highest DH (23.54%) was obtained when the pH was 7.5, which was selected to optimize the following enzymatic parameters. Similar to that of pH value, the DH of grass carp bone protein significantly (\( p < 0.05 \)) increased and then decreased with the elevated enzymatic temperature (Figure 1(c)). At the temperature of 50°C, the highest DH of 23.54% was obtained. The DH increased significantly (\( p < 0.05 \)) with the continuing increase of pH. Effects of initial pH (\( x_1 \)) and reaction temperature (\( x_2 \)) are illustrated that the lack of fit was not significant, which confirmed the goodness of fit and hence suitability of the regression model for predicting the DG of Maillard reaction of GCBPH under any combination of values of the independent variables. The coefficient of multiple determinations (\( R^2 \) of 0.9891) revealed that 98.91% of the variation in the DG was due to the independent variables and only 1.09% of the total variation was not explained by the model. Table 1 also demonstrates that the independent variables selected (\( X_1, X_2 \)) exerted significant effects on DG within a 95% confidence interval (\( p < 0.05 \)). The quadratic terms (\( X_1X_1, X_2X_2, \) and \( X_1X_2 \)) as well as the interaction terms (\( X_2X_3 \)) were also significant. Parity plot (Figure 2) showed an acceptable level of agreement. The strong correlation between the actual and the predicted results confirmed that the response model was accurate to reflect the expected optimization. All these results confirmed the predictability of the model for DG of Maillard reaction of GCBPH.

The response surface and contour plots drawn by the Box-Behnken design are shown in Figure 3, which provided a method to visualize the mutual effects of test variables at different levels and the reciprocal interactions between the test variables on the DG. The mutual interactions of initial pH (\( x_1 \)) and reaction temperature (\( x_2 \)) are illustrated in Figures 3(a) and 3(b). The pattern of Figure 3(a), which witnessed a slow rise in DG at first, unexpectedly declined with the continuing increase of pH. Effects of initial pH (\( x_1 \)) and reaction temperature (\( x_2 \)) are illustrated in Figures 3(c) and 3(d), which showed that, with the prolonging of reaction time, the DG changed in a certain range. What is more, the effects of reaction temperature (\( x_2 \)) and time (\( x_3 \)) are illustrated in Figures 3(e) and 3(f).

According to the results in Figure 3 and Table 1, the response surface had both the highest point and the contour of the response surface within the experimental range of the independent variables. According to the established mathematical response surface design method, the optimum conditions of Maillard reaction derived from interactions of
The three independent variables were initial pH of 7.07, the temperature of 118.33°C, and the time of 1.75h. To confirm the validity of the model, three assays were performed under the optimum conditions and the average data of three tests notified that the DG was 69.05%, which was closely associated with the predicted value (70.45%) calculated by equation (5). The strong correlation between the actual and the predicted results confirmed that the response model was accurate to reflect the expected optimization of Maillard reaction.

Figure 1: Single factor experimental results of enzymolysis conditions: (a) substrate concentration, (b) pH value, (c) temperature, (d) enzyme-to-substrate ratio, and (e) time. Means with different superscripts are significantly different ($p < 0.05$).
3.3. Biological Activity. Figure 4 shows the DPPH radical scavenging rate and ACE inhibitory rate of different products. The DPPH radical scavenging rate of MRP increased significantly \((p < 0.05)\) compared to that of GCBPH and TDP. These results were in consistency with the literatures [18, 22]. The reason why the antioxidant activity of MRP rose markedly may be due to the generation of increased antioxidant products during Maillard reaction rather than the thermal degradation reaction of protein hydrolysate.

The ACE inhibitory rate of MRP had no significant difference \((p > 0.05)\) compared to that of GCBPH. However, the ACE inhibitory rate of TDP (with its value of 39.67%) decreased by 8.9% in contrast to that of GCBPH, which indicated that thermal degradation methods reduced the ACE inhibitory activity of protein hydrolysate significantly \((p < 0.05)\). The reason might be that thermal treatment degraded some of the active proteins or peptides into inactive fragment. Furthermore, Maillard reaction could generate more compounds and substances with strong ACE inhibitory activities, which resulted in no difference of ACE inhibitory activity between MRP and GCBPH.

3.4. Amino Acid Composition. The selectivity and specificity of flavourzyme to hydrolyze the fish bone protein resulted in a considerable amount of peptide and amino acids production. Amino acids were highly correlated with taste characteristics in foods peptides and related to some mechanisms of functionality, such as umami taste or umami-enhancing properties [23, 24]. The flavour peptides were a group of peptides related to the feelings of taste and smell, which would be of varying structures and length and possess unique taste properties [25]. In order to evaluate the flavour compounds of different treatment methods on the fish bone, the amino acid compositions of GCBPH, TDP, and MRP were analyzed.

The results of total amino acid and free amino acid are shown in Table 2. In GCBPH, the contents of TAA and FAA were 566.15 and 210.32 mg/g, respectively, which suggested that the ratio of FAA to TAA was 37.15%, of which the umami-sweet taste amino acids much lower than individual thresholds expected for Glu, Asp, and Arg accounted for 35.47% of FAA. In TDP, the total amino acid content (505.33 mg/g) was lower than that of GCBPH, which might result from the generation of new compound during thermal treatment. However, the FAA content and its ratio to TAA and the ratio of umami-sweet taste amino acids to FAA did not change significantly \((p > 0.05)\). This might be attributed to the crack of some of the amino acids existent in peptide into free amino acid during the high-temperature treatment. In MRP, the FAA proportion of TAA slumped to 31.82% after the Maillard reaction of hydrolysate, which proved that more FAA engaged in the Maillard reaction, compared to the amino acids present in the peptide form. Among all the FAA, Lys (9.51 mg/g), Arg (11.49 mg/g), and Leu (14.25 mg/g) presented dominant position in Maillard reaction, which decreased by 15.14, 6.16, and 13.94 mg/mL compared to that of GCBPH, respectively. This indicated that Lys, Arg, and Leu of FAA probably were mainly free amino acids involved in the Maillard reaction. In addition, umami-sweet taste amino acids of FAA were of 41.32%, which increased significantly \((p < 0.05)\) compared to those of GCBPH. These results showed that simple heating hydrolysis did not affect the ratios of FAA and umami-sweet taste amino acids. The decreased ratio of FAA to TAA and increased ratio of umami-sweet taste amino acids to FAA were ascribed to the Maillard reaction rather than the heat treatment during reaction processing.

3.5. Volatile Compounds. The flavourzyme hydrolysis is valuable due to the large number of natural fragrances and volatile compounds produced in the hydrolysis. The role of flavourzyme is to increase the concentrations of most of the volatile compounds that are predominantly chemical families in the hydrolysate [26]. Also, Maillard reaction can modify the odour of protein hydrolysate [14]. Accordingly, the volatile compounds of GCBPH, TDP, and MRP were detected and quantified using the DHS-GC-MS technique.

Table 3 declares the total detected individual volatile compounds with 37 flavour compounds of GCBPH, which were categorized into seven groups, namely, aldehydes, alcohol, ketone, hydrocarbon, furans, phenol, and esters, where each was composed of 9, 8, 3, 5, 4, and 3 individuals, respectively. Aldehydes, indicated as final oxidation products of ω-3 fatty acids for seafood and marine raw materials, were important aroma compounds in food stuffs [27]. Results illustrated that pentanal, hexanal, heptanal, and 2-hexenal with low threshold values were the major volatiles in hydrolysate which mainly associated with tallow aroma. Moreover, hexanal was the aldehyde with the highest relative content of 5.21%, which could be resulting from a degradation process of preformed volatiles, such as 2-octenal [28]. The 2-Methyl-butanal and 3-Methyl-butanal, representing 1.56% and 2.68% of the total volatile compounds, have been
Figure 3: Response surface plots (a, c, and e) and contour plots (b, d, and f) for the interactive effects of initial pH ($x_1$), temperature ($x_2$), and time ($x_3$) on the DG of the Maillard reaction.
identified as spoilage index. Benzaldehyde, with a relative content of 1.26%, characterized by sweet and oily flavour, was reported in meat, bone meal, and sukiyaki flavour [29]. In alcohol, the 1-Octen-3-ol, identified and characterized as lipid oxidation product in fish oil, was an important flavour contributor due to its low odour threshold [28, 30]. So far, 1-Octen-3-ol as a predominant alcohol of hydrolysate (with relative amount of 4.10%) revealed a pungent, soil, and fruit odour. It has been mentioned as the most distinct volatile characterizing fresh sardine and other fatty fish aromas [31]. Compared to short straight chain alcohols, long straight chain alcohols like 1-pentanol, 1-hexanol, and heptanol, as well as branched-chain alcohols like 1-Penten-3-ol and (Z)-2-Octen-1-ol, contributed aroma to the products with relatively lower threshold value [32]. The 2,4-Di-tert-butylphenol was found to be the most abundant volatile (17.10%) in the hydrolysate, which revealed a fermented sausage odour and contributed to antibiofilm activity [33].

| AA     | Taste threshold | TAA (mg/g) | FAA (mg/g) |
|--------|-----------------|------------|------------|
|        | GCBPH           | TDP        | MRP        | GCBPH | TDP | MRP |
| Pro    | 300             | 87.65      | 84.18      | 68.17 | 6.38 | 5.95 | 2.87 |
| Trp    | —               | 67.58      | 58.5       | 37.65 | 11.77 | 14.37 | 4.94 |
| Leu    | 90              | 38.35      | 32.94      | 22.65 | 28.19 | 24.28 | 14.25 |
| Val    | 150             | 17.97      | 11.7       | 8.55  | 12.04 | 6.63  | 5.62 |
| lle    | 380             | 17.86      | 11.88      | 6.2   | 11.65 | 8.8   | 6.25 |
| Phe    | 150             | 18.52      | 17.23      | 13.16 | 13.54 | 14.13 | 6.38 |
| Met    | 30              | 14.87      | 12.75      | 9.57  | 9.05  | 9.48  | 6.58 |
| His    | 20              | 15.69      | 14.78      | 12.49 | 3.85  | 4.5   | 2.17 |
| Glu    | 5               | 64.51      | 59.45      | 33.84 | 12.18 | 13.12 | 5.85 |
| Asp    | 3               | 41.69      | 39.37      | 24.63 | 13.24 | 12.45 | 8.57 |
| Arg    | 10              | 34.16      | 28.38      | 21.29 | 17.65 | 14.83 | 11.49 |
| Ala    | 60              | 23.97      | 23.64      | 12.47 | 9.85  | 9.39  | 5.57 |
| Gly    | 110             | 28.48      | 24.17      | 19.5  | 5.83  | 6.22  | 3.45 |
| Thr    | 260             | 17.41      | 16.23      | 9.34  | 9.85  | 8.73  | 5.21 |
| Ser    | 150             | 9.2        | 8.64       | 5.62  | 6.01  | 6.08  | 4.87 |
| Cys    | —               | 8.74       | 5.35       | 2.65  | 2.85  | 2.57  | 1.25 |
| Lys    | 50              | 42.85      | 40.79      | 24.69 | 24.65 | 23.62 | 9.51 |
| Tyr    | —               | 16.65      | 15.35      | 9.49  | 11.74 | 11.46 | 4.02 |
| Total  | 566.15          | 505.33     | 341.96     | 210.32| 196.61| 108.8 |
| FAA ratio |            | 37.15      | 38.91      | 31.82 | 35.47 | 36.02 | 41.32 |
| USAA  | 219.42          | 199.88     | 126.69     | 74.61 | 70.82 | 44.96 |
| USAA ratio |          | 38.76      | 39.55      | 37.05 | 35.47 | 36.02 | 41.32 |

TAA represents total amino acids; FAA represents free amino acids; USAA represents umami-sweet taste amino acids, including Glu, Asp, Arg, Ala, Gly, Thr, Ser.
| Compound | Rta | Percentage of total area (%) |
|----------|-----|-----------------------------|
|          |     | GCBPH | TDP   | MRP   |
| **Aldehydes** |     |       |       |       |
| 1        | 2-Methyl-propanal | 6.91  | 0.27  |       |
| 2        | Butanal          | 7.92  | 0.14  |       |
| 3        | 2-Methyl-butanal | 8.69  | 1.56  | 2.12  |
| 4        | 3-Methyl-butanal | 8.69  | 2.68  | 3.21  |
| 5        | Pentanal         | 10.21 | 1.68  | 1.44  |
| 6        | 2-Butenal        | 11.81 | 0.18  | 0.21  |
| 7        | Hexanal          | 12.71 | 5.21  | 7.84  |
| 8        | (E)-2-Pentenal   | 13.13 | 0.19  |       |
| 9        | Heptanal         | 14.96 | 0.33  | 0.33  |
| 10       | 2-Hexenal        | 15.8  | 1.05  | 0.86  |
| 11       | (Z)-2-Heptanal   | 17.77 | 2.27  | 2.41  |
| 12       | 2-Methyl-2-heptenal | 18.27 | 0.43  |       |
| 13       | Nonanal          | 18.82 | 0.75  | 1.08  |
| 14       | (E, E)-2,4-Hexadienal | 19.08 | 0.21  |       |
| 15       | (E, E)-2,4-Heptadienal | 19.95 | 1.95  |       |
| 16       | 3-(Methylthio)-propanal | 19.84 | 0.57  | 1.23  |
| 17       | Furfural         | 19.89 | 0.75  | 0.42  |
| 18       | Benzaldehyde     | 20.91 | 1.26  | 1.71  |
| 19       | (E,Z)-2,6-Nonadienal | 21.64 | 0.83  |       |
| 20       | (E,E)-2,4-Nonadienal | 23.11 | 0.59  |       |
| 21       | 4-Ethyl-benzaldehyde | 23.3  | 0.41  | 0.48  |
| 22       | (E,E)-2,4-Decadienal | 24.41 | 1.85  |       |
| 23       | α-Ethylidene-benzenecetaldehyde | 26.08 | 0.42  |       |
|          | Subtotal         | 14.87 | 22.46 | 26.04 |
| **Alcohol** |     |       |       |       |
| 24       | (E)-2-Pentenol   | 13.92 | 1.22  | 0.10  |
| 25       | 1-Penten-3-ol    | 14.55 | 3.25  | 2.89  |
| 26       | 1-Pentanol       | 16.31 | 2.70  | 3.04  |
| 27       | 1-Hexanol        | 18.05 | 2.45  | 2.24  |
| 28       | 1-Octen-3-ol     | 19.46 | 4.10  | 4.43  |
| 29       | Heptanol         | 19.59 | 2.11  | 1.71  |
| 30       | 2-Ethyl-1-hexanol| 20.06 | 0.69  | 1.06  |
| 31       | 1-Octanol        | 20.99 | 0.88  |       |
| 32       | (Z)-2-Octen-1-ol | 21.75 | 0.83  | 1.29  |
| 33       | 6-Undecanol      | 22.4  | 2.45  |       |
| 34       | Benzyl alcohol   | 25.12 | 0.39  |       |
|          | Subtotal         | 17.35 | 16.77 | 16.70 |
| **Ketone** |     |       |       |       |
| 35       | 2-Heptanone      | 15.04 |       | 1.09  |
| 36       | 3-Octen-2-one    | 19.12 | 0.89  |       |
| 37       | 2-Ethylidene-cyclohexaneone | 19.37 | 1.44  |       |
| 38       | 3,5-Octadien-2-one | 21.46 | 7.67  | 4.88  |
| 39       | 1H-Inden-1-one   | 24.4  | 0.73  |       |
| 40       | 2-Ethyl-cyclopentanone | 28.47 | 1.56  | 1.95  |
|          | Subtotal         | 10.00 | 6.44  | 5.29  |
| **Hydrocarbon** |     |       |       |       |
| 41       | Hexamethyl-cycloctasiloxane | 7.33  | 0.37  | 2.72  |
| 42       | Octamethyl-cyclotetrasiloxane | 10.07 | 8.64  | 7.17  |
| 43       | p-Xylene        | 14.05 |       | 0.77  |
| 44       | D-Limonene      | 15.09 |       | 0.46  |
| 45       | 1,3-Cyclooctadiene | 18.69 | 0.34  |       |
| 46       | 1,3,6-Octatriene | 22.6  | 5.80  | 2.71  |
| 47       | Hexamethyl-cycloctasiloxane | 23.42 | 0.34  |       |
| 48       | 2,6-Dimethyl-2,4,6-octatriene | 24.53 |       | 1.67  |
|          | Subtotal         | 15.48 | 12.60 | 10.23 |
| **Furans** |     |       |       |       |
| 49       | 2-Ethyl-furan    | 9.53  | 3.87  | 3.34  |
| 50       |                 |       | 2.69  |       |
providing green bean and butter flavours, could be generated from sugar caramelization and carbohydrate degradation [28] and had been identified in fish oil and fish sauce [30, 34]. Esters were identified in contributing to pleasant seafood aromas in general [31].

During the thermal degradation process, the flavour compounds of sample were 40 kinds, with eleven new substances added in aldehydes, pyrazine, and other categories, and eight substances disappeared in ketone, hydrocarbon, furans, and phenol. In addition to taste-eliciting compounds such as peptides and amino acids, aroma compounds were generated by Maillard browning reaction, Strecker degradation, and so forth [35]. The volatile compounds identified in the MRP with 62 kinds are presented in Table 3, which included 22 aldehydes, 10 alcohol, 4 ketone, 5 hydrocarbon, 5 furans, 3 phenol, 3 esters, 5 pyrazine, and 5 others. The volatile aldehydes class contributed the major volatiles in the MRP, which were of relative higher amounts 26.04%, which were much higher than those of GCBPH. Straight and branched-chain aldehydes generally provided herbaceous, grassy, and pungent aromas, while unsaturated aldehydes were linked with vegetal and fishy notes and each of these compounds possesses its own specific odours. For instance, 2-Methyl-Propanal (value of 0.27%) gave a pungent smell and fishy and grassy aroma was provided by hexanal (value of 6.05%). Moreover, 2-Methyl-butanal (value of 1.86%) and 3-Methyl-butanal (value of 2.25%) produced nutty odours. Furthermore, unsaturated n-2-alkenals, such as (Z)-2-Heptenal, (E, E)-2,4-Heptadienal, and (E,E)-2,4-Decadienal, might play a more important role in the formation of species-specific flavour [36].

The volatile ketones were most likely the products of lipid and/or amino acid degradation. Only four ketone compounds were detected in the MRP samples, which may contribute to the cheesy odour of MRP with relative lower total amount. All of these factors contributed to the overall aroma of the MRP. The pyrazines, furans, and esters were the most desirable compounds in food products to enhance the aroma of the products. Compared with volatile components in GCBPH, the compounds in MRP increased from 8 to 13 kinds. The

| Compound | Rta | Percentage of total area (%) |
|----------|-----|----------------------------|
|          |     | GCBPH  | TDP  | MRP  |
| 2-Pentyl-furan                     | 15.88 | 3.43   | 2.97 | 1.91 |
| (E)-2-(1-Pentenyl)-furan           | 17.04 | 1.76   |
| cis-2-(2-Pentenyl)furan            | 17.18 | 4.20   | 3.39 | 1.64 |
| 2-Methyl-furan                     | 20.16 |       | 0.98 |
| (E)-2-(1-Pentenyl)-furan           | 25.51 | 0.65   | 1.59 | 4.99 |
| Subtotal                            | 13.90 | 11.29  | 12.20 |
| 4-Ethyl-phenol                     | 15.53 | 0.49   |
| 2-Ethyl-phenol                     | 15.65 | 1.04   |
| 4-(1-Methylpropyl)-phenol          | 25.03 | 1.54   |
| 2,6-Bis(1,1-Dimethylethyl)-4-(1-oxopropyl)phenol | 25.43 | 1.85 | 0.34 |
| 2,4-Di-tert-butylphenol            | 32.37 | 17.10  | 9.74 | 1.96 |
| Subtotal                            | 20.98 | 9.74   | 3.34 |
| Pentyl ester                       | 20    | 1.05   | 1.11 |
| Diocetyl phthalate                 | 20.99 | 2.64   | 1.79 |
| Benzyl benzoate                    | 24.89 | 3.25   | 2.86 |
| Bis(2-Ethylhexyl)ester             | 27.27 |       | 0.61 |
| Butylisohexylester                 | 29.28 | 1.71   | 2.29 |
| Methyl palmitate                   | 30.44 | 1.52   | 0.56 |
| Subtotal                            | 7.41  | 7.97   | 4.01 |
| Methyl-pyrazine                    | 16.83 | 5.33   | 4.12 |
| 2,6-Dimethyl-pyrazine              | 17.86 |       | 0.93 |
| 3-Ethyl-2,5-Dimethyl-pyrazine      | 19.75 | 1.85   |
| 2,5-Dimethyl-3-Propyl-pyrazine     | 20.7  |       | 0.87 |
| 2-Butyl-3,5-Dimethyl-pyrazine      | 22.53 |       | 2.87 |
| Subtotal                            | 5.33  | 10.63  |
| Butanamide                          | 13.52 |       | 3.22 |
| 1,3-Diazine                         | 15.72 |       | 0.81 |
| 2-Chloro-4-(4-Methoxyphenyl)-6-(4-Nitrophenyl)pyrimidine | 16.56 | 4.73 | 3.32 |
| 4-Methylthiazole                    | 22.26 | 1.31   | 1.87 |
| 2-Methylpiperidine                  | 28.47 | 1.36   | 2.35 |
| Subtotal                            | 7.40  | 11.56  |
| Total                               | 100   | 100    | 100  |
relative content of pyrazine in the volatile components was 10.63%. Pyrazine, due to its large size and low taste threshold, was considered to be a significant volatile flavour in many heat-processed products and gave a pleasant nutty and baking flavour. The 4-Methylthiazole in the sulfur-containing compound accounted for 1.87% of the total amount, which was regarded as a perfume intermediate that might come into being during the process of Maillard reaction consisting of amino acids and sugars or compounds containing carbonyl groups. Compared to that of GCBPH and TDP, MRP contained more kinds of flavour volatile compounds of aldehydes, alcohol, ketone, pyrazine, and others. Maillard reaction was then indicated to be capable of perfecting the flavour and aroma of the hydrolysate from grass carp protein.

4. Discussion

In this paper, the fish bone of common species of fish, grass carp, was used as a sample to investigate its high value utilization using different methods. The preparation of bioactive peptide and flavour compounds by enzymatic method was an effective method to make full use of protein resources. However, it still has a relatively low biological activity and low content of flavour compounds. Many researches showed that Maillard reaction not only can increase the bioactivities but also can improve the flavour of protein hydrolysate [15, 22]. As a consequence, the Maillard reaction of hydrolysate from grass carp bone protein was used for preparation of bioactive compounds and identification of characteristic flavour compounds using thermal degradation method as the positive control. Compared with the thermal degradation methods, Maillard reaction probably generated more compounds and substances with higher DPPH radical scavenging activity and ACE inhibitory activities. However, thermal degradation methods significantly reduced the ACE inhibitory activity of protein hydrolysate in contrast to those of GCBPH and MRP. The reason might be that thermal treatment degraded some of the active proteins or peptides into inactive fragment.

The flavour and taste quality of foods are the most important factors affecting consumers’ choice and acceptance. In terms of aquatic products, the flavour, as the main factor, determines its quality to a great extent. These flavours include not only the soft and pleasant smell but also the unpleasant odour. In the products related to protease hydrolysis, some peptides with sensory properties, such as sweet peptides, bitter peptides, acid peptides, salty peptides, and flavour-enhancing peptides, play a vital role in improving sensory characteristics and flavour enhancement of hydrolysates. Therefore, the proportion of umami-sweet taste amino acids in free amino acids directly affects the flavour and quality of hydrolysates. In this experiment, Maillard reaction can lead to the increase of the umami-sweet taste amino acids ratio in free amino acids and improve the flavour by increasing the volatile compounds. It indicated that Maillard reaction was an effective way to process fish bone, which provided a new direction for the application of fish bone.

In conclusion, compared to grass carp bone protein hydrolysate and its thermal degradation products, Maillard reaction significantly increased the DPPH radical scavenging activity of hydrolysate and the proportion and variety of umami-sweet taste amino acid in total free amino acids of grass carp protein hydrolysate. These findings suggested that fish bone protein hydrolysate could be effectively prepared by enzymolysis and Maillard reaction could improve its utilization by increasing antioxidant activity and flavours.

Data Availability

The data supporting the results of this study are available within the paper or are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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