The dual functions of flavor and antioxidant potential of porcine bone marrow extract (PBME)

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Abstract To improve and confirm the dual functions of flavor and antioxidant potential of porcine bone marrow extract (PBME). Response surface methodology and Box-Behnken design was employed to optimize the conditions for enzymatic hydrolysis of PBME. The optimal hydrolysis conditions were: hydrolysis time, 3h; temperature, 55 °C; substrate concentration, 375g/L; and amount of enzyme, 0.4%. $L_{16}(3^5)$ orthogonal experimental was utilized to obtain the optimal Maillard reaction conditions for PBME and enzymatic hydrolysate of PBME (EH-PBME). The optimal conditions for PBME were: components, 4% glucose, 2% xylose, 1.5% Tyr, 1.5% Ala, and 4% VB₁; reaction time, 40 min; and reaction temperature, 115 °C. The optimal conditions for EH-PBME were: components, 2% glucose, 2% xylose, 3% Ala, and 5% VB₁; reaction time, 40 min; temperature, 110 °C. The antioxidant activities for PBME, EH-PBME, Maillard reaction products of PBME (MPRs A) and Maillard reaction products of EH-PBME (MPRs B) were 50%, 86%, 84% and 41% respectively. The content of taste-active substances and volatile compounds were also determined. Finally, PLSR was employed to evaluate the correlation between flavor compounds and sensory data.

Keywords Porcine bone marrow extract (PBME) · Enzymatic hydrolysis · Maillard reaction · Sensory evaluation · Antioxidant · Partial least squares regression (PLSR)

Introduction

Porcine bone marrow extract (PBME) is a pasty condiment extracted from porcine bone; its main ingredient is bone collagen (structural protein), which is a helical molecular structure consisting of three polypeptide chains, peptides and amino acids (Gao et al. 2018; Mohammadkhah et al. 2017; Oechsle et al. 2016). It not only is enriched in umami substances, but also retains the natural odor of poultry meat and has high nutritional value (Zhao et al. 2010). PBME has been widely used in the production and processing of meat products and seasonings (Zhao et al. 2010; Tao 2011). According to the statistics reported by the National Bureau of Statistics of China, the domestic porcine production was increased by 0.8% in the fiscal year of 2017 (National Bureau of Statistics of China. 2018). The global demand for foods with high protein content has also been increased (Mullen et al. 2017). Porcine, bovine and chicken bones are by-products; their uses can add value to the products, thus can prevent the production of waste while promote in the development of healthy pork products (Ryder et al. 2016).

Liu and his team showed that peptides with molecular weights of 400–2000Da (4–16 amino acids) had strong antioxidant activity (Liu et al. 2016). Other study also revealed that the quality of free amino acids, taste nucleotides and minerals in PBH could be improved through the enzymatic hydrolysis method (Yang et al. 2007). Deer bone collagen was prepared, and its properties and antioxidant activities were studied. Peptides with

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molecular weights of >5kDa, 1–5kDa and <1kDa had strong antioxidant activity (Yu 2016). By analyzing the molecular distribution of peptide, it was concluded that the peptide can be thermally degraded upon heating, and both thermal degradation and polymerization of peptide coexist during the Maillard reaction. In general, peptides with smaller molecular weights lead to higher Maillard reaction activity, and as a result, the antioxidant activities of Maillard Reaction Products (MRPs) are increased (Su 2012; Cui et al. 2018). Recently, a study was conducted to investigate the enhancement of structural, functional and antioxidant properties of fish gelatin films by using Maillard reaction (Kchaou et al. 2018; Lafarga et al. 2016).

Partial least squares regression (PLSR) provides a regression modeling method for multiple dependent variables, and effectively solves the problems of multiple correlations between these variables. It is suitable for a regression model where the sample size is less than the number of variables. The method has been used in various data analyses, according to several scientific studies (Ergon 2014; Wang 1999). The combination of front-face fluorescence and PLSR provides a fast and non-invasive procedure for the quantification of theobromine and caffeine in dark chocolate, as an effective alternative for the traditional HPLC method (Tan et al. 2019). PLSR was used to get the relationship between peptides, amino acids and aroma-active compounds (Alim et al. 2018). PLSR models were calibrated and externally tested. Meanwhile, these models based on FTIR fingerprints of fish skin exhibited a good performance (Fengou et al. 2019).

Researchers worldwide have independently studied collagen flavors and antioxidant properties of various poultry, livestock and aquatic products. However, to date, a comprehensive research studying the attribution of flavors and antioxidant properties has not been conducted (Chen et al. 2016; Sun et al. 2015; Tan et al. 2018). Thus, this study aims at investigating umami taste-active compounds and antioxidant activities of different samples (PBME, EH-PBME, and MRPs of PBME and EH-PBME). The relationship between amino acid content, peptide distribution, nucleotide content and volatile compounds were analyzed by PLSR.

Materials and methods

Chemicals and reagents

Porcine bone marrow extract (PBME) was obtained from Fushun Dufengxuan Bone God Biotechnology (Fushun, Liaoning, China). ProtamexTM and FlavourzymeTM were purchased from Novozymes Biotechnology (Tianjin, China). 2,2-Diphenyl-1-picrylhydrazyl was purchased from

Alfa Aesar (Shanghai, China). Amino acids mixture standard reagent, o-phthalaldehyde (OPA), 9-fluorene methyl chloroformate (FMOC) and borate buffer solution were purchased from Agilent Technology (California, America). Papain PSM500 was purchased from Belgian Enzyme Products Company. (Brussels, Belgium) Alkaline protease was purchased from China Pharmaceutical Foreign Trade Corporation (Beijing, China). Trypsin, neutral protease and pepsin were purchased from Shanghai Yuanye Biological Technology (Shanghai, China). Potassium ferricyanide, trichloroacetic acid and ferric chloride were purchased from Shanghai Maclean Biochemical Technology (Shanghai, China). 2-methyl-3-heptanone (GR), n-alkanes (C7–C30) (GR) and n-hexane (GR) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Sensory evaluation with choosing of enzyme

As shown in Table 1(a), the sensory evaluation panel consisting of five males and five females with ages between 20 and 45 years old were trained on sensory evaluation for three months as said by the reference definition (Pepe et al. 2016; Skelton et al. 2010). The contribution of each taste was scored as follows: a total of 10 points was given when sour taste score was -0.2, meat flavor score was 0.3, bitterness score was -0.2, salty taste score was 0.2, umami taste score was 0.6, and kokumi taste score was 0.3 (Kang and Song 2017). Sensory evaluation of seven different enzyme and mixed enzyme hydrolysates was conducted with taste score as an index. Then, the best enzyme was chosen for next steps. In addition, the sensory panel were provided with PBME, EH-PBME, MRPs A and MRPs B for sensory analysis.

The optimization of enzymatic hydrolysis of PBME by response surface method

Response surface method (RSM) seeks the optimal process parameters by analyzing the contour of the response surface, and uses the multivariate two regression equation to fit a statistical method of the function relationship between the factor and the response value (Dou et al. 2007). Central composite design (CCD) and Box-Behnken design (BBD) are the most common design methods. For a start, CCD is suitable for multi-factor and multi-level experiments and has continuous variables. However, BBD is suitable for less factor levels (generally less than 5 and level 3). Meanwhile, three methods were used in data analysis. Residual analysis of correlation, fit and reliability; variance analysis of its rationality, relevance and credibility and significant analysis of interaction. For data processing, if the residual value fits well with real value, conforms to the normal distribution, so the model constructed is better. In

Table 1 Sensory evaluation training standard and design of optimized experiment

Sensory evaluation		Reference definition				
(a)						
Taste						
Sour		20 mmol/L Lactic acid				
Bitter		1 mmol/L Caffeine				
Salty		20 mmol/L NaCl				
Umami		10 mmol/L Sodium glutamate				
Kokumi		10 mmol/L Glutathione solution				
Odor						
Meat		Boiled meat aroma, consistent with 2-methyl-3-furanthiol				
Roast		Dilute the 2, 5-dimethylpyrazine standard with water 100 times				
Burnt		Dilute furfuryl alcohol standard with water for 100 times				
Fishy		Boil fish with 10 mmol/L NaCl for 2h to prepare fish soup solution				
Caramel		Dilute furfural alcohol standard with water for 100 times				
Enzymatic hydrolysis time (h)		Enzymatic hydrolysis temperature (°C)			Substrate concentration (g/L)	
(b)						
1	2.00	55.00			500.00	
2	4.00	55.00			500.00	
3	4.00	50.00			375.00	
4	2.00	60.00			375.00	
5	3.00	55.00			375.00	
6	3.00	55.00			375.00	
7	4.00	60.00			375.00	
8	4.00	55.00			250.00	
9	3.00	55.00			375.00	
10	3.00	60.00			250.00	
11	3.00	55.00			375.00	
12	2.00	55.00			250.00	
13	2.00	50.00			375.00	
14	3.00	50.00			250.00	
15	3.00	60.00			500.00	
16	3.00	55.00			375.00	
17	3.00	50.00			500.00	
Sample	Number	A reducing sugar addition (%)	B amino acid addition (%)	C VB ₁ addition (%)	D reaction time (min)	E temperature (°C)
(c)						
PBME	1	3	1	2	20	105
	2	4	2	3	30	110
	3	5	3	4	40	115
EH-PBME	1	3	1	2	20	100
	2	4	2	3	30	105
	3	5	3	4	40	110

Samples are porcine bone marrow extract (PBME), enzymatic hydrolysate of PBME (EH-PBME), Maillard reaction products of PBME (MRPs A) and Maillard reaction products of EH-PBME (MRPs B) (a) Sensory evaluation form and evaluation training standard; (b) Response surface experiment of PBME; (c) L16 (3⁵) Orthogonal experiments

addition, the higher determination coefficient of RSM, the better the model fits. (Wang et al. 2018). RSM was programmed based on single factor variables and Box-Behnken design, which include duration of enzymatic hydrolysis, enzymatic hydrolysis temperature and substrate concentration. The response value was calculated based on the taste score of 50%, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) clearance of 40% and the degree of hydrolysis of 10% (Kang and Song 2017; Wong et al. 2017). After the optimal conditions for the enzyme hydrolase were obtained, subsequent validation experiments were carried out. The sequence of experiments generated based on the system is listed in Table 1(b).

Calculation of degree of hydrolysis (DH)

Whereas the FlavourzymeTM is an exopeptidase, other six enzymes (pepsin, neutral protease, alkaline protease, papain PSM500, ProtamexTM and trypsin) are endopeptidases. The degree of hydrolysis (DH) was calculated as follows when different enzymes are at the optimum pH value. Due to the amphoteric property of amino acids, formaldehyde was added to fix the basicity of amino group; the mixture was then titrated with sodium hydroxide standard solution. The amino acid content (AAN) was calculated by indirectly monitoring and controlling the titration endpoints, indicated by the pH value measured using a pH meter (Hakalin et al. 2018).

$$\text{AAN(g/mL)} = \frac{(V_1 - V_2) * C * 0.014}{\frac{5}{100} * 20} * 100$$

Where V_1 is the volume (mL) of NaOH standard solution consumed during the titration of sample to the end-point (pH 9.2), V_2 is the volume (mL) of NaOH standard solution consumed after the titration of blank sample to the end point, C is the concentration of NaOH standard solution, which equals to 0.05 mol/L, and 0.014 is the molar mass of nitrogen (mg/mol).

The nitrogen content was calculated by Kjeldahl method using the following equation:

$$\text{DH (\%)} = \frac{\text{AAN}}{\text{Nitrogen content before enzymatic hydrolysis}} * 100\%$$

Determination of *in vitro* antioxidant activity

The antioxidant activity of the samples was determined based on the DPPH clearance method. Ten milliliters of samples were mixed with 6 mL of 50 µg/mL DPPH solution in a 10-mL centrifuge tube for 1 minute. The reaction was carried out in the dark for 30 min. The ultraviolet spectrophotometer was turned on for at least 15 min, and

the wavelength was adjusted to 517 nm. The samples were measured at this wavelength (Bursal and Köksal 2011), and the DPPH clearance [the higher the clearance rate, the lower the absorbance] was calculated by the following formula:

$$\text{DPPH clearance} = \frac{A_0 - (A_i - A_j)}{A_0} * 100\%$$

Where A_i is the wavelength of sample and reagent, A_0 is the wavelength of reagent, and A_j is the wavelength of sample.

The optimization of Maillard reaction by Orthogonal experiment

Orthogonal experiment was designed and programmed to optimize the conditions of Maillard reaction of different samples (PBME, EH-PBME), as shown in Table 1(c) (Zhou et al. 2015). The orthogonal experiment was designed according to the previously selected single factor experiment. The five selected factors were A (reducing sugar addition), B (amino acid), C (vitamin B₁), D (Reaction time) and E (Reaction temperature). Whereas reducing sugars, glucose and xylose were added at a constant ratio of 1:1 into PBME and EH-PBME mixtures named as (a) and (b), respectively, the ratio of amino acid was varied. While both Tyr and Ala were present in (a) at a ratio of 1:1, only alanine was present in (b). The comprehensive score was calculated based on the following criteria: 50% for taste, 40% for DPPH clearance and 10 % for degree of browning.

Determination of degree of browning

An ultraviolet spectrophotometer was utilized to determine the degree of browning of the samples. The measurement was conducted at a wavelength of 420 nm, which is the maximum absorption peak for Maillard reaction products, according to previous studies (Xu 2017).

Analysis of free amino acid

Free amino acid analysis was carried out by reverse phase-high performance liquid chromatography (RP-HPLC, Agilent 1100) using a Zorbax Eclipse-AAA column (Agilent Technologies, Palo Alto, CA, USA). The amino acid standard mixture had a concentration of 1 nmol/µL, and a standard curve of 16 amino acids (Asp, Glu, Ser, His, Gly, Thr, Arg, Glu, Tyr, Cys, Val, Met, Phe, Ile, Leu, Lys) were diluted with multiples of 0.125, 0.25, 0.5, 0.75 and 1. The values were then multiplied with the dilution factor, given that 0.1 mol/L HCl was added. Samples were first treated with o-phthalaldehyde and 3-mercaptopropionic acid in borate buffer (OPA), and 9-fluorenylmethylchloroformate

in acetonitrile (FMO). Operating conditions were as follows: solution A, 0.04 mol/L NaH_2PO_4 (pH 7.8); solution B, methanol/acetonitrile/ H_2O (45/45/10, v/v/v); injection volume, 0.5 μL , column temperature, 40 $^\circ\text{C}$; and DAD detection wavelength, 338 nm.

Quantification of nucleotides

The quantification of nucleotides was performed on an Agilent 1100 RP-HPLC, equipped with a Zorbax Eclipse XDB-C18 column (4.6 mm $\times$ 250 mm, 5 μm) (Agilent Technologies, Palo Alto, CA, USA). All samples and solutions were filtered through a 0.22- μm nylon membrane filter. Other operating conditions were as follows: mobile phase, 0.02 mol/L KH_2PO_4 (pH 3.80); injection volume, 1 μL ; column temperature; 35 $^\circ\text{C}$; and DAD detection wavelength, 254 nm.

Analysis of peptide distribution

The peptide distribution was analyzed using an Agilent 1100 RP-HPLC, equipped with a TSK gel 2000 SW_{XL} column (300 mm $\times$ 7.8 mm) (Tosoh Co., Tokyo, Japan). The eluent consisted of 20% acetonitrile aqueous solution (pH 4.0) and 0.1% formic acid, and the column temperature and DAD detection wavelength were set at 30 $^\circ\text{C}$ and 220 nm, respectively.

Analysis and quantification of volatile compounds

The volatile compounds in the samples were isolated using head space-solid phase micro extraction (HS-SPME) coupled with gas chromatography-olfactometry-mass spectrometry (GC-O-MS). First, 10 g of samples and 1 μL of internal standard (2-methyl-3-heptanone, 0.816 $\mu\text{g}/\mu\text{L}$) were added into a 20-mL SPME vial, and was then placed in a water bath at 55 $^\circ\text{C}$ for 20 min. After that, an extraction fiber (DVB/CAR/PDMS) was inserted into the head space of the SPME vial to absorb the volatiles at 55 $^\circ\text{C}$ for 40 min. The SPME extraction fiber was then inserted into the front inlet of the GC injection system, at which the samples were thermally desorbed at 250 $^\circ\text{C}$ for 5 min. The identification and quantification of the volatile compounds were carried out by an Agilent GC-MS (7890A/7000B, Agilent Technologies, Palo Alto, CA, USA), which was equipped with an olfactory detector port (Sniffer 9000, Brechbühler, Schlieren, Switzerland) and a DB-5MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) (J&W Scientific, Folsom, CA, USA).

The oven temperature for GC-O-MS was maintained at 40 $^\circ\text{C}$ for 3 min, and was then raised to 200 $^\circ\text{C}$ at 5 $^\circ\text{C}/\text{min}$, followed by 230 $^\circ\text{C}$ at 10 $^\circ\text{C}/\text{min}$ for 3 min. Ultrahigh-purity helium was used as carrier gas at a flow rate of 1.2 mL/min. The conditions for the mass spectrometer were as follows: ion source, electron impact (EI); electron energy, 70 eV;

transmission line temperature, 280 $^\circ\text{C}$; quadrupole temperature, 150 $^\circ\text{C}$, ion source temperature, 230 $^\circ\text{C}$; and mass (m/z) scan range, 40–450 amu. The GC-O temperature was installed at 200 $^\circ\text{C}$. For the sniffing test of each group of samples were carried out by at least 5 sniffers, while that of each volatile compound was conducted by at least three times. Qualitative analysis of unknown volatile compounds was performed by three methods including: calculation of RI values, which was then compared with the odor compound retention index (RI) reported in the literature; mass spectral library search by NIST 14 software; and comparison of sniffing data. The RI value of volatile odor compound was calculated by the following formula:

$$\text{RI} = 100N + 100n \frac{t_{Ra} - t_{RN}}{t_{R(N+n)} - t_{RN}}$$

Where N is the number of carbon atoms of (a) for the normal paraffin on the left side of the odor compound to be tested; n is the number of carbon atoms between the two alkanes on the other side (n=1); t_R is the retention time of the corresponding compound.

Internal standard method was employed in semi-quantification. The concentration of each compound was calculated by the following formula:

$$C_i = \frac{C_{is} * A_i}{A_{is}}$$

Where C_i is the concentration of the tested compound ($\mu\text{g}/\mu\text{L}$), C_{is} is the concentration of the internal standard, A_i is the peak area of an unknown compound, and A_{is} is the peak area of the internal standard.

Statistical analysis

Numerous statistical tools, including SPSS 17.0 (IBM, New York, NY, USA) for the analysis of variance (ANOVA) with Duncan's multi-range test and Origin 8.0 (Northampton, MA, USA), were employed to verify the experimental data. PLSR is a method for multivariate statistical data analysis and is a more effective when the variables have high linearity, compared to the principal component (PC) regression. Correlations between sensory evaluation, amino acid content, molecular weight distribution of the peptides and antioxidant activities were analyzed by PLSR using SIMCA-P+11 (Umea, Sweden).

Results and discussion

Choice of enzyme

Previous studies have demonstrated that using exopeptidases to digest the samples can dramatically increase

Fig. 1 Taste-weighted scores of 8 species different enzymatic hydrolysates and PBME. Sample names are as follows, F, PE, N, A, PS, PT, T, EH-PBME respectively means FlavourzymeTM, pepsin, neutral protease, alkaline protease, papain (PSM500), ProtamexTM, Trypsin and FlavourzymeTM mixing papain hydrolysates. Results with different letters (a–g) were significantly different in content

umami taste while reduce bitterness (Pepe et al. 2016). As shown in Fig. 1, the samples denoted as F, N, A, PS, PT and T had taste scores of higher than 5.5 but lower than 7. Taste scores of all enzymatic hydrolysates other than PE were higher than that of PBME, which had a score of 2. The hydrolysate resulted from enzymatic a single enzyme digestion by FlavourzymeTM had higher taste of 6.8. Among all samples, EH-PBME had the best taste with a score of 7.2. The taste of amino acids is linked with the hydrophobicity of its side chain (R group). When amino acids have side chains with low hydrophobicity, such as glycine and alanine, they contribute mainly to sweet taste (Cheung et al. 2015). Moreover, the exopeptidase FlavourzymeTM can also reduce the bitter taste in N. To achieve hydrolysates with the highest taste scores, the two proteases (exo- and endo-protease) at the ratio of 1:1 were selected to hydrolyze PBME.

Optimal conditions obtained from response surface methodology

As shown in Fig. 2 and Table 2(a), the linear effects of B (enzymatic hydrolysis temperature) and C (substrate concentration) on the comprehensive scores were significant ($P < 0.05$). By contrast, the linear effect of A (enzymatic hydrolysis time) on the comprehensive score was not significant ($P > 0.05$). The effects of AC, BC, A^2 , B^2 and C^2 were significant with a p-value of 0.0009, which is considered as extreme significance ($P < 0.001$). The p-value for the out-of-fitting item was 0.0859, which was not significant, indicating that the model can suitably reflect the

relationship between various factors and the comprehensive score.

Comprehensive scores = $-856.4 - 14.4A + 30.6B + 0.75C + 0.54AB + 0.03AC - 6.94BC - 4.8A^2 - 0.27B^2 - 6.68C^2$. Correlation coefficient (R^2) = 0.9499.

Based on the analysis of regression model and the evaluation of comprehensive score, the optimal enzymatic hydrolysis conditions were as follows: reaction temperature and time, 55 °C and 3 h; substrate concentration, 375 g/L; and percentage of enzyme, 0.4%. The comprehensive score obtained by the confirmatory experiment carried out at these conditions was 77, which is consistent with the theoretical value. These data demonstrate that the optimization by the model is reliable and feasible, thus could have high practical values.

Optimal conditions for Maillard reaction obtained from orthogonal experiment

As listed in Table 2(b)(c), the five factors had no significant effects on the results, and the influence of each factor on the comprehensive score could be ranked as follows: $D > E > C > B > A$. The optimal conditions achieved from the orthogonal experiment carried out at 115 °C for 40 min were $A_2B_3C_2D_3E_3$ (i.e., 4% glucose, 2% xylose, 1.5% Tyr, 1.5% Ala, 4% vitamin B₁). This result was confirmed by the validation experiment, and MPRs A was found to be the best product. MPRs A had a strong umami with slight salty taste; its overall taste score was higher than the overall taste score of PBME. Additionally, its DPPH clearance was as high as 0.84.

As shown in Table 2, as indicated by (d) and (e), the L_{16} (3^5) orthogonal experimental range and the variance analysis showed that the effect of reaction temperature on the comprehensive score was significant ($P < 0.05$), while those of other four factors were not significant ($P > 0.05$). The influence of each factor on the comprehensive score could be ranked as follows: $E > C > A > B > D$. The optimal conditions obtained from the orthogonal experiment conducted at 110 °C for 40 min was $A_2B_3C_3D_3E_3$ or 2% glucose, 2% xylose, 3% Ala, 5% vitamin B₁. This result was finally confirmed by the validation experiment, and MPRs B was found to be the best product. It had strong overall umami, bitter and salty tastes with the values higher than the PBME values; however, it had elevated DPPH clearance value of 0.41.

Change of taste and DPPH clearance

The optimal formula and two indexes of reaction samples are shown in Table 3(a). The taste scores of the samples could be ranked as follows: EH-PBME > MPRs A > MPRs B > PBME. Their DPPH clearance could be ranked as

Fig. 2 Response surface and contour plots of **a, b** enzymatic hydrolysis time (min) and enzymatic hydrolysis temperature ($^{\circ}\text{C}$); **c, d** enzymatic hydrolysis time (min) and substrate concentration (g/L); **e, f** enzymatic hydrolysis temperature ($^{\circ}\text{C}$) and substrate concentration (g/L)

follows: EH-PBME>MRPs A >PBME>MRPs B. These data indicate that the taste scores and DPPH clearance increase after enzymatic hydrolysis and Maillard reaction,

except for the DPPH clearance of MRPs. Additionally, EH-PBME was the best sample in terms of both taste and antioxidant activity. Similarly, to EH-PBME, MPRs also

Table 2 The result of response surface experiment and L16 (3⁵) orthogonal experiment

Variance	Quadratic sum	Degree of freedom	Mean square	F value	P value
(a)					
Model	1242.24	9	138.03	14.75	0.0009
A-time	2.50	1	2.50	0.27	0.6210
B-temperature	124.48	1	124.48	13.30	0.0082
C-concentration of substrate	104.59	1	104.59	11.17	0.0124
AB	29.66	1	29.66	3.17	0.1183
AC	71.32	1	71.32	7.62	0.0281
BC	75.30	1	75.30	8.05	0.0252
A ²	97.07	1	97.07	10.37	0.0146
B ²	201.29	1	201.29	21.51	0.0024
C ²	458.43	1	458.43	48.98	0.0002
Residual error	65.51	7	9.36		
Lack of fit	50.91	3	16.97	4.65	0.0859
Pure error	14.60	4	3.65		
Summation	1307.76	16			

Number	A (Reducing sugar)	B (Amino acid)	C (VB ₁)	D (Reaction time)	E (Reaction temperature)	Comprehensive score
(b)						
1	1	2	1	3	1	48.15
2	1	1	2	1	3	65.83
3	3	1	3	2	1	51.20
4	1	3	3	3	3	86.43
5	1	1	1	1	1	59.76
6	1	1	3	1	2	40.23
7	2	1	1	3	2	78.13
8	1	2	2	2	2	44.83
9	2	2	3	1	1	75.83
10	2	3	2	1	1	62.11
11	3	3	1	1	2	63.24
12	3	1	2	3	1	84.99
13	1	1	1	1	1	54.66
14	1	3	1	2	1	49.32
15	2	1	1	2	3	57.25
16	3	2	1	1	3	61.01
K ₁	56.15	61.51	58.94	60.33	60.75	
K ₂	68.33	57.46	64.44	50.65	56.61	
K ₃	65.11	65.27	63.42	74.43	67.63	
R	12.18	7.82	5.50	23.77	11.02	

Source	Type III square sum	df	Mean square	F	Sig.
(c)					
Calibration model	2091.873 ^a	10	209.187	1.282	0.414
Intercept	42925.291	1	42925.291	263.011	0.000
A (Reducing sugar content)	467.553	2	233.776	1.432	0.322
B (AA content)	122.312	2	61.156	0.375	0.705
C (VB ₁)	101.690	2	50.845	0.312	0.746
D (Reaction time)	1149.919	2	574.959	3.523	0.111
E (Reaction temperature)	250.398	2	125.199	0.767	0.512
Error	816.037	5	163.207		

Table 2 continued

Source		Type III square sum		df	Mean square	F	Sig.
Sum		63299.047		16			
Sum of correction		2907.910		15			

Number	A (Reducing sugar)	B (Amino acid content)	C (VB ₁)	D (Reaction time)	E (Reaction temperature)	Comprehensive score
(d)						
1	2	1	2	2	2	65.86
2	1	1	1	1	1	48.78
3	1	2	2	3	1	55.97
4	1	1	1	1	1	43.92
5	1	1	1	1	1	57.23
6	2	2	1	1	3	58.07
7	3	1	3	3	3	70.57
8	1	3	1	2	3	67.05
9	3	2	1	1	2	58.80
10	2	3	3	1	1	64.54
11	3	3	2	1	1	59.98
12	1	1	2	1	3	59.98
13	3	1	1	2	1	48.29
14	1	1	3	1	2	56.02
15	2	1	1	3	1	58.62
16	1	2	3	2	1	55.08
K1	55.50	56.51	55.10	56.26	54.40	
K2	61.77	56.98	60.45	59.07	59.48	
K3	59.41	62.20	61.55	60.60	63.92	
R	6.27	5.69	6.46	4.34	9.52	

Source		Type III square sum		df	Mean square	F	Sig.
(e)							
Calibration model		657.392 ^a		10	65.739	3.633	0.084
Intercept		39801.158		1	39801.158	2199.435	0.000
A (Reducing sugar content)		114.652		2	57.326	3.168	0.129
B (AA content)		92.487		2	46.243	2.555	0.172
C (VB ₁)		141.896		2	70.948	3.921	0.095
D (Reaction time)		55.750		2	27.875	1.540	0.301
E (Reaction temperature)		252.608		2	126.304	6.980	0.036
Error		90.480		5	18.096		
Sum		54659.912		16			
Sum of correction		747.872		15			

(a) Regression model analysis of variance in response surface; (b) The result of Maillard reaction of PBME; (c) Testing of intersubjective effects in Maillard reaction of PBME; (d) The result of Maillard reaction of EH-PBME; (e) Testing of intersubjective effects in Maillard reaction of EH-PBME

had strong taste and high antioxidant activity; nonetheless, their values were different.

Changes of amino acid, peptides and nucleotide

Four samples containing twelve amino acids are listed in Fig.3(a). While serine was detected in PBME sample, it

was detected in other three samples. This may be due to the enzymatic hydrolysis and Maillard reaction, which can break down peptide bonds, generating free amino acids. Moreover, serine was found to contribute to both sweet and umami flavors to the solution. In addition, the contents of His and Tyr were also found to vary among all four samples. The Maillard reaction products, MRPs A and MRPs

Table 3 The optimum formula and basic indexes of the optimized 4 samples. Samples are (MRPs B) (a) The result of taste score and DPPH clearance in 4 samples in optimum formula; porcine bone marrow extract (PBME), enzymatic hydrolysate of PBME (EH-PBME), Maillard reaction products of PBME (MRPs A) and Maillard reaction products of EH-PBME

Sample	Optimum formula	Taste score	DPPH clearance					
(a)								
PBME	Substrate concentration of 375g/L	4.2±0.1 ^a	0.50±0.03 ^a					
MRPs A	4% glucose, 2% xylose, 1.5% Tyr, 1.5% Ala, 4% VB ₁ and reaction 40min with temperature 115 °C	5.5±0.3 ^c	0.84±0.02 ^a					
EH-PBME	Substrate concentration of 375g/L, 0.4% enzyme, 3h, 55°C	6.6±0.3 ^d	0.86±0.01 ^a					
MRPs B	2% glucose, 2% xylose, 3% Ala, 5% VB ₁ and reaction 40min with temperature 110 °C	5.0±0.6 ^b	0.41±0.04 ^a					
No	Compound name	Compound concentration (µg/L)						
		PBME	EH-PBME	MRPs A	MRPs B			
(b)								
1	Hexanal	893	Grass, tallow, fat	MS, RI	0	2.04±0.21 ^b	0.11±0.08 ^a	7.58±0.45 ^c
2	Furfural	925	Bread, almond, sweet	MS, RI, O	0	0	301.09±18.22 ^a	554.37±11.28 ^b
3	Pyrazine, 2,5-dimethyl-	1004	Cocoa, roasted nut, roast beef, medicine	MS, RI, O	8.43±0.98 ^b	3.63±0.34 ^a	19.13±1.05 ^c	25.61±1.43 ^d
4	Benzaldehyde	1055	Almond, burnt sugar	MS, RI, O	0	54.84±9.86 ^a	108.41±21.75 ^b	200.18±14.93 ^c
5	1-Octen-3-ol	1075	Mushroom	MS, RI, O	0	0	5.77±0.99 ^a	0
6	Furan, 2-pentyl-	1087	Green bean, butter	MS, RI, O	4.33±0.13 ^a	24.14±1.21 ^b	38.17±4.11 ^c	75.08±6.21 ^d
7	2-Acetylthiazole	1114	Roast, nut, sulfur	MS, RI, O	0	0	1.82±0.33 ^a	0
8	Dihydrocarvyl acetate	1127	Mint, camphor, medicine	MS, RI, O	0	0	10.17±0.85 ^a	0
9	Benzeneacetaldehyde	1140	Hawthorne, honey, sweet	MS, RI, O	0	4.93±1.11 ^a	39.56±2.32 ^b	0
10	Pyrazine, 2-ethyl-3,5-dimethyl-	1184	Potato	MS, RI	0	0	6.85±1.04 ^a	0
11	Nonanal	1201	Fat, citrus, green	MS, RI, O	61.24±7.32 ^a	221.08±10.95 ^c	82.50±8.43 ^b	295.75±22.54 ^d
12	2-Nonenal, (E)-	1257	Cucumber, fat, green	MS, RI, O	0	9.94±1.05 ^a	12.89±2.62 ^b	19.27±3.21 ^c
13	Octanal	1267	Fat, soap, lemon, green	MS, RI, O	0	0	3.10±0.05 ^a	0
14	Cyclohexanol 2-methyl-5-(1-methylethenyl)-	1291	Mint, spice	MS, RI	0	0	8.17±1.03 ^a	0
15	Benzaldehyde, 4-(1-methylethyl)-	1297	Acid, sharp	MS, RI	0	0	4.11±0.85 ^a	0
16	Decanal	1302	Soap, orange peel, tallow	MS, RI, O	7.62±0.09 ^b	10.94±0.98 ^c	7.39±0.53 ^a	15.80±0.44 ^d
17	Benzoic acid, ethyl ester	1270	Camomile, flower, celery, fruit	MS, RI, O	6.46±0.52 ^a	0	0	0
18	2-Decenal, (E)-	1359	Tallow	MS, RI, O	0	5.30±3.23 ^a	10.04±1.12 ^b	40.75±2.44 ^c
19	Dodecanal	1404	Lily, fat, citrus	MS, RI, O	0	1.65±0.15 ^a	3.64±0.45 ^b	4.69±0.84 ^c
20	2-Undecenal	1461	Sweet	MS, RI, O	0	5.03±0.63 ^a	7.84±1.17 ^b	33.50±2.98 ^c
21	Styrene	987	Balsamic, gasoline	MS, RI, O	0	0.21±0.04 ^a	0	0
22	Disulfide, dimethyl	822	Onion, cabbage, putrid	MS, RI	0	0.38±0.01 ^b	0	0.18±0.02 ^a
23	Heptanal	994	Fat, citrus, rancid	MS, RI, O	1.32±0.07 ^a	3.00±0.63 ^b	0	9.06±0.94 ^c

Table 3 continued

No	Compound name	RI (DB-5)	Volatile property	Method of identification	Compound concentration (µg/L)			
					PBME	EH-PBME	MRPs A	MRPs B
24	Estragole	1297	Licorice, anise	MS, RI, O	5.42±0.33 ^b	3.51±0.08 ^a	0	0
25	2-Octenal, (E)-	1155	Green, nut, fat	MS, RI	0	6.32±0.09 ^a	0	0
26	Undecanal	1404	Oil, pungent, sweet	MS, RI	0	2.46±0.12 ^a	0	8.82±1.56 ^b
27	2,4-Decadienal, (E,E)-	1414	Fried, wax, fat	MS, RI	0	18.23±1.03 ^a	0	91.86±9.47 ^b
28	2-Octen-1-ol, (E)-	1074	Soap, plastic	MS, RI	0	0	0	11.12±1.45 ^a
29	p-Cymene	1122	Solvent, gasoline, citrus	MS, RI, O	0	0	0	2.80±0.05 ^a
30	2-Dodecenal, (E)-	1154	Green, fat, sweet	MS, RI, O	0	0	0	19.30±3.08 ^a
31	Benzothiazole	1326	Gasoline, rubber	MS, RI, O	0	0	0	6.67±0.14 ^a
32	Benzaldehyde, 4-methoxy-	1263	Mint, sweet	MS, RI, O	0	0	0	11.96±1.09 ^a
33	5-Thiazoleethanol, 4-methyl-	1379	Sulfur	MS, RI, O	0	0	0	18.03±1.79 ^a
34	Caryophyllene	1528	Wood, spice	MS, RI	0	0	0	2.23±0.06 ^a
35	α-Terpineol	1291	Oil, anise, mint	MS, RI	10.26±1.44 ^a	0	0	0

All data were expressed as mean values (mean ± SD). Results with different letters (a–c) were significantly different in content

All data were expressed as mean values (mean ± SD). Results with different letters (a–d) were significantly different in content

B, contained the highest content of His, indicating that it may be responsible for increased taste and bitterness of the two samples (Song et al. 2013). However, as an essential amino acid, His has great health benefits for patients suffering from uremia and hypertension (Kopple and Swendseid 1975; Wang et al. 2008). Tyr was also found to have the largest proportion and variation among the four samples; its content was decreased after the enzymatic hydrolysis. Although Tyr is a colorless and odorless amino acid, it is highly beneficial to the formation of black color in Maillard reaction (Xu 2017). Gly has been reported to be the most important amino acid owing to its contribution to sweet taste, as well as its function as an antioxidant (Zhao et al. 2016; Luo et al. 2011). The content of Gly in the enzymatic hydrolysate EH-PBME was higher than those in other three samples, due to previous research shown that Gly is responsible for the increased antioxidant activity and improved taste of EH-PBME (Rajapakse et al. 2005). Other amino acids that may be produced by the Maillard reaction include Ala, which was found to improve the sweet taste of MRPs A and B, and Glu, which contributed to sour and umami tastes. Figure 3(b) shows the change of peptides in the four samples. The content of peptides with molecular weights of <1kDa in PBME was lower than compared with those in other three samples, especially for EH-PBME and MRPs B. This may be due to that the enzymatic digestion of peptides and Maillard reaction can degrade the macromolecular peptides into smaller peptides. Flavor peptides are oligopeptides with molecular weights of <5kDa, and it can include the characteristic taste peptides and flavor precursor peptides. Several studies have shown that peptides with molecular weights of <1kDa or <3kDa contribute to better tastes and higher antioxidant activity (Wu et al. 2017; Zhang et al. 2016). According to Fig. 3(c), the contents of all four nucleotides, except for IMP, were slightly changed and were nearly unnoticeable. After enzymatic hydrolysis and Maillard reaction, both the contents of CMP and GMP were increased to some extent. Whereas the content of AMP was dramatically increased after enzymatic hydrolysis, the content of IMP was increased by nearly three folds after Maillard reaction. According to a research conducted by a team of Japanese scientists, the four nucleotides contribute to umami taste, which is the desirable taste in food (Jacquelin 2005).

Change of volatile compounds

As shown in Table 3(b), thirty-five key volatile compounds from four different samples produced from PBME were identified by HS-SPME. Three common volatile compounds (2,5-dimethylpyrazine, nonanal, decanal) were detected in all four samples, and their contents were changed. Key odors captured in the samples include cocoa,

Fig. 3 Changes of amino acids, peptides and nucleotides before and after enzymatic hydrolysis and Maillard reaction **a** amino acids; **b** peptides; **c** nucleotides. **d** Correlation of amino acids, nucleotides,

peptides, taste score, bitter, meat, umami and DPPH clearance by PLSR; **e** Correlation of aroma compound content and flavor by PLSR

roasted nut, roast beef, medicine, fat, citrus, green, soap, orange peel and tallow. The highest numbers of volatile compounds with high concentrations were detected in MPRs B. The numbers of types of odors and their contents in the samples could be ranked in the following order: MPRs B>MPRs A>EH- PBME >PBME. However, careful analysis of the odors shown in the table showed that although MPRs B had the highest numbers of flavor compounds, the proportion of off-flavor compounds were

also higher compared to other three samples. MPRs A and B obtained after Maillard reaction contained only one same volatile compound furfural, which is commonly smell as bread, almond or sweet. Additionally, pleasant odors, including 1-octen-3-ol (mushroom), dihydrocarvyl acetate (mint, camphor, medicine), 2-ethyl-3,5-dimethyl pyrazine (potato), 2-methyl-5-(1-methylethenyl) and cyclohexanol (mint, spice) were detected only in MPRs A.

These data suggest that volatile compounds are generated from the degradation of amino acids, while other chemical reactions may also occur within the system. Oxidative degradation of fat produces fatty taste, and the degradation of cysteine releases H_2S , which then participates in the Maillard reaction to generate the volatile sulfur-containing compounds (e.g., thiol) contributing to roasted meat flavor (Kerscher and Grosch, 1997). However, there can be a risk of sulfur odor due to dimethyl sulfide. The typical meaty substance 2-methyl-3-furanthiol, which tends to further generate hydrogen sulfide and reducing sugar dehydration product, and 5-methyl-4-hydroxy-3, which ultimately forms (2H) furan through the degradation of cysteine, were not detected (Xie et al. 2004; Christoph and Davidek 2003). This may be due to insufficient Cys content in the solution or the use of insufficient reaction time.

Correlation analysis by PLSR

To determine the effects of enzymatic hydrolysis and Maillard reaction on the contents of amino acids, nucleotides, peptides, antioxidant activity and various tastes scores in the four samples, PLSR was employed to analyze the correlation between the first and the second factors for X and Y matrices. As shown in Fig.3(c), the model fitted well with the indicators with a variance of up to 89.66%; therefore, PC1 and PC2 were subsequently analyzed. The four samples were distributed along the PC2 axis: PBME are shown on the left, while other three samples are shown on the upper and lower right of the loading diagram. PBME and MPRs can be found near PC2. The X variable, except for the 3-5kDa and 1-3kDa, is shown on the left of the PC1 axis, while other variables, except for the salty taste, are shown on the right of the diagram. The diagram shows that PBME is positively correlated with salty, and peptide fragments of 3-5kDa and 1-3kDa, while is inversely correlated with other three samples. Additionally, MPRs A is positively correlated with Asp, Glu, IMP, peptide fragment of >5kDa, meat, bitter and umami tastes, and DPPH clearance (Cho et al. 2010). The contents of peptides in MPRs A with molecular weights between 3 and 5kDa were decreased, while those with molecular weights of >5kDa were increased, which may be caused by the cross-linking between sugars and peptides during the Maillard reaction. Asp, Glu and IMP were found to cause significant changes in umami, meaty and bitter tastes. Asp contributes to strong sweet and umami tastes (Zhao et al. 2016). Glu is a sour-and umami-taste amino acid that is abundant in MPRs (Liao et al. 2009). IMP is the nucleotide contributing to high umami taste score. In addition, EH-PBME and MPRs B lie near the center of the loading diagram, indicating that it has weak correlation with other samples. Whilst EH-

PBME is positively correlated with Gly and umami taste, MPRs B is positively correlated with Met and Thr. Gly is the most important amino acid that contributes to sweet taste while functions as an antioxidant (Zhao et al. 2016; Luo et al. 2011). Whereas Met is a complex amino acid contributing to both bitter and umami tastes, Thr contributes to rich sweet taste with slight bitterness (Zhao et al. 2016; Liao et al. 2009). These observations show that the samples underwent Maillard reaction had overall increased tastes or flavors. According to Fig.3(e), the model fitted well with the indicators with the variance of up to 89.53%; thus, PC1 and PC2 were further analyzed. The analysis showed that most volatile compounds were positively correlated with burnt, roasted, caramel, meat and sulfur flavors (the right side of the diagram), as indicated by MPRs B sample. Other five volatile compounds, such as 2-Acetylthiazole (roast, nut, sulfur), 1-Octen-3-ol (mushroom) etc. were significantly positively correlated with MPRs A. As shown on the left of the plot, benzoic acid ethylester (camomile, flower, celery, fruit) and α -terpineol (oil, anise, mint), which contribute to fishy taste, were positively correlated with PBME sample. In contrast, because EH-PBME and dimethyl disulfide were located near the Y-axis, they were not highly correlated with others. Whilst pyrazine (2,5-dimethylpyrazine, 2-ethyl-3, 5-dimethylpyrazine) and thiazole (2-acetylthiazole, benzo thiazole) mainly contribute to roasted odor, benzaldehyde contributes to the typical caramel odor. The data showed that MPRs B contained the highest flavor-active compounds (both favorable and unfavorable), while PBME contained the least.

Conclusion

In the present study, the optimal conditions for the enzymatic hydrolysate and Maillard reaction were obtained via RSM and orthogonal experiment. The optimal conditions for the hydrolysis of EH-PBME obtained from RSM were: reaction time and temperature, 3 hours and 55°C; substrate concentration, 375 g/L; and amount of enzyme, 0.4%. In addition, the optimal conditions obtained from orthogonal experiment were as follows: (i) PBME containing 2% glucose, 2% xylose, 1.5% Tyr, 1.5% Ala and 4% vitamin B₁, with reaction temperature and time of 115°C and 40 min, respectively; and (ii) Maillard reaction at 110°C for 40 min using the enzymatic hydrolysate supplemented with 2% glucose, 2% xylose, 3% Ala and 5% vitamin B₁. In terms of flavor and DPPH clearance, EH-PBME and MPRs A performed better than other samples, with EH-PBME being particularly prominent.

After the enzymatic hydrolysis, the bitterness was decreased while the umami taste was increased, which may

be due to the changes of Gly content and numbers of different peptides. Gly has sweet taste, thus can alleviate the sour and alkali tastes, while mask the bitter taste. Additionally, other taste-active amino acids were also increased after the Maillard reaction. These amino acids, which have been reported to have two-taste profile, i.e. sweet and bitter tastes, resulted in the increased overall taste of the samples. The antioxidant activities were also improved. Based on the analysis of characteristics of different samples, it can be concluded that PBME resulted in the best taste and oxidation resistance, thus, EH-PBME and MRPs A should be selected for further analysis. Researchers worldwide have independently studied collagen flavors and antioxidant properties of various poultry, livestock and aquatic products. However, to date, a comprehensive research studying the attribution of flavors and antioxidant properties has not been conducted. Combining with flavor and oxidation resistance, this work may have high practical value.

However, future experiments may be carried out to improve the typical meat flavor by the Maillard reaction, and to explore new directions for investigating the relationship between the taste and the antioxidant peptides.

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