



Preparation process of chicken small peptide by enzymatic hydrolysis

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ABSTRACT

The study optimized the enzymatic hydrolysis conditions for the production of small peptides composed of 2~3 amino acids from chicken. Experiment adapted 4 factors 3 levels orthogonal design, and result analysis showed that enzymatic hydrolysis condition has an effect on total nitrogen (T-N), total amino nitrogen (A-N), degree of hydrolysis (DH), nitrogen recovery (NR), average peptide chain length (APL) and average peptides molecular mass (APM). By main effect analysis, enzymolysis time was the main influencing factor of the DH, APL and APM. Solid-liquid ratio was the main influencing factor of NR, A-N and T-N. From quadratic curve between temperature and NR, optimal temperature can be obtained. The small peptides samples were analyzed using HPLC to find out the peptide content. These results indicated that method with simple equipment, mild reaction, and easy control, this provides a further theoretical basis for the production of meat peptides.

Keywords: Chicken, Enzymatic hydrolysis, Protease, Small peptide

China is rich in chicken resources, and chicken breast meat has high protein, low fat and high nutritional value. The traditional protein metabolic pattern holds that the protein must be hydrolyzed into free amino acids and then absorbed into the body by the amino acid transport carrier. However, in the 1970s, studies found that small peptides containing 2 and 3 amino acids were the main product of proteolysis (Adibi *et al.* 1973) which have many special physiological functions and play a very important role in the process of protein metabolism (Sun *et al.* 2011). The peptides can be absorbed directly and completely with the help of intestinal transporters, which is characterized by fast absorption speed, unsaturated carrier, low energy consumption and high efficiency. In addition, the supply of amino acids in the form of small peptides can avoid the inhibitory effect of free amino acids due to competitive binding sites and promote the absorption and transformation of protein feed (Gilbert *et al.* 2008). Enzymatic hydrolysis is one of the most frequently used approaches to prepare peptides (Jang *et al.* 2017, Alice and Fitzgerald 2017). The study explores process conditions for neutral protease enzyme hydrolysis of 2 to 3 amino acids of small peptide, in order to develop easy, safe and reliable method to obtain rich nutrition small peptides products, which also provides

reference for research in other enzymolysis technology for meat protein.

MATERIALS AND METHODS

Sample preparation: Fresh chicken (breast chicken) was obtained from subtropical plateau monsoon climate area. All samples were used after mincing (machine broken). Neutral proteases were obtained from Kunming Aike biotechnology Co., Ltd. (≥ 50000 U/g).

Test design: Experiment with 4 factors 3 levels orthogonal experiment design (Table 1), the dosage of neutral proteases were compared: 1000 U/g, 3000 U/g, 5000 U/g, and solid-liquid ratio 1:1, 1:1.5, 1:2. temperature 40°C,

Table 1. Orthogonal experimental design

Group	Enzymatic hydrolysis (time/h)	Enzyme dosage (U/g)	Temperature (°C)	Material liquid ratio
1	4	1000	40	1:01
2	4	3000	48	01:01.5
3	4	5000	56	1:02
4	6	1000	48	1:02
5	6	3000	56	1:01
6	6	5000	40	01:01.5
7	8	1000	56	01:01.5
8	8	3000	40	1:02
9	8	5000	48	1:01

Analysis of breast chicken hydrolysate.

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48°C and 56°C. The time of enzymatic hydrolysis was: 4 h, 6 h, 8 h.

Total nitrogen content of chicken hydrolysate: Total nitrogen (T-N) content of the hydrolysate was determined by micro-Kjeldahl analysis (AOAC 2011). Amino nitrogen (A-N) was measured by analyzing the terminal amino groups with formaldehyde titration.

Degree of hydrolysis (DH) was decided as per Himani *et al.* (2018).

Average peptide length (APL) was calculated as per Adler-Nissen (1986).

Average peptides molecular mass was calculated (APM) as per Suh (2000). The average molecular weight of the amino acids was 120.

Nitrogen recovery rate (NR) was calculated as per the following formula (Yang P 2008):

$$\text{Nitrogen recovery rate \%} = \frac{\text{Total nitrogen content in chicken hydrolysate}}{\text{Total nitrogen content in raw materials}} \times 100\%$$

Small peptide compositional analysis

Amino acid analysis: According to the above optimum enzymatic hydrolysis conditions, chicken small peptides were enzymatically hydrolyzed, which was concentrated to obtain product. Using the freeze-dried powder to detect the contents of acid hydrolyzed amino acid, alkaline hydrolyzed amino acid (Tryptophan) and free amino acid, amino acids in HPLC, samples were pre-treated by acid hydrolysis method. The instrument was Shimazu HPLC (LC-20A). Column chromatography detection conditions were as per Huang *et al.* (2018). Content of small peptides was calculated as per Qiu *et al.* (2011).

RESULTS AND DISCUSSION

Effects of different hydrolysis conditions on the enzymolysis of peptides: According to orthogonal test design based on method of 1.3, enzymatic hydrolysis of chicken and the A-N and T-N in the enzymatic hydrolysis solution was determined respectively. The NR, DH, APL and APM were calculated. The results are shown in the Fig. 1.

It can be seen from Fig. 1. (a-f) that the enzymolysis conditions have an effect on the A-N, T-N, DH, NR, APL and APM in the treatment groups ($P < 0.05$).

A-N (Fig. 1a) showed no significant difference between groups 2, 3, 4, 7, 8 and 9, and the content was relatively high. There was no significant difference between group 6 and 5 ($P > 0.05$). A-N was highest in group 4, which was higher than group 1, 5 and 6, it as 88.28% ($P < 0.05$), 39.31% ($P < 0.05$) and 46.06% ($P < 0.05$).

T-N (Fig. 1b) showed no significant difference between groups 2, 3, 4 and 8 ($P > 0.05$), and the content was relatively high. There was no significant difference between groups 4, 5, 6, 7 and 9 ($P > 0.05$), and no significant difference between groups 1, 5, 6 and 7 ($P > 0.05$). T-N was highest in group 3, which was higher than groups 1, 5, 6, 7, and 9, it as 47.72% ($P < 0.05$), 30.97% ($P < 0.05$), 16.16% ($P < 0.05$),

18.28% ($P < 0.05$), 16.37% ($P < 0.05$).

DH (Fig. 1c) showed no significant difference between groups 4, 5, 7 and 9 ($P > 0.05$), no significant difference between groups 2, 3, 5 and 8 ($P > 0.05$), and no significant difference between groups 1, 2, 3 and 6 ($P > 0.05$). The degree of hydrolysis is highest in group 4, which was higher than that groups 1, 2, 3 and 6, it as 36.25% ($P < 0.05$), 18.81% ($P < 0.05$), 20.13% ($P < 0.05$), 34.46% ($P < 0.05$).

NR (Fig. 1d) showed no significant difference between groups 2, 3, 7 and 8 ($P > 0.05$), and the content was higher. There was no significant difference between groups 4, 5, 6 and 9 ($P > 0.05$), and no significant difference between groups 1 and 5 ($P > 0.05$). NR was the highest in group 8, which was higher than groups 1, 4, 5, 6, and 9, it as 47.71% ($P < 0.05$), 26.83% ($P < 0.05$), 30.32% ($P < 0.05$), 15.57% ($P < 0.05$), 15.47 ($P < 0.05$).

APL (Fig. 1e) showed no significant difference among groups 1, 2, 3 and 6, and the content was higher. There was no significant difference between groups 2, 3, 5 and 8 ($P > 0.05$), and no significant difference between groups 4, 5, 7, 8 and 9 ($P > 0.05$). APL in group 1 was the longest, which was longer than the groups 4, 5, 7, 8, it as 36.65% ($P < 0.05$), 19.93% ($P < 0.05$), 35.57% ($P < 0.05$), 24.78% ($P < 0.05$), 36.65% ($P < 0.05$).

APM (Fig. 1f) showed no significant difference among groups 1, 2, 3, and 6, and the content was higher. There was no significant difference between groups 2, 3, 5 and 8 ($P > 0.05$), and no significant difference between groups 4, 5, 7, 8 and 9 ($P > 0.05$). The average peptide molecule mass in group 1 was the longest, which was higher than the groups 4, 5, 7, 8, it as 36.65% ($P < 0.05$), 19.93% ($P < 0.05$), 35.57% ($P < 0.05$), 24.78% ($P < 0.05$), 36.65% ($P < 0.05$).

Because we attempted to prepare small peptide composed of 2–3 amino acids, the average peptide chain length should be between 2 and 3, the average molecular weight of the peptide should be between 240 and 360, and the selected hydrolysis degree should be between 33% and 50% that can meet the requirements. Group 2, 3, 4, 5, 7, 8, 9 can meet the requirements, and NR have no significant difference among group 2, 3, 7, 8 ($P > 0.05$). The group 7 had the minimal enzyme dosage. So we chose group 7 as the optimal enzymatic hydrolysis conditions.

DH as the index formula was used for preparation of small peptide nutrient solution, by orthogonal test to determine the optimal hydrolysis conditions of three kinds of enzymes, the hydrolysis degree of crassostrea gigas meat with three enzymes was up to 52.97%, according to the vertical plate polyacrylamide gel electrophoresis, relative molecular mass of small peptide was between 300–1000 (Fang *et al.* 2012). Higher DH means smaller peptides, which were expected to have proportionally more polar residues and the ability to form hydrogen bonds with water, thereby improving the solubility (Gbogouri *et al.* 2004). Deng and Feng *et al.* (2001) used dextran gel (Sephadex G-25) column chromatography and HPLC to study relationships between determination of molecular mass of soybean peptide and degree of hydrolysis, with the increase

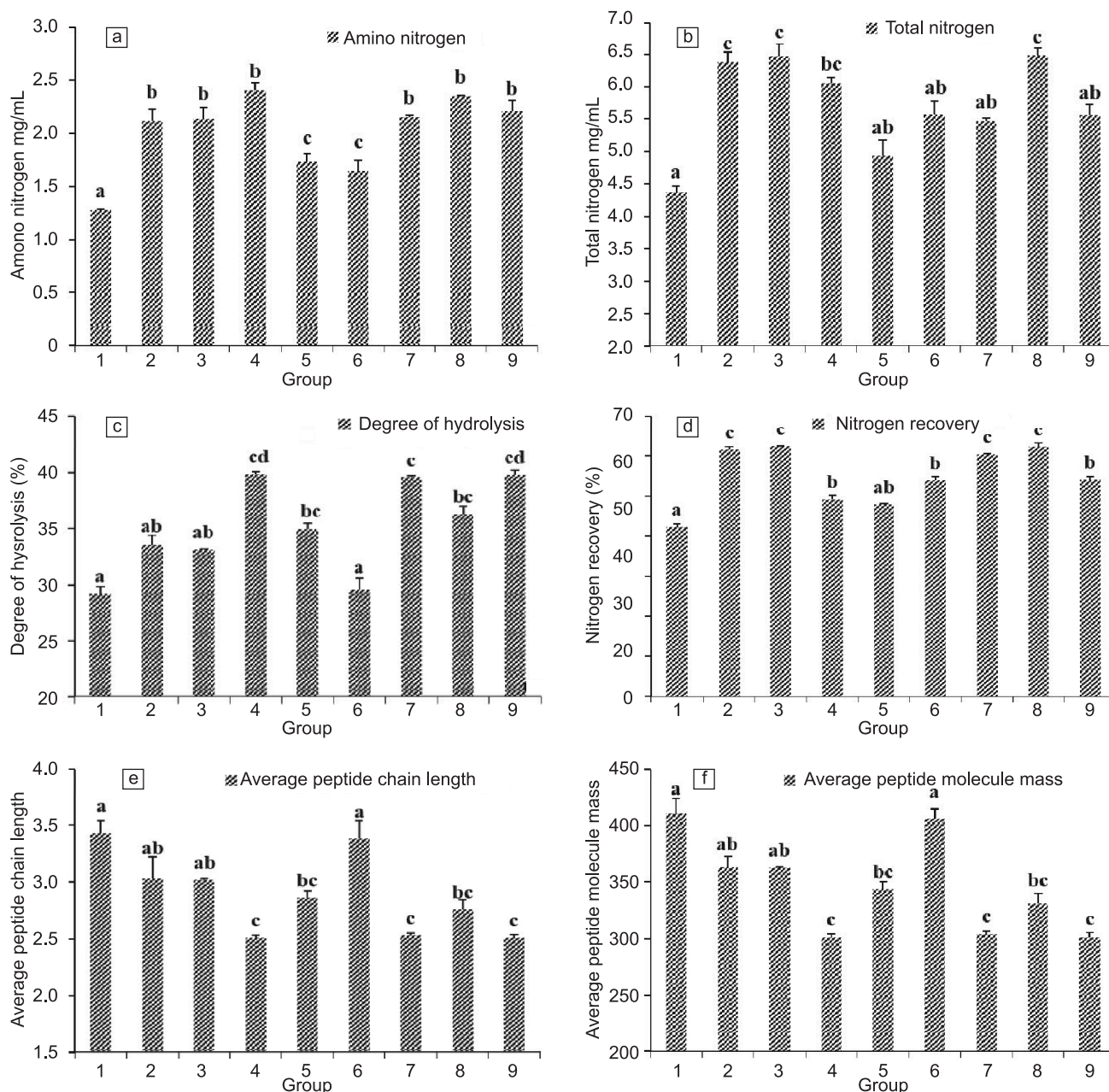


Fig. 1. Effect of enzymolysis conditions on (a) Amino nitrogen; (b) Total nitrogen; (c) Degree of hydrolysis; (d) Nitrogen recovery; (e) Average peptide chain length; and (f) Average peptide molecule mass.

of soy protein hydrolysis, the molecular mass of soybean peptide mixture decreases, and further verify the feasibility of using hydrolysis degree judgment peptide molecular mass. It was used for polynomial regression to approximate and predict DH as a function of independent variable. SDS-PAGE combined with MALDI TOF method was successfully applied to determine the molecular weight distribution of the hydrolysate of collagen peptides from fish skin and the results showed that the developed peptide extract contained peptides in the range below 2 kDa (Hema *et al.* 2017).

NR refers to the degree of nitrogen loss during enzymatic hydrolysis of proteins. Theoretically, the higher the nitrogen recovery rate is, the higher the nitrogen utilization rate will

be, and the less the loss will be. It can also use the degree of hydrolysis and nitrogen recovery as indicators to evaluate hydrolysate (Liu *et al.* 2014).

Main effect analysis of hydrolysis effect under different hydrolysis conditions: In order to compare the order of the influences of different hydrolysis conditions on the A-N content, T-N content, NR, DH, and other factors, the range method was used to determine the order of the influences of various factors by the range value. The results are shown in Table 2.

It can be seen that from Table 2, enzymolysis time was the main influencing factor of the DH, APL and APM of peptide, and solid-liquid ratio was the main influencing factor of T-N, A-N and NR.

Table 2. Main effect analysis of hydrolysis effect index under different hydrolysis conditions

Indicator	Range				Main effect analysis
	Time (R ₁)	Protease dosage (R ₂)	Temperature (R ₃)	Solid-liquid ratio (R ₄)	
Total amino nitrogen	0.39	0.11	0.51	0.56	R ₄ >R ₃ >R ₁ >R ₂
Total nitrogen	0.22	1.31	0.95	1.37	R ₄ >R ₂ >R ₃ >R ₁
Degree of hydrolysis	6.57	1.38	6.00	2.20	R ₁ >R ₃ >R ₄ >R ₂
Nitrogen recovery	3.03	6.06	5.19	13.04	R ₄ >R ₂ >R ₃ >R ₁
Average peptide chain length	0.56	0.15	0.22	0.20	R ₁ >R ₃ >R ₄ >R ₂
Average peptide molecule mass	66.57	17.76	60.61	26.01	R ₁ >R ₃ >R ₄ >R ₂

In order to intuitively understand the influence law and trend of reaction enzymolysis conditions on test indexes, the enzymolysis conditions were used as the abscissa, and the average value of T-N, A-N, DH and APL was used as the ordinate to draw the trend chart of the indexes, as shown below in the Fig. 2 (a-d).

It can be seen (Fig. 2a) that with the prolongation of enzymatic hydrolysis time, T-N and DH gradually increased, while T-N and APL gradually decreased. With the increase of enzyme dosage, DH and APL gradually increased, T-N

decreased first and then increased, while A-N increased first and then decreased. With the increase of temperature, T-N, A-N and DH increased first and then decreased, while the APL gradually decreased. With the increase of solid-liquid ratio, the T-N, A-N and DH increased gradually, and the APL decreased gradually.

The study used enzymatic hydrolysis to prepare soybean small peptide, with increase of dosage of protease, content of low polypeptide and DH in soybean small peptides gradually improved, this was because with the increase of

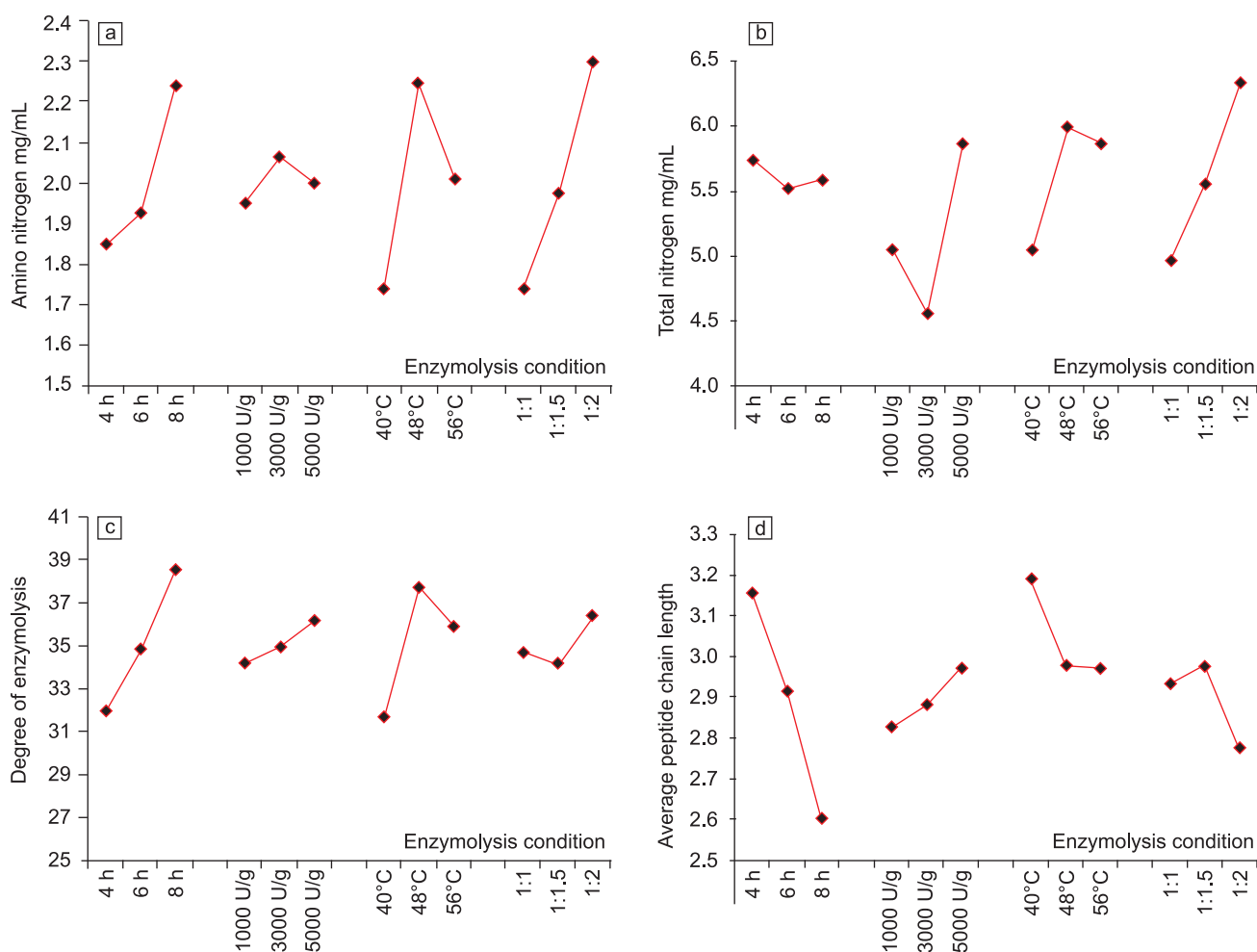


Fig. 2. Trend chart effects of enzymolysis conditions on (a) Amino nitrogen; (b) Total nitrogen; (c) Degree of enzymolysis; and (d) Degree of average peptide chain length.

dosage of protease, the contact rate between enzyme and substrate increased, and increasing number of peptide bonds hydrolyzed (Zhao *et al.* 2010). It was also found in our study that with the increase of enzyme amount, the DH and the APL gradually increased, which was due to the increase of enzyme amount, the possibility of protein peptide bond being cut off was greater. The increase in enzyme quantity will improve the capacity of protein. With the extension of the enzymatic hydrolysis time, the large molecular weight peptides in the products decreased and the small molecular weight peptides increased. Within 24 hours of enzymatic hydrolysis, the content of peptides with molecular weight of 1000~3000 Da was relatively low on the whole, and the content showed a decreasing trend with the extension of the enzymatic hydrolysis time (Li *et al.* 2012).

In immobilized enzyme amount of 1000 U/g, and solid-liquid ratio of 1:2 was adopted for single factor experiment design to compare 45°C, 50°C and 55°C enzyme solution of NR.

The influencing tendency of different enzymatic hydrolysis conditions on A-N revealed (Table 3) that temperature has an effect on the NR. The relationship between temperature and nitrogen recovery was studied through regression analysis and it was found that there was an extremely significant regression relationship between temperature and the NR. The relationship between temperature and the nitrogen recovery rate was a quadratic curve. On the basis of the immobilized dosage of proteases, temperature significantly affected the NR; with the increase of temperature, NR appears gradually upward and downward trend, that because temperature can lead to

Table 3. Comparison of nitrogen recovery rate

Group	Temperature (°C)	Nitrogen recovery rate %
1	45	61.65±1.40 ^a
2	50	71.38±0.73 ^b
3	55	52.79±0.78 ^c

Note: In same column, values with same letter superscripts mean no significant difference ($P>0.05$), while with different letter superscripts mean significant difference ($P<0.05$).

enzyme activity changes. If temperature x was 49.25°C, it can obtain the maximum NR with 71.79%.

Analysis of nutrition composition and content of small peptide: The hydrolyzed amino acid and free amino acid composition of the peptide was analyzed, and samples of HPLC figure are shown in Fig. 3 (a-d), and nutrition of small peptide are given in Table 4.

The content of small peptides in product could reach 37.97%. In terms of hydrolyzed amino acids, the content of umami amino acids was higher than that of sweet amino acids, with umami amino acids accounting for 38.42% and sweet amino acids 30.93% of total amino acids. Umami and sweet taste amino acids accounted for 69.35% of the total hydrolyzed amino acids. In terms of free amino acids, the content of umami amino acids was also higher than that of sweet amino acids. Umami amino acids account for 28.01% of total free amino acids, while sweet amino acids account for 22.94%. Umami and sweet amino acids account for 50.95% of total free amino acids.

From the results it is concluded that the enzymatic hydrolysis resulted in the development of chicken small

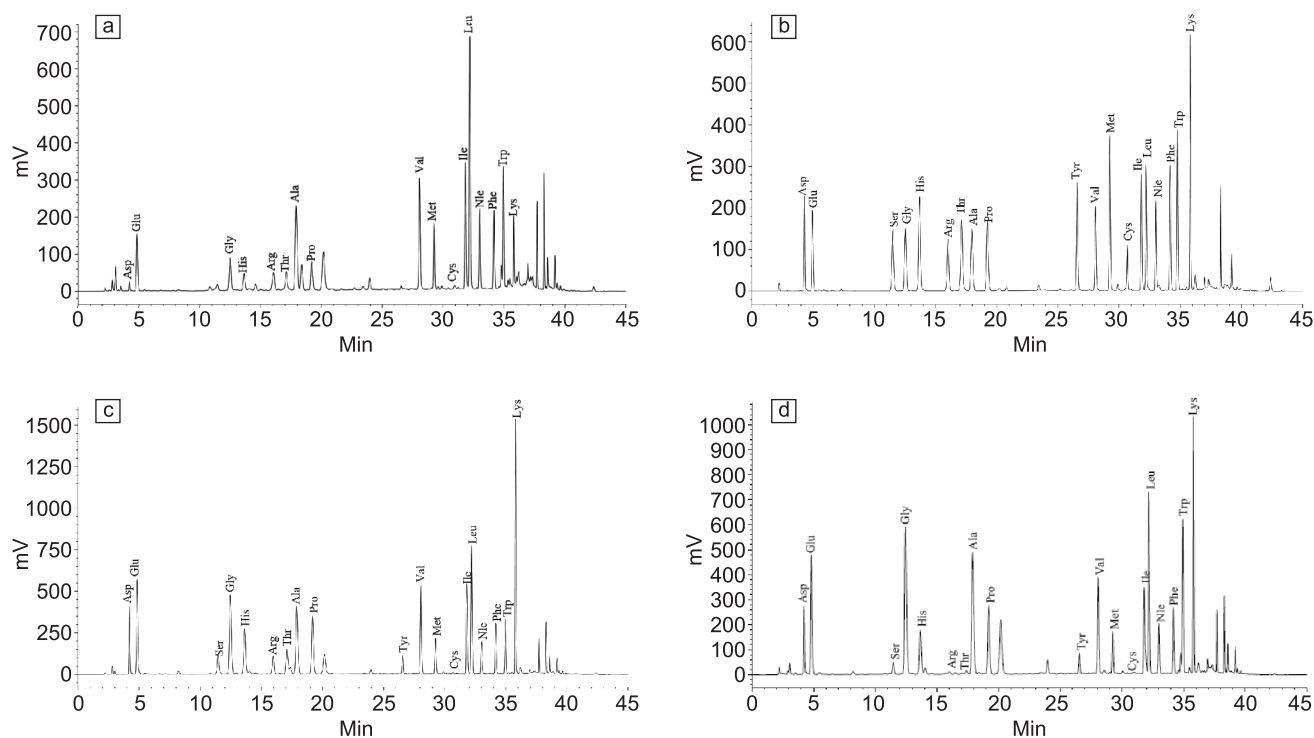


Fig. 3. Chromatogram of (a) Amino acid (standard); (b) Free amino acid of small peptide; (c) Acids hydrolyzed by small peptide; and (d) Alkaline hydrolyzed by peptides.

Table 4. Nutritional composition analysis of small peptide, dry sample %

Indicator	Hydrolyzed amino acid	Free amino acid
Aspartic acid [#]	5.43±0.38	0.41±0.06
Glutamic acid [#]	9.51±0.34	2.94±0.52
Serine ^S	1.69±0.11	0.52±0.11
Glycine ^{#S}	3.45±0.24	0.67±0.12
Histidine	3.19±0.16	0.60±0.07
Arginine [#]	2.84±0.21	1.62±0.17
Threonine ^{S^}	1.82±0.29	0.98±0.14
Alanine ^{#S}	4.15±0.20	2.21±0.23
Proline ^S	3.28±0.20	0.81±0.07
Tyrosine	1.22±0.15	0.17±0.02
Valine [^]	5.00±0.29	3.18±0.32
Methionine [^]	1.55±0.13	1.14±0.13
Cysteine	0.07±0.04	0.08±0.01
Isoleucine [^]	3.93±0.22	2.42±0.29
Leucine [^]	5.51±0.29	4.57±0.56
Phenylalanine [^]	1.93±0.08	1.37±0.14
Lysine ^{S^}	6.04±0.39	1.24±0.32
Tryptophan [^]	5.39±0.26	3.10±0.26
Total amino acid content (TAA)	66.06±4.01	28.03±0.47
Essential amino acid (EAA)	31.17±1.87	18.00±0.13
EAA/TAA	47.23±0.30	64.22±0.09
Umami amino acid	25.38±0.29	7.85±0.14
Sweet amino acid	20.43±0.21	6.43±0.21
Content of small peptide	37.97±0.27	

Note: #, umami amino acids; ^S, Sweet amino acid; [^], Essential amino acid.

peptides composed of 2–3 amino acids. The chicken peptides produced in this study have the advantages of simple equipment, mild reaction, and easy control, high peptide content and good quality. It provides a further theoretical basis for the production of meat peptides.

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