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Optimisation of preparation of active peptides by enzymatic hydrolysis of soybean processing wastewater and their antioxidant activity

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ABSTRACT: Papain enzymatic hydrolysis of soybean processing wastewater (SPWW) was used to prepare a useful soybean active peptide. Based on single-factor experiments, the peptide content and degree of hydrolysis were used as experimental indicators to optimise the process of preparing active peptides by enzymatic hydrolysis of SPWW, following which their antioxidant activity was determined. The results showed that the optimal enzymatic hydrolysis conditions were: temperature, 53 °C; concentration ratio of papain to SPWW, 2.0 g/100 ml; initial pH, 5.0; hydrolysis time, 7 h. Under these conditions, the degree of hydrolysis of SPWW was 2.801% and the peptide yield was 115.0%. The antioxidant results showed the half-maximum scavenging concentration (EC_{50}) of the peptide for 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radicals to be 6.459 and 1.871 mg/ml, respectively. This study provides a theoretical basis for the further utilisation of SPWW.

KEYWORDS: papain, soybean processing wastewater, active peptide, antioxidant

INTRODUCTION

Soybean processing wastewater (SPWW) is a mixed liquid containing protein, fat, sugar, organic acids and other beneficial ingredients that form during the production of soybean products, with high nutritional value [1]. The processing of 1 t of soybeans will produce about 5 t of SPWW; thus, the direct discharge of SPWW not only pollutes the environment but also wastes resources. Yang showed that the protein content of SPWW can be as high as 4.08 g/l [2]. The enzymatic hydrolysis of SPWW can yield soybean peptides with many health benefits including nutritional [3], enhanced immunity [4], lower blood pressure [5], antioxidant [6], anti-fatigue, etc. Thus, it has good development prospects in food, medicine and other industries.

Currently, many kinds of enzymes can be used for hydrolysis. Compared with other hydrolytic proteases, papain has the advantages of strong stability, high activity, non-toxicity, no side effects, safety and hygiene, rich resources and convenient extraction. Papain is abundant in the juice of immature papaya [7].

Research on the proteins in SPWW is of great significance. At present, economical and environmentally friendly methods of protein processing and utilisation are mainly via enzymatic hydrolysis. Chen investigated three different enzymes for the enzymatic hydrolysis of tartary buckwheat protein to determine the optimal hydrolysis conditions and its antioxidant activity [8]. The use of microbial fermentation to produce peptides has also been reported. Yin used lactic acid bacteria for the enzymatic hydrolysis of SPWW to prepare active peptides [9], which provided a basis for the development and utilisation of SPWW. However, microbial fermentation takes a long time and it is difficult to isolate and purify peptides. Enzymatic hydrolysis has the advantages of controllable conditions, rapid reaction and easy separation of products, and is widely used in the production of biologically active polypeptides. In this study, papain was selected to optimise the process of preparing active peptides via the enzymatic hydrolysis of SPWW. The aim was to provide parameters for the industrial utilisation of soybean liquid.

MATERIALS AND METHODS

Reagents and instruments

SPWW was provided by our laboratory. Reagents and enzyme were purchased from various companies: papain (5000 U/g), from Nanning Pangbo Biological Engineering Co., Ltd. (Guangxi Zhuang Autonomous Region, China); trichloroacetic acid, from the Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China); ABTS, from Hefei BASF Biotechnology Co., Ltd. (Anhui Province, China); DPPH, from Shanghai Ruji Biotechnology Co., Ltd. (Shanghai, China).

The following instruments were used. Model 722 visible spectrophotometer, Shanghai Sunny Heng-

ping Scientific Instrument Co., Ltd. (Shanghai, China). SY-1210 constant temperature water bath, Nanjing Changxiang Instrument Equipment Co., Ltd. (Jiangsu Province, China). 3H16RI benchtop high-speed refrigerated centrifuge, Hunan Herxi Instrument Equipment Co., Ltd. (Hunan Province, China). SCIENTZ-18N freeze dryer, Ningbo Xinzhi Biotechnology Co., Ltd. (Zhejiang Province, China).

Methods

Process flow

SPWW → adjust pH (NaOH, HCl) → enzymatic hydrolysis by papain (55 °C, 5 h) → inactivate enzyme → determine degree of hydrolysis and peptide yield → membrane purification → freeze-dry → crude peptide product → antioxidant activity analysis.

Single-factor test

The effects of the enzymatic hydrolysis temperature (45, 50, 55, 60, 65 °C), enzyme to substrate ratio (1.5, 2.0, 2.5, 3.0, 3.5 g/100 ml), initial pH (4.5, 5.0, 5.5, 6.0, 6.5), and enzymatic hydrolysis time (4, 5, 6, 7, 8 h) on the yield of peptide were investigated according to the process flow diagram. Each experiment was performed in triplicate.

Response surface optimisation of the peptide production process

Based on the single-factor test results, the enzymatic hydrolysis temperature (A), enzyme to substrate ratio (B), initial pH (C) and enzymatic hydrolysis time (D) were selected as variables. The Box-Behnken central combination test response surface analysis was used to design and optimise the process of producing peptides from SPWW.

Determination of degree of hydrolysis

The degree of hydrolysis was measured by the method of Cui et al [10] with appropriate modifications. The nitrogen determination method via spectrophotometry is described in the national standard GB 5009.5-2016.

Determination of peptide yield

To the sample solution was added 10% (w/v) of trichloroacetic acid (TCA) solution in a 1:1 (v/v) ratio. This mixture was mixed well and allowed to stand for 10 min at room temperature before centrifugation at 7000 rpm/min for 15 min. The supernatant was removed and mixed well with biuret reagent in a 1:4 (v/v) ratio. The resulting mixture was allowed to stand at room temperature for 30 min, following which the absorbance was determined (using deionised water as control). The peptide yield (D%) was calculated using Eq. (1).

$$D\% = \left(\frac{c_1}{c_0} - 1\right) \times 100\%, \qquad (1)$$

where c_0 and c_1 represent the polypeptide content before and after hydrolysis (mg/ml), respectively.

Antioxidant analysis

The product obtained from the enzymatic hydrolysis of SPWW under the optimum process conditions was dried in a vacuum freeze dryer for 48 h, yielding a solid polypeptide sample. The antioxidant analysis using ABTS and DPPH was according to references [11] and [12], respectively.

Data processing

Each experiment was performed in triplicate. Origin 9.0 was used to prepare figures, Design Expert v10 was used for data processing and response surface analysis and SPSS was used for data significance analysis.

RESULTS AND DISCUSSION

Standard curve

The standard curves of free amino acid content and protein concentration were obtained by the ninhydrin method (Fig. 1). The absorbance of the solution at 570 nm was positively correlated with the concentration of the solution. For amino acids, the following equation was obtained: $A_1 = 0.0363\,x_1 + 0.0137$; the R_1^2 value of 0.9985 indicated that the correlation was good. For protein, the following equation was obtained: $A_2 = 0.0103\,x_2 + 0.0064$; the R_2^2 value of 0.9932 also indicated a good correlation.

Single-factor analysis of papain hydrolysis of SPWW

Chemically, papain is a protein. When the temperature is too high or too low, the binding site of the active site of the enzyme can be distorted, thereby reducing the yield of enzymatic hydrolysis products [13]. The effect of enzymatic hydrolysis temperature on the polypeptide yield is shown in Fig. 2a, indicating that the optimum temperature for papain hydrolysis of SPWW was 55 °C. Below 55 °C, the enzymatic hydrolysis rate

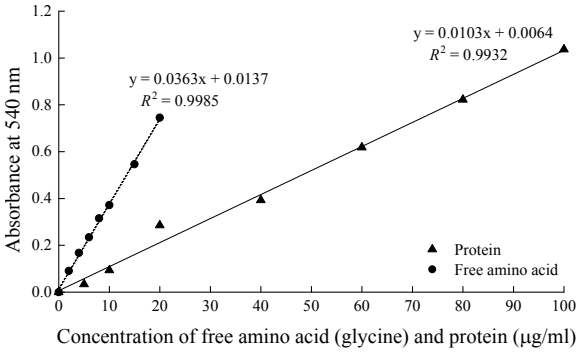

Fig. 1 Standard concentration curve for free amino acid and protein.

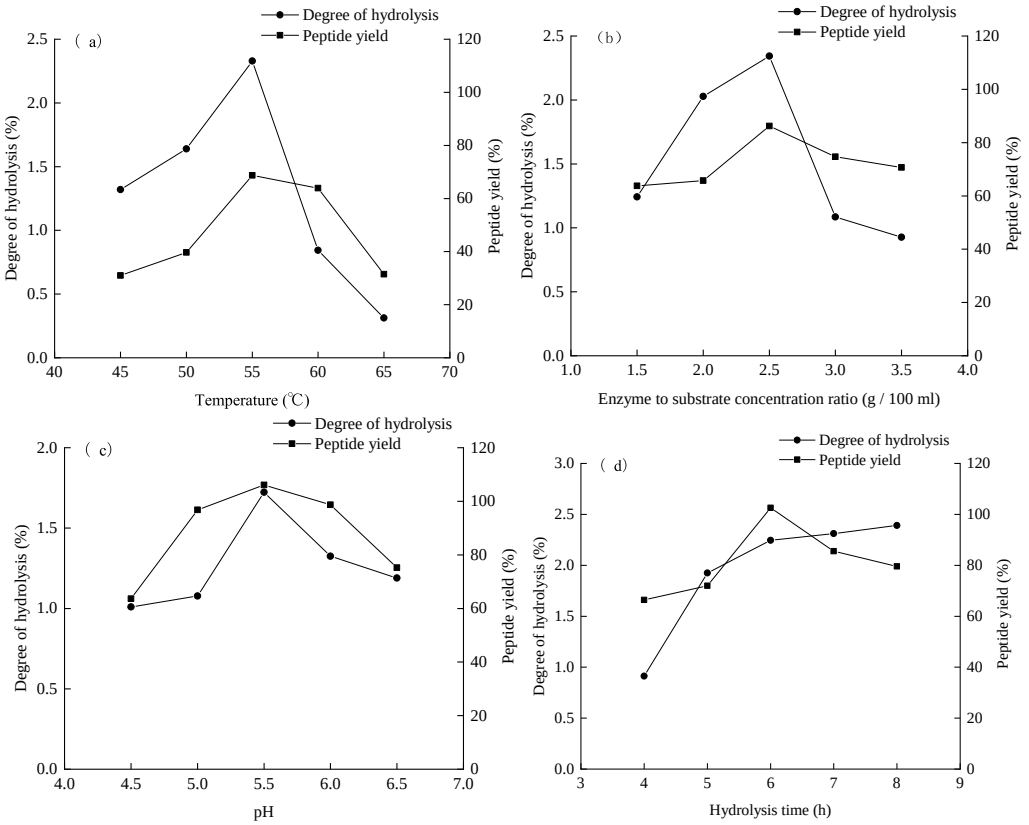

Fig. 2 Single-factor experiments for papain hydrolysis of SPWW.

continued to increase with increasing temperature but above 55 °C the hydrolysis rate decreased with increasing temperature. This was likely due to the increase in the enzymatic hydrolysis temperature causing partial breakage of the enzyme protein chains, showing the rate of hydrolysis and decreasing the polypeptide yield [14]. An optimal hydrolysis temperature of 55 °C was selected.

The effect of the ratio of enzyme to substrate concentration on the degree of hydrolysis and peptide yield is shown in Fig. 2b. When the concentration of enzyme to substrate ratio was less than 2.5 g/100 ml, the enzymatic hydrolysis rate continued to increase as the enzyme to substrate ratio increased. This may have been because the enzyme had not reached saturation below 2.5 g/100 ml [15]. As the concentration of enzyme to substrate ratio gradually increased beyond 2.5 g/100 ml, the amount of enzyme exceeded that required for hydrolysis of the substrate and became redundant [16]. Therefore, the optimal concentration of enzyme to substrate ratio for papain hydrolysis of SPWW was 2.5 g/100 ml.

Previous studies have shown that pH mainly impacts the stability of an enzyme by affecting its spatial structure, the dissociation state of the enzyme group, the enzyme active centre and the enzyme binding site of the substrate. Thus, pH affects the enzymatic hydrolysis reaction [17]. The effect of the initial enzymatic hydrolysis pH on the polypeptide yield is shown in Fig. 2c. The optimal initial pH was 5.5; more acidic or alkaline pH levels decreased the enzymatic activity. As proteins, peptides and amino acids are all ampholytes, they have different dissociation states with changes in pH and the active part of the enzyme can only act on one dissociated state of the substrate. Additionally, the charged state of the enzyme molecule would also change at different pH values, which could inhibit the hydrolysis reaction and affect the protein hydrolysis by the enzyme [18].

The effect of time on the enzymatic hydrolysis of SPWW is shown in Fig. 2d. When the enzymatic hydrolysis time was less than 6 h, the yield of polypeptide increased as the enzymatic hydrolysis time increased. When the enzymatic hydrolysis time exceeded 6 h, the yield of polypeptide decreased as the enzymatic hydrolysis time increased. This may have been due to the further hydrolysis of the polypeptide into amino acids, thereby reducing the polypeptide yield. Therefore, to maximise the yield and economic outcome, a hydrolysis time of 6 h was selected for subsequent

experiments.

Response surface optimisation of papain-hydrolysed SPWW

Table of factors and levels of response surface analysis

Table 1 Factors and levels of response surface analysis.

Level	A Temp. (°C)	B Concentration ratio of papain to SPWW (g/100 ml)	C pH	D Hydrolysis time (h)
−1	50	2.0	5.0	5
0	55	2.5	5.5	6
1	60	3.0	6.0	7

The factors and levels of the response surface analysis are shown in Table 1. According to the results of the single-factor experiments, peptide yield and the degree of hydrolysis were used as the response values and enzymatic hydrolysis temperature (A), enzyme to substrate concentration ratio (B), pH (C) and enzymatic hydrolysis time (D) were used as the variables. Response surface optimisation was performed for the production of peptides by the enzymatic hydrolysis of SPWW. The Box-Behnken experimental design and results are shown in Table 2.

Table 2 Experimental design and results of Box-Behnken.

No.	Factor				Peptide yield (%)	Hydrolysis (%)
	A (°C)	B (g/100 ml)	C pH	D (h)		
1	60	2.5	6.0	6	42.38	1.43
2	50	2.5	5.5	5	73.55	1.71
3	55	3.0	6.0	6	41.55	1.88
4	55	2.5	5.5	6	83.93	2.37
5	55	3.0	5.5	5	95.98	3.64
5	50	3.0	5.5	6	84.35	1.59
6	55	2.0	5.5	7	74.38	2.78
7	60	2.5	5.5	7	84.35	1.06
8	55	2.5	5.5	6	72.71	1.67
9	55	2.5	6.0	5	47.79	1.80
10	55	2.5	5.5	6	70.64	1.80
11	55	2.5	5.5	6	73.55	2.08
12	50	2.5	5.5	7	65.24	1.47
13	50	2.5	5.0	6	86.43	1.67
14	60	3.0	5.5	6	97.64	1.43
15	55	2.0	5.5	5	88.50	2.04
16	55	2.5	5.5	6	62.74	1.84
17	55	2.0	6.0	6	35.74	1.02
18	55	2.0	5.0	6	113.85	1.67
19	60	2.5	5.0	6	96.40	1.51
20	50	2.0	5.5	6	91.41	1.59
21	55	2.5	5.0	7	96.81	2.49
22	60	2.5	5.5	5	88.09	1.88
23	55	2.5	5.0	5	108.03	2.45
24	55	3.0	5.5	7	66.07	2.08
25	55	2.5	6.0	7	36.98	2.41
26	50	2.5	6.0	6	27.43	0.73
27	55	3.0	5.0	6	56.10	1.92
28	60	2.0	5.5	6	90.16	1.59
29	60	2.5	6.0	6	42.38	1.43

Regression equation model

Establishment of the regression equation and significance analysis

Using Design-Expert to perform multiple regression fitting on the results in Table 2, the multiple regression equations for peptide yield and the degree of hydrolysis in response to enzymatic hydrolysis temperature (A), enzyme to substrate concentration ratio (B), pH (C) and enzymatic hydrolysis time (D) were as follows:

$$\text{Peptide yield} = 1009.31383 - 28.66203\,A - 512.08167\,B + 295.91967\,C - 58.23267\,D + 1.454\,AB + 0.498\,AC + 0.2285\,AD + 63.56\,BC - 7.89500\,BD + 0.205\,CD + 0.20085\,A^2 + 24.23533\,B^2 - 48.88467\,C^2 + 4.81383\,D^2.$$

$$\text{Degree of hydrolysis} = -57.34725 + 2.36207\,A + 2.71167\,B - 0.44433\,C - 2.283\,D - 0.016\,AB + 0.086\,AC - 0.029\,AD + 0.61\,BC - 1.15\,BD + 0.285\,CD - 0.023807\,A^2 + 0.40433\,B^2 - 0.72067\,C^2 + 0.42358\,D^2.$$

The variance analysis results for the peptide yield and the degree of hydrolysis are shown in Table 3 and Table 4, respectively. The p-values of the model ($p < 0.01$) and the lack of fit ($p > 0.05$) indicated that the model was extremely significant while the lack of fit was not significant, respectively. The correlation coefficient (R^2) indicated that there was no significant difference between the predicted values and experiment results (Table 3, Table 4). The conditions providing the optimal predicted peptide yield and degree of hydrolysis were as follows: temperature, 53.41 °C; enzyme to substrate concentration ratio, 2.0 g/100 ml; pH, 5.0; enzymatic hydrolysis time, 7 h. The predicted peptide yield was (115.9 ± 0.506)%, which was not significantly different ($p > 0.05$) to the experimental peptide yield (115.0%). The predicted degree of hydrolysis was (2.866 ± 0.506)%, which was not significantly difference ($p > 0.05$) to the experimental degree of hydrolysis (2.801%).

Compared with our previous research on the preparation of peptides from the microbial fermentation of SPWW [9], the peptide yield in this study was higher but its antioxidant capacity was lower than that obtained using lactic acid bacterial fermentation. This may have been because lactic acid bacteria utilised part of the protein to provide the energy required by the organisms during fermentation, decreasing the availability of raw materials for peptide preparation; however, some other fermentation metabolites produced by the lactic acid bacteria enhanced the antioxidant capacity.

Antioxidant analysis

The antioxidant activity of peptides has become a research hotspot in recent years. Antioxidant peptides derived from food proteins not only have good antioxidant activity but also are extremely safe, so they are widely used to develop new functional foods or healthy products.

DPPH is one of the few free radicals that can re-

Table 3 Variance analysis of peptide yield.

Source	Sum of square	Degrees of freedom	Mean square	*F*-value	*p*-value	Significance
model	12994.26	14	928.16	8.13	0.0002	**
A	415.48	1	415.48	3.64	0.0771	
B	228.38	1	228.38	2.00	0.1790	
C	8842.76	1	8842.76	77.48	<0.0001	**
D	508.43	1	508.43	4.45	0.0533	
AB	52.85	1	52.85	0.46	0.5073	
AC	6.20	1	6.20	0.054	0.8191	
AD	5.22	1	5.22	0.046	0.8337	
BC	1009.97	1	1009.97	8.85	0.0100	**
BD	62.33	1	62.33	0.55	0.4721	
CD	0.042	1	0.042	3.68×10^{-4}	0.9850	
A^2	163.55	1	163.55	1.43	0.2512	
B^2	238.12	1	238.12	2.09	0.1706	
C^2	968.80	1	968.80	8.49	0.0113	*
D^2	150.31	1	150.31	1.32	0.2704	
Residual	1597.82	14	114.13			
Lack of fit	1367.54	10	136.75	2.38	0.2098	ns
Pure error	230.28	4	57.57			
Total difference	14592.08	28				
$R^2 = 0.8905$		$R^2_{\text{Adj}} = 0.781$		CV = 14.39%		

* Significant difference ($p < 0.05$); ** very significant difference ($p < 0.01$).

Table 4 Variance analysis of degree of hydrolysis.

Source	Sum of square	Degrees of freedom	Mean square	*F*-value	*p*-value	Significance
model	7.23	14	0.52	3.92	0.0077	**
A	1.63×10^{-3}	1	1.63×10^{-3}	0.012	0.9129	
B	0.29	1	0.29	2.16	0.1634	
C	0.50	1	0.50	3.76	0.0728	
D	0.13	1	0.13	0.96	0.3446	
AB	6.40×10^{-3}	1	6.40×10^{-3}	0.049	0.8288	
AC	0.18	1	0.18	1.40	0.2559	
AD	0.084	1	0.084	0.64	0.4377	
BC	0.093	1	0.093	0.71	0.4149	
BD	1.32	1	1.32	10.04	0.0068	**
CD	0.081	1	0.081	0.62	0.4455	
B^2	0.066	1	0.066	0.50	0.4898	
C^2	0.21	1	0.21	1.60	0.2269	
A^2	2.30	1	2.30	17.44	0.0009	**
D^2	1.16	1	1.16	8.83	0.0101	*
Residual	1.84	14	0.13			
Lack of fit	1.54	10	0.15	2.01	0.2617	ns
Pure error	0.31	4	0.077			
Total difference	9.08	28				
$R^2 = 0.7967$		$R^2_{\text{Adj}} = 0.5935$		CV = 19.64%		

* Significant difference ($p < 0.05$); ** very significant difference ($p < 0.01$).

main stable at room temperature. When DPPH comes into contact with proton donating compounds such as antioxidants, the radical is scavenged, leading to a reduction in the absorbance of its ethanol solution and a change in colour from light brownish to yellow [19]. Thus, the DPPH assay is a non-physiologically relevant assay that has been widely used to test the ability of natural compounds to act as free radical scavengers or hydrogen donors as a means of evaluating their antioxidant capacities. The ABTS assay is based on the ability of radical scavenging compounds to reduce the green-blue ABTS radical cation into the non-coloured form. Multiple enzymes have been immobilised on magnetic composites using natural cross-linking agents to affect the hydrolysis of corn cobs, producing a potential nutraceutical product with antioxidant activity [20].

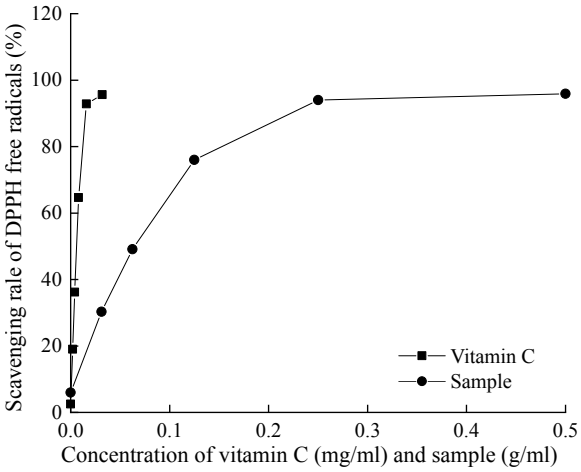

Fig. 3 DPPH free radical scavenging by peptides.

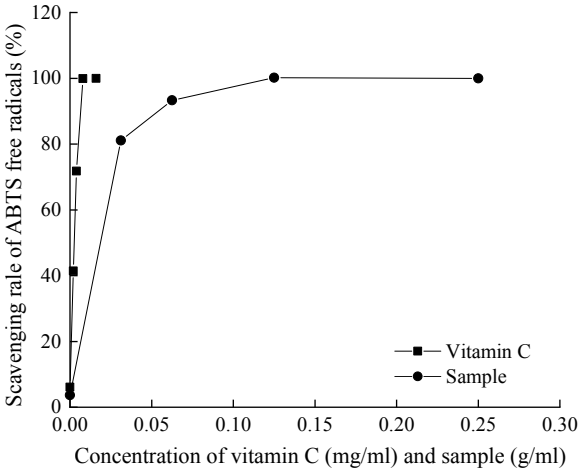

Fig. 4 ABTS free radical scavenging by peptides.

The free radical scavenging ability of the peptides produced by the hydrolysis of SPWW is shown in Fig. 3 and Fig. 4. When the peptide concentration was between 0 and 0.025 g/ml, the DPPH free radical scavenging rate increased with increasing peptide concentration. When the concentration exceeded 0.025 g/ml, the DPPH free radical scavenging rate stabilised. The calculated EC_{50} of the DPPH scavenging by peptides was 6.459 mg/ml (Fig. 3), which indicated that the peptides had some antioxidant capacity but it was lower than V_C. When the peptide concentration was below 0.0125 g/ml, the ABTS free radical scavenging rate also increased with increasing concentration. When the concentration exceeded 0.0125 g/ml, the ABTS free radical scavenging rate was close to 100%. The EC_{50} of the ABTS scavenging by peptides was 1.871 mg/ml.

CONCLUSION

Bioactive peptides have recently gained research attention as potential therapies for the management of bodily disorders and metabolic syndromes. In this study, based on single-factor experiments and the Box-Behnken design, effective antioxidant peptides were produced by enzymatic hydrolysis of SPWW with papain. This work shows the health potential of peptides produced via the enzymatic hydrolysis of SPWW.

Although increasing numbers of studies have confirmed that peptides have a scavenging effect on free radicals, to date there is no definitive conclusion about the antioxidant mechanism of antioxidant peptides and its influencing factors. Additionally, the conserved sequences of antioxidant peptides have not been determined. Thus, the relationship between antioxidant activity and the amino acids in peptides needs to be further explored. Exploring the antioxidant mechanism not only provides a theoretical basis for the structure-activity relationship but also has significance for the extraction or synthesis of antioxidant peptides with stronger effects. With the continuous elucidation of peptide structures, the in-depth study of functional relationships and the development of peptide synthesis methods, theoretical research will usher in a brighter future for the practical application of peptides.

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