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ARTICLE

Preparation and Characterization of Antioxidant Peptides from Fermented Goat Placenta

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Abstract

The goat placenta was fermented by *Bacillus subtilis* and the optimal fermentation parameters of strongest antioxidant capacity of peptides were obtained using response surface methodology (RSM). The effects of fermentation time, initial pH value and glucose content on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity of the goat peptides were well fitted to a quadric equation with high determination coefficients. According to the data analysis of design expert, the strongest DPPH radical scavenging capacity value was obtained with the following conditions: content of glucose was 2.23%, initial pH value was 7.00 and fermentation time was 32.15 h. The DPPH radical scavenging capacity commonly referring antioxidant activity showed a concentration dependency and increased with increasing peptide concentration. The effects of temperature and pH were assessed to determine the stability of antioxidant peptides prepared from goat placenta. Antioxidant peptides showed good stabilities when temperature was lower than 70°C. However, the antioxidant peptides lost antioxidant activities rapidly under alkaline and excessive acid condition. Ultrafiltration technique was performed to separate fermentation broth with different Mw (molecular weight). It was found that peptides in the range of < 3 KDa mainly accounted for the antioxidant activities.

Keywords: *Bacillus subtilis*, goat placenta, antioxidant, peptide, fermentation

Introduction

In recent years, many researchers have focused on antioxidant peptides prepared from various bio-resources (Li *et al*., 2013; Luo *et al*., 2013). Antioxidant peptides are widely used in food and medicinal materials which possess the ability to reduce oxidative damage associated with many diseases, including cancer, cardiovascular diseases and atherosclerosis (Bougatef *et al*., 2010; Chen and Li, 2012).

The role of natural antioxidants has attracted much attention. Hydrolysed peptides from animals, by-products and plant sources have been found to possess antioxidant activity, such as corn gluten meal (Zhuang *et al*., 2013), silver carp (Zhong *et al*., 2011), tilapia skin gelatin (Zhang *et al*., 2012), chickpea (Zhang *et al*., 2011a), sweet potato (Zhang *et al*., 2014) and loach (You *et al*., 2011). Goat placenta has long been used in Chinese medicine for the treatment of human organs, and the latest studies have demonstrated that it is an animal recourse rich in biological and therapeutic components (Chakraborty and Bhattacharyya, 2005; Chakraborty *et al*., 2006; Park *et al*., 2010; Teng *et al*., 2011). During processing, the water-soluble immuno-active peptides were extracted as health care ingredient; the rest was used to prepare bioactive peptides. The preparation of goat placenta peptides using enzymatic hydrolysis has been carried out; nevertheless, little information is known about goat placenta peptides prepared by fermentation. This article, the goat placenta antioxidant active peptides were prepared through fermentation with *Bacillus subtilis*.

For a long time, microbial fermentation has been one of the most important sources of proteolytic enzymes which can effectively hydrolyze proteins to prepare bioactive peptides (He *et al*., 2012). Various peptides with antioxidant and ACE-inhibitory activity derived from rapeseed, milk, peanut meal, whey protein, and they were prepared by microbial fermentation (He *et al*., 2012; Otte *et al*., 2011; Pan and Guo, 2010; Zhang *et al*., 2014). Zhang *et al*. (2011b) have reported that *Bacillus subtilis* fermentations of peanut meal are characterized by extensive hydrolysis of protein to peptides, resulting in improvement of the antioxidant properties. This article, the goat placen-

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tal peptides with immune and antioxidant activity were prepared through fermentation with *B. subtilis*.

The objective of the present work was to investigate the effects of fermentation parameters (content of glucose, initial pH and fermentation time) on DPPH radical scavenging capacity for the strongest antioxidant activity of the fermentation broth using response surface methodology (RSM). In addition, characterization of active antioxidant peptides derived from goat placenta was investigated.

Materials and Methods

Materials

Goat placenta was obtained from ewes at parturition and preserved by freezing in -45°C with immersion freezing (China). In order to protect the activity of protein in the production process, liquid nitrogen is added into the beating process of goat placentas to reduce temperature. After the beating process, the goat placentas were homogenized with five times the weight of physiological saline. The mixture was stirred for 4 h at 20°C. The water-soluble proteins were extracted by centrifugation at 2.84×*g*, and the part of precipitation was used as fermentation material (Fang *et al*., 2007). The composition of precipitation was shown in Table 1.

A freeze-dried culture of *B. subtilis* was kindly donated by Henan University of technology (China). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich (USA). All other reagents were of analytical grade and were purchased from Guangzhou Qi Yun biological technology co., LTD (China). Filtering centrifuge tubes were purchased from Millipore Co., (USA). 722S visible spectrophotometer was purchased from Electrical and Instrument Analysis Instrument Co., Ltd. of Shanghai (China).

Preparation of pre-cultures

The *B. subtilis* strain with high protease activity was rejuvenated and maintained on a nutrient agar slope at 4°C. The inoculum was prepared by adding a loopful of cells to 100 mL of sterile culture medium, which contained 10 g/L glucose, 10 g/L peptone, and then was incubated in an air bath shaker for 36 h at 37°C and rotation speed 2.84×*g*.

Fermentation experiments

The microbial cells were harvested in their exponential phase, and were transferred into the peptide production medium, which contained 1 g/L $CaCl_2$, 2 g/L Na_2HPO_4 and 25 g/L goat placentas solids. Glucose was used as the carbon source with the concentrations varied from 1 to 3 g/L. The pH varied within 5.5 to 8.5 and the fermentation time varied from 20 h to 40 h. The soluble peptides were extracted by centrifugation at 2.84×*g* and for 20 min after the fermentation progress. The other conditions were the same as the pre-culture conditions. Each experiment was conducted in triplicate.

Experiment of fermentation conditions

Based on the preliminary experiments the main factor and levels of the independent parameters were determined, and then fermentation conditions were optimized using RSM. RSM includes a group of empirical techniques to find the relationship between controlled experimental independent factors and the measured responses (dependent variables). The fermentation conditions, including initial pH of culture media (x_1), content of glucose (x_2) and fermentation time (x_3) were optimized for the antioxidant activity of the fermentation broth using a Central Composite design (design expert 8.0). The Central Composite design with three factors in one block encompassing 20 runs was used for this study (Ren *et al*., 2008).

The Central Composite design contained three levels for each process parameter, coded as -1, 0 and +1. The central design was applied based on three different initial pH (5.5, 7.0, and 8.5), as well as three different content of glucose (1, 2, and 3 g/L), and fermentation time (20, 30, and 40 h). The pre-cultured *Bacillus subtilis* cells were inoculated into 5% (w/v) reconstituted fermentation medium (adjusted to the design pH). These mixtures were incubated at the designated temperature and time. All the experiments were done in triplicate according to the experimental design (Table 2). Three second-order polynomial equations were used to express the sti- mulus index (Y). Functions of the independent variables are as follows:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij} X_i X_j + e_i \quad (1)$$

Table 1. Composition of the precipitation

Items	Moisture	Protein	Fat	Carbohydrate	Ash
Content	73.143%	6.721%	0.062%	0.017%	0.023%

Where Y is the response variable; b_0 is the constant coefficient (intercept); b_i is the linear coefficient (main effect); b_{ii} is the quadratic coefficient; b_{ij} is the two factors interaction coefficient and e_i is the random error.

Scavenging effect on DPPH free radical

DPPH method was found to be used mostly for the in vitro antioxidant activity evaluation purpose (Alam *et al*., 2013). The molecule DPPH is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole. When a solution of DPPH is mixed with that of a substrate (AH) that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color, characterized by an absorption band in ethanol solution centered at about 517 nm. The DPPH radical-scavenging capacity of goat placenta peptides was carried out using a previously method (Bey *et al*., 2013; Lim *et al*., 2010; Zhang *et al*., 2011b) with minor modification. Briefly, sample (0.5 mL) was mixed with 2.5 mL of distilled water and 3 mL 0.1 mM DPPH in ethanol and kept for 30 min in the dark at room temperature. The ab- sorbance was measured at 517 nm of the resulting solution against a blank control. Ethanol was used to calibrate the spectrophotometer. The DPPH radical-scavenging capacity was calculated as follows:

DPPH radical scavenging capacity (%)

$$RSA = \left(1 - \frac{Abs_{sample} - Abs_{sample\ control}}{Abs_{blank}}\right) \times 100 \quad (2)$$

Where Abs_{sample} is the absorbance of the sample with DPPH solution; $Abs_{sample\ control}$ is the absorbance of the sample without DPPH solution; Abs_{blank} is the absorbance of the distilled water and the DPPH solution.

Preparation of peptide fractions

Amicon filters with different Mw. were used to separate goat placenta peptides. All recovered fractions (fractions from above 100 KDa, 50 K to 100 KDa, 30 K to 50 KDa, 10 K to 30 KDa, 3 K to 10 KDa and below 3 KDa) were dissolved to the initial volume in water. The antioxidant activity was measured according the methods above.

Effect of temperature on antioxidant activity of peptides

The thermal stability of the goat placenta peptides was determined by incubating at 20, 30, 40, 50, 60, 70, 80, 90, and 100°C in a water bath for 30 min. The heated samples were immediately cooled in iced water. The remaining specific antioxidant activity was determined.

Effect of pH on antioxidant activity of peptides

The effect of pH on the stability of the goat placenta peptides was investigated from pH 2.0 to 11.0 and maintained at room temperature for 1 h. The pH was adjusted through 1 M HCl or 1 M NaOH.

Statistical analysis

All of the stimulation index and antioxidant assays in this study were conducted with three replicates and the data were expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was conducted for the analysis of the response values obtained by the RSM model.

Results and Discussion

Optimization of the fermentation conditions by RSM

RSM was used to optimize the fermentation conditions for the preparation of antioxidant peptides from goat placenta. The influence of content of glucose, initial pH and fermentation time on the DPPH radical scavenging capacity of the fermentation broth was shown in Table 2.

The following empirical regression Eq. (3) represents the DPPH radical scavenging capacity (RSA) of the fermentation broth as a function of initial pH (X_1), content of glucose (X_2) and fermentation time (X_3).

$$RSA = 63.75 + 0.87 \times X_1 + 5.14 \times X_2 + 1.23 \times X_3 - 2.47 \times X_1 \times X_2 - 1.30 \times X_1 \times X_3 + 1.75 \times X_2 \times X_3 - 13.78 \times X_1^2 - 12.06 \times X_2^2 - 3.78 \times X_3^2 \quad (3)$$

The statistic analysis for the response surface model was provided in Table 3. The p value for the model showed the model was significant and could be used to monitor the optimization ($p<0.0001$). Among the three independent variables, the p value of content of glucose was less than 0.0001, which indicated the content of glucose was the highest significant variable. The effect of fermentation time exerted the higher significant effect within a 95% confidence interval ($0.01 < p=0.0398 < 0.05$). But the effect of initial pH was not significant ($p=0.1279 > 0.05$). Therefore, it was concluded that content of glucose and fermentation time had a relatively higher significant effect on the DPPH radical scavenging capacity of the fermentation broth as compared with the initial pH. As well, the quadratic terms, X_1^2, X_2^2 and X_3^2 ($p<0.05$), as

Table 2. Experimental values for the optimization of the fermentation conditions by RSM

Run order	Coded unit			Experimental values			RSA (%)
	X_1	X_2	X_3	Initial pH	Content of glucose (%)	Fermentation time (h)	
1	1	-1	1	8.5	1	40	30.94
2	-1	-1	1	5.5	1	40	26.66
3	1	0	0	8.5	2	30	50.92
4	0	0	0	7	2	30	63.62
5	0	0	0	7	2	30	63.25
6	-1	-1	-1	5.5	1	20	25.24
7	1	1	-1	8.5	3	20	36.23
8	1	1	1	8.5	3	40	39.43
9	-1	1	1	5.5	3	40	46.42
10	0	0	0	7	2	30	62.19
11	-1	1	-1	5.5	3	20	36.62
12	0	0	0	7	2	30	65.82
13	1	-1	-1	8.5	1	20	33.34
14	-1	0	0	5.5	2	30	47.25
15	0	0	-1	7	2	20	58.92
16	0	0	1	7	2	40	59.24
17	0	1	0	7	3	30	55.26
18	0	0	0	7	2	30	66.28
19	0	0	0	7	2	30	64.92
20	0	-1	0	7	1	30	46.34

Table 3. Statistic analysis for the response surface quadratic model obtained from RSM design

Source	Sum of Squares	df	Mean Square	F Value	P	
Model	3704.271035	9	411.586	150.9040518	< 0.0001	**
A (initial pH)	7.51689	1	7.517	2.755997692	0.1279	
B (content of glucose)	264.60736	1	264.607	97.01582348	< 0.0001	**
C (fermentation time)	15.22756	1	15.228	5.583043015	0.0398	*
AB	48.8072	1	48.807	17.8947052	0.0017	**
AC	13.57205	1	13.572	4.976065696	0.0498	*
BC	24.43005	1	24.430	8.957050244	0.0135	*
A^2	521.8142188	1	521.814	191.3183221	< 0.0001	**
B^2	399.9699	1	399.970	146.6452377	< 0.0001	**
C^2	39.2931	1	39.293	14.40644906	0.0035	**
Residual	27.27466	10	2.727			
Lack of Fit	14.65552667	5	2.931	1.16137347	0.4368	
Pure Error	12.61913333	5	2.524			
Total	3731.545695	19				
Adj R^2	0.9861					
Pred R^2	0.9681					
Adeq precision	33.2979					

*Significant within a 95% confidence interval, **Significant within a 99% confidence interval.

well as the interaction term $X_1 \cdot X_2$, $X_1 \cdot X_3$ and $X_2 \cdot X_3$ ($p<0.01$) were also significant.

The ANOVA analysis for the model (Table 3) showed the "lack of fit" was not significant (p=0.4368 > 0.05), indicating that the model indeed represented the actual relationships of fermentation parameters. The model F-value of 150.904 implies the model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant. The "Pred R^2" of 0.9681 is in reasonable agreement with the "Adj R^2" of 0.9861. "Adeq Precision" measures the signal to noise ratio, a ratio greater than 4 is desirable. Adeq Precision of 33.298 indicates an adequate signal, indicating this model can be used to navigate the design space.

To determine the optimal levels of each variable for antioxidant activity peptide production, three-dimensional response surface plots were constructed by plotting the response (DPPH radical scavenging capacity of fermentation broth) on the Z-axis against any two independent variables, while maintaining other variables at their optimal

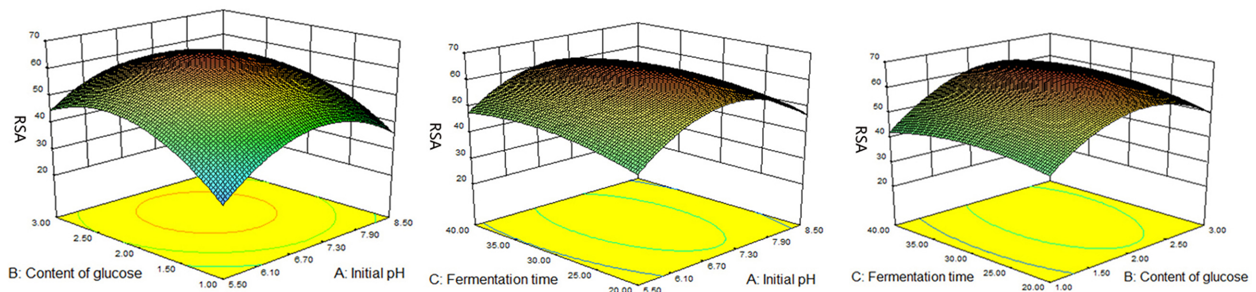

Fig. 1. Response-surface plots for the effect of variables on antioxidant activity: (a) Content of glucose and initial pH. The fermentation time was set at the centre of its level viz. 30 h; (b) Fermentation time and initial pH. Content of glucose was set at the centre of its level viz. 2%; (c) fermentation time and content of glucose. Initial pH was set at the centre of its level viz. 7.0.

levels (Fig. 1).

Optimization and model validation

According to the data analysis of design expert, the highest DPPH radical scavenging capacity value of 64.29% was obtained with the following conditions: content of glucose was 2.23%, initial pH value was 7.00 and fermentation time was 32.15 h. To confirm the validity of the model, three assays were performed under the optimal conditions. Comparative analysis of the predicted value and experimental values using paired t-test indicated no significant (p<0.01) difference between the two values, thereby establishing validity of the generated model.

Antioxidant activity of goat placenta peptides on different concentration

The antioxidant properties of goat placenta peptides are due to their free radical scavenging activities. As shown in Fig. 2, the DPPH radical scavenging capacity commonly referring antioxidant activity showed a concentration dependency and increased with increasing peptide concentration.

The DPPH scavenging activity of goat placenta peptides reached 85.43% at a concentration of 1.4 μg/mL, which was lower than that of the VC at 35 μg/mL that were used as standard (He *et al*., 2012). The activity increased steadily at the concentration range of 0-1.4 μg/mL, while reaching a maximum plateau from 1.4 to 1.8 μg/mL for goat placenta peptides. The 50% inhibition concentration (IC_{50}) value of peptides was calculated by nonlinear regression to be 0.84 μg/mL, which is lower than the 3.63 and 4.11 mg/mL for peptides produced by solid state fermentation (Wang *et al*., 2012) and is lower than the 2.62 mg/mL for a novel antioxidant peptide derived from blue mussel protein hydrolysates (Wang *et al*., 2013).

Effect of temperature on antioxidant activity of goat placenta peptides

During the processing of goat placenta peptides, concentration and drying process related to temperature were used (Escudero *et al*., 2014). In this case, it is necessary to consider the stability of the antioxidant activity among the typical temperature processing conditions. The temperature changes of the extracts were conducted by heating at different temperature for 30 min (Fig. 3).

As shown in Fig. 3, the antioxidant activity of goat placenta peptides kept steadily at the temperature range of 20-40°C, while reaching a maximum plateau from the temperature range of 40-50°C, and then showed a sharp decline between 50°C and 100°C. Low molecular weight peptides don't have the tertiary and quaternary structure. Only those proteins having molecular weight ≥ 50 KDa can form the quaternary structure; However, they still can form secondary structures, which are the key factors affecting the antioxidant activity. The extremely high temperature would affect the secondary structure, which would lead to the instability of antioxidant activity (Zhu *et al*., 2014). These results are consistent with those found in

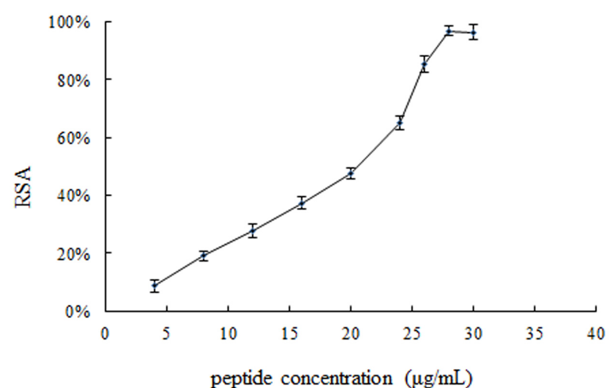

Fig. 2. Effect of peptide concentrations on antioxidant activity.

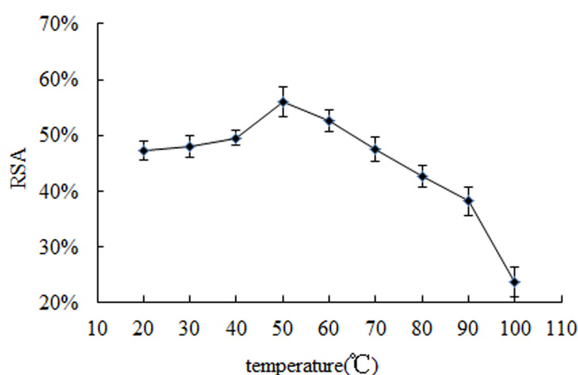

Fig. 3. Effect of temperature on antioxidant activity of goat placenta peptides.

antioxidant activity peptides derived from whey proteins which lose almost 90% of their free radical-scavenging activity after heating at 70°C for 15 min (Tong *et al.*, 2000). Also, peptides derived from curry leaves were also shown to have a reduced activity after being heated at 95°C for 20 min (Ningappa and Srinivas, 2008).

Effect of pH on antioxidant activity of goat placenta peptides

The antioxidant activity of peptides from goat placenta under different pH values is shown in Fig. 4. Peptides at the pH of 4 exhibited the strongest DPPH radical scavenging activity, and there was no significant decrease from the pH of 3 to 6. However, the DPPH radical scavenging activity of the peptides decreased significantly under the alkaline condition and excessive acidic conditions. When the pH was increased to 11, the DPPH radical scavenging activity sharply declined, the activity was reduced by 58.07% compared with that under the pH of 4. There are several factors that could account for the loss of antioxidant activity under alkaline condition. The first reason is the occurrence of racemization. Under alkaline conditions, racemization reactions will occur possibly, forming a mixture of L- and D-isomers known that differences in biological activities existed between isomers. When peptide was in alkaline condition, it is likely that racemization reaction occurs. Secondly, the loss of activity could be the result of a deamination reaction. Deamination is promoted at higher pH values resulting in changes with structure, conformation and loss of antioxidant activity. The activation energy of peptide degradation varies with changing pH is determined to the third possibility. Different pH values will affect the actual degradation pathway used. Generally speaking, each peptide has its proper pH range. During this pH range, the structure is relatively stable as well as the antioxidant activity. In addition, the side chains of some small peptides can be hydrolyzed by alkaline catalyzed (Zhu *et al.*, 2014). Therefore, alkaline conditions are unfavorable to maintain the antioxidant activity of peptides from goat placenta.

Fractionation of fermentation broth and its antioxidant activity

Ultrafiltration technique was performed to separate fermentation broth with different Mw. As shown in Fig. 5, the fermentation broth obtained at optimum parameters was then fractioned into six fractions. It was found that peptides in the range of < 3 KDa mainly accounted for the antioxidant activity. There are certain correlation between the bioactivity of goat placenta peptides and its Mw. It is recognized that, due to their lower molecular mass, peptides can be more reactive than those with higher Mw (Korhonen and Pihlanto, 2006), and the bioactivity ability of peptides is also related to the material characteristics, restricted enzyme sites and its space structure. This finding is in agreement with previous studies which support

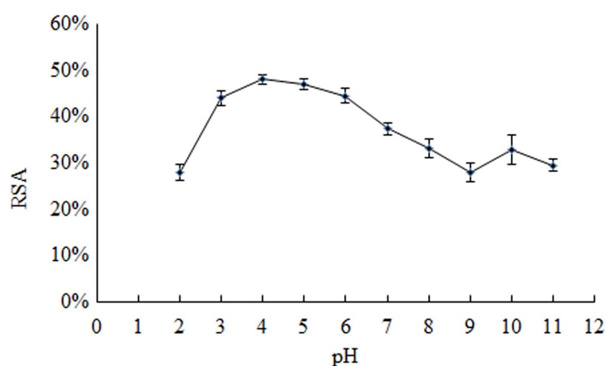

Fig. 4. Effect of pH on antioxidant activity of goat placenta peptides.

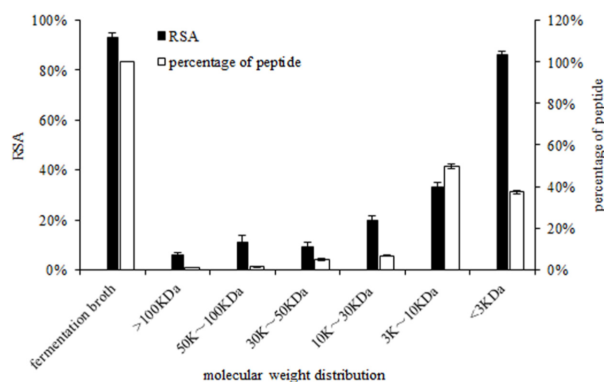

Fig. 5. Antioxidant activity of the components with different molecular weights.

the fact that the bioactivity ability of peptides is related to molecular mass (Cian *et al*., 2012; Kim *et al*., 2007; Pan and Guo, 2010; Teng *et al*., 2011; Tsai *et al*., 2008; Zhou *et al*., 2012).

Conclusions

Our study first reported the optimized fermentation conditions of antioxidant peptides from goat placenta by using response surface methodology (RSM). Therefore, it was concluded that content of glucose and fermentation time had a relatively higher significant effect on the DPPH radical scavenging capacity of the fermentation broth as compared with the initial pH. According to the data analysis of design expert, the highest DPPH radical scavenging capacity value of 64.29% was obtained with the following conditions: content of glucose was 2.23%, initial pH value was 7.00 and fermentation time was 32.15 h. The DPPH radical scavenging capacity showed a concentration dependency and increased with increasing peptide concentration. The DPPH scavenging activity of goat placenta peptides reached 85.43% at a concentration of 1.4 μg/mL. The 50% inhibition concentration (IC_{50}) value of peptides was calculated by nonlinear regression to be 0.84 μg/mL. Antioxidant peptides showed good stabilities when temperature was lower than 70°C and under the condition of moderate acidity. Peptides in the range of < 3 KDa mainly accounted for the antioxidation. These results suggested that goat placenta peptides had the potential to be used as natural antioxidants in enhancing antioxidants properties of functional foods and in preventing oxidation reactions in food processing. Further research should be done in order to purify and identify the antioxidative peptides of the fraction below 3 KDa, and more detailed studies on physiological functions, pharmacological effects and structure-activity relationship of the purified peptides will also be needed.

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