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Production optimization and characterization of immunomodulatory peptides obtained from fermented goat placenta

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Abstract

The goat placental immunomodulatory peptides were produced by fermentation with *Aspergillus Niger*. The objective of the present study was to investigate the effects of fermentation parameters (carbon source content, pH, and time) on spleen lymphocyte proliferation for the highest immune activity of the fermentation broth using response surface methodology (RSM). According to the data analysis by the Design-Expert® software, the stimulation index value (23.51%), which is the maximum immune activity, was obtained under the following conditions: content of carbon source 1.97 g·L^{-1}, initial pH 5.0, and 74.43 h of fermentation time. Under the optimized fermentation conditions, at a certain concentration range, the fermentation broth produced a significant effect on the proliferation of mouse spleen lymphocytes. Ultrafiltration technique was performed to separate the fermentation broth with different MW (molecular weight). It was found that peptides in the range of <10 KDa were the main bioactivity fractions for the immunomodulatory and antioxidant activities.

Keywords: *Aspergillus Niger*; goat placenta; immune modulation; antioxidant; bioactive peptide.

1 Introduction

Bioactive peptides play an important role in metabolic regulation and modulation, including antioxidant, antihypertensive, antimicrobial and immunomodulatory activities (Anyogu et al., 2014; Kim & Wijesekara, 2010; Korhonen & Pihlanto, 2006; Zheng et al., 2014). Immunomodulatory peptides can enhance immune cell functions such as lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis, and cytokine regulation (Singh et al., 2014). Many immunomodulatory peptides have been isolated from enzymatic digests of various food proteins such as those found in fish, soy, and whey (Halldorsdottir et al., 2014; Kong et al., 2008; Saint-Sauveur et al., 2008).

Goat placenta has long been used in Oriental medicine for the treatment of physiological abnormalities in human organs, and recent studies have demonstrated that it is a rich source of biological and therapeutic compounds (Chakraborty & Bhattacharyya, 2005; Chakraborty et al., 2006; Park et al., 2010; Teng et al., 2011). Although numerous activities of goat placental peptides have been reported so far, studies have not been reported on the immune active peptides prepared by fermentation.

Basically, bioactive peptides can be prepared from precursor proteins in multiple ways, including enzymatic hydrolysis and microbial fermentation (Zhang et al., 2014). Microbial fermentation is one of the most important sources of enzyme production. Therefore, microbial fermentation could be used as a source of proteolytic enzymes that can effectively hydrolyze proteins to prepare peptides (He et al., 2012). Various peptides with antioxidant and ACE-inhibitory activity are derived from rapeseed, whey protein, milk and peanut meal and are prepared by microbial fermentation (He et al., 2012; Otte et al., 2011; Pan & Guo, 2010; Zhang et al., 2014). In this study, the goat placental peptides with immune and antioxidant activity were prepared by fermentation with *Aspergillus Niger*.

The objective of the present study was to investigate the effects of fermentation parameters on spleen lymphocyte proliferation for the highest immune activity of the fermentation broth using response surface methodology (RSM). The mixture of immune-active peptides prepared under the optimal conditions was separated with ultrafiltration (UF) and the immune activity and antioxidant capacity of most effective fraction were determined.

2 Materials and methods

2.1 Materials and chemicals

Goat placenta was obtained from ewes at parturition and preserved by freezing at –45 °C(immersion freezing). A freeze-dried culture of *Aspergillus Niger* was kindly donated by Henan University of Technology; 6-8 week-old female BALB/c mice were purchased from the laboratory animal center of the Southern Medical University. All experimental procedures were carried out in accordance with standard guidelines for the care of animals and approved by the Welfare Committee of the Centre of Experimental Animal, Guangzhou, China.

Concanavalin A (Con A), lipopolysaccharides (LPS), and 3 - (4, 5 – dimethylthiazol – 2 - yl) - 2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co., USA. RPMI-1640 and Fetal Bovine Serum (FBS) were obtained from Gibco Co., USA. Filtering centrifuge tube was purchased

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from Millipore Co., USA. All other reagents used in this study were of analytical grade.

2.2 Preparation of pre-cultures

The *Aspergillus Niger* strain with high protease activity was reanimated 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 30 g·L^{-1} glucose, 20 g·L^{-1} peptone, 2 g·L^{-1}$MgSO_4$, 1 g·L^{-1} K_2HPO_4 and 2 g·L^{-1}KH_2PO_4 at pH 5.5, which was then incubated in an air-bath rotary shaker for 36 h at 30 °C and rotation speed 2.84 × g.

2.3 Preparation of fermentation material

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 physiological saline(five times the weight of goat placentas), the mixture was stirred for 4 hours at 20 °C. The water-soluble immune-active peptides were extracted by centrifugation at 4000 × g, and the precipitate was used as fermentation material.

2.4 Fermentation experiments

The microbial cells were harvested in their exponential phase and were transferred into the peptide production medium, which contained 1 g·L^{-1} $CaCl_2$, 1 g·L^{-1}NH_4Cl, 2 g·L^{-1} Na_2HPO_4, and 25 g·L^{-1} goat placenta solids. Glucose was used as the carbon source with the concentrations ranging from 1 to 3 g·L^{-1}. The pH varied between 4 and 6, and the fermentation time varied from 60 h to 80 h. The other conditions were the same as the pre-culture conditions. Each experiment was conducted in triplicate.

2.5 Optimization of fermentation conditions

The fermentation conditions, including the initial content of glucose (x_1) and fermentation time (x_2), pH of culture media (x_3), were optimized for the immune activity of the fermentation broth using a Central Composite design. A Central Composite design with three factors and one block and 20 runs was used in 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 content of glucose (1 g·L^{-1}, 2 g·L^{-1}, 3 g·L^{-1}), three different fermentation times (60 h, 70 h, 80 h), and initial pH (pH 4.0, pH 5.0, pH 6.0). The pre-cultured *Aspergillus Niger* cells were inoculated into 10% (w/v) reconstituted fermentation medium (adjusted to the experimental design pH). These mixtures were incubated at the designated temperature and time. All experiments were conducted in triplicate according to the experimental design (Table 1). The second-order polynomial equation was used to express the stimulus index (Y). Functions of the independent variables are as follows (Equation 1):

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

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.

Table 1. Experimental values for the optimization of the fermentation conditions by RSM.

	Coded unit			Experimental values			
Run	X_1	X_2	X_3	Glucose Content (g·L^{-1})	Fermentation time (h)	Initial pH	Stimulation index (%)
1	0	0	0	2	70	5	22.59
2	0	–1	0	2	60	5	16.02
3	–1	1	1	1	80	6	15.85
4	0	0	0	2	70	5	22.7
5	1	1	–1	3	80	4	16.83
6	0	0	0	2	70	5	22.39
7	1	–1	1	3	60	6	13.09
8	0	0	–1	2	70	4	19.48
9	1	1	1	3	80	6	17.77
10	–1	0	0	1	70	5	19.89
11	–1	–1	–1	1	60	4	11.28
12	0	1	0	2	80	5	21.88
13	0	0	0	2	70	5	22.48
14	1	0	0	3	70	5	19.81
15	0	0	1	2	70	6	20.84
16	1	–1	–1	3	60	4	8.43
17	0	0	0	2	70	5	23.16
18	0	0	0	2	70	5	21.43
19	–1	1	–1	1	80	4	19.09
20	–1	–1	1	1	60	6	12.35

2.6 Splenocyte preparation

Immunomodulation activity of the fermentation broth was evaluated by measuring their effect on spleen lymphocytes proliferation in vitro (Hou et al., 2012). The splenocytes were isolated from 6-8 week-old female BALB/c mice. The mice were sacrificed by cervical dislocation. The spleens were removed and placed in individual sterile culture dishes containing 3 mL of RPMI-1640 medium under aseptic conditions. The splenocytes were dissociated by gently pressing the organ through a 200 mesh cell strainer. The cells suspension was collected in sterile 15 ml conical tubes and centrifuged (135 × g) at room temperature for 8 min. The pellets were resuspended in 6 mL of 0.87% NH_4Cl to remove erythrocytes by osmotic shock. One minute later, twice the volume of the RPMI medium was added to stop the reaction. The mononuclear cell suspension prepared was washed twice with RPMI and centrifuged at 111×g for 8 min. Cell viability was assessed by the trypan blue exclusion method. The cell suspension was then adjusted to 2×10^6 viable cells/mL in RPMI medium (Bao et al., 2002; Yuan et al., 2009).

2.7 Splenocyte lymphocyte proliferation assay

Splenocyte lymphocyte proliferation was determined by the MTT colorimetric assay method (Hou et al., 2012). The cells were added to 96-well flat-bottom plates and treated with the fermentation broth at the same concentrations. Control wells received the same volume of the RPMI medium. The effect of the fermentation broth and the co-effect of that with Con A and LPA were also evaluated in the same experiments. The microplates with and without Con A and LPA were incubated at 37 °C under 5% CO_2 atmosphere at 90% relative humidity for 48 h. MTT (20 µl of 5 mg/ml) was added to each well of the multiwell plates, and the plates were incubated for another 4 h period under the same conditions. Next, the plates were centrifuged at 1000g for 8 min, and the medium was removed. Finally, dimethyl sulfoxide was added to each well, and the plates were agitated on a plate shaker for 10 min. Absorbance was determined at 570 nm using a PerkinElmer Victor1420 micro plate reader. Data were expressed as a stimulation index (SI) and were calculated using in the following Equations 2 and 3:

SI (without Con A and LPA)

$$SI = \frac{Fluorescence_{cells+sample} - Control_{cells}}{Control_{cells}} \quad (2)$$

SI (with Con A or LPA)

$$SI = \frac{Fluorescence_{cells+sample+ConA\ or\ LPA} - Control_{cells}}{Control_{cells}} \quad (3)$$

2.8 Scavenging effect on DPPH free radical

The DPPH radical-scavenging capacity assay of goat placental peptides was carried out using a previously method (Blois, 1958) with minor modification. Briefly, the sample (0.5 mL) was mixed with 2.5 mL of distilled water and 3 mL 0.2 mM DPPH in ethanol and kept for 30 min in the dark at room temperature. The absorbance of the resulting solution was measured at 517 nm against a blank control. Ethanol was used to calibrate the spectrophotometer. The DPPH radical-scavenging capacity was calculated as follows (Equation 4):

DPPH radical scavenging capacity (%)

$$= \left(1 - \frac{Abs_{sample} - Abs_{sample\ control}}{Abs_{blank}}\right) \times 100 \quad (4)$$

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

2.9 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 KDa to 100 KDa, 30 KDa to 50 KDa, 10 KDa to 30 KDa, 3 KDa to 10 KDa, and below 3 KDa) were dissolved to the initial volume in water. The immune and antioxidant activity were measured according the methods described above.

2.10 Statistical analysis

The stimulation index determinations and the antioxidant assays were conducted in 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.

3 Results and discussion

3.1 Optimization of the hydrolysis conditions by RSM

RSM was used to optimize the fermentation conditions for the preparation of goat placenta bioactivity peptides. The influence of glucose content, fermentation time, and initial pH on the spleen lymphocyte proliferation of the fermentation broth was shown in Table 1. Statistical analysis of the response surface model is shown in Table 2. The p value shows that the model was significant and could be used to monitor the optimization of the hydrolysis conditions ($p<0.0001$). Among the three independent variables, the p value of fermentation time was less than 0.0001, which indicates that the fermentation time was the variable with the highest significance level. The effect of initial pH had the most significant effect within a 99% confidence interval ($p= 0.0064 < 0.01$). However, the effect of glucose content was not significant ($p=0.1000 > 0.05$). Therefore, it was concluded that fermentation time and initial pH had a greater significant effect on the spleen lymphocyte proliferation of the fermentation broth as compared with that of the glucose content. The quadratic terms, $X_1 \cdot X_1$, $X_2 \cdot X_2$ and $X_3 \cdot X_3$ ($p < 0.05$) and the interaction terms $X_1 \cdot X_3$, $X_2 \cdot X_3$ ($p < 0.01$) were also significant.

The following empirical regression (Equation 5) represents the stimulation index (Y) of the fermentation broth as a function of glucose content (X_1), fermentation time (X_2), and initial pH (X_3). The ANOVA for the response surface quadratic model is shown in Table 2.

$$Y=22.42-0.25\times X_1+3.02\times X_2+0.48\times X_3+0.22\times X_1\times X_2+ 0.97\times X_1\times X_3-1.00\times X_2\times X_3-2.50\times X_1^2-3.40\times X_2^2-2.19\times X_3^2 \quad (5)$$

Table 2. Statistical analysis for the response surface quadratic model of the RSM design.

Source	Sum of Squares	df	Mean Square	F Value	P
Model	352.3192966	9	39.14658851	200.8935556	<0.0001**
A-content of glucose (%)	0.64009	1	0.64009	3.284831728	0.1000
B-fermentation time	91.50625	1	91.50625	469.5943278	<0.0001**
C-initial pH	2.29441	1	2.29441	11.77451728	0.0064**
AB	0.3916125	1	0.3916125	2.009687958	0.1867
AC	7.5466125	1	7.5466125	38.72791667	<0.0001**
BC	8.0601125	1	8.0601125	41.3631103	<0.0001**
A^2	17.21250909	1	17.21250909	88.33163458	<0.0001**
B^2	31.82400909	1	31.82400909	163.3153381	<0.0001**
C^2	13.21118409	1	13.21118409	67.79752326	<0.0001**
Residual	1.948623409	10	0.194862341		
Lack of Fit	0.317940076	5	0.063588015	0.194973524	0.9515
Pure Error	1.630683333	5	0.326136667		
Total	354.26792	19			
R- squared	0.9945				
Adj R- squared	0.9895				
Pred R-squared	0.9856				
Adeq precision	45.008				

**Significant within a 99% confidence interval.

The ANOVA analysis for the model (Table 2) showed that the "lack of fit" was not significant (p=0.9515 > 0.05), indicating that the model indeed represented the actual relationships of fermentation parameters. The F-value of the model was 200.89, which indicates that the model is significant. Values of "Prob > F" less than 0.0500 indicate that the model terms are significant. The "Pred R-Squared" of 0.9856 is in reasonable agreement with the "Adj R-Squared" of 0.9895. "Adeq Precision" measures the signal to noise ratio; a ratio greater than 4 is desirable. Adeq Precision of 45.008 indicates an adequate signal showing that this model can be used to navigate the design space.

To determine the optimal levels of each variable for immune activity peptide production, three-dimensional response surface plots were constructed by plotting the response (stimulation index of fermentation broth) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels (Figure 1). As shown in Figure 1, there is a close relationship between the stimulation index and initial pH or glucose content. Excessively high and low content of glucose or initial pH led to a decrease in the stimulation index. When initial pH or glucose content was similar to that of the medium, the stimulation index increased. Fermentation time had a positive linear effect on the stimulation index. However, the negative quadratic effect became significant during long time fermentation.

3.2 Optimization and model validation

According to the data analysis by the Design-Expert® software, the highest stimulation index value of 23.51% was obtained under the following conditions: fermentation time of 74.43 h, initial pH value of 5.00, and glucose content of 1.97%. To confirm the model validity, 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.

3.3 Immune activity of fermentation broth

The immunoactivity effect of the fermentation broth was evaluated in vitro by measuring its effect on lymphocyte proliferation. As shown in Figure 2, the stimulation index commonly associated with immune activity increased in a concentration dependent way. The stimulation index increased quickly from the concentration of 6.25 mg/mL to 100 mg/mL, and at the concentration of 6.25-100 mg/mL; the stimulation index of the fermentation broth showed a good linear relationship with a linear R^2 of 0.991. Then, the stimulation index increased gradually with the increase in concentration of 100-200 mg/mL, in agreement with enzymatic hydrolysates of Alaska Pollock frame according to the study reported by Hou et al. (2012). These results suggested that the fermentation broth might act as a mitogen for murine splenic lymphocytes.

3.4 Effect of fermentation broth with Con A or LPS on spleen lymphocyte proliferation

Well-known as T-cell and B-cell mitogens, Con A and LPS are used as the positive control, markedly stimulated the proliferation of murine splenic lymphocytes. The effects of the fermentation broth with different concentration on lymphocytes proliferation were evaluated in stimulated cells (with mitogens, Con A or LPS), as shown in Figure 3. The fermentation broth had the synergistic activity with Con A and LPS. At the concentration of 6.25-25 mg/mL, the synergistic effect of the fermentation broth with Con A was better than that of LPS; however, at the concentration of 50-200 mg/mL, contrary results were obtained.

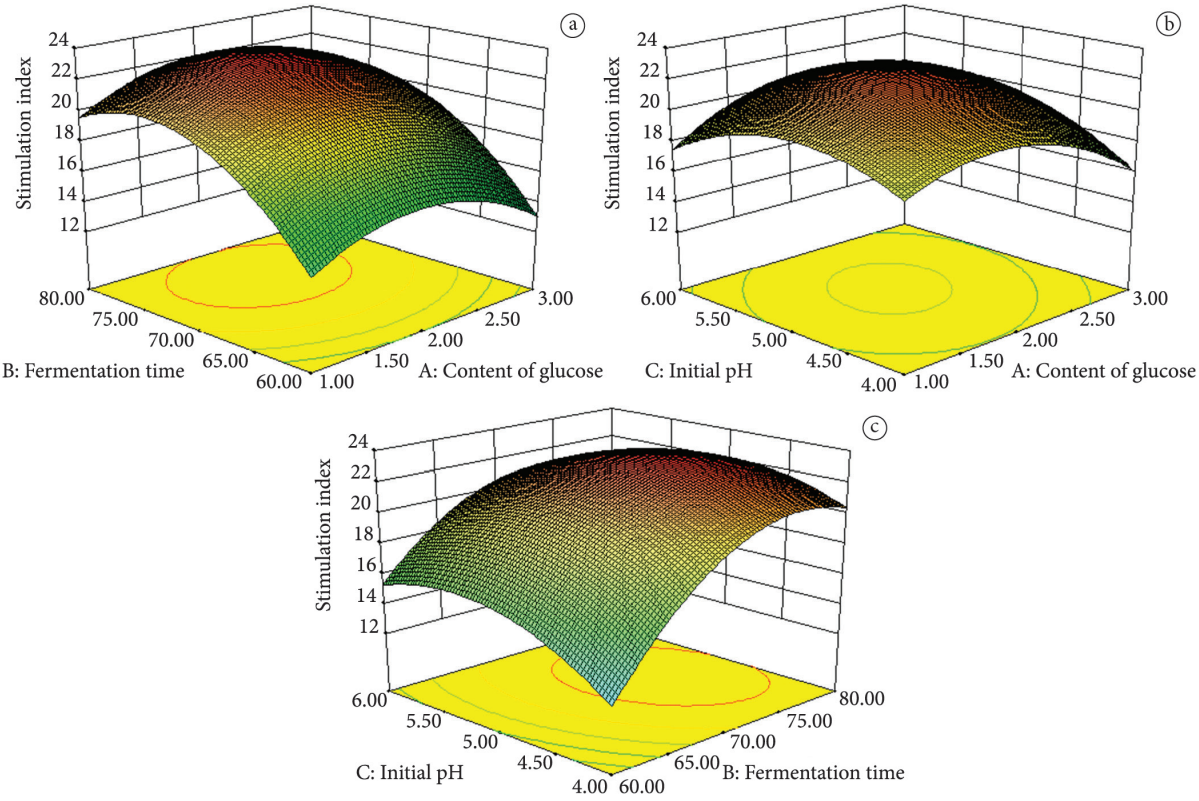

Figure 1. Response-surface plots for the effect of variables on immune activity: (a) Glucose Content (A) and fermentation time (B). The initial pH (C) was set at the centre of its level viz. 6.0; (b) Glucose Content (A) and initial pH (C). Fermentation time (B) was set at the centre of itslevel viz. 70h; (c) fermentation time (B) and initial pH (C). Glucose Content (A) was set at the centre of its level viz. 2%.

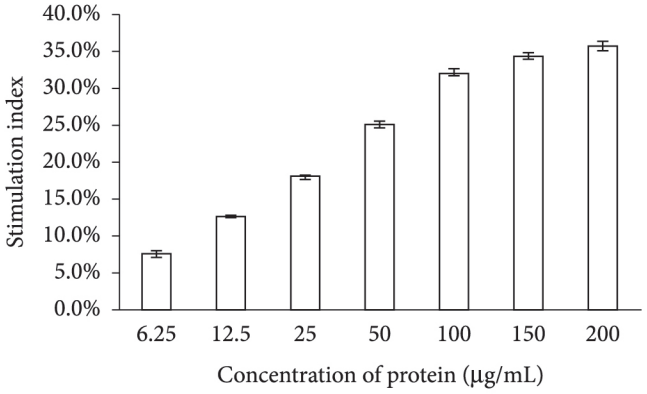

Figure 2. Effect of fermentation broth with different protein concentrations on spleen lymphocyte proliferation.

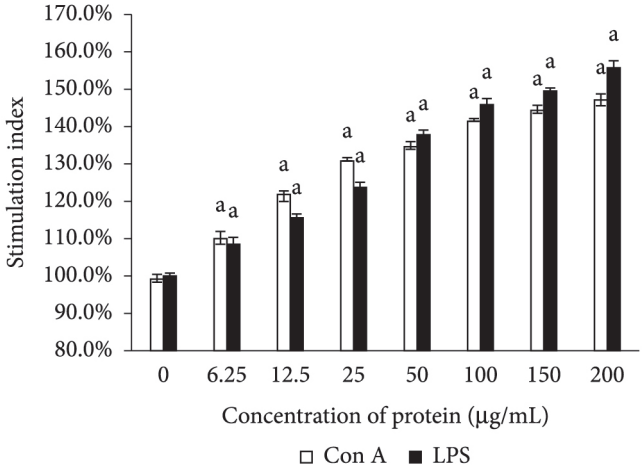

Figure 3. Effect of the fermentation broth with LPS and Con A on spleen lymphocyte proliferation. The concentration of LPS or Con A was 10 mg/mL; each value is expressed as mean ± SD of 6 separate experiments; P values are shown as $^{a}p < 0.05$, $^{aa}p < 0.01$compared with those of the control group.

3.5 Fractionation of fermentation broth and its bioactivity

Ultrafiltration technique was performed to separate the fermentation broth with different MW. As shown in Table 3, the fermentation broth obtained at optimum parameters was then fractioned into six fractions. It was found that peptides in the range of 3KDa-10 KDa were the main bioactivity fractions for the immune activity and peptides in the range of 3K-10KDa, and <3KDa showed the highest antioxidant activity. Immunoregulatory peptides in the range of 3KDa-10 KDa extracted from goat placenta could enhance humoral immunity function and recover the ability of humoral immunity in mice with immunodeficiency (Liaoqiong et al., 2005). Bezerra et al. (2013) demonstrated that the activity level was higher in permeate peptides (<3KDa) than in retentate peptides (>3KDa). These authors used ultrafiltration technique to evaluate the

influence of peptides molar mass molecular weight on biological activities. The analyzed peptides were present in caprine casein hydrolysates, which were obtained by papain enzyme use.

There is a certain correlation between the bioactivity of goat placental 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 & Pihlanto, 2006), and the bioactivity of peptides is also related to their space structure, the characteristics of precursor proteins, and restricted enzyme sites. This finding is in agreement with those of previous studies which support the fact that the bioactivity of peptides is related to molecular mass (Cian et al., 2012; Kim et al., 2007; Pan & Guo, 2010; Teng et al., 2011; Tsai et al., 2008; Zhou et al., 2012).

4 Conclusion

Goat placental protein was effectively fermented using *Aspergillus Niger* to obtain peptides with strong immunoactivity. It was shown that a second-order polynomial model was sufficient to properly describe and predict the responses of immunoactivity of the fermentation broth. The linear term of fermentation time and initial pH and the quadric term of initial pH along with the interaction of fermentation time and glucose content significantly affected the immunoactivity of the fermentation broth. The stimulation index value for the fermentation broth increased with the increase in concentration of 6.25-200 mg/mL. The fermentation broth had the synergistic activity with Con A and LPS. Peptides in the range of <10 KDa showed the highest immunoactivity and antioxidation. These results suggest that the fermentation broth might act as a mitogen for murine splenic lymphocytes.

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