OPTIMIZING PARAMETERS FOR OBTANING AN ANTIHYPERTENSIVE PEPTIDE FROM SHEEP BONE PROTEIN BY ENZYMATIC HYDROLYSIS

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Introduction

The rapid growth of meat-processing leads to large amounts of animal bones with high quality components that may be used for human consumption. These by-products are important protein and mineral sources, and they can be converted to value-added products by enzymatic hydrolysis, which is widely applied to improve and upgrade the functional and nutritional properties of proteins. Recently, several studies have been reported on utilization of fish[1], chicken[2] and veal[3] by-products by enzymatic hydrolysis for the recovery of all valuable components, but few data on sheep bone.

Bioactive peptides can be released by enzymatic proteolysis of animal bone proteins and may act as potential physiological modulators of metabolism during the intestinal digestion of the diet. The possible regulatory effects of peptides relate to nutrient uptake, osteoporosis defense, bacteria inhibitor, skin tender and antihypertensive activities. Sheep bone, one of the major fractions of sheep by-product, contains c.a.12% protein which could be good candidate as nutraceuticals. Therefore, the objective of this study was to optimize parameters of enzymatic hydrolysis to obtain more active antihypertensive peptide.

Materials and Methods

Materials: sheep bones was kindly donated by ZhongAo Food Co. (TianJin, China), and stored at -25℃ until use. Commercially available food grade enzyme was procured from Aono Pharmaceutical Co.; angiotensin converting enzyme (ACE) , hippuryl-histidyl-leucine (HHL) as a substrate peptide of ACE from Sigma Co; Ultrafiltration membrane (UF) reactor were purchased from Millipore Co. All other reagents used were of Analar grade.

Methods: To produce antihypertensive peptide from sheep bone, enzymatic hydrolysis was firstly performed using various enzymes with their optimal conditions, and then reaction parameters of selected enzyme was optimized using a statistical experimental design technique called the response surface methodology (RSM). ACE inhibition activity (y) was considered as the dependent output variable. The different levels of independent variables are given in Table2. A 2^4 full factorial central composite rotatable design (CCRD) for four independent variables each at five levels was adopted in this study. A total of 30 experiments were necessary for the estimation of the 14 coefficients of the model. Sheep bone samples was pretreated mainly under a pressure of 0.1MPa with steam for 1.5 h and subsequent powdered by crusher to be less than 250 um in diameter. A mixture of 1share of the bone powder and 4 share of sterilized water were considered as potential hydrolytes. The ratio of enzyme to substrate solution was 1:100 (w/v). The enzymatic hydrolysis was stopped by boiling for 5 min. The degree of hydrolysis (DH) was estimated by measuring the nitrogen content soluble in 10% trichloroacetic acid (TCA) as discussed by Kim et al[4]. Ultrafiltration system：large molecular weight fraction was removed from sheep bone hydrolysates by ultrafiltration at 4℃by PM-10 membrane. The ACE inhibitory activity was measured by the method of Cushman and Cheung[5] with slight modifications.

Results and discussion

Optimization of protease: DH determined at various incubation times with aforementioned enzymes is shown in Fig.1. After 24 h incubation, all hydrolysates showed over 50% of DH. In particular, neutrase showed a higher DH level at all incubation times and a high ACE inhibition activity at 6h (table1). Although pepsin showed the highest DH level, it's reaction condition is not mildness. Thus, neutrase was selected as potent protease for further research.

Optimization of reaction parameters of neutrase: The application of RSM yielded the following regression equation which is an empirical relationship between the response variable and the test variables in coded units:

$$Y=95.08-0.42X_1-0.92X_2-5.91X_3+6.49X_4-4.75X_1^2-14.42X_2^2-4.51X_3^2-13.39X_4^2-1.31X_1X_2-5.07X_1X_3-15.23X_1X_4-7.42X_2X_3+8.51X_2X_4-1.80X_3X_4$$

(R^2=0.9019; F-Value(model)=9.85; (Prob > F) < 0.0001)

The coefficient of determination (R^2) is a measure of total variation of observed ACE inhibition activity values about the mean explained by the fitted model. The value of R^2 is 0.9019 suggesting that observed variation in ACE inhibition activities can be explained by the fitted mode. The Model F-value of 9.85 implies the model is significant. There is only a 0.01% chance that a "Model F-Value"could occur due to noise. The multistage Monte Carlo optimization was used to solve coded values of the independent variables. The optimum conditions were

found to be as amount of enzyme, 250U/g, Concentration of substrate, 0.29%; temp., 45℃; time, 5.75h. In addition, this technique was instrumental in understanding the interaction effect between amount of enzyme and other parameters. The contour plots that give the variation of ACE inhibition activity with independent variables are shown in Fig.2. Each contour curve represent an infinite number of combinations of two test variables with the other two maintained at their respective zero-level. As can be seen ACE inhibition activity is mainly dependent of the enzyme amount and reaction time. There is a strong interaction effect between the enzyme amount and reaction time and temperature, while the interaction effect between the enzyme amount and concentration of substrate is almost negligible.

Table 1. ACE inhibitory activity of enzymatic hydrolysates at different incubation hours at optimum temperatures (unit:%)

protease	Hours				
	3	6	9	12	24
a-chymotrypsin	23.6	50.2	0	0	0.74
neutrase	31.2	62.8	17.5	0	0
papain	1.63	40.0	3.86	0	0
animal protease	0	0	0	0	0.82
pepsin	25.8	55.4	0	0	0

Table2. Experimental range and levels of independent variables

Xi	range and levels				
	-2	-1	0	1	2
X_1(u/gbonepowder)	100	150	200	250	300
X_2(%)	0.15	0.20	0.25	0.3	0.5
X_3(˚C)	40	45	50	55	60
X_4(h)	4	5	6	7	8

X_1, amount of enzyme; X_2, Concentration of substrate ;
X_3, reaction temperature; X_4, reaction time

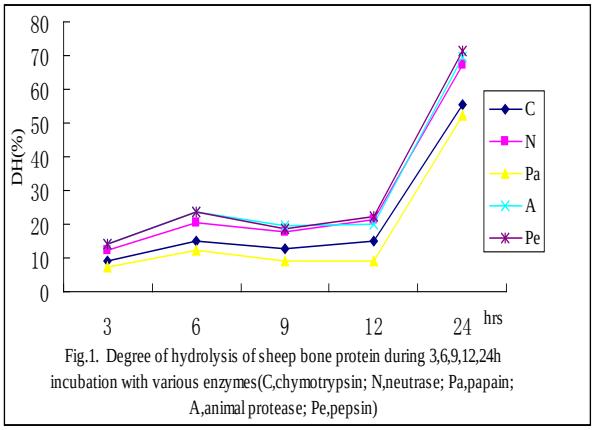

Fig.1. Degree of hydrolysis of sheep bone protein during 3,6,9,12,24h incubation with various enzymes(C,chymotrypsin; N,neutrase; Pa,papain; A,animal protease; Pe,pepsin)

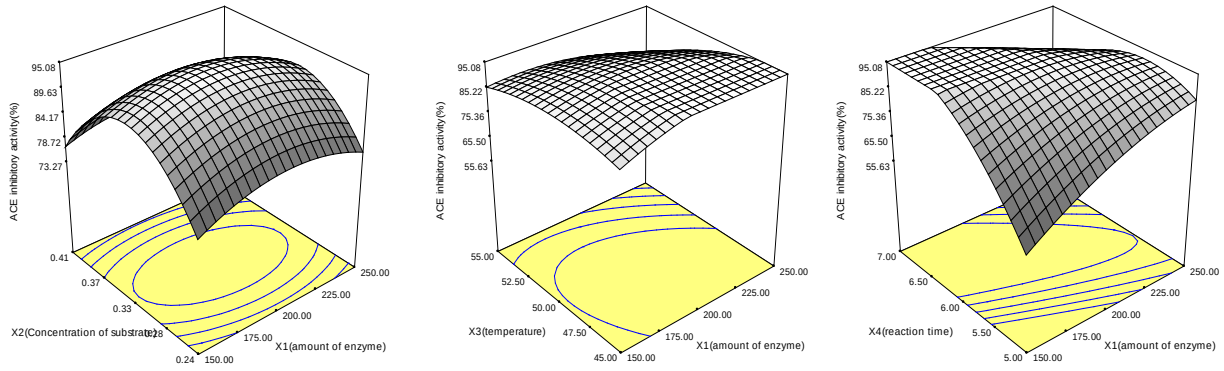

Figure 2. Surface response plot showing the interaction effect of independent variables

Conclusions

The enzymatic hydrolysis parameters of sheep bone were successfully optimized using response surface methodology. The optimum conditions were found to be as amount of enzyme, 250U/g, Concentration of substrate, 0.29%; temp., 45℃; reaction time, 5.75h. The actual maximum ACE inhibitory activity of sheep bone protein hydrolysates with under 10 kD size peptide separations after ultrafiltration is 89.92% . Further studies on purification and identification of angiotensin converting enzyme inhibitory peptides remain to be done.

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