XVII[th] World Congress of the International Commission of Agricultural and Biosystems Engineering (CIGR)

Hosted by the Canadian Society for Bioengineering (CSBE/SCGAB)
Québec City, Canada June 13-17, 2010

APPLICATION OF RESPONSE SURFACE METHODOLOGY TO THE OPTIMISATION OF *IN VITRO* ENZYMATIC DIGESTION OF SOY PROTEIN ISOLATE OF HIGH HYDROSTATIC PRESSURE PROCESSING

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CSBE101313 – Presented at Section VI: Postharvest Technology, Food and Process Engineering Conference

ABSTRACT Enzymatic digestion of soy protein isolate (SPI) using high hydrostatic pressure processing was studied by response surface methodology. A central composite design (CCD) with three independent variables: pre-treatment pressure (337, 400, 500, 600 and 663MPa), pepsin-substrate ratio (0.16, 0.5, 1.0, 1.5 and 1.8%) and pancreatin-substrate ratio (1.3, 2.0, 3.0, 4.0 and 4.7%) were used to study the response variable (degree of hydrolysis, DH). A predictive polynomial quadratic equations model was developed in SAS 6.0 software. Regress equations, response analysis, and the mathematical model showed good fit with the experimental data. The R^2 value indicated that 96.6% of the variability within the range of values studied could be explained by the model. A pre-treatment pressure of 590MPa, a pepsin-substrate ratio of 1.1 % and a pancreatin-substrate ratio of 3.2% were the optimal conditions achieving the highest DH.

Keywords: High hydrostatic pressure; Soy protein isolate; *In vitro* enzymatic digestion, Response surface methodology; Optimisation

INTRODUCTION Soybean proteins have been widely applied in the food industry as an important ingredient due to their nutritious and desirable bioactive properties. Enzymatic soybean protein digests are absorbed more readily in the intestine than intact proteins (Ziegler et al., 1998), thus making them a more nutritional source of amino acids or peptides. However, most peptide bonds in soybean protein are located in the interior of globular proteins making them inaccessible for proteolytic enzyme digestion. This means that moderate pre-treatment is required for protein denaturation to expose a greater number of peptide bonds. Traditional heat treatment effectively promotes the hydrolysis of soy protein, but it damages the covalent bonds causing a decrease in the taste and nutritive value of protein.

The industrial process of high hydrostatic pressure (HHP) is one of the most promising non thermal techniques for food preservation in the last decade. (Arroyo,Sanz, and Pre′stamo, 1997, 1999; Cheftel,1995; Pre′stamo,Arabas,Fonberg-Broczek, and Arroyo, 2001), In HHP, pressure can be applied at ambient or moderate temperatures and is

transferred throughout food instantly and uniformly. Therefore HHP treatment is independent of food size and geometry. The low energy levels involved in pressure processing may explain why covalent bonds of food constituents are usually less affected than weak interactions (Cheftel and Culioli, 1997). Most often, it produces a decrease in microbial populations, and normally maintains the nutritional and sensory properties of the food as the raw products (Morild,1981; Rademacher, 1998; Hinrichs and Kessler,1998). Recently, HHP treatment has been used in the enzymatic hydrolysis of proteins by enhancing the proteolysis, as has been reported by several authors (Bonomi,et.al., 2003; Maynard, Weingand and Hau et.al, 1998; Stapelfeldt, Petersen, Kristiansen, Qvist and Skibsted, 1996). The application of pressure less than 800 MPa leads to the disruption of the native structures of most proteins in solution because the volume of the system becomes smaller when the protein adopts an unfolded conformation. Protein unfolding can expose new cleavage sites to enzymatic hydrolysis making it vulnerable to proteolysis (Tabilo-Munizaga, Moyano, Simpson, Barbosa-Canovas, and Swanson, 2004).

Response surface methodology (RSM) has become a popular and successful method for optimization of the enzymatic hydrolysis process (Bhaskar, Benila, Radha, and Lalitha, 2008; Xia Wang, and Xu, 2007; Ferreira-Dias, Correia, and Fonseca, 2003; Ribeiro ,Manha, and Brito, 2006; Kapat, Rakshit, and Panda,1996; King, 1993). It is a combination of a mathematical and a statistical techniques used to evaluate the relative significance of several factors affecting the system with a minimum number of experiments and even works in the presence of complex interactions (Giovanni, 1983). The main objective of RSM is to determine the optimal operational conditions of the process or to locate a region that meets the operating specifications.

The objective of this research was to study and optimize *in vitro* enzymatic digestion of soy protein isolate of high hydrostatic pressure processing by pepsin and pancreatin. The goal is to produce a high degree of hydrolysates (DH). Three independent factors: pre-treatment pressure, pepsin-substrate ratio and pancreatin-substrate ratio were investigated according to the central composite design (CCD) with the aim of assessing the effects of these variables and their interactions on the degree of hydrolysis.

2 Materials and methods

2.1 Materials The SPI was obtained from Harbin High –Tech(Group)Co., Ltd and contains a 91.4% protein content (dry basis). To perform *in vitro* enzymatic digestion, pepsin (catalog # P-6887) from porcine stomach mucosa and pancreatin (catalog # P-1625) from porcine pancreas were obtained from Sigma-Aldrich (Oakville, Ontario) and prepared in 0.01 M HCl and phosphate buffer pH 7.0, respectively. All reagents were of analytical grade.

2.2 High hydrostatic pressure treatment HHP processing was carried out in a 3L reactor unit equipped with temperature and pressure regulation. The maximum pressure level achievable by the equipment is 800MPa. The pressure chamber, which contained the sealed bags with the SPI solutions, was filled with oil that used as the pressure transmitting medium in the vessel. The temperature of the pressurization medium was monitored by a thermocouple attached to a data logger during the pressure treatment.

Temperature was being kept constant at 20℃ during the pressure processing. Prior to pressure processing, the SPI were dissolved in double distilled water to make 5% solutions (w/v) with a suitable volume are vacuum-conditioned in a polyethylene bag. In each experiment, the indicated pressure was achieved within 2-3 min, held for 20 min and released to atmospheric pressure within 2-3 min. Each sample was run in triplicate. After HP treatment, the protein solutions were then freeze-dried, lyophilized proteins were placed into aliquots in polyethylene bag (5mL) and stored in a deep freezer at -80℃ for further *in vitro* enzymatic digestion. Repeated freeze thaw cycles were avoided.

2.3. *In vitro* enzymatic digestion The *in vitro* enzymatic digestion method to simulate the *in vivo* gastrointestinal digestion of milk proteins was performed as previously described by Vilela et al. (2006). The freeze-dried SPI were diluted in double distilled water to a concentration of 3 mg protein/mL and the pH of the solution was adjusted to 1.5 with HCl by drop-wise addition of 1.0 M HCl. The 0.3 percent (w/v) SPI solution were placed into 10 ml plastic tubes, which were added into a shaking water bath at 37℃. Pepsin solution (5 mg/ml in 0.01 M HCl) was added to the protein solutions to start the reaction. By the end of 30 min, 2 drops of 1.0M NaOH were added to terminate the enzymatic reaction. The peptidic digests were placed into a water bath at 40^{o}C and the pH was adjusted to 7.8 for the optimal reaction of the pancreatin enzyme. Freshly prepared pancreatin stock solution (5 mg/ml in sodium phosphate buffer pH 7.0) was added to the digests, which were incubated in a water bath for 60 min with gentle shaking. By the end of 60 min digestion, 70 µl of 150 mM Na_2CO_3 solution was added to the pancreatic digests to interrupt the enzymatic digestion and the tubes were placed in an ice bath to rapidly decrease the temperature of the solution of the pancreatic hydrolysates.

2.4. Experimental design The effect of three independent variables X_1 (pre-treatment pressure), X_2 (pepsin-substrate ratio), and X_3 (pancreatin-substrate ratio) at five levels on degree hydrolysates of SPI (dependent variable) were investigated by RSM, using a central composite experimental design. In this regard, a set of 19 experiments was carried out with five replicates at the center of the design. The center point and the different levels considered in central composite design are given in Table 1.

Table 1.Coded levels of the experimental factors used in central composite design

variables	coded	Range and levels				
		-r(-1.682)	-1	0	1	r(1.682)
Pre-treatment pressure MPa	x_1	337.2	400	500	600	662.8
Pepsin-substrate ratio %	x_2	0.159	0.5	1.0	1.5	1.841
Pancreatin-substrate ratio %	x_3	1.318	2.0	3.0	4.0	4.682

The full experimental design, with respect to the real values of the independent variables and attained values for the response (DH) is presented in Table 2.

Table 2.Central composite design for independent variables X_1 (pre-treatment pressure),

X_2 (pepsin -substrate ratio), X_3 (pancreatin-substrate ratio) and their response (DH,%) (n=3).

Run No.	Variables levels			Observed response
	X_1	X_2	X_3	Y
	pre-treatment pressure (MPa)	pepsin-substrate ratio (%)	pancreatin-substrate ratio (%)	DH （%）
1	1(600)	1(1.5)	1(4)	78.38
2	1(600)	1(1.5)	-1(2)	70.46
3	1(600)	-1(0.5)	1(4)	69.28
4	1(600)	-1(0.5)	-1(2)	63.22
5	-1(400)	1(1.5)	1(4)	70.61
6	-1(400)	1(1.5)	-1(2)	65.12
7	-1(400)	-1(0.5)	1(4)	64.80
8	-1(400)	-1(0.5)	-1(2)	56.97
9	1.682(663)	0(1.0)	0(3)	85.98
10	-1.682(337)	0(1.0)	0(3)	69.08
11	0(500)	1.682(1.8)	0(3)	76.32
12	0(500)	-1.682(0.2)	0(3)	55.89
13	0(500)	0(1.0)	1.682(4.7)	71.06
14	0(500)	0(1.0)	-1.682(1.3)	58.53
15	0(500)	0(1.0)	0(3)	81.84
16	0(500)	0(1.0)	0(3)	82.19
17	0(500)	0(1.0)	0(3)	79.71
18	0(500)	0(1.0)	0(3)	80.55
19	0(500)	0(1.0)	0(3)	78.40

All experiments were carried out in randomized order to minimize the effect of unexplained variability in the observed responses due to extraneous factors (Wanasundara and Shahidi, 1996).

2.5. Determination of the degree of hydrolysis The degree of hydrolysis (DH, %) during digestion process was determined by the TCA-NSI method (Iwami,Sakakibara and Ibuki, 1986), with some modifications. A 10mL volume of digested mixtures were mixed with 10m L of 10% (w/v) TCA. The mixture was incubated for 30 minutes at room temperature, then the insoluble protein was centrifuged at 5,000×*g* for 30 min and washed with 10mL of TCA(10%,w/v). Protein precipitates were obtained a second time by centrifugation under the same conditions. The TCA-soluble fraction and the supernatant of reaction mixture were each analyzed for nitrogen to determine the protein content by the micro- Kjeldahl method (N×6.25). The results were reported as:

$$\text{TCA soluble N}(\%) = \frac{(N_0 - N_t) \times 100}{N_{tot}} \quad (1)$$

Where t is the digestion time(min), N_t (mg) is the TCA-insoluble N after the digestion for t min, N_o is the TCA-insoluble N in the protein sample, and N_{tot} (mg) is the total N in protein sample. All results are reported as means of triplicates.

2.6. Statistical analysis Data of central composite design (Table 2) were analyzed for response surface regression according to Wanasundara and Shahidi (1996) using SAS software (SAS Institute Inc. 6.0, from Statsoft, USA). RSM usually contains three steps: (1) design and experiments; (2) response surface modeling through regression; (3) optimization. The main objective of RSM is to determine the optimal operational conditions of the process or to determine a region that meets the operating specifications (R.H. Myers, and D.C. Montgomery, 2001). A quadratic polynomial regression model was assumed for predicting Y response, the model proposed for response (Y) was:

$$y = (b_0 + \varepsilon) + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} b_{ii} x_i^{\,2} + \sum_{i=1}^{3} \sum_{j=i+1}^{3} b_{ij} x_i x_j \qquad (2)$$

where b_0 is the value for the fixed response at the central point of the experiment, b_i, b_j, b_{ii} and b_{ij} are the linear, quadratic and cross product coefficients. Both linear and quadratic effects of the three variables under study, as well as their interactions on degree of hydrolysis were calculated. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Furthermore, three-dimensional surface and contour plots were developed using the fitted full quadratic polynomial equations obtained by holding one of the independent variables at a constant value and changing the levels of the other two variables. This procedure illustrates the relationship between the response and experimental levels of each factor and to deduce the optimum conditions fitted to the experimental data.

3 Results and discussion

3.1 Model fitting To evaluate the degree of hydrolysis (see Table 2), the application of RSM yields the second order regression Eq.(3), which represents an empirical relationship between the response (DH) and the tested variables (in coded units):

$$Y(\%) = 80.59 + 3.827x_1 + 4.743x_2 + 3.542x_3 - 1.373x_1^{\,2} - 0.298x_1x_2 - 5.411x_2^{\,2} + 0.0825x_1x_3 - 0.06x_2x_3 - 5.874x_3^{\,2} \qquad (3)$$

Where is the Y is predicted response (DH, %); x_1 the coded value of variable X_1 (pre-treatment pressure), x_2 the coded value of variable X_2 (pepsin-substrate ratio)and x_3 the coded value of variable X_3 (pancreatin-substrate ratio).

An analysis of variance (ANOVA) tested for the effects of the variables and their possible interactions (Table 3). ANOVA for model terms and its significance ($p \leq 0.05$) showed that the largest effect has the linear term of pepsin-substract (x_2), followed by the linear term of pre-treatment pressure (x_1) and the linear term of pancreatin-substract (x_3) .The interaction terms did not have significant influence ($p>0.1$), which means that the interaction between the different factors did not influence the response.

Table 3. ANOVA regression coefficients testing for degree of hydrolysis.

Variables	Regression coefficient	Standard error	t-value	p-value

Intercept	80.59	1.054	76.46	<.0001*
Linear				
X_1	3.827	0.6385	5.99	0.0002*
X_2	4.734	0.6385	7.41	<.0001*
X_3	3.542	0.6385	5.55	0.0004*
Quadratic				
X_1*X_1	-1.373	0.6386	-2.15	0.0600
X_2*X_2	-5.411	0.6386	-8.47	<.0001*
X_3*X_3	-5.874	0.6386	-9.20	<.0001*
Interaction				
X_1*X_2	0.2975	0.8343	0.36	0.7296
X_2*X_3	0.08250	0.8343	0.10	0.9234
X_1*X_3	-0.06000	0.8343	-0.07	0.9442

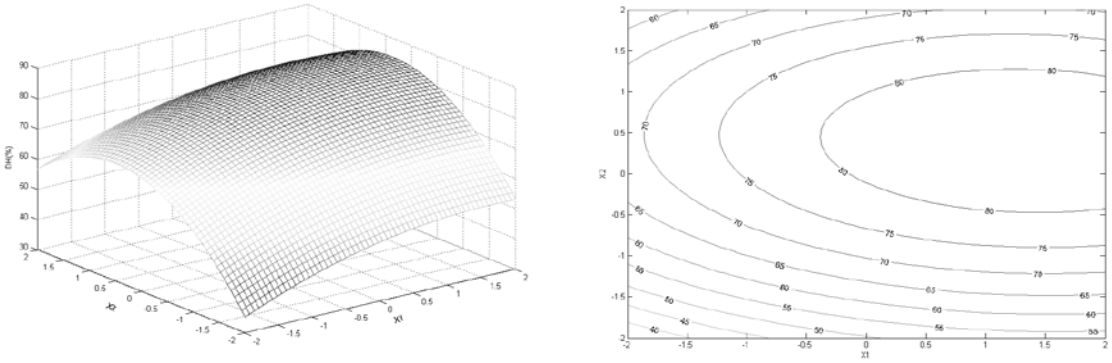
Coefficients of a full model were evaluated by regression analysis. The analysis of variance (Table 4) demonstrates that the regression model for data was highly significant ($p<0.0001$). The model has shown a good fit with the experimental data, since the coefficient of determination R^2 had a value of 0.966. This means that the fitted model could explain 96.6% of the total variability within the range of values studied. Error analysis showed that the lack of fit was not significant ($p>0.05$) thereby indicating that the predicted model was adequate.

Table 4.Analysis of variance for the predicted model of *in vitro* enzymatic hydrolysis of SPI.

Source	D.f	SS	MS	F-value	p-value
Model	9	1438.3	159.8	28.7	<0.0001*
Error	9	50.11	5.568		
Corrected Total	18	1488.4			
Lack of Fit	5	40.43	8.087	3.34	0.1329
Pure Error	4	9.681	2.420		
Total Error	9	50.11	5.568		

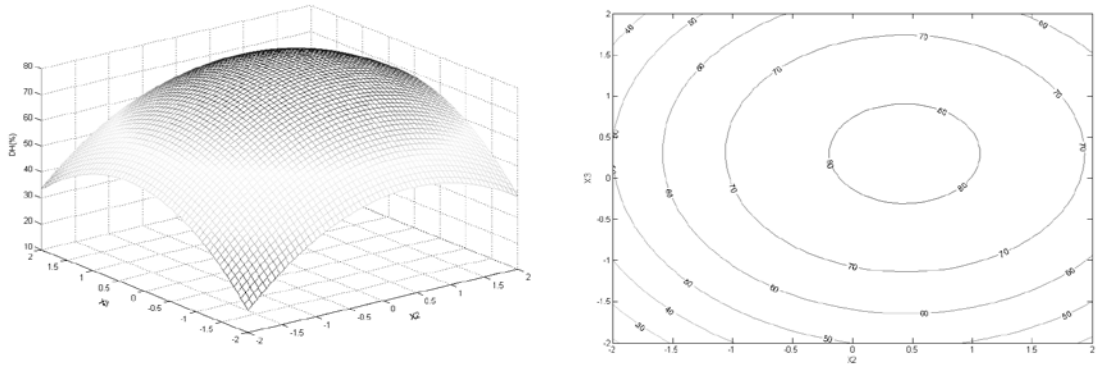
3.2 Optimization study Eq. (3) shows that degree of hydrolysis has a complex relationship with the independent variables that encompass both first- and second-order polynomials and may have maximum point. Analysing the response surface and contour plots for Y is the best way to evaluate the relationships between responses and variables and interactions that exist herein (Xu, Fomuso, & Akoh, 2000). Figures 2 through 4 show the relationship between independent and dependent variables in three-dimensional representations, as a function of the interactions of any two of the variables by holding the other one at center level, whose regression coefficients are given in Table 3.

Fig.1 shows the effect of pre-treatment pressure and pepsin-substrate ratio on the DH value at center level of pancreatin-substrate ratio. It can be seen that DH increased in pre-treatment pressure, but after 480 MPa, a stationary area of DH is observed. Higher values of DH occur when the pepsin-substrate ratio is between 0.75% to 1.6%. Fig.2 shows the effect of pepsin-substrate ratio and pancreatin-substrate ratio on the DH value at center level of pre-treatment pressure. DH increases in pepsin-substrate ratio and also increases with the increased of pancreatin-substrate ratio. Fig.3 shows the effect of pre-treatment pressure and pancreatin-substrate ratio on the DH value at center level of pepsin-substrate ratio. The general form of three-dimensional relationship is similar to Fig.1.



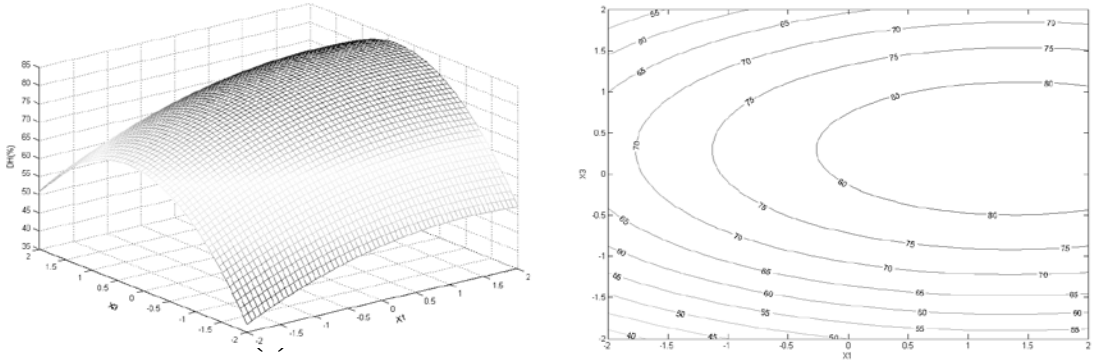
(a) (b)

Fig. 1. Response surface(a) and respective contour plot(b), fitted to the experimental data points, corresponding to DH, as a function of the pre-treatment pressure, pepsin-substrate ratio (pancreation-substract ratio was set at it's center level).



(a) (b)

Fig. 2. Response surface(a) and respective contour plot(b), fitted to the experimental data points, corresponding to DH, as a function of pepsin-substrate ratio**,** pancreatin-substrate ratio (pre-treatment pressure was set at it's center level).



(a) (b)

Fig.3. Response surface(a) and respective contour plot(b), fitted to the experimental data points, corresponding to DH, as a function of pepsin to substrate ratio, pancreatin - substrate ratio (pepsin -substract ratio was set at it's center level).

These results show that the response surface has a stationary (maximum) point within the experimental range of the independent variables. The stationary point (maximum) of the fitted model is: 0.8646, 0.2828, 0.1839.The optimum conditions for the *in vitro* enzymatic digestion are presented in Table 5. It could be seen under the optimal conditions achieved DH is 85.05%.

Table 5. Optimum conditions for SPI hydrolysis obtained applying RSM.

Independent variables	Optimum conditions
X_1(pre-treatment pressure, MPa)	587
X_2(pepsin-substrate ratio, %)	1.14
X_3 (pancreatin-substrate ratio, %)	3.17

3.3 Experimental Verification To confirm the validity of the suggested mathematical model, an additional experiment was conducted under the predicted optimal condition obtained from the above study (the pre-treatment pressure of 590MPa, pepsin -substrate ratio of 1.1 % pancreation- substrate ratio of 3.2%). Five probes were run in parallel and DH values were measured as shown in table 6.

Table 6.Predicted and observed values of the validation experiments.

No	1	2	3	4	5	Mean value
DH (%)	87.27	84.62	86.98	86.05	85.63	86.11

The mean of five replicate determinations was 86.11 % (SE=0.47). The degree of hydrolysis using a regression model with these parameters was 85.05%. There is no significant difference between the measured values and the calculated value for DH. This indicates that the second order polynomial model (Eq.(3)) can be used to predict DH if different levels of pressure, pepsin-substrate ratio, and pancreatin-substrate ratio are chosen as control factors for the *in vitro* digestion of SPI of high pressure treatment.

CONCLUSION RSM was successfully applied to model and optimize *in vitro* enzymatic digestion of SPI of HHP processing. Main effects and interactions between the three reaction parameters were successfully elucidated through the RSM models. The response surface analysis and contour map analysis were used to determine the stability of point maxima. The optimal conditions were determined as follows: pre-treatment pressure of 590MPa, pepsin-substrate ratio of 1.1%, and pancreatin-substrate ratio of 3.2%. Under optimal conditions (the pressure of 590MPa, pepsin-substrate ratio of 1.1 %, pancreatin-substrate ratio of 3.2%) the tests used in this study show that the predictive value in the regression model is closer to the measured values and further regression model proved to be an excellent adaptation.

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