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Analytical Methods

Enzymatic hydrolysis of protein isolate from hull-less pumpkin oil cake: Application of response surface methodology

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ABSTRACT

Enzymatic hydrolysis of protein isolate from hull-less pumpkin (*Cucurbita pepo* L.) oil cake was studied by response surface methodology, using a central-composite experimental design. The hydrolysis was carried out with an acid protease, at temperature of 30 °C and pH 3.00. Second-order polynomial model was proposed with regard to of effect of time, enzyme and NaCl concentration. The mathematical model showed good fit with the experimental data, since the R^2 of 0.946 indicated that 94.6% of the variability within the range of values studied could be explained by the model. A hydrolysis time of 32.5 h, enzyme concentration of 0.137% (v/v) and NaCl concentration of 0.84% (w/v) were found to be the optimal conditions to achieve the highest value of degree of hydrolysis (DH).

1. Introduction

Enzymatic hydrolysis has become widely used biotechnological process to obtain plant proteins with improved functional properties and commercial protein hydrolysates. Plant protein hydrolysates are mostly used as protein ingredients or supplements in food and beverages (Panyam & Kilara, 1996) or as ingredients in special formulation for clinical nutrition (Clemente, 2000). Enzymatic hydrolysis could result peptides with biological activity (Aluko & Monu, 2003; Korhonen & Pihlanto, 2006) or might reduce allergenic potential of some intact plant proteins (Tsumura, Kugimiya, Bando, Hiemori, & Ogawa, 1999). Recently, increasing attention is focused on plant protein hydrolysates with antioxidative properties (Cumby, Zhong, Naczk, & Shahidi, 2008; Li, Jiang, Zhang, Mu, & Liu, 2007). The usage of protein hydrolysates depends on their nutritional value and functional properties. Specific properties of the hydrolysates are dependent upon degree of hydrolysis (DH), which is influenced by the specific activity of the protease, physical and chemical character of the protein substrate and reaction conditions. To produce hydrolysates with desirable properties it is necessary to undertake studies to find the right proteolytic enzyme for a protein substrate. It is, also, necessary to select factors with major effects on the proteolysis (enzyme and substrate concentration, time, temperature, pH and ionic strength). Response surface methodology (RSM) has become a very popular and successful method for the optimisation of enzymatic hydrolysis processes with different substrates and proteases (Bhaskar, Benila, Radha, & Lalitha, 2008; Cao, Zhang, Hong, & Ji, 2008; Guerard, Sumaya-Martinez, Laroque, Chabeaud, & Dufossé, 2007; Xia, Wang, & Xu, 2007). Generally, RSM defines the effect of the independent process parameters, alone or in combinations, and generates a mathematical model that describes the overall process (Bas & Boyaci, 2007).

According to criteria of nutritional quality and cost, many legumes, oilseeds and their by-products of the oil industry are suitable as protein sources to produce protein hydrolysates for food or non-food application. In recent years, pumpkin (*Cucurbita* sp.) has received great attention, because the seed proteins have great potential to be used in food and pharmaceutical industry (Caili, Huan, & Quanhong, 2006; El-Adawy & Taha, 2001; El-Soukkary, 2001; Zdunczyk, Minakowski, Frejnagel, & Flis, 1999). Traditional industrial oil processing by pressing pumpkin seeds leaves oil cake as a by-product with 60–65% proteins. The pumpkin oil cake (PuOC) has been used only for animal feed. Since the oil production from hull-less and hulled pumpkin seeds has been growing in Austria, Slovenia, Hungary and Serbia, it is of practical, economic and environmental interest to valorize its main by-product.

Due to high protein content, availability and low cost, PuOC is a valuable raw material to obtain high-value protein products. Bioconversion of PuOC by enzymatic proteolysis is one of the alternatives to produce protein hydrolysates with increased solubility and enhanced functionality.

The objective of this research was to study and optimise hydrolysis conditions of hull-less pumpkin (*Cucurbita pepo* L. c. v. "Olinka") oil cake protein isolate by a commercial fungal acid

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protease (Aspergillopepsin I), with regard to produce high soluble protein hydrolysates. Three independent factors: time, enzyme and NaCl concentration were investigated by RSM to identify their influence on the degree of hydrolysis (DH) and their optimum levels. Also, the conducted study was performed to prove the usability of RSM to optimise the process of enzymatic hydrolysis, which is a complex process and its main parameters and their interactions must be defined in different systems for the same substrate and enzyme.

2. Materials and methods

2.1. Materials

The PuOC was acquired by the "Pan-Union", Novi Sad, Serbia. It was stored at the temperature of 4 °C and ground in a coffee-grinder before use. The enzyme was a commercial, fungal acid protease–Aspergillopepsin I (Allyzme Vegpro), produced by *Aspergillus niger*. It was provided by Alltech Hungary with a declared activity of 7500 HUT/g.

2.2. Preparation of protein isolate (PI)

The protein isolate (PI) was prepared according to the method described by Nkosi, Opoku, & Terblanche, 2005. The grounded PuOC was defatted with hexane. The defatted PuOC was suspended in water (pH 10.00) and the slurry filtered. The pH of filtrate was adjusted to 5.00. After centrifugation the resulting residue was collected and air dried at room temperature (20–23 °C).

2.3. Experimental design

In preliminary studies, time, amount of enzyme and NaCl concentration were found to have a significant influence on the degree of hydrolysis (DH). The effect of three independent variables on the DH and their optimum levels were investigated by RSM, using a central–composite experimental design. In this regard, a set of 15 experiments was carried out (with three replicates at the centre of the design). The range and central point values of the independent variables (Table 1) were assorted based on the results of preliminary experiments. 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.

2.4. Protein isolate hydrolysis

The enzymatic hydrolysis was carried out as stated in Table 1, according to the experimental plan in Table 2. PI was dissolved with Glycine/HCl buffer pH 3.00 (0.2 mol/l) to a final concentration of 2% w/v. and NaCl was added according to the experimental plan (Table 2). The hydrolysis was performed in a glass reactor under controlled conditions (temperature and stirring speed). The mixtures were incubated at the appropriate temperature of 30 °C. The hydrolysis reaction was started by the addition of the enzyme (to the required concentration according to the experimental plan). Aliquots (1.5 ml) were taken out after 5, 22 and 39 h. The reaction mixtures were immediately heated (100 °C, 5 min) to end the reaction. The mixtures with inactivated enzyme were centrifuged at 4.5 rpm (Ependorf Mini spin plus) for 5 minutes and the supernatant was used for further analysis.

Table 1
Real and coded levels of the independent variables used in RSM study for optimising hydrolysis conditions of PuOC protein isolate with Aspergillopepsin I.

Independent variables	Symbol	Levels		
		−1	0	1
Time, (h)	x_1	5	22	39
Enzyme, (%) v/v	x_2	0.06	0.13	0.2
NaCl, (%) w/v	x_3	0	0.75	1.5

Table 2
Central-composite experimental design of the independent variables along with the observed values for the response, (Y_o).

Experiment	Independent variables (real values)			Response
	x_1	x_2	x_3	Y_o (%)
1	5	0.06	0.75	30.58
2	5	0.13	0	22.63
3	5	0.13	1.5	36.69
4	5	0.2	0	29.83
5	22	0.06	0	42.12
6	22	0.06	1.5	40.01
7	22	0.2	0	35.85
8	22	0.2	1.5	39.31
9	39	0.06	0.75	38.37
10	39	0.13	0	46.62
11	39	0.13	1.5	48.65
12	39	0,2	0	43.34
13	22	0.13	0.75	47.42
14	22	0.13	0.75	47.42
15	22	0.13	0.75	43.30

2.5. Determination of the degree of hydrolysis

The degree of hydrolysis was determined according to the method by Tsumura et al. (1999). To a 0.5 ml aliquot of the supernatant obtained after hydrolysis, an equal volume of 0.44 mol/l trichloroacetic acid (TCA) was added. The mixture was incubated for 30 minutes at room temperature. Thereafter, the mixture was centrifuged at 4.5 rpm (Ependorf Mini spin plus) for 5 min. The obtained 0.22 mol/l TCA-soluble fraction and the supernatant of reaction mixture were each analysed to determine the protein content by the method of Lowry, Rosenbrough, Fair, and Randall (1951), using bovine serum albumin as the standard protein. The DH value was calculated as the ratio of 0.22 mol/l TCA-soluble protein to total protein in the supernatant of reaction mixture, expressed as a percentage.

2.6. Statistical analysis

Data from the central-composite design (Table 2) were analysed by the least squares method to fit the second–order polynomial model, given by the equation:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_1^2 + b_5x_2^2 + b_6x_3^2 + b_7x_1x_2 + b_8x_1x_3 + b_9x_2x_3 \quad (1)$$

where Y is the predicted response variable (degree of hydrolysis, DH), b_0 is the regression coefficient of intercept term, b_1, b_2, b_3 are linear regression coefficients, b_4, b_5, b_6 are squared regression coefficients and b_7, b_8, b_9 are interaction regression coefficients. The proposed model equation predicts the response as a function of the different levels of independent variables (x_1, x_2, x_3). The significance of each coefficient of the resulted model was determined by using the Student t-test and p-value. The adequacy of the polynomial model is expressed by the coefficient of multiple determination, R^2 and analysis of variance was employed for the determination of the significance of the model. Further, the fitted polynomial equation was presented as surface plots in order to visualise the relationship between the response and experimental levels of each factor and to deduce the optimum conditions.

Statistical analysis was performed by using Statistical software (StatSoft, Statistica 7). Surface plots were drawn using the same program.

3. Results and discussion

3.1. Model fitting

The application of RSM yields the regression Eq. (2), which represents an empirical relationship between the response (DH) and the tested variables (in coded units):

$$Y = 46.046 + 7.157x_1 - 0.343x_2 + 2.179x_3 - 5.5931x_1^2 - 4.92x_2^2 - 1.803x_3^2 + 1.427x_1x_2 - 3.007x_1x_3 + 1.395x_2x_3 \quad (2)$$

The significance of each coefficient was determined using the Student t test and p value in Table 3. The largest effect has the linear term of time (x_1), followed by the quadratic term of time (x_1^2) and the quadratic term of enzyme concentration (x_2^2). 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. Considering our results, among the independent variables time played dominant role in the extent of hydrolysis.

The model has shown a good fit with the experimental data, since the coefficient of determination R^2 had a value of 0.946. This means that the fitted model could explain 94.64% of the total variability within the range of values studied. The analysis of variance (Table 4) showed high *F*-value, while *p*-value was less than 0.01, which implied that the model itself is significant. In further analysis, each of the observed value for the degree of hydrolysis (Y_o) was compared with the adequate predicted value (Y). Parity plot (Fig. 1) shows an acceptable level of agreement. All these results imply a satisfactory mathematical description of the hydrolysis process by the fitted model (Eq. (2)).

3.2. Optimisation of the process

The three-dimensional response surface graphs were drawn to illustrate the main and interactive effects of the independent variables on the degree of hydrolysis (DH). The response surface, whose regression coefficients are given in Table 3, is shown in Fig. 2. Fig. 2a shows the effect of time and enzyme concentration on DH, (NaCl concentration at the central of its level); quadratic effect for both variables was observed, though time had greater influence on the response. DH increased in time, but after 30 h of hydrolysis a stationary area of DH could be noticed. After 39 h of hydrolysis, DH started to decrease. Also, higher values of DH could be noticed when the enzyme concentration took place between 0.12 and 0.16%. Fig. 2b shows the effect of time and NaCl concentration (enzyme concentration at the central of its level); quadratic effect of time and a linear effect of NaCl concentration on the response can be noticed. DH increased in time and also increased with the increase of NaCl concentration. In Fig. 2c quadratic effect of both variables, enzyme and NaCl concentration (time at the central of its level), was presented.

Table 3
Significance of regression coefficients for degree of hydrolysis.

Variables	Regression coefficient	Computed *t*-value	Significance level, *p*-value
Intercept	46.046	19.829	0.000006
Linear			
x_1	7.157	5.03	0.004
x_2	−0.343	−0.24	0.819
x_3	2.179	1.532	0.186
Quadratic			
x_1^2	−5.593	−2.672	0.044
x_2^2	−4.92	−2.35	0.065
x_3^2	−1.803	−0.861	0.428
Interaction			
$x_1 \cdot x_2$	1.427	0.709	0.509
$x_1 \cdot x_3$	−3.007	−1.495	0.19
$x_2 \cdot x_3$	1.395	0.693	0.518

Table 4
Analysis of variance for the predicted model of the PuOC protein isolate hydrolysis using Aspergillopepsin I.

Effect	SS	d*f*	MS	*F*-value	*p*-value
Regression	24072.3	10	2407.2	148.8	0.000015
Residual	80.89	5	16.2		
Total	24153.3	15			
Corrected total	776.3	14			
Regression vs. corrected total	24072.3	10	2407.2	43.4	0.000000

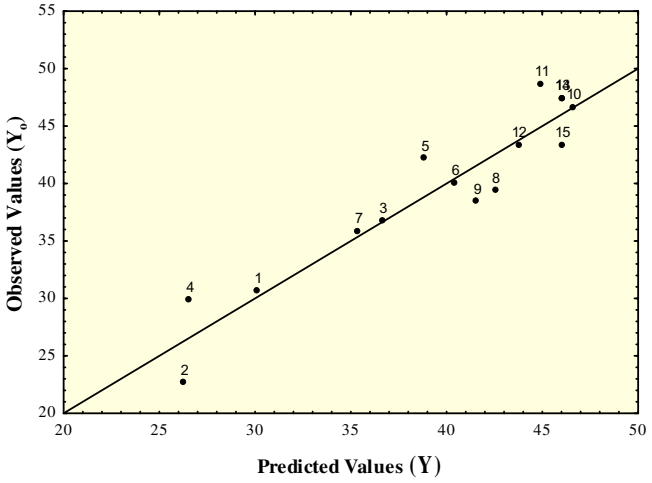

Fig. 1. Relationship between the observed and predicted values of the degree of hydrolysis (Parity plot).

These results have shown that the response surface had a maximum point within the experimental range of the independent variables. The precise coordinates of optimum, the levels for three independent variables were obtained by analytical procedure. The stationary point (maximum) of the fitted model was found by deriving first derivatives of the function (2), as follows:

$$\begin{aligned} &7.157 - 11.187x_1 + 1.427x_2 - 3.007x_3 = 0 \\ &-0.343 - 9.84x_2 + 1.427x_1 + 1.395x_3 = 0 \\ &2.179 - 3.606x_3 - 3.007x_1 + 1395x_2 = 0 \end{aligned} \quad (3)$$

The system of linear Eq. (3) was solved and following results were obtained: $x_1 = 0.6174$, $x_2 = 0.071$ and $x_3 = 0.1169$. The calculated values (x_1, x_2 and x_3) correspond to the coded values of the independent factors for the maximum value of the response (DH). The optimum conditions for the hydrolysis are presented in Table 5. It could be seen that under the optimal conditions achieved DH is close to 50%.

To confirm the validity of the suggested mathematical model, an additional experiment was conducted under the predicted optimal condition. Two parallel probes were performed and two DH values (46.34% and 47.374%) were measured. The mean of two replicate determinations was 46.855%, at the sample standard deviation of 0.517. Using the *t*-distribution, for the one-sided test, the critical value of the lower estimate, at a level of $\alpha = 0.05$, was obtained (46.09316%). As the mean value (46.885%) is higher than the critical value (46.09316), there is no significant difference between the measured values and the calculated value for DH, at

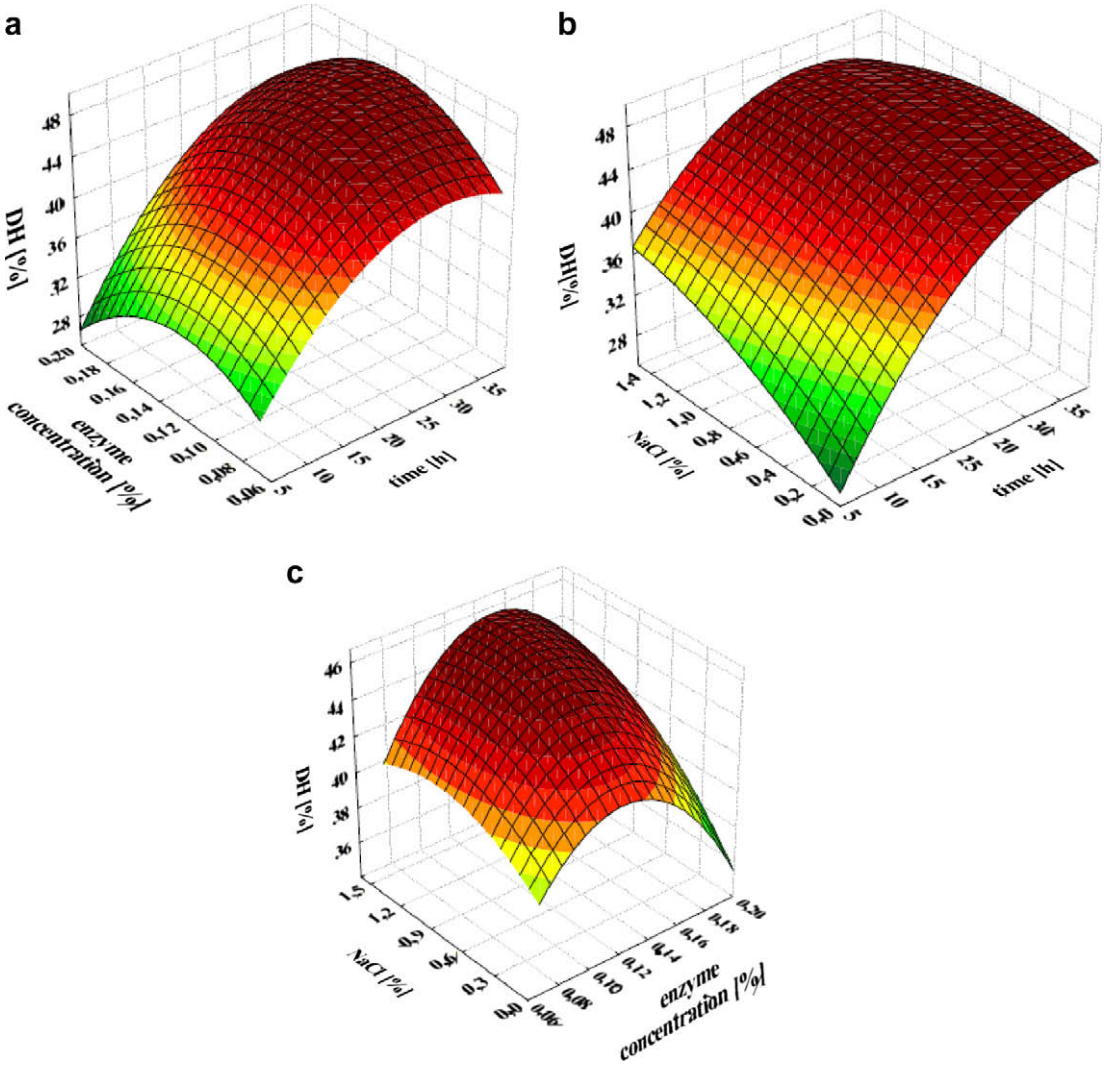

Fig. 2. Response surface graphs for DH as a function of: (a) time and enzyme concentration enzyme concentration (NaCl concentration at the central of its level), (b) time and concentration NaCl (enzyme concentration at the central of its level), (c) enzyme and NaCl concentration (time at the central of its level).

Table 5
Optimum conditions for PuOC protein isolate hydrolysis by Aspergillopepsin I, obtained applying RSM.

Independent variables	Optimum conditions
Time (h)	32.49
Enzyme (%) v/v	0.137
NaCl (%) w/v	0.84

the optimal conditions (Table 5). This indicate that the second-order polynomial model (Eq. (2)) can be used to predict DH, if different levels of time, enzyme and NaCl concentration are chosen as control factors for the hydrolysis of PuOC protein isolate with Aspergillopepsin I, at temperature of 30 °C and pH 3.00. On the other hand, enzymatic hydrolysis has been shown as effective tool to produce high soluble protein hydrolysates, which have potential to be used in different food products. Further investigation of functional, nutritive and pharmacological (antioxidant) properties of the obtained protein hydrosylates takes place in order to complete the data basis on the enzymatic processing of PuOC.

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