

Hydrolysis of Hull-Less Pumpkin Oil Cake Protein Isolate by Pepsin

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Abstract—The present work represents an investigation of the hydrolysis of hull-less pumpkin (*Cucurbita Pepo L.*) oil cake protein isolate (PuOC PI) by pepsin. To examine the effectiveness and suitability of pepsin towards PuOC PI the kinetic parameters for pepsin on PuOC PI were determined and then, the hydrolysis process was studied using Response Surface Methodology (RSM). The hydrolysis was carried out at temperature of 30°C and pH 3.00. Time and initial enzyme/substrate ratio (E/S) at three levels were selected as the independent parameters. The degree of hydrolysis, DH, was measured after 20, 30 and 40 minutes, at initial E/S of 0.7, 1 and 1.3 mA/mg proteins. Since the proposed second-order polynomial model showed good fit with the experimental data ($R^2 = 0.9822$), the obtained mathematical model could be used for monitoring the hydrolysis of PuOC PI by pepsin, under studied experimental conditions, varying the time and initial E/S. To achieve the highest value of DH (39.13 %), the obtained optimum conditions for time and initial E/S were 30 min and 1.024 mA/mg proteins.

Keywords—Enzymatic hydrolysis, Pepsin, Pumpkin (*Cucurbita Pepo L.*) oil cake protein isolate, Response surface methodology.

I. INTRODUCTION

THE usage of plant proteins, especially from cereals and oilseeds, has been growing intensively over the last decades. They have been used as the alternative for animal proteins in human nutrition, functional agents and bioactive components in food as well in cosmetics and other pharmaceutical products.

Plant proteins are usually exploited as protein isolates or concentrates, but their usage could be limited by their unsuitable functional properties, such as low solubility [1]. In intention to expand the field of their usage, there have been a number of alternative techniques investigated to improve the

functional characteristics of proteins, which could be attended by increasing of nutritive value and bioactivity. Up till now, plant protein modifications can be made by chemical [2], [3], [4] and enzymatic modifications [5], [6], [7], [8], [9] or physical treatments using high temperature, pressure and ultrasonic waves [10], [11].

Nowadays, enzymatic hydrolysis has become the most frequent used modification on plant proteins [12]. Enzymatic hydrolysates have a wide range of applications; as protein ingredients or supplements in food and beverages [12], or in clinical nutrition [13], bioactive components (ACE - inhibitors, natural antioxidants) in functional food or pharmaceutical products [14], [15].

The nutritive and functional properties of final hydrolysates depend on their molecular mass and structural characteristics. The molecular characteristics of hydrolysates are influenced by protein substrate characteristics, protease specificity and hydrolysis conditions (enzyme/substrate ratio, temperature, pH, time). Therefore, enzymatic hydrolysis performed under selective conditions make possible to transform proteins into hydrolysates with desirable properties.

Pumpkin seeds are known to be rich protein sources, which have great potential to be used in food and pharmaceutical industry [16], [17], [18], [19]. In Serbia and adjacent countries, the pumpkin seeds are mostly used for pumpkin oil production. After the oil extraction the by-product is a protein rich (60-65%) pumpkin oil cake (PuOC). Up till now, this by-product has been used mainly for animal feeding. In regard to valorize this by-product, which is of great economical and environmental interest, PuOC might be consider as promising and attractive source of proteins. In development of processes to produce value-added protein products from PuOC, enzymatic hydrolysis on isolated proteins could be employed [20].

Enzymatic hydrolysis is a complex process and many preliminary studies should be undertaken to realize an effective process. The choice of protease should be based on kinetic studies, which affirms the effectiveness of protease towards the selected substrate. The process parameters (temperature, pH, initial E/S, time) should be selected properly, depending on their influence on protease activity and substrate behavior. RSM has been proved and successfully used method for the optimization of enzymatic hydrolysis process with different substrates and proteases [22], [23], [24], [25]. Using this methodology the effect of the independent process parameters, alone or in combinations could be defined, as well the

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mathematical model that describes the overall hydrolysis process [26].

The aim of this study was an investigation of hydrolysis of PuOC PI using pepsin. Pepsin is an acid protease, with activity at low pH (1.5-3). In order to determine the effectiveness and suitability of pepsin towards PuOC PI, a kinetic study was performed to determine the apparent kinetic parameters maximal reaction rate (v_{\max}), Michaelis-Menten constant (K_m), catalytic constant (k_{cat}) and catalytic effectiveness k_{cat}/K_m . Further, the hydrolysis with pepsin was carried out and the process was studied using RSM. The influence of time and initial E/S on DH was examined, in regard to establish the mathematical model and optimize the hydrolysis.

II. MATERIALS AND METHODS

A. 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. Pepsin was obtained by “Kemika” Zagreb, Croatia, with a declared activity of 1 mAnson / mg.

B. Preparation of protein isolate (PI)

The protein isolate (PI) was prepared according to the method described by Nkosi, Opoku and Terblanche [27]. 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).

C. Determination of kinetic parameters

The apparent kinetic parameters for pepsin towards PuOC PI were determined at 30°C and pH 3.00. The substrate (PI) concentration was varied between 0.3 and 4 mg/ml in Glycine/HCl buffer pH 3.00 (0.2 mol/l), while the enzyme concentration was kept constant. In order to determine the initial rate of reaction (v_0) the reaction was terminated during 20 minute, by addition of trichloroacetic acid (TCA). The reaction mixtures were centrifuged at 10 000 rpm for 5 minute. The protein content in supernatants was determined according to method of Lowry, Rosenbrough, Fair and Randall [28]. The reaction rates (v) were calculated as the concentration of trichloroacetic acid – soluble proteins in obtained supernatans, expressed in [g/l].

D. Enzymatic hydrolysis

To examine the hydrolysis process affected by time and initial enzym/substrate ratio using RSM, a full factorial 3^2 experimental design was performed. The actual and coded levels at which the independent parameters were employed is presented in Table 1.

The hydrolysis was carried out in a glass reactor, under controlled conditions (pH, temperature and stirring speed).

TABLE I
REAL AND CODED LEVELS OF THE INDEPENDENT VARIABLES

Independent parameters	Coded values		
	-1	0	1
Time [min] (X_1)	20	30	40
E/S [mA/mg] (X_2)	0.7	1	1.3

The experiments were performed according to the experimental design described in Table 2. The reaction was started by addition of the required amount of enzyme solution

TABLE II
FUL FACTORIAL 3^2 EXPERIMENTAL DESIGN
(THE INDEPENDENT VARIABLES ALONG WITH
THE OBSERVED VALUES FOR THE RESPONSE).

Independent variables (Coded values)		Response DH [%]
x_1	x_2	Y
-1	-1	25.634
-1	0	37.703
-1	1	30.878
0	-1	29.583
0	0	39.167
0	1	32.358
1	-1	29.358
1	0	35.683
1	1	28.908

to the preheated PI solution in Glycine/HCl buffer pH 3.00 (0.2 mol/l). Aliquots (3 ml) were taken out after 20, 30 and 40 minute. The reaction mixtures were immediately heated (100°C, 5 minutes) to inactivate the enzyme. The mixtures were centrifuged at 14 500 rpm (Ependorf Mini spin plus) for 5 minutes and the supernatant was used for further analysis to determine the degree of hydrolysis.

E. Determination of the degree of hydrolysis

The degree of hydrolysis was determined according to the method by Tsumura, Kugimya, Bando, Hiemori and Ogawa [28]. 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 14 500 rpm (Ependorf Mini spin plus) for 10 minutes. The obtained 0.22 mol/l TCA-soluble fraction and the supernatant of reaction mixture were each analyzed to determine the protein content by the method of Lowry *et al* [28], 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.

F. Statistical analysis

The results of hydrolysis experiments (Table 2) were analyzed by the least squares method to fit the second-order polynomial model, given by the equation:

$$Y_0 = b_0 + b_1 \cdot x_1 + b_2 \cdot x_2 + b_3 \cdot x_1^2 + b_4 \cdot x_2^2 + b_5 \cdot x_1 \cdot x_2 \quad (1)$$

where Y_0 is the predicted response (DH), b_0 is the regression coefficient of intercept term, b_1 , b_2 are linear regression coefficients, b_3 and b_4 are squared regression coefficients and b_5 is the interaction regression coefficient. The proposed model equation predicts the response as a function of the different levels of the independent variables (x_1 and x_2). The significance of each coefficient in the resulted model was determined by using the Student t -test and p -value. The adequacy of the model is expressed by the coefficient of multiple determination R^2 and analysis of variance was employed for determination of the significance of the model. Statistical analysis was performed by using Statistical software (StatSoft, Statistica 8). The fitted polynomial equation was presented as 3D- surface plot using the same program.

III. RESULTS AND DISCUSSION

A. Kinetic study

The kinetic study of pepsin towards PuOC PI was performed as it is described in materials and methods section. The final results are present in Table 3. The apparent V_{\max} and K_m were calculated from X and Y intercept of the

TABLE III

APPARENT KINETIC PARAMETERS FOR PEPSIN, WHEN PUOC PI IS USED AS SUBSTRATE, AT PH 3.00 AND TEMPERATURE AT 30°C. THE INITIAL SUBSTRATE CONCENTRATION VARIED FROM 0.3 TO 4 g/l.

E_0 [g/l]*	$V_{\max} \cdot 10^3$ [g/l s]	K_m [g/l]	$k_{cat} \cdot 10^3$ [1/s]	$k_{cat}/K_m \cdot 10^3$ [(g/l) ⁻¹ s ⁻¹]
1.125	5.376			
2.25	9.173	4	4.399	1.1
3.375	1.466			

*Img of enzyme is equivalent to 1mA unit (according to declared activity of enzyme).

Lineweaver-Burk plot, where the reciprocal substrate concentration, $1/s$ ((g/l)⁻¹) was plotted against the reciprocal initial reaction rate, $1/v_0$ (g/l s⁻¹) (data not shown). The apparent catalytic constant k_{cat} was calculated as V_{\max}/E_0 , where the E_0 is the initial enzyme concentration (g/l). The apparent catalytic effectiveness represents the ratio k_{cat}/K_m .

In our laboratory studies, comparing the relative kinetic parameters for three acid proteases (pepsin, aspergillopepsin I and *P. roquefortii* acid protease) (results not shown), pepsin showed good catalytic effectiveness to PuOC PI, higher than did aspergillopepsin I, but lower than *P. roquefortii* acid protease.

B. Modeling of hydrolysis process

The obtained values for the response (Y) at different codes for the time and enzyme/substrate ratio is presented in Table 2. According to the statistical analysis the hydrolysis process could be presented by the mathematical model described by follow equation (2):

$$Y_0 = 39.079 - 0.044 x_1 + 1.262 x_2 - 2.342 x_1^2 - 8.064 x_2^2 - 1.423 x_1 \cdot x_2 \quad (2)$$

TABLE IV
SIGNIFICANCE OF REGRESSION COEFFICIENTS IN MATHEMATICAL MODEL FOR THE DH

	Estimate	Standard error	t-value df=3	p-level
b_0	39.079	0.728	53.696	0.000014
b_1	-0.044	0.399	-0.111	0.918434
b_2	1.262	0.399	3.165	0.050673*
b_3	-2.342	0.691	-3.392	0.042709**
b_4	-8.064	0.691	-11.680	0.001348***
b_5	-1.423	0.488	-2.915	0.061716*

*** Significant within a 99% confidence interval, **Significant within a 95% confidence interval, * Significant within a 90% confidence interval.

TABLE V
ANALYSIS OF VARIANCE (ANOVA) OF THE PREDICTED MATHEMATICAL MODEL.

	Sum of Square	df	Mean Squares	F-value	p-value
Regression	9456.295	6	1576.05	1653.1	0.000023
Residual	2.860	3	0.953		
Corrected Total	161.563	8			
Regression vs. Corrected Total	9456.295	6	1576.05	78.040	0.000001
R^2	0.9823				
R	0.9911				

The regression coefficients (Table 4) showed significant influence of the independent parameters on DH. The most significant term was the quadratic term of initial E/S (x_2^2), followed by linear term of E/S (x_2), quadratic term of time (x_1^2) and the interactive term ($x_1 \cdot x_2$).

The analysis of variance for the model is presented in Table 5. The model has shown a good fit with the experimental data, since the coefficient of determination R^2 had a value of 0.9822. This means that the fitted model could explain 98.22% of the total variability within the range of values studied. The F-value for the model was at significant level, while p -value was less than 0.01 (Table 5). These results implied that the model by itself is significant. Further, each of the observed value for the degree of hydrolysis (Y) was compared with the adequate predicted value (Y_0). 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).

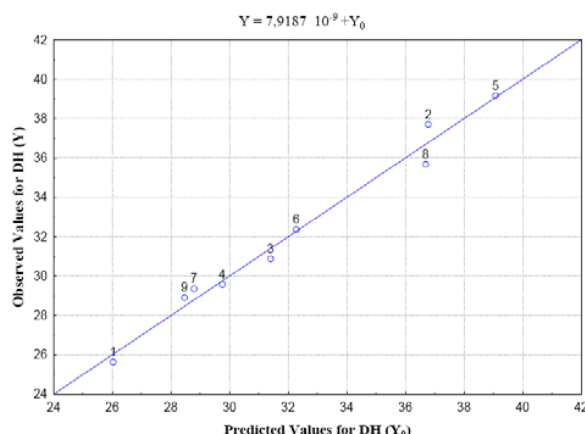


Fig.1. Parity Plot. The predicted values for the DH (Y_0), compared with the experimentally observed values for DH (Y).

C. Optimization of the hydrolysis process

The three-dimensional response surface graph was drawn to illustrate the main and interactive effects of the independent variables on the degree of hydrolysis (DH). In Fig. 2 is presented the mathematical model of the hydrolysis process, with regression coefficients given in Table 3.

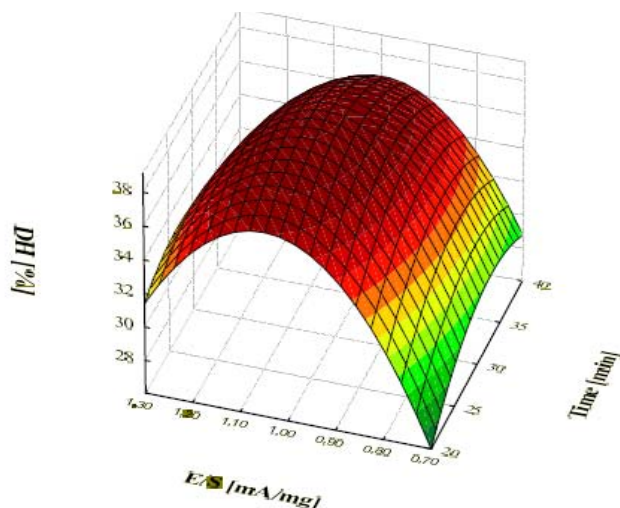


Fig. 2. 3D-Response surface graph for the hydrolysis of PuOC PI by pepsin; influence of time and initial E/S on DH.

In Fig.2 could be seen that the progress of hydrolysis is mainly affected by initial E/S (x_2). At low values of E/S (under 1 mA), DH increase with increasing of time, as well with increasing of E/S. however, since the quadratic effects of time and E/S are significant there are certain points of the values of these independent parameters where the progress of hydrolysis starts to decrease, with increasing of E/S. The results implied that the response surface had a maximum point within the experimental range of studied independent

variables. The precise coordinates of optimum levels for the independent variables were obtained by analytical procedure.

TABLE VI
THE OPTIMUM CONDITION FOR HYDROLYSIS OF PUOC PI BY PEPSIN.
PREDICTED AND EXPERIMENTALLY OBTAINED VALUES FOR DH.

Independent parametrs	Time [min]	30
	E/S [mA/mg]	1.024
Response	DH [%]	39.1
	Predicted	
	DH [%]	38.537± 2

The stationary point (maximum) of the fitted model was found by deriving first derivatives of the Eq. (2), as follows:

$$\frac{\partial Y_0}{\partial x_1} = 0 \quad -0.04435 - 4.684 \cdot x_1 - 1.4234 \cdot x_2 = 0 \quad (3)$$

$$\frac{\partial Y_0}{\partial x_2} = 0 \quad 1.26166 - 16.1286 \cdot x_2 - 1.4234 \cdot x_1 = 0$$

The system of linear equations (3) was solved and following results were obtained: $x_1 = -0.03415$ and $x_2 = 0.0812$. The calculated values (x_1 and x_2) correspond to the coded values of the independent parameters for the maximum value of the response (DH). Under the optimum conditions DH reached to 39.1%. To confirm the validity of the statistical experimental strategies, three additional experiments were performed under the predicted optimal condition. The measured DH values were close to the predicted value for DH using RSM. The optimum conditions for the hydrolysis, predicted and observed values for DH are showed in Table 6.

IV. CONCLUSION

The kinetic parameters in this present work shows, that pepsin has good affinity to PuOC PI and therefore could be used to produce protein hydrolysates from PuOC PI. The influence of independent parameters on progress of hydrolysis could be successfully establish and the process described by mathematical model using RSM. Application of mathematical modelling enables of efficiency and easily realization of the hydrolysis in practice. The nutritive, functional and bioactive properties of protein hydrolysates from PuOC PI should be determined in regard to prewise their potential use. These investigations have been conducted already in our laboratory.

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