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

Ivan Živanović, Žužana Vaštag, Senka Popović, Ljiljana Popović and Draginja Peričin

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 (R² = 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...
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 ($v_{\text{max}}$), Michaelis-Menten constant ($K_{m}$), catalytic constant ($k_{\text{cat}}$) and catalytic effectiveness $k_{\text{cat}}/K_{m}$. 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 5 minutes and the supernatant was used for further analysis to determine the degree of hydrolysis.

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

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 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 apparent kinetic parameters for pepsin towards PuOC PI, a kinetic study was performed to determine the apparent kinetic parameters

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:

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

(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 DISSCUSSION

#### 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 $V_{\text{max}}$ and $K_m$ were calculated from $X$ and $Y$ intercept of the Lineweaver-Burk plot, where the reciprocal substrate concentration, $1/(\text{[g/l]})$ was plotted against the reciprocal initial reaction rate, $1/v_0$ ($\text{g/l s}^{-1}$) (data not shown). The apparent catalytic effectiveness represents the ratio $k_{\text{cat}}/K_m$.

<table>
<thead>
<tr>
<th>$E_0$ [g/l]</th>
<th>$V_{\text{max}}\times 10^3$ [g/l s]</th>
<th>$K_m$ [g/l]</th>
<th>$k_{\text{cat}}\times 10^5$ [1/s]</th>
<th>$k_{\text{cat}}/K_m \times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.125</td>
<td>5.376</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.25</td>
<td>9.173</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3.375</td>
<td>1.466</td>
<td></td>
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</tr>
</tbody>
</table>

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

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

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

$$Y_0 = 39.079-0.044 \times x_1 + 1.262 \times x_2 - 2.342 \times x_1^2 + 8.064 \times x_2^2 - 1.423 \times x_1 \times x_2$$  \hspace{1cm} (2)
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.

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

\[
\frac{\partial Y_0}{\partial x_1} = 0 - 0.04435 - 4.684 \cdot x_1 - 1.4234 \cdot x_2 = 0
\]

\[
\frac{\partial Y_0}{\partial x_2} = 0 - 1.26166 - 16.1286 \cdot x_2 - 1.4234 \cdot x_1 = 0
\]

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.

### Table VI

<table>
<thead>
<tr>
<th>Independent parameters</th>
<th>Time [min]</th>
<th>E/S [mA/mg]</th>
<th>DH [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>1.024</td>
<td></td>
</tr>
<tr>
<td>Response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH [%]</td>
<td>Predicted</td>
<td>39.1</td>
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<tr>
<td>DH [%]</td>
<td>Observed</td>
<td>38.537± 2</td>
<td></td>
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</tbody>
</table>

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 established 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 previse their potential use. These investigations have been conducted already in our laboratory.

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