PRODUCTION OF WHEAT GLUTEN HYDROLYSATES
WITH IMPROVED FUNCTIONAL PROPERTIES:
OPTIMIZATION OF OPERATING PARAMETERS BY STATISTICAL DESIGN

Jelena Jovanović1, Andrea Stefanović1, Nataša Šekuljica2, Zorica Knežević-Jugović1*

1Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade, Serbia
2Innovation Center of the Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade, Serbia
*e-mail: zknez@tmf.bg.ac.rs

Abstract

Native wheat gluten is a deluxe bread improver and may be utilized as a functional protein additive in multifarious non-bakery foodstuffs due to its desirable structure-enhancing properties. Also its utilization would be economically interesting, but lack of some desirable functional properties limited their expanding utilization in foodstuff formulations. This study was designed to examine the relationship between process parameters and functional properties of the obtained hydrolysates using Box-Benken experimental design and response surface methodology (RSM). The hydrolysate showing the highest improvement of solubility and foaming ability was further separated by sequential ultrafiltration to obtain molecular weight distribution profile.

The progress of wheat gluten hydrolysis was followed by monitoring the degree of hydrolysis (DH) using the pH-stat method and functional properties were measured by our methods already adopted. The effects of process parameters (pH, T, [S], [E]/[S] ration) and their interactions were investigated by the means of the four-factor Box-Behnken experimental design with 29 experimental points (5 central points). Experiments were carried out in triplicates and expressed as means with SD. The effects of different parameters under the significance level of p < 0.05 were examined using one-way analysis of variance (ANOVA) and Student t-test. The coefficients of the response function and their statistical significance were evaluated by the response surface regression analysis, using the Design software. Non-significant terms (p ≥ 0.05) were deleted from the second order polynomial and a new polynomial has been recalculated. The Fisher test (F-value) was used to determine whether the second-order model was adequate to describe the obtained data while the goodness of fit of the model was evaluated by the determination coefficient (R²).

The obtained results showed that the second-order models developed for DH, solubility and foaming properties of gluten hydrolysates were significant (p < 0.05) with a high value of coefficients of determination (0.944 - 0.981). The statistical analysis showed that each variable had a significant effect on degree of hydrolysis and the functional properties of tested system. Almost the linear increase in DH was observed with the rise in temperature at the highest substrate concentration, while on the other hand increasing the concentration of the substrate leads to a decrease in DH. In terms of foaming properties results showed that foam capacity range are in the range of 24.2 - 80.3%, depending on the independent variables that were tested.

Results are relevant to the protein ingredient industry because of the economic importance of novel gluten-based functional products and can provide useful information for the design an efficient enzymatic process for their production in high yield and with improved functionality.

Key words: Wheat gluten protein, Enzymatic hydrolysis, Solubility, Foaming properties, Box-Benken experimental design.
1. Introduction

In recent years, cereals and their active constituents are recognized as functional foods due of providing dietary: fibers, proteins, energy, minerals and vitamins necessary for human health and among them, wheat products are notified the most common cereal based functional foods. Gluten, the major wheat protein, has attracted the heed of food processors for new product development or for use in existing food products. It is an economically important co-product in the recovery of wheat starch in wet processing of wheat flour, and presents an abundant plant protein source. It is also well known for their high nutritional value and multilateral functional properties in food products. In addition, native gluten is a de-luxe bread improver and may be utilized as a functional protein additive in multifarious non-bakery foodstuffs due to its desirable structure-enhancing properties. Also, its utilization would be economically interesting, but lack of some desirable functional properties such as insolubility limited their expanding utilization in foodstuff formulations. Wheat gluten is a rather complex protein composed of two seed storage proteins, gliadins and glutenins. Glutenins, the major proteins, are poorly soluble in alcohols because they are capable to form large polymers that are stabilized by intermolecular disulfide bonds and hydrophobic interactions. In the opposite, gliadins are soluble in aqueous alcohol and are mainly present in gluten as monomers interacting by non-covalent forces [1]. Especially, bad techno-functional properties such as emulsifying properties or solubility, notably close to its isoelectric point at pH 6 - 7 may limit the its use in many other applications such as nutraceuticals, cosmetics and drugs [2].

Because of the above said, researchers have been focusing on chemical and enzymatic modifications of wheat gluten proteins to enhance their functional and nutritional properties. Some of these modifications are impractical for the commercial use of wheat gluten proteins in foodstuffs. Enzymatic hydrolysis of plant proteins, notably gluten proteins, is a more desirable tool for obtaining hydrolysates with specific and preserved polypeptides compared to the traditional chemical acid or alkali route, due to the high selectivity and mild conditions of enzymatic processes. Their release from related intact proteins has been shown to be affected by various factors such as: protein source, pretreatment, type and amount of enzyme, substrate concentration, hydrolysis degree, temperature, pH, and process operating conditions [3, 4, and 5]. Production of wheat protein hydrolysates may bid a cheap protein source for use in various food and nutritional supplements. It has been shown that functional and nutritional properties of food proteins can be improved by enzymatic hydrolysis. Protein hydrolysates possess properties that make them more attractive than native proteins and useful for special nutrition, such as diets for: elderly and patients with impaired gastrointestinal absorption, hypoallergenic infant formulas, sports nutrition, and weight-control diets, as well as in consumer products for general use. Anyway, it has been shown that protein hydrolysates should be rich in low molecular weight peptides which offer advantages for dietary purposes [6, 7]. Response surface methodology (RSM) and Box-Behnken experimental design have been already proven useful for optimization of process parameters relevant for the enzymatic hydrolysis and scaling-up of future processes, aiming to obtain protein hydrolysates with improved, accurately defined and desired, functional properties [8, 9].

In order to enhance the functional properties of gluten hydrolysates and confirm the gluten potential for inclusion into functional foods, the impact of four key selected process conditions including gluten concentration, temperature, pH and enzyme-gluten (E/S) ratio on the enzymatic reaction was investigated by applying a RSM and Box-Behnken experimental design from the viewpoint of hydrolysis degree and functional properties determined by two methods. Namely, this study was designed to examine the relationship between hydrolysis process parameters and functional properties such as solubility, foam capacity and foam stability. Finally, the gluten hydrolysate which showing the highest improvement of solubility and foaming properties was separated by sequential ultrafiltration using three cellulose membranes (pore cut-off 30, 10 and 3 kDa) to obtain molecular weight distribution profile of polypeptides mixture, viz. hydrolysate.

2. Materials and Methods

2.1 Materials

Gluten from wheat (moisture content: 6.8%, protein content (N 5.70): 78.52% on dry basis) was purchased from MP Biomedicals (Santa Ana, California, USA). The commercial food-grade protease Alcalase 2.4L (endo-peptidase from Bacillus licheniformis) was obtained from Sigma Aldrich (St. Louis, MO, USA). The enzyme activity was ≥ 2.4 Anson Units (U)/g, where one U is defined as the amount of enzyme which, under specified conditions, digests urea-denatured hemoglobin at an initial rate such that there is liberated an amount of TCA-soluble product per minute, giving the same color with Folin-Ciochateu Phenol reagent as one milliequiv-alent of tyrosine at 25 °C and at pH 7.50. The ultrafiltration (UF) stirred cell unit and cellulose membranes with 30, 10 and 3 kDa molecular weight cut-off (MWCO) for the preparation and fractionation of hydrolysates was from Millipore Co. (Bedford, MA, USA). The deionized water (18.2 MQ) used for the experiments was produced using a Thermo Scientific Barnstead Smart2Pure water purification system. Other chemicals used in this research were of analytical grade.
2.2 Preparation of gluten hydrolysates

The batch bioreactor apparatus was consisted of a stirred tank reactor equipped with an impeller-agitator, heating unit, a pH meter, thermometer and burette. The stirred tank reactor was contained of a 600 mL glass vessel with an inner diameter of 8 cm, flat bottom and at a working volume of 330 mL. The distance from the bottom wall was kept constant at 3.2 cm throughout the experiments. The substrate for enzymatic hydrolysis was aqueous dispersion of untreated wheat gluten (typically 5% w/protein/v) which was adjusted to optimum pH for enzyme activity with 0.8 M HCl or 0.8 M NaOH, then stirred and allowed to equilibrate to the working temperature for 20 min. The reactions were started by adding the appropriate amount of alcalase.

Enzymatic hydrolysis was carried out at constant pH, temperature and agitation (typically 200 rpm) while the progress of the reaction was followed using a pH-stat method. When the reaction achieved an equilibrium state, the enzyme was inactivated by heat treatment at 90 °C for 15 min. The hydrolysates were then rapidly cooled to 25 °C, and then centrifuged at 12,000 × g for 15 min. at room temperature. The supernatants were collected and kept frozen (-20 °C) for further analysis. Protein content of each gluten sample was assessed using the Lowry method with BSA as the standard [10]. The initial reaction rate (r, h⁻¹) was calculated as follows:

\[ r = \left( \frac{dDH}{dt} \right)_{t=0} \]  

(1)

Where DH presents the degree of hydrolysis (%) at time t (min).

The DH was calculated according equation [11]:

\[ DH = \frac{h \cdot 100}{h_{tot}} = \frac{N_b \cdot B \cdot 100}{a \cdot m_p \cdot h_{tot}} \]  

(2)

Where: h represents the number of equivalents of peptide bonds hydrolyzed at the time expressed in meq/g, \( h_{tot} \) is the theoretical amount of peptide bonds in the protein per weight unit of a protein (meq/g) and can be calculated from its amino acid composition (for wheat gluten protein \( h_{tot} \) is 8.38 mmol/g of protein [12]), B is the consumption of the base in mL, \( N_b \) is the normality of the base, \( m_p \) is the mass of protein in g, and \( a \) is the degree of dissociation of the α-amino groups (a=0.926 at 60 °C and pH 8.0 [11]).

2.3 Optimization study

2.3.1 The effects of process parameters on functional properties by the means of an experimental design

The effects of four key process parameters on selected functional properties of hydrolysates obtained in the alcalase-catalyzed wheat gluten hydrolysis such as gluten concentration (\( X_1 \); 1 - 9% w/v), temperature (\( X_2 \); 40 - 60 °C), pH (\( X_3 \); 7 - 9) and enzyme/substrate ratio, E/S ratio (\( X_4 \); 0.25 - 0.75 AU/g of protein) were investigated by the means of an experimental design. These variables were chosen based on the results obtained in a preliminary study and are the most commonly used for modeling enzymatic hydrolytic reactions. The degree of hydrolysis and several functional properties like solubility, foam capacity (FC) and foam stability (FS) were taken as the response variable. The design of experiments employed as well as the variables and their levels selected for developing the model are presented in Table 1. To avoid bias, 29 runs were performed in a totally random order.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Symbol</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluten concentration, %</td>
<td>( X_1 )</td>
<td>0.1</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>( X_2 )</td>
<td>40</td>
</tr>
<tr>
<td>pH</td>
<td>( X_3 )</td>
<td>7</td>
</tr>
<tr>
<td>E/S ratio, AU/g of gluten</td>
<td>( X_4 )</td>
<td>0.25</td>
</tr>
</tbody>
</table>

The data obtained were fitted to a second-order polynomial equation:

\[ y = \beta_{00} + \sum_{i=1}^{4} \beta_{i}X_i + \sum_{i=1}^{4} \beta_{ii}X_i^2 + \sum_{j=i+1}^{4} \sum_{i=1}^{4} \beta_{ij}X_iX_j \]

Where: \( \beta_{00}, \beta_i, \beta_{ii}, \text{and } \beta_{ij} \) are regression coefficients for intercept, linear, quadratic and interaction terms, respectively and \( X_i \) and \( X_j \) are independent variables.

The coefficients of the response function and their statistical significance were evaluated by the response surface regression analysis, using the software. Non-significant terms (p ≥ 0.05) were deleted from the second order polynomial and a new polynomial has been recalculated. The Fisher test (F value) was used to determine whether the second-order model was adequate to describe the obtained data while the goodness of fit of the model was evaluated by the determination coefficient (R²).

2.4 Functional properties of wheat gluten hydrolysates

2.4.1 Protein solubility

Functional properties of hydrolyzed wheat gluten proteins were determined as described according to our previous studies [13, 14]. For determination the solubilization of gluten hydrolysates, test of solubility was performed at different pH. Namely, samples of hydrolysates were suspended in water (1 % w/w), and pH was...
adjusted to: 2, 4, 6, 8, 10, and 12, using 0.5 M HCl or 0.5 M NaOH while stirring at room temperature for 1 h. The samples were then centrifuged at 12,000 × g for 10 min. The protein contents in the supernatant were determined by Lowry method. Solubility was expressed as the percentage of protein remaining in the supernatant as compared to the total protein content in the sample after dissolution.

2.4.2 Determination of foam capacity and stability

The wheat gluten protein hydrolysates were diluted to 1% (w/w) to prepare foams. The initial volume of 50 mL (20 °C) foaming solution was placed in the plastic beaker of 700 mL (diameter 7.2 cm) and whipping for 4 min. with a laboratory homogenizer at a speed of 9,500 rpm and ambient temperature. Foam capacity (FC) was expressed as foam expansion at 0 min, which was calculated according to the following equation:

\[
FC(\%) = \frac{A - B}{A} \times 100
\]

Where: \( A \) is the volume after whipping (mL) and \( B \) is the volume before 4 min of whipping (mL).

The foam stability (FS) was defined as the percentage of liquid still present in the foam after 30 min. compared to the solution at 4 min. after whipping:

\[
FC(\%) = \frac{A - B}{A} \times 100
\]

Where \( A \) is the volume of foam after 30 min standing (mL) and \( B \) is the volume before 4 min whipping (mL).

2.5 Fractionation of gluten hydrolysate using membrane ultrafiltration

The selected hydrolysate has been further separated by sequential ultrafiltration into three major gluten fractions (GF), GF I (10 - 30 kDa), GF II (3 - 10 kDa) and GF III (< 3 kDa). The ultrafiltration was performed using an ultrafiltration stirred cell unit through cellulose membranes. During the ultrafiltration process, the pressure was applied with nitrogen, as indicated by the manufacturer of the membranes. The protocol of separation gluten hydrolysates on fractions by ultrafiltration was previously described by Jovanović et al., [15] and schematically presented in Figure 1. Retentates and permeates was collected and stored in the freezer until required for further analysis such as determination of protein contents.

2.6 Statistical analysis

In this study, all experiments according to the functionality were carried out in triplicates and expressed as means with standard deviation. The effects of different parameters under the significance level of \( p < 0.05 \) were examined using one-way analysis of variance (ANOVA) and Student t-test. Analysis of variance, followed by the Tukey test was performed to examine the effects of different pretreatments under the significance level of \( p < 0.05 \). All statistical analyses including calculations were conducted using OriginPro 8.5 (OriginLab Corp., Mass., USA).

3. Results and Discussion

3.1 Optimization of the process parameters regarding functional properties of wheat gluten hydrolysates

Generally, the optimization of enzymatic reactions implies varying one parameter at a time, while keeping the all others constant. This approach does not provide insight into the existence and nature of interactions between factors. On the other hand, statistical tools including RSM and experimental design are very useful, not only in process optimization, but also in explaining qualitatively and quantitatively the relationship between the important reaction parameters.

The effects of well-defined process parameters for the alcalase-catalyzed gluten hydrolysis and their interactions were investigated by the means of the four-factor Box-Behnken experimental design with 29 experimental points (5 central points) as shown in Table 2. During the hydrolysis in batch bioreactor the stirring rate (200 rpm) and running time (2 h) were kept constant. The degree of hydrolysis and several functional properties including solubility, foam capacity and foam stability were set as response variables.
3.2 Response surface methodology for DH

Based on the results from the RSM analysis, the final second-order polynomial model was developed to describe the degree of hydrolysis by considering only the significant coded terms:

\[
Y_i = 24.92 - 1.36 \cdot x_1 + 2.33 \cdot x_2 + 7.50 \cdot x_3 + 6.50 \cdot x_4 + 3.02 \cdot x_1 x_2 + 1.54 \cdot x_1^2 - 1.18 \cdot x_2^2 - 3.51 \cdot x_3^2 - 1.74 \cdot x_4^2 + 2.55 \cdot x_2 + 3.02 \cdot x_1 x_2 + 1.54 \cdot x_1^2 - 1.18 \cdot x_2^2 - 3.51 \cdot x_3^2 - 1.74 \cdot x_4^2
\]  

Significant model terms included gluten concentration, temperature, pH, and E/S ratio in their linear and quadratic terms. Obviously, only gluten concentration and temperature showed a strong interaction effect, whereas the interactions among other parameters were insignificant (Figure 2). The coefficient of determination ($R^2$) is of 0.981, which indicated an adequate adjustment of the experimental data, showing that more than 98% of the data variability was explained in the proposed empirical equation.
The shape of the 3D surface plot representing DH versus gluten concentration and temperature is shown in Figure 2a. It is apparent an increase or decrease in one axis and decrease or increase in the other axis, detecting that both parameters may affect reaction rate in opposite ways. The DH gradually increased as temperature increased at higher gluten concentrations, pointing out that in such conditions the kinetic effect was dominant. At gluten concentration of 1%, the DH initially slightly increased with the temperature, passing through a maximum at around 50°C and then decreased. This was probably due to the enzyme denaturation at higher temperature which was pronounced at lower substrate concentration. The highest DH value of 30.05% was achieved at 60°C and high level of gluten concentration of 9% (Figures 2a and 2c).

On the other hand, the DH increased with an increase in E/S ratio (Figures 2b and 2d). As expected, higher DH (31.51%) was achieved at higher studied E/S ratio (0.75 AU per g of protein).

It is evident that in this optimization, the influence of pH was much prominent than the influence of the E/S ratio. For an illustration, at pH 9, the DH varied from 24.75 to 32.32% on increasing the E/S ratio. Meanwhile, at E/S ratio of 0.5, it even increased from 13.60 to 28.72% with pH increase from 7 to 9, indicating that it is possible to achieve a high DH level with low amounts of alkalase at high pH, which could be useful from the economic view point (Figure 2b). Discussed results was similar or higher than the results reported previously, in which authors reported DH values widely ranging from 4.7 to 26% after 3.5 to 24 h for alcalase-catalyzed reaction, depending on reaction system, operating and reaction conditions [16]. For example, Zhang et al. [17] found that the batch hydrolysis of gluten performed with alcalase was considerably enhanced by the addition of very small amounts of cysteine. Authors were explained this observation by the influence of cysteine on the structural and rheological properties of wheat gluten, altering a typical gluten viscoelastic behavior (ranging from more solid-like to more fluid-like) and increasing its solubility.

Noticeable increase in DH in this study can be attributed to a regularly designed reactor setup and efficient mixing. Namely, the enzymatic hydrolysis of wheat gluten often carried out in the absence of mixing in mechanically agitated bioreactors, resulting in substrate and product concentration gradients and mass transfer limitations.

3.3 Response surface modeling for functional properties of gluten hydrolysates

The results of the effect of selected parameters on techno-functional properties of the gluten protein hydrolysates (solubility, foam capacity (FC) and stability...
(FS)) are shown in Table 2. The results of the second-order response surface models obtained by analysis of variance (ANOVA) representing the empirical relation between these functional properties and variables are presented in Table 3. Hydrolysis of wheat gluten by alcalase was optimized regarding several functional properties and three response equations were obtained, making it possible to predict selected functional properties from known values of the four main factors.

The results of the second-order response surface model were examined by analysis of variance (ANOVA) and Fischer’s F-test. Based on the obtained response surfaces (Figures 3, 4 and 5), it is easier to analyze the effect of temperature, pH of hydrolysis, as well as the concentration of gluten and E/S ratio on the observed responses. The fit of the models was checked by the $R^2$, which was calculated to be in the range of 84.4 to 98.1, indicating that 84.4 - 98.1% of the variability in the response could be explained by the model (Table 4). The models also showed statistically insignificant lack of fit, as is evident from the lower calculated F values than the theoretical F value at 5% level. Results obtained by the statistical analyses are shown in Table 4.

### 3.3.1 Influence of process parameters on the solubility

The solubility is an important functional aspect that must be taken into account due of its influence on other techno-functional properties and quality of the end hydrolysate. For example, to obtain optimum functionality in foods that requires gelation and foaming properties, a highly soluble protein is preferable. Model parameters $X_1, X_2, X_3, X_4, X_1^2$ and the $X_1 X_4$ interaction are significant at a level of significance of 95% (the results are not shown), while the effects of all others parameter interaction and square effects $X_2^2, X_3^2, X_4^2$ have no statistically significant effect on solubility of gluten hydrolysate. p-values at a level of significance of 95% indicate that the largest effect on this response function has the interaction of the substrate concentration and the E/S ratio ($p < 0.0001$). Because that Figure 3 shows only influence of gluten concentrations and E/S ratio in 3D graph. The obtained solubility model, characterized by the coefficient of determination $R^2 = 0.9440$, proved to be adequate ($p < 0.05$), with only 5.6% of the total number of variations that cannot be explained by the model.

### Table 3. Response equations for selected functional properties of gluten hydrolysates

<table>
<thead>
<tr>
<th>Response functions</th>
<th>The empirical second-order polynomial equations with significant factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>$Y_2 = 89.33 - 0.98 \cdot x_1 - 0.91 \cdot x_2 - 0.41 \cdot x_3 - 1.96 \cdot x_4 + 11.03 \cdot x_1 x_4 - 5.93 \cdot x_1^2$</td>
</tr>
<tr>
<td>Foam capacity (FC)</td>
<td>$Y_3 = 45.16 + 4.67 \cdot x_1 + 2.01 \cdot x_2 - 6.83 \cdot x_3 + 2.28 \cdot x_4 + 5.00 \cdot x_1 x_2 - 7.13 x_1 x_3 - 1.55 \cdot x_1 x_4 + 0.046 x_2 x_3 - 6.93 x_2 x_4 - 0.068 x_3 x_4 + 4.87 \cdot x_1^2 + 3.31 x_2^2 + 4.42 x_3^2 + 3.76 x_4^2$</td>
</tr>
<tr>
<td>Foam stability (FS)</td>
<td>$Y_4 = 24.23 + 4.64 \cdot x_1 + 1.57 \cdot x_2 + 4.58 \cdot x_3 + 0.70 \cdot x_4 + 5.45 x_1 x_2 + 4.21 x_1 x_3 + 2.94 x_1 x_4 + 5.51 x_2 x_3 - 2.53 x_2 x_4 - 0.043 \cdot x_3 x_4 - 6.53 x_1^2 - 6.20 x_2^2 - 1.68 x_3^2 - 0.63 x_4^2$</td>
</tr>
</tbody>
</table>

### Table 4. Results obtained by the statistical analyses (ANOVA)

<table>
<thead>
<tr>
<th>Response functions</th>
<th>Determination coefficient $R^2$</th>
<th>Probability (p-value)</th>
<th>Fisher test, $F$-value</th>
<th>Coefficient of variation (CV)</th>
<th>Lack of fit</th>
<th>Adequate precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Y_2$ - Solubility</td>
<td>0.9440</td>
<td>&lt; 0.0001</td>
<td>2.93</td>
<td>7.78</td>
<td>0.0679</td>
<td>7.82</td>
</tr>
<tr>
<td>$Y_3$ - Foam capacity (FC)</td>
<td>0.8440</td>
<td>&lt; 0.0001</td>
<td>4.64</td>
<td>4.64</td>
<td>0.0529</td>
<td>17.232</td>
</tr>
<tr>
<td>$Y_4$ - Foam stability (FS)</td>
<td>0.9812</td>
<td>&lt; 0.0001</td>
<td>6.25</td>
<td>3.71</td>
<td>0.0517</td>
<td>18.416</td>
</tr>
</tbody>
</table>
their increase (T), or decrease (pH) were reduced hydrolysates solubility. The obtained results can be explained by the high alkaline affinity according to gluten and the ability to hydrolyze peptide bonds by forming a large amount of soluble peptides. In addition, the analysis reveals that all tested variables have a significant effect on the solubility, within the experimentally tested ranges (data not shown). The most relevant variable seems to be E/S ratio, but the effects of gluten concentration, pH, temperature and gluten concentration and E/S interactions are also significant. The quadratic term of gluten concentration is also significant, indicating that a response is a quadratic function with a local maximum. High solubility of wheat protein (>60%) achieved in the pH range of 2 - 12 showed Kong at all., [18]. Mejri at all., [19] were tested the influence of different additives on the solubility of wheat gluten and obtained almost 100% solubility using 0.5% cysteine solution at pH 2 - 4. Wheat protein solubility of 81.01% was achieved by Drag and Gonzalez at pH 4 for 2 h. Also, it has been shown that high solubility of 53.74% and 73.88%, is achieved at pH 6.5 and 9, respectively [20].

It can be concluded that the high solubility (98.85%) of the wheat gluten hydrolysates is achieved at a medium reaction temperature (50 °C) and a pH of 9, using minimal amounts of substrate and a rather low amount of enzymes, since the optimal E/S ratio seems to be 0.5 AU/g of gluten. Based on both results obtained for DH and presented results, it is obvious that the enzymatic hydrolysis of wheat gluten has improved gluten solubility since the positive correlation between the DH and solubility is remarked. In this way, hydrolysis influenced the reduction of the molecular weight and the hydrophobicity of wheat protein as well as the augmented content of polar and ionizing groups, resulting in a notable increase in the solubility. In general, it is obvious that an increase in DH has been influenced by the appearance of smaller gluten peptides with the content of a large number of polar amino acid residues relative to non-hydrolyzed gluten, and consequently stronger hydrogen bonds with water molecules could be formed and become more soluble in aqueous solutions.

3.3.2 Influence of process parameters on the foam capacity and stability

The foaming ability of the proteins and their hydrolysates is one of the most important technological-functional properties. The basic prerequisites that a proteins and their hydrolysates have the ability to form a foam is to quickly adsorb during the mixing process at the water-air interface, then quickly change their conformation and the distribution of the functional groups on the surface of the molecules, as well as having the possibility of forming a cohesive viscous-elastic film using intermolecular interactions. The obtained results of the foaming ability of the wheat gluten hydrolysate prepared by alkalase are presented as foam capacity (FC) and foam stability (FS) and are illustrated in Figures 4 and 5.
The obtained results reveal that the foam capacity range of the wheat gluten hydrolysates are in the range of 24.24 - 80.31% (Table 2), depending on the independent variables that were tested. Overall, it is apparent that the gluten hydrolysates have a relatively good foam capacity, since in a large number of experiments this ability has been higher than 50%. The value of the coefficient of determination of 0.9812 indicates that the fitted second-order polynomial well approximates the experimental results for the interval tested since only 1.88% of the variation could not be ascribed by the model. Also, the goodness of fit and significance of the model (p < 0.05) are confirmed. All parameters, $X_1, X_2, X_3$, and interactions like $X_1 X_2, X_1 X_3, X_2 X_3$, and $X_1^2, X_2^2, X_3^2$ are significant at the level of 0.05 (p < 0.05). From Figure 4, it seems that the most relevant variables for the FC are gluten concentration and E/S ratio. The effects of temperature and pH level are also statistically significant (p < 0.05). It appears that while substrate concentration, temperature and pH have a positive effect, the gluten concentration and E/S ratio and pH and E/S ratio interactions have a significant negative influence on FC. Among the various experiments, the highest FC of 80.31% is achieved in run No. 1 (gluten concentration of 1%, 40°C, pH 8 and E/S ratio 0.5). Experimental values for FC are found to be in good agreement with predictions.

From Figure 5, it seems that the most relevant variables for the FS are gluten concentration and pH. The effects of temperature and E/S ratio are statistically significant (p < 0.05). It appears that while substrate concentration, temperature and pH have a positive effect, the gluten concentration and E/S ratio interaction has a significant negative influence on FS. Among the various experiments, the highest FS of 35.06% is achieved in run No. 6 (gluten concentration of 5%, 50°C, pH 9 and E/S ratio 0.5). Experimental values for FS are found to be in good agreement with predictions. Additionally it is very interesting noticed that temperature showed an interactive effect with pH (Figure 5c). It appears that the surface is smooth showing increase or decrease in one axis and decrease or increase in the other axis, which reflect that the temperature may affect reaction rate in opposite ways. Specifically, as the temperature increases, the expected increase in reaction rate resulting from more productive molecule collisions per unit time is offset by the increasing rate of enzyme denaturation at higher pH value. At intermediate and low levels of pH, however, a different behavior is observed as the surface increases when reaction temperature increases. This could be result of a negative temperature-pH interaction, probably caused by thermal inactivation of enzyme which is more pronounced at higher pH value. The maximum yield of FS, from the aspect of temperature and pH, could be obtained when working at medium temperatures and high level of pH (also, exp. No. 6).

The researchers have been examined the possibility of preparation of gluten hydrolysates with wheat-bug (Eurygaster spp.) protease and improvement of functional properties of hydrolysates with 3 and 5% degree of hydrolysis. They were found that foaming capacity (FC) values of gluten hydrolysates were significantly higher (p < 0.05) at pH level 6, 7, 8, while the foam stability (FS) values of gluten hydrolysates were significantly higher (p < 0.05) at pH 6 and 7. In addition, they have determined significant correlations (p < 0.001) between solubility FC and FS values of gluten and its hydrolysates with only 3% and 5% DH [21]. Based on the discussed results (Figure 4 and 5), mentioned literature data are significantly worse, because obtained hydrolysates can be considerable as favorable for use in functional foods.

3.4 Molecular weight distribution profile of the gluten hydrolysate fraction

The hydrolysate obtained in experiments No. 8 and 19 showing the most satisfactory values of solubility and both foaming properties, respectively, has been...
The degree of hydrolysis of the wheat gluten hydrolysate was 31.51 and 32.23% (exp. No. 8 and 19, respectively) much higher comparing to with the results of Kong et al., [22]. A moderate degree of hydrolysis may contribute to significant improvement of foaming ability. According to the molecular weight distribution, most peptides with high molecular weight (> 30 kDa) were enzymatically hydrolyzed into smaller peptides for both hydrolysates. The protein contents of the fractions GF II (10 - 30 kDa), and GF III (3 - 10 kDa) were notable higher than first fraction; 24.5 and 29.1% for hydrolysate with DH 31.51%, and 28.5 and 24.6% for hydrolysate with DH 32.23%. Based on the presented results of mass fraction with alcalase-catalyzed hydrolysis the small peptides below 3 kDa were appeared, accounting for approximately 24 and 20% of the total hydrolysate, exp. No. 8 and 19, respectively. Summary, the relative percentage of the peptides released with molecular weight of over 30 kDa decreased with increasing enzymatic hydrolysis viz. degree of hydrolysis, while those with molecular weight below 10 kDa increased significantly (p < 0.05).

4. Conclusions

- The aim of the research was to find the optimal operational and process parameters for the enzymatic hydrolysis of wheat gluten in a batch stirred bioreactor regarding the degree of hydrolysis and functional properties of the obtained hydrolysates. The impact of selected process conditions including gluten concentration, temperature, pH and enzyme-gluten (E/S) ratio on the enzymatic reaction was further investigated by applying a Box-Behnken experimental design from the viewpoint of the degree of hydrolysis (DH). The coefficient of determination (R²) is of 0.981, which indicated an adequate adjustment of the experimental data, showing that more than 98% of the data variability was explained in the proposed empirical equation. The statistical analysis showed that each variable had a significant effect on degree of hydrolysis. It appeared that only gluten concentration and temperature showed strong interaction effect whereas the interactions among other parameters were insignificant.

- The impact of process conditions including gluten concentration, temperature, pH and enzyme-gluten (E/S) ratio on the enzymatic reaction was also investigated by applying a Box-Behnken experimental design from the viewpoint of the selected functional properties like solubility, foam capacity, foam stability. The analysis revealed that all tested variables had a significant effect on the solubility, within the experimentally tested ranges. The most relevant variable seemed to be E/S ratio, but the effects of gluten concentration, pH, temperature and gluten concentration and E/S interactions were also significant. The quadratic term of gluten concentration was also significant, indicating that a response was a quadratic function with a local maximum. It can be concluded that the high solubility of the wheat gluten hydrolysates was achieved at a low temperature of the reaction of enzymatic hydrolysis (40 °C) and a pH of 9, using minimal amounts of substrate and a rather low amount of enzymes, since the optimal E/S ratio seemed to be 0.5 AU/g of gluten. The obtained results revealed that the foam capacity range of the wheat gluten hydrolysates were in the range of 24.2 - 80.3%, depending on the independent variables that were tested. Overall, it was apparent that the gluten hydrolysates had a relatively good foam capacity, since in a large number of experiments this ability was higher than 50%. It seemed that the most relevant variables for the foam stability were gluten concentration and pH. The effects of temperature and E/S ratio were also significant (p < 0.05). It appeared that while substrate concentration and all other parameters had a positive effect, the gluten concentration and E/S ratio interaction had a significant negative influence on foam stability. According to the statistical analysis, the maximum foam stability can be obtained at high level of gluten concentration and pH value.

- Overall, presented results are relevant to the protein ingredient industry because of the economic importance of novel gluten-based bioactive products and can provide useful information for the design an efficient enzymatic process for their production in high yield and with improved functionality. The findings of our study shown enzymatic hydrolysis is a suitable route to improve the functional properties of wheat
gluten proteins, and the molecular weight distribution of wheat gluten hydrolysates showed there occurred lots of smaller polypeptides, thus further study on the bioactivities of wheat gluten hydrolysates is in process. Based on this, wheat gluten protein’s hydrolysates unique functional and nutritional properties may offer enormous possibilities for use not only in existing food applications but also in new food product formulations.

Acknowledgment

The authors wish to extend their appreciation to the Ministry of Education, Science and Technological Development of the Republic of Serbia for its financial support within the EUREKA Project E19936 and Innovation Project “LAVGLU-Innovative processes of production cereals-based functional products enriched with non-allergenic proteins and bioactive peptides”.

5. References


