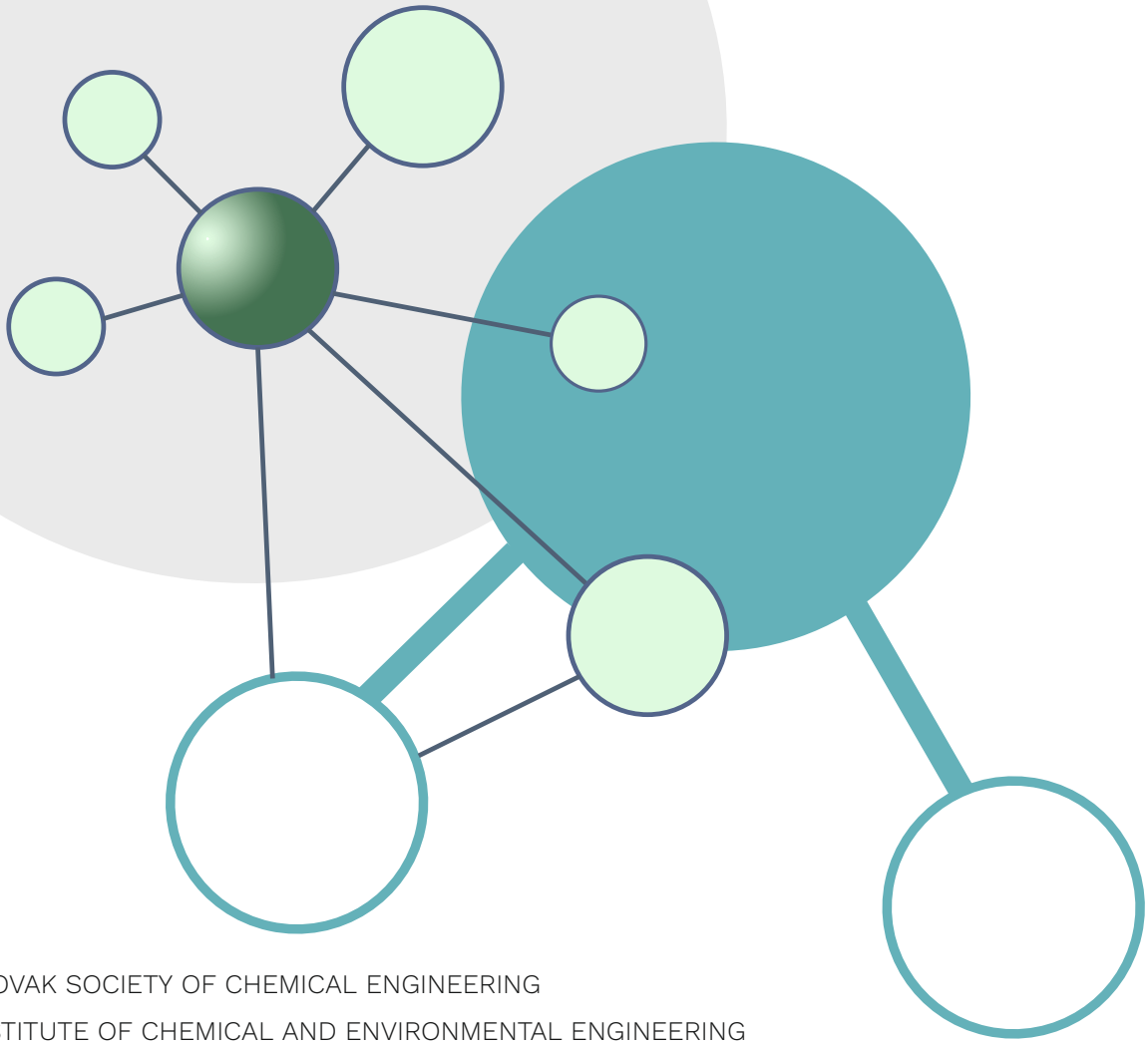
SSCHE 2019

46th International Conference of the Slovak Society of Chemical Engineering



SLOVAK SOCIETY OF CHEMICAL ENGINEERING
INSTITUTE OF CHEMICAL AND ENVIRONMENTAL ENGINEERING
SLOVAK UNIVERSITY OF TECHNOLOGY IN BRATISLAVA

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Slovak Society of Chemical Engineering

Institute of Chemical and Environmental Engineering
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46th International Conference of the Slovak Society of Chemical Engineering

Hotel Hutník I

Tatranské Matliare, High Tatras, Slovakia, May 20 - 23, 2019

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MODIFICATION OF EMULSIFYING PROPERTIES AND METAL-ION CHELATING ABILITY OF GLUTEN HYDROLYSATES BY PARTIAL ENZYMATIC HYDROLYSIS

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Key words: wheat gluten protein, enzymatic hydrolysis, emulsification activity, emulsification stability, optimization, experimental design, metal-ion chelating ability

Abstract

Wheat gluten represents the major protein fraction present in wheat flour and as protein-rich material posses cohesive and viscoelastic properties which permit the retention of gas bubbles in the dough. It could be obtained as a by-product during the separation of starch from wheat flour and as such may be utilized like a functional protein additive in various non-bakery foodstuffs due to its desirable structure-enhancing properties. However, wheat gluten is hardly soluble in water, which limits their expanding utilization.

In the regard, the aim of this research was to investigate the correlation between process parameters of wheat gluten hydrolysis and emulsification activity and stability of the prepared hydrolysates. The hydrolysates showing the greatest enhancement of the emulsifying properties which are closed correlated with solubility improvement was further separated by sequential ultrafiltration to obtain molecular weight distribution profile and peptide fraction with higher metal-ion chelating ability. The hydrolysis was performed by using commercial endopeptidase from *Bacillus licheniformis* and process was followed by monitoring the degree of hydrolysis, emulsifying properties and metal-ion chelating activity. The effects of some relevant process parameters such as gluten concentration (1-9% w/v), temperature (40-60 °C), pH (7-9), enzyme/substrate ratio (0.25-0.75 AU g^{-1} of protein) and their interactions were investigated by the means of the four-factor Box-Behnken experimental design with 29 experimental points (5 central points). The obtained results showed that the second-order models developed for emulsification activity and stability of gluten hydrolysates were significant (p<0.05) with a high value of the coefficients of determination (0.965-0.995). The statistical analysis showed that each variable had a significant effect on the emulsifying properties of the tested system. In terms of emulsifying properties results showed that gluten concentration and temperature have had a positive effect on the increase of emulsification activity, while the enhancement of emulsification stability was achieved with the highest gluten concentration and pH 9. It appeared that the hydrolysate with great emulsifying properties had the highest percentage of peptides with medium molecular weight (3-10 kDa) which had the ability to strongly chelating prooxidant metal ions such as Fe^{2+} at level 99.3%.

Results are substantial because they give useful information for the design an efficient process of gluten hydrolysis for production in high peptide yields with improved emulsifying properties. Also, may be suggesting that there are peptides with considerable size presenting a remarkable metal-ion chelating ability.

Introduction

The increasing human population, over the last few decades, has great influence to the progressive increase in demand for value-added food products. Substantial effort has expressly focused on various relatively inexpensive sources of plant proteins that are considered as an economic and an adaptable substitute for animal proteins in functional foodstuff formulations. With the expansion of wheat starch production, wheat gluten, as a by-product from the wet processing of wheat flour, is a renewable and an abundant plant protein source available in large amounts with relatively moderate prices. Wheat gluten represents, major protein fraction present in wheat flour, as protein-rich material posses cohesive and viscoelastic properties which permit the retention of gas bubbles in the dough. It could be obtained as a by-product during the separation of starch from wheat flour and as such may be utilized like a functional protein additive in various non-bakery foodstuffs due to its desirable structure-enhancing properties. However, wheat gluten is hardly soluble in water, which limits their expanding utilization [1, 2]. In particular, 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 [3]. According to the chemical structure, 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].

Due to the foregoing, researchers have been focusing on the 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. Partial and limited enzymatic hydrolysis of plant proteins, notably gluten proteins, is a more desirable tool for obtaining hydrolysates with specific 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 [4-6]. Enzyme type and degree of hydrolysis (*DH*) are substantial factors which affect the hydrolysates' performances, such as amino acid distribution, peptides molecular weight distribution and amount of residual intact protein, thus influencing their functionality. Protein hydrolysates possess improved and upgrade functional and nutritional 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 [7].

Despite the industrial importance of gluten as a multifunctional food ingredient, in the literature, very little information is found about the production of bioactive peptides of wheat gluten and the relationship between working conditions, *DH* and antioxidant ability. As very appropriate, the response surface methodology (RSM) and Box-Behnken experimental design have been already used for optimization of process parameters relevant for the controlled enzymatic hydrolysis and scaling-up of future processes, aiming to obtain protein hydrolysates with improved, accurately defined and desired properties [8].

In order to upgrade 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 *DH* and

emulsifying properties. Namely, this study was designed to examine the relationship between hydrolysis process parameters and functional properties, emulsification activity and stability. Finally, the gluten hydrolysate which showing the highest improvement of emulsifying 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. After that, the ability of gluten hydrolysate and polypeptide fractions metal-ion to chelating prooxidant metal ions such as Fe^{2+} was evaluated.

Materials and Methods

Materials

Gluten from wheat (moisture content: 6.8%, protein content: 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-Ciocalteu Phenol reagent as one milliequivalent 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). Other chemicals used in this research were of analytical grade.

Enzymatic hydrolysis of wheat gluten protein

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 400 mL glass vessel with an inner diameter of 8 cm, flat bottom and at a working volume of 200 mL. The distance from the bottom wall was kept constant at 2 cm throughout the experiments (Figure 1). The substrate for enzymatic hydrolysis was aqueous dispersion of untreated wheat gluten (1-9% $w_{protein}/v$) 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 [9]. The initial reaction rate (r, h^{-1}) was calculated as follows:

$$r = \left(\frac{dDH}{dt}\right)_{t\to 0} \tag{1}$$

where *DH* present the degree of hydrolysis (%) at time *t* (min). The *DH* was calculated according equation [10]:

$$DH = \frac{h\cdot 100}{h_{tot}} = \frac{N_b\cdot B\cdot 100}{\alpha\cdot m_p\cdot h_{tot}} \tag{2}$$

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 [11]), 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 α is the degree of dissociation of the α-amino groups at adequate temperature and pH [10]).

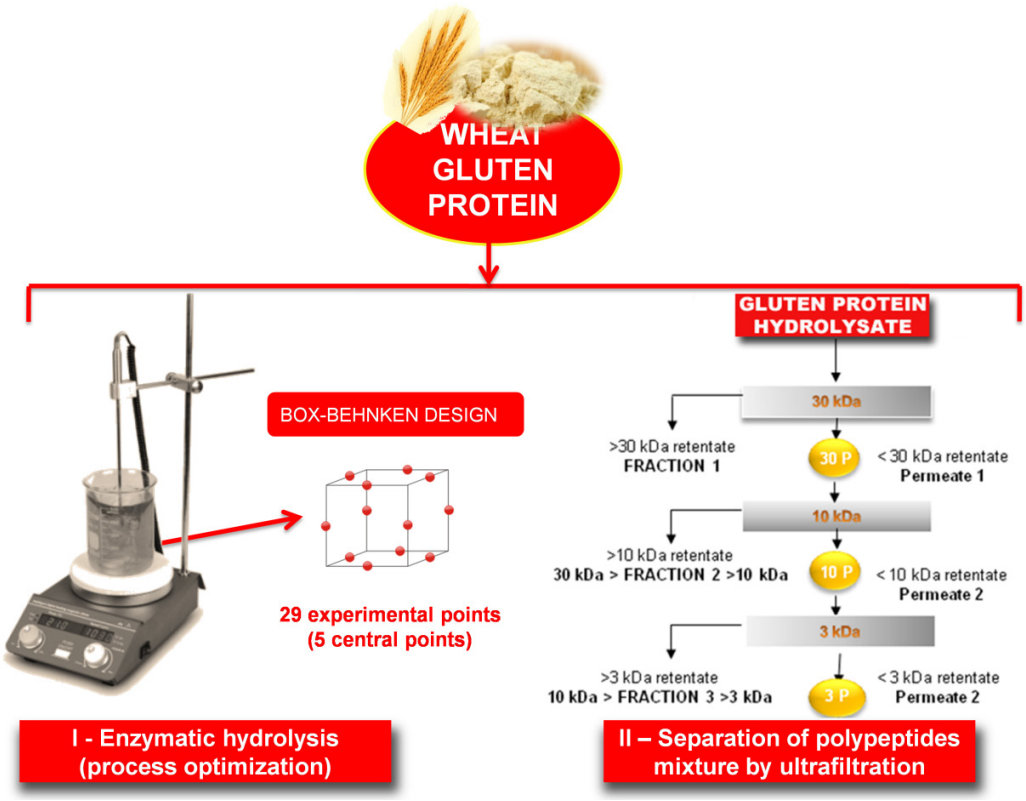

Figure 1 Illustration of the enzymatic hydrolysis with used bath bioreactor and schematic representation of gluten hydrolysate separation on the peptide fractions by utilization of ultrafiltration

Optimization study

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

The effects of four pivotal 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 gluten) were investigated by the means of an experimental design. The degree of hydrolysis (*DH*) and functional properties like emulsification activity index (*EAI*) and emulsification stability index (*ESI*) 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 1 Experimental setup for three-level, four-factor Box-Behnken experimental design with 29 experimental points in terms of coded and actual values of variables

Factors	Symbol	Level		
		-1	**0**	**1**
Gluten concentration, %	X_1	0.1	0.5	0.9
Temperature, °C	X_2	40	50	60
pH	X_3	7	8	9
***E*/*S* ratio, AU/g gluten**	X_4	0.25	0.50	0.75

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

$$y = \beta_{k0} + \sum_{i=1}^{4} \beta_{ki} X_i + \sum_{i=1}^{4} \beta_{kii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{kij} X_i X_j$$

here β_{k0}, β_{ki}, β_{kii} and β_{kij} 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 softver. Non-significant terms ($p \geq 0.05$) 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 (R^2).

Functional properties of wheat gluten hydrolysates

Emulsification activity and stability

Prepared gluten protein hydrolysates were analyzed by the turbidimetric technique for emulsion activity index and emulsion stability index as described by Pearce and Kinsella [12]. Emulsions of the each hydrolysate dispersion (1% w/w) were prepared with sunflower oil in molar ratio 1:2 and mixing for 90 sec with laboratory homogenizer. The absorbance of the diluted emulsions was measured by a UV-Vis spectrophotometer at 500 nm and it was read initially. The turbidity was calculated by the following equation:

$$T = 2.303 \frac{A}{l} \tag{3}$$

where T is turbidity, A is absorbance and l is a path length (m).

The emulsion activity index (*EAI*) was then calculated as:

$$EAI = 2 \cdot T \frac{r}{c \cdot \theta} \tag{4}$$

where T is turbidity calculated from Eq. (3), θ is the fraction of oil used to form the water-in-oil (mL), c is the weight of protein per unit volume of aqueous phase before emulsion is formed (g) and r is dilution factor.

For determining emulsion stability, the SPI dispersions were kept at 4 °C for 24 h and analyzed for emulsion activity as previously described. An **emulsion stability index (*ESI*)** was calculated by the following formula:

$$ESI = \frac{T \cdot \Delta t}{\Delta T} \tag{5}$$

where T is turbidity value at 0 h, ΔT is change in turbidity during 24 h and Δt is time interval (24 h).

Fractionation of gluten hydrolysate by 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 and during the process the pressure was applied with nitrogen (40 psi), 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. [13] and schematically presented in Figure 1. Retentates and permeates were collected and stored in the freezer for further analysis.

Metal-ion chelating ability of gluten hydrolysate and peptide fractions

The metal-ion chelating ability was determined by the protocol established by Decker and Welch [14] with slight modification. The samples of gluten hydrolysate and peptide fraction were dissolved in double distilled water at the final protein concentration of 2 mg/mL. The 0.20 mL of aliquot was mixed with 0.80 mL of double distilled water and 0.1 mL of 2 mM $FeCl_2$ and intensively mixed on the vortex. After 3 min inbubation at room temperature, the reaction between $FeCl_2$ and peptide was inhibited by the addition 0.10 mL of 5 mM ferrozine solution. The mixture was stirred and kept further at room temperature for 10 min. The absorbance of the resulting solution was measured at 562 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. Metal-ion chelating ability (%) was then calculated as follows:

$$MJHA\ (\%) = \frac{A_c - A_s}{A_c} \cdot 100 \qquad (6)$$

where: *A*s represents the absorbance of the sample solution and *A*c is the absorbance of the control solution. All experiments were carried out in triplicate.

Statistical analysis

In presented 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 (Origin Lab Corp., Mass. USA).

Results and discussion

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

The optimization of enzymatic hydrolysis implicates varying one parameter at a time, while keeping the all others constant and like that doesn't give comprehension 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. Herein, 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 show 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 functional properties including emulsification activity and stability were set as response variables.

Table 2 Experimental setup for three-level, four-factor Box-Behnken design in terms of coded, actual values of variables and experimental data of degree of hydrolysis, emulsification activity and stability

No.	RSM experimental variables				**Experimental responses**		
	X_1 (A)	**X_2 (B)**	**X_3 (C)**	**X_4 (D)**	***DH*, %**	***EAI*, m^2/g**	***ESI*, h**
1.	1(-1)	40(-1)	8(0)	0.5(0)	27.32	2245.20	19.42
2.	9(1)	40(-1)	8(0)	0.5(0)	19.29	1670.07	43.42
3.	1(-1)	60(1)	8(0)	0.5(0)	25.41	786.51	45.73
4.	9(1)	60(1)	8(0)	0.5(0)	28.34	763.81	36.71
5.	5(0)	50(0)	7(-1)	0.25(-1)	9.29	1000.58	55.62
6.	5(0)	50(0)	9(1)	0.25(-1)	24.75	826.53	43.02
7.	5(0)	50(0)	7(-1)	0.75(1)	15.51	1509.56	61.73
8.	5(0)	50(0)	9(1)	0.75(1)	29.52	1140.53	76.05
9.	1(-1)	50(0)	8(0)	0.25(-1)	25.16	2236.84	54.35
10.	9(1)	50(0)	8(0)	0.25(-1)	21.16	1115.96	48.80
11.	1(-1)	50(0)	8(0)	0.75(1)	27.21	2737.97	86.07
12.	9(1)	50(0)	8(0)	0.75(1)	26.60	1366.36	83.32
13.	5(0)	40(-1)	7(-1)	0.5(0)	8.85	1550.32	46.33
14.	5(0)	60(1)	7(-1)	0.5(0)	17.14	1525.68	77.64
15.	5(0)	40(-1)	9(1)	0.5(0)	25.16	840.67	59.90
16.	5(0)	60(1)	9(1)	0.5(0)	31.01	1050.91	48.89
17.	1(-1)	50(0)	7(-1)	0.5(0)	16.32	2782.92	67.64
18.	9(1)	50(0)	7(-1)	0.5(0)	13.60	1233.65	53.01
19.	1(-1)	50(0)	9(1)	0,5(0)	31.50	2038.42	60.83
20.	9(1)	50(0)	9(1)	0,5(0)	28.72	1129.60	77.05
21.	5(0)	40(-1)	8(0)	0.25(-1)	17.20	1795.31	45.28
22.	5(0)	60(1)	8(0)	0.25(-1)	19.90	798.37	59.68
23.	5(0)	40(-1)	8(0)	0.75(1)	22.04	1137.33	58.09
24.	5(0)	60(1)	8(0)	0.75(1)	27.20	1352.42	60.95
25.	5(0)	50(0)	8(0)	0.5(0)	24.76	1281.71	71.09
26.	5(0)	50(0)	8(0)	0.5(0)	25.16	970.57	21.49
27.	5(0)	50(0)	8(0)	0.5(0)	24.93	1124.35	54.23
28.	5(0)	50(0)	8(0)	0.5(0)	25.01	1058.32	67.52
29.	5(0)	50(0)	8(0)	0.5(0)	24.75	991.46	70.21

** Experiments from 25-29 presented central points.*

Response surface methodology for DH

According to the results from the RSM analysis, the second-order polynomial model (Eq. 7) is established and described *DH* taking into account only significant coding terms.

$$Y_1 = 24.92 - 1.36 \cdot x_1 + 2.33 \cdot x_2 + 7.50 \cdot x_3 + 2.55 \cdot x_4 + 3.02 \cdot x_1 x_2 + 1.54 \cdot x_1^2 - 1.18 \cdot x_2^2 - 3.51 \cdot x_3^2 - 1.74 \cdot x_4^2 \quad (7)$$

This significant model terms involved gluten concentration, temperature, pH and *E*/*S* ratio in their linear and quadratic terms. Manifestly, only gluten concentration and temperature showed a strong interaction effect, since the interactions among other two parameters were not significant (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 justified in the suggested empirical Eq. 7.

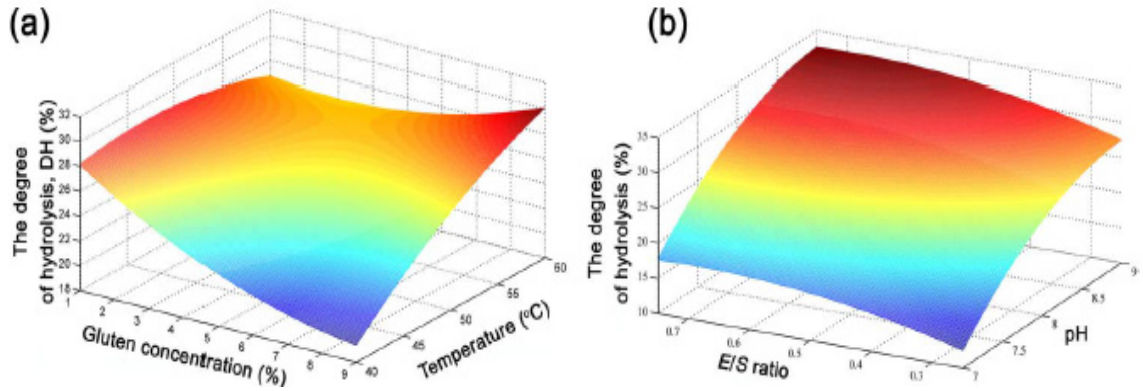

Figure 2 Response surface showing the effect of gluten concentration and temperature (a) and *E*/*S* ratio and pH (b) on the achieved degree of hydrolysis

From the 3D surface plot illustrated in the Figure 2a, it is obvious an enhancement or reduction in one axis and reduction or enhancement in the other axis. Namely, both parameters gluten concentration and temperature may influence on the reaction rate in opposite ways. Herein, 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 reduced. 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% (Figure 2a). Besides, the *DH* increased with an increase in *E*/*S* ratio (Figure 2b). As expected, higher *DH* (31.51%) was achieved at higher studied *E*/*S* ratio (0.75 AU per g of gluten). Additional, it can be emphasized that the effect of pH was more notable than the effect of the *E*/*S* ratio. Thus, 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).

Demonstrated results (Figure 2) was similar or higher than the research study 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 [15]. Zhang et al. [16] found that the batch hydrolysis of gluten performed with alcalase was considerably enhanced by the addition of very

small amounts of cysteine, which was explained by the influence of cysteine on the structural and rheological properties of gluten such as altering a gluten viscoelastic behavior and increasing its solubility.

Response surface modeling for emulsifying properties of gluten hydrolysates

The effects of four pivotal process parameters on emulsifying properties of the gluten protein hydrolysates are shown in Table 2. The results of the second-order response surface models obtained by analysis of variance representing the empirical relation between emulsifying properties and variables are presented in Table 3. Given the fact that the alcalase-catalyzed hydrolysis of wheat gluten was optimized regarding degree of hydrolysis and emulsifying properties, the obtained response equations make it possible to predict functional properties from known values of the four main factors.

Table 3 Response equations for selected functional properties of gluten hydrolysates

Response functions	**The empirical second-order polynomial equations with significant factors**
Emulsification activity index, *EAI*	$\boldsymbol{Y_2} = 1151.54 - 622.34 \cdot x_1 - 1.28 \cdot x_2 - 208.27 \cdot x_3 + 18.05 \cdot x_4 + 301.60 \cdot x_1 x_2 + 457.11 x_1^2 + 229.57 x_4^2$
Emulsification stability index, *ESI*	$\boldsymbol{Y_3} = 46.72 + 1.91 \cdot x_1 + 5.13 \cdot x_2 - 0.11 \cdot x_3 + 9.13 \cdot x_4 - 8.26 x_1 x_2 + 7.71 x_1 x_3 + 4.37 x_1 x_4 - 10.58 x_2 x_3 - 4.00 x_2 x_4 + 6.51 x_3 x_4 - 2.82 x_1^2 - 5.12 x_2^2 + 11.79 x_3^2 + 9.98 x_4^2$

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 and 4), 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 0.965 to 0.992, indicating that 96.5-99.2% of the variability in the response could be explained by the proposed 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 summarized in Table 4.

Table 4 Results obtained by the statistical analyses (ANOVA)

Response functions	**Determination coefficient R^2**	**Probability (*p*-value)**	**Fisher test, *F* -value**	**Coefficient of variation (CV)**	**Lack of fit**	**Adequate precision**
$\boldsymbol{Y_2}$ – emulsification activity index (*EAI*)	0.9915	<0.0001	46.3	4.42	0.0676	36.214
$\boldsymbol{Y_3}$ – emulsification stability index (*ESI*)	0.9651	<0.0001	23.76	4.31	0.4824	41.497

Influence of process parameters on the emulsification activity index

The activity of creating protein emulsions is the ability of proteins/peptides to participate in the process of emulsion generation, and *EAI* represents the size of the surface of an intermediate oil-water that can stabilize one gram of protein.

The most relevant variable on the emulsification activity index seems to be interaction of the gluten concentration and temperature ($p<0.0001$), thus this interaction is graphically represented in the Figure 3. The quadratic terms of gluten concentration and temperature are also significant, indicating that a response is a quadratic function with a local maximum. Herein, it is obvious an increase or decrease in one axis and decrease or increase in the other axis. Specifically, both parameters gluten concentration and temperature may influence on the reaction rate in opposite ways. At gluten concentration of 1%, the *EAI* initially slightly increased with the temperature, passing through a maximum at around 45 °C and then reduced. The highest value of *EAI* of 2782.9 m²/g was reached at pH 8.0 and at a temperature of 50 °C, *E/S* of 0.75 AU/g gluten and substrate concentration of 1%. In general, it is accepted that the protein hydrolysates, like gluten hydrolysates, display better emulsifying properties than the native or non-hydrolyzed protein ones, due to the higher exposure of hydrophobic amino acid residues to the surface of molecules. However, this depends on the size of protein chains. For emulsifying activities and the possibility of protein migration to the oil/water surface, protein chains with a molecular weight greater than 10 kDa are needed, as they contribute to the production of better emulsions than chains of less molecular weight.

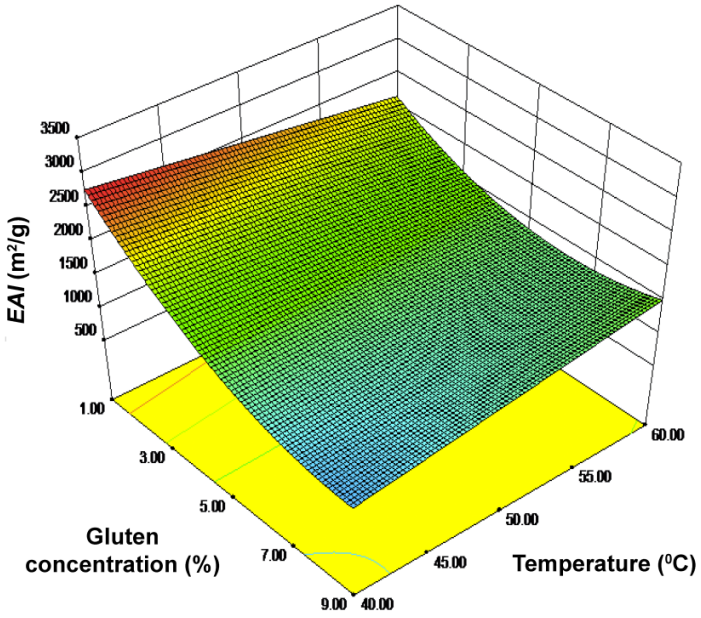

Figure 3 Response surface showing the effect of temperature and gluten concentration on the emulsion activity index (actual factors pH and *E/S* ration were 8.00 and 0.75 AU/g gluten, respectively)

Influence of process parameters on the emulsification stability index

The influence of the analyzed variables (gluten concentration, temperature and pH reaction, E/S ratio) on the emulsification stability index of wheat protein hydrolysates prepared by alkalase is shown on Figure 4. The *ESI* in this case represents the time during which the proteins/peptides can continue on the surface of the oil-water and affects the maintenance of stability of the formed emulsions.

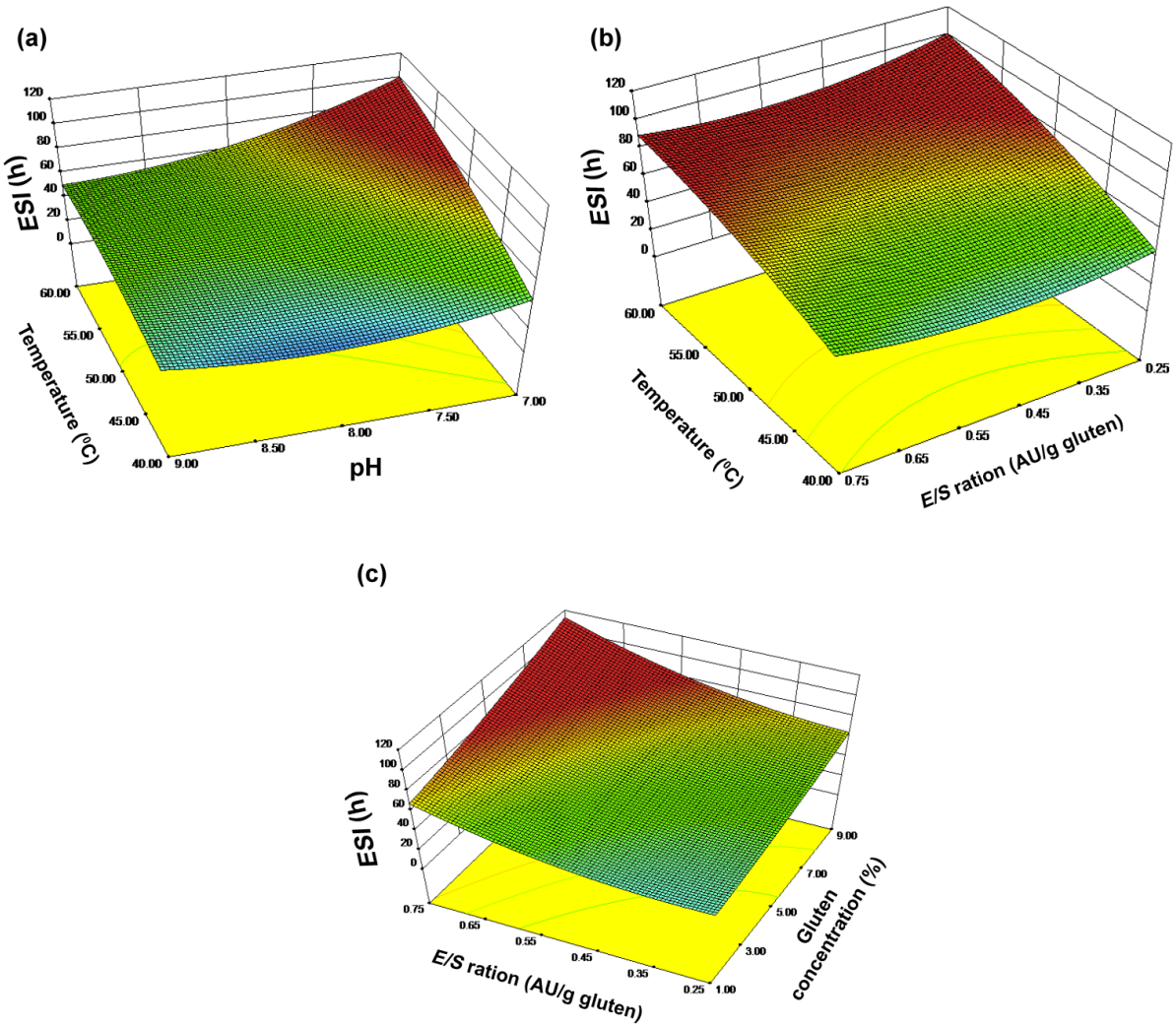

Figure 4 Response surface showing the effect of (a) temperature and pH (actual factors *E/S* ration and gluten concentration were 0.25 AU/g and 1%, respectively); (b) temperature and *E/S* ratio (actual factors gluten concentration and pH were 1% and 7.00, respectively); (c) *E/S* ration and gluten concentration (actual factors temperature and pH were 40 °C and 9.00, respectively) on the emulsification stability index

According to the represented surface responses and statistical dates from the Table above, the high value of the coefficient of determination of 0.9651 was noticed which indicate that the second-order polynomial model perfect fits the experimental results for the *ESI*, since only 3.49% of the variation could not be described by the model. The all tested parameters and their interactions as well as quadratic value of parameters are significant at the level of 0.05 (p<0.05). Herein, it is obvious an increase or decrease in one axis and decrease or increase in the other axis (Figure 4a). Specifically, both parameters pH and temperature may influence on the reaction rate in opposite ways. At gluten concentration of 1%, the *ESI* initially slightly increased with the temperature, passing through a maximum at around 50 °C and then reduced. The highest value of *ESI* of 100 h was reached at pH 7.0 and at a temperature of 60 °C, *E/S* of 0.25 AU/g gluten and substrate concentration of 1%. From the surface illustration data can be emphasized that the greatest emulsifying stability of protein hydrolysate was achieved higher substrate concentration of 9 % and at higher *E/S* of 0.75 AU/g of gluten (Figure 4b and 4c). Besides, it was apparent that the emulsifying stability of gluten hydrolysates was in almost linear functional dependence of pH and gluten concentration.

Previous studies have shown that the controlled and partial enzymatic hydrolysis could be successfully applied to improve and upgrade the functional properties of gluten, but the excessive hydrolysis affects the functional properties negatively and may reduce their application for human consumption. For example, the authors have stated that the *DH* should be even lower, <5% in order to obtain desirable emulsifying properties. Thus, the degree of hydrolysis should be controlled because the *ESI* or *EAI* increase with increasing the degree of hydrolysis (decreasing peptide chain length) up to a critical value after which the efficiency decreases [7, 17]. The researchers were found, during the optimization of hydrolysis process of wheat gluten with protease from *Aspergillus usamii*, that hydrolysates possesses a significant improvement in the emulsifying activity compared to the non-hydrolyzed gluten one, but although there was an improvement in the activity, the ability to stabilize the formed emulsion remained the same as well as for non-hydrolyzed gluten [18]. Wang at al. [19] have determined that the emulsifying activity and stability of gluten hydrolysate significantly improves even twice in relation to the native gluten.

Molecular weight distribution profile of the gluten hydrolysate fraction

The hydrolysate obtained in experiment No. 16 showing the most satisfactory values of both emulsification ability and stability index, has been additional separated by ultrafiltration into four major peptide fractions: GF I (>30 kDa), GF II (10–30 kDa), GF III (3–10 kDa) and GF IV (<3 kDa). Prepared fractions based on their protein content and metal-ion chelating activity were compared and the results are illustrated in Figure 5.

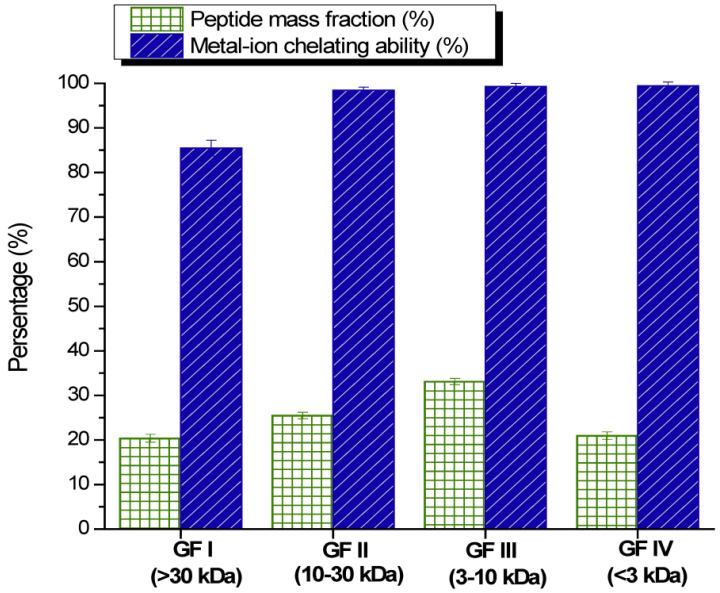

Figure 5 The molecular weight distribution profile of ultrafiltrated fractions of wheat gluten hydrolysate and profile of their metal-ion chelating ability

The degree of hydrolysis of the selected wheat gluten hydrolysate was 31.01% (exp No. 16) much higher comparing to with the results of Kong et al. [17, 20]. A moderate degree of hydrolysis may contribute to significant improvement of emulsification activity and stability. According to the molecular weight distribution, most peptides with high molecular weight (> 30 kDa) were enzymatic hydrolyzed into smaller peptides. The protein contents of the fractions GF II (10–30 kDa) and GF III (3–10 kDa) were notable higher than first fraction (20.4%); 25.5 and 33.1%, respectively. Based on the presented results of mass fraction with alcalase-catalyzed hydrolysis the small peptides below 3 kDa were appeared, accounting for approximately 20% of the total hydrolysate.

It appeared that the selected hydrolysate with great emulsifying properties had the highest percentage of peptides with medium molecular weight (3-10 kDa) which had the ability to strongly chelating prooxidant metal ions such as Fe^{2+} at level 99.3% compared to the other peptide fractions. Namely, it was emphasized that peptide fractions exhibited very higher ability to chelating the ions of transitions metals which were 85.5%, 98.4%, 99.3% and 99.2% for fractions GF I (>30 kDa), GF II (10–30 kDa), GF III (3–10 kDa) and GF IV (<3 kDa), respectively. Furthermore, peptide fraction GF III seemed to be primarily responsible for prooxidant ability according to Fe^{2+}. Summary, these results suggested that a certain retained protein structure was needed in order to have good chelating activity. These results were similar to those found by Kong et al. [20] and Cian et al. [21] for wheat gluten hydrolyzates and peptide fractions, who also reported that peptides of medium size were primarily responsible for scavenging activity and inhibition of the autoxidation of linoleic acid, indicating that the sequence of peptides and peptide conformation, were mainly responsible for antioxidant activity peptides isolated from gluten.

Conclusion

The aim of this study was to find the optimal operational and process parameters for the enzymatic hydrolysis of wheat gluten from the viewpoint of *DH* and emulsification properties. For this propose the impact of process conditions including gluten concentration, temperature, pH and enzyme-gluten ratio was investigated by applying a Box-Behnken experimental design.

The statistical analysis showed that each analyzed variable had a significant effect on *DH*. It appeared that only gluten concentration and temperature showed strong interaction effect whereas the interactions among other parameters were insignificant. The analysis revealed the emulsification activity and stability shown that all tested variables had a significant effect within the experimentally tested ranges. The most relevant variable seemed to be interaction of the gluten concentration and temperature (p<0.0001). The quadratic terms of gluten concentration and temperature were also significant, indicating that a response was a quadratic function with a local maximum. It appeared that the highest *EAI* of 2782.9 m²/g was achieved at pH 8.0 and at a temperature of 50 °C, *E/S* of 0.75 AU/g gluten and a substrate concentration of 1%. The emulsifying stability of protein hydrolysates was in almost linear functional dependence of pH and substrate concentration, wherein the maximum of *ESI* of 105 h was achieved at pH 9.0 and the substrate concentration of 9.0%. Summary, *DH* must be controlled because the *ESI* or *EAI* increase with increasing *DH* (decreasing peptide chain length) up to a critical value after which the efficiency decreases. Further, the most adequate hydrolysate from the aspect of emulsification activity and retained stability was very efficiently separated on the bioactive peptide fractions among which the fraction with the highest peptide yield with molecular weight 3-10 kDa, at equal peptide concentration 2 mg/mL, exhibited the highest ability to chelating prooxidant ferrous ions.

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.

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