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FACULTY OF TECHNOLOGY ZVORNIK

V

INTERNATIONAL CONGRESS

V

ENGINEERING, ENVIRONMENT AND MATERIALS IN PROCESSING INDUSTRY

PROCEEDINGS

JAHORINA
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ANTIOXIDANT PROPERTIES OF HYDROLYSATES OF WHEAT GLUTEN AS INFLUENCED BY PROCESS CONDITIONS

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Abstract

The effects of some relevant process parameters for the Alcalase-catalyzed wheat gluten hydrolysis such as gluten concentration (X_1; 1-9% w/v), temperature (X_2; 40-60 ^oC), pH (X_3; 7-9) and enzyme/substrate ratio, [E]/[S] ratio (X_4; 0.25– 0.75 AU g^{-1} of protein) were investigated by the means of an experimental design. The second-order models developed for the degree of hydrolysis, DH, DPPH and ABTS radical scavenging activity of gluten hydrolysates were significant ($p<0.01$) with a high value of coefficients of determination (0.981-0.992).
The statistical analysis showed that each variable had a significant effect on degree of hydrolysis and the antioxidant capacity of both tested systems. Hydrolysis up to around DH 15 improved DPPH radical scavenging activity, while excessive hydrolysis worsened it. It seemed there was not a correspondence between degree of hydrolysis and ABTS activity at different protein concentrations, suggesting that there were peptides with considerable size presenting a good antioxidant capacity.

Keywords: *wheat gluten, enzymatic hydrolysis, optimization, experimental design, antioxidant activity*

Introduction

Wheat (Triticum *spp*.) remains the most important food grain source for human consumption due to high nutritional value, availability, low cost and ease of converting grain into flour. The rapid growth of wheat flour production leads to large amounts of by-products with high quality components that may be used for human consumption. Wheat gluten is a protein-rich co-product of the starch industry (60–80%), primarily sold as a low-value animal feed. There is an apparent interest from industry for its alternative valorization routes [1].

It appears that gluten may find application in the production of functional foods or food supplements with health benefits, because individual peptides have been shown to have important physiological functions, such as prevention of diabetes complications, anti-inflammatory, immunostimulating, anti-hypertensive and antitumour effects [2]. It is possible to modify the properties of gluten by means of enzymatic hydrolysis as to enhance its performance as desired.

Gluten is a complex biopolymer composed of two seed storage proteins, gliadins and glutenins. Glutenins are insoluble in water, salt solution, aqueous alcohols and soluble in dilute acids or bases containing a high proportion of glutamic acid and hydrophobic amino acids, such as leucine, proline, and glycine. They are both cohesive and elastic, and are responsible for dough strength and elasticity. On the other side, Gliadins are water-soluble monomeric proteins having a similar amino acid`s content.

Many variables affect gluten hydrolysis. The time of hydrolysis, the temperature, the gluten concentration, *E*/*S* ratio and pH seem to be influential. Several studies have verified that the enzymatic hydrolysis of wheat gluten using proteases and controlled conditions improve its functional properties, such as foaming capacity, emulsifying properties or solubility, especially close to its isoelectric point at pH 6-7, but few works have dealt with the aim of its application as an antioxidative food ingredient [3-5].

Usually, the optimization of enzymatic reactions involves varying one parameter at a time, while keeping the others constant. Such approach does not offer insight in the existence and the nature of interactions between factors. On the other hand, statistical tools including response surface methodology (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. RSM has been previously successfully applied in the optimization of protein hydrolysis from different natural sources, such as pumpkin seed [6], egg-white [7], sesame cake [8], whey and soy proteins, marine shrimp etc. However,

systematic investigations of the effects of process operating conditions on the wheat gluten hydrolysis regarding antioxidant capacity of the obtained hydrolysates have not been reported.

The aim of the study was to find the optimal operational parameters to obtain the highest output for the hydrolysis of wheat gluten in a batch stirred bioreactor regarding both degree of hydrolysis and antioxidant capacity. Reaction parameters including gluten concentration, temperature, pH and enzyme-substrate ratio, *E*/*S* on degree of hydrolysis (*DH*), DPPH scavenging activity and ABTS radical scavenging activity were optimized using Box-Behnken response surface methodology.

Materials and methods

Material

Wheat gluten (moisture content: 6.8%, protein content (N 5.70): 78.52% on dry basis), was from MP Biomedicals (Santa Ana, CA). Protease from *Bacillus licheniformis*, Alcalase®, (EC 3.4.21.14), with the claimed activity of 2.4 Anson unit (AU) g^{-1} was purchased from Sigma Aldrich (St. Louis, USA). Other reagents were obtained from Sigma Aldrich, St. Louis, USA and were analytical grade.

Preparation of gluten hydrolysates

An aqueous dispersion of wheat gluten (typically 1-9% $w_{protein}/v$) was stirred and pretreated by thermal treatment. After that, the obtained suspension was allowed to equilibrate at the working temperature (40-60 ^{o}C) for 20 min. Thereafter, the pH and temperature of the suspension was adjusted and then the enzyme was added at a level of 0.25-0.75 AU/g of gluten protein according to the experimental plan (Table 1).

Enzymatic hydrolysis was carried out at constant pH, temperature and agitation (240 rpm) while the progress of the reaction was followed using a pH-stat method. The DH was calculated according the Equation 1 (Adler-Nissen, 1978):

$$DH\ (\%) = \frac{h \cdot 100}{h_{tot}} = \frac{N_b \cdot B \cdot 100}{\alpha \cdot m_p \cdot h_{tot}} \quad (1)$$

where h represents the number of equivalents of peptide bonds hydrolyzed at the time per weight unit; h_{tot} is the total amount of peptide bonds per weight unit of a protein and can be calculated from its amino acid composition (for wheat gluten protein h_{tot} is 8.38 mmol g^{-1} of protein), B is the volume of base necessary to keep pH constant (mL), N_b is the normality of the base, α is the average degree of dissociation of the α-amino groups and m_p is the mass of protein in g.

When the reaction achieved an equilibrium state, the enzyme was inactivated by heat treatment at 95 °C for 15 min. The hydrolysates were then rapidly cooled to 25 °C, and then centrifuged (10 min, 4332 *x* g). The supernatants were collected and kept frozen (-20 °C) for further analysis.

Experimental design and statistical analysis

Box-Behnken RSM was used to study the effect of four independent variables X_1 (gluten concentration), X_2 (hydrolysis temperature, °C), X_3 (pH), and X_4 (enzyme-substrate ratio, *E*/*S*, AU/g of gluten), at three levels on the degree of hydrolysis and DPPH and ABTS radical scavenging activity. The experimental design was shown in Table 1.

Antioxidant capacity measured by DPPH radical scavenging method

Measurement of antioxidant capacity of hydrolysates by DPPH method is based on their ability to reduce stable DPPH method by accepting an electron or hydrogen. All experiments were carried out according to the method previously described elsewhere [9]. Namely, 0.5 cm^3 of hydrolysate and 0.5 cm^3 of 0.1 mM methanolic DPPH solution were mixed and left in dark for 30 min and then absorbance was measured at 517 nm.

$$\mathbf{DPPH}(\%) = \mathbf{100} \cdot \left[\mathbf{1} - \left(\frac{A_s - A_b}{A_c}\right)\right] \quad (2)$$

where, A_s represents the absorbance of the sample solution in the presence of the DPPH, A_b is absorbance of the sample solution without DPPH, and A_c is absorbance of the control solution with DPPH.

Antioxidant capacity measured by ABTS radical scavenging method

Antioxidant capacity expressed as the ability of the hydrolysates to scavenge $ABTS^{\bullet+}$ radical was based on the $ABTS^{\bullet+}$ radical cation decolourization. $ABTS^{\bullet+}$ radical was produced in the reaction between 7 mM ABTS solution and 2.45 mM potassium persulfate (final concentration), which were left in dark for 12-16 hours. Produced $ABTS^{\bullet+}$ solution was diluted with PBS, pH 7.4, until the absorbance of 0.700 (± 0.05) was reached. Hydrolysates, 5 µl were mixed with 500 µl of prepared $ABTS^{\bullet+}$ solution and after 5 min absorbance was measured at 734 nm.

$$\mathbf{ABTS}\ (\%) = \mathbf{100} \cdot \left(\frac{A_c - A_s}{A_c}\right) \quad (3)$$

where, A_s represents the absorbance of the sample solution in the presence of

the $ABTS^{\bullet+}$, A_b is absorbance of the sample solution without $ABTS^{\bullet+}$, and A_c is absorbance of the control solution with $ABTS^{\bullet+}$.

Results and discussion

The Box-Benken experimental design with the independent variables (substrate concentration X_1, temperature, X_2, pH, X_3 and E/S ratio, X_4) and the results for degree of hydrolysis and antioxidant activities are presented in Table 1.

Table 1. Box-Benken experimental design with real values for the variables and responses for degree of hydrolysis (DH%) and DPPH and ABTS antioxidant activities of gluten hydrolysates.

Run No.	RSM experimental variables				Experimental responses		
	X_1	X_2	X_3	X_4	DH (%)	DPPH (%)	ABTS (%)
1	1	40	8	0.5	28.45	47.61	74.09
2	9	40	8	0.5	19.29	82. 71	89.73
3	1	60	8	0.5	25.41	35.96	61.65
4	9	60	8	0.5	28.34	56.35	91.47
5	5	50	7	0.25	9.29	89.89	95.73
6	5	50	9	0.25	24.75	48.23	87.99
7	5	50	7	0.75	15.51	87.23	95.58
8	5	50	9	0.75	29.52	54.43	99.42
9	1	50	8	0.25	25.16	37.41	70.46
10	9	50	8	0.25	21.16	82.27	98.89
11	1	50	8	0.75	27.21	55.14	92.73
12	9	50	8	0.75	26.6	64.36	99.53
13	5	40	7	0.5	8.85	82.99	95.26
14	5	60	7	0.5	17.14	88.12	84.11
15	5	40	9	0.5	25.16	71.10	78.67
16	5	60	9	0.5	31.01	18.08	92.96
17	1	50	7	0.5	16.32	52.84	85.47
18	9	50	7	0.5	13.60	94.50	97.16
19	1	50	9	0.5	31.5	35.22	74.41
20	9	50	9	0.5	28.72	49.38	97.79
21	5	40	8	0.25	17.2	72.69	91.94
22	5	60	8	0.25	19.9	53.41	77.09
23	5	40	8	0.75	22.04	82.00	92.68
24	5	60	8	0.75	27.20	58.16	99.37
25	5	50	8	0.5	24.76	67.91	98.26
26	5	50	8	0.5	25.16	64.89	98.42
27	5	50	8	0.5	24.93	65.13	97.96

28	5	50	8	0.5	25.01	64.97	98.02
29	5	50	8	0.5	24.75	65.83	97.84

The highest value obtained for degree of hydrolysis of 31.01% was observed in run 16 (5% substrate, 60 ^{o}C, pH 9, *E*/*S* 0.5), and the lowest was 13.60, detected in run 18 (9% substrate, 50 ^{o}C, pH 7 and *E*/*S* 0.5). It appeared that optimum conditions for obtaining hydrolysates with antioxidant capacity measured by two different methods differed in great deal. In DPPH assay, the maximum antioxidant activity was observed in the hydrolysate presenting low DH (13.60%) obtained at higher gluten concentration (run No. 18). In contrast, for ABTS assay the highest values of antioxidant activity was detected in the hydrolysate obtained at also high gluten concentration in the run 12, where the degree of hydrolysis was 26.6%. The differences between optimum conditions for achieving the highest ABTS and DPPH radical scavenging activities were probably the consequence of different solubility and diffusivity of tested radicals. $ABTS^{\bullet+}$ is water soluble, and $DPPH^{\bullet}$ is oil-soluble radical and the reason for higher $ABTS^{\bullet+}$ radical scavenging activity is probably due to the better diffusivity of the radical in the aqueous assay conditions compared to oil-soluble $DPPH^{\bullet}$, antioxidant peptides are more accessible to the $ABTS^{\bullet+}$ radical.

The effect of reaction conditions on degree of hydrolysis of the wheat gluten

The response surface analysis showed that *DH*% was significantly ($p < 0.01$) dependent on the linear and quadratic terms of all variable tested, while the interaction between variables generally had no significant effect:

$$\boldsymbol{Y_1} = 24.92 - 1.36 \cdot x_1 + 2.33 \cdot x_2 + 7.50 \cdot x_3 + 2.55 \cdot x_4 + 3.02 \cdot x_1 x_2 + 1.54 \cdot x_1^2 - 1.18 \cdot x_2^2 - 3.51 \cdot x_3^2 - 1.74 \cdot x_4^2$$

A) B)

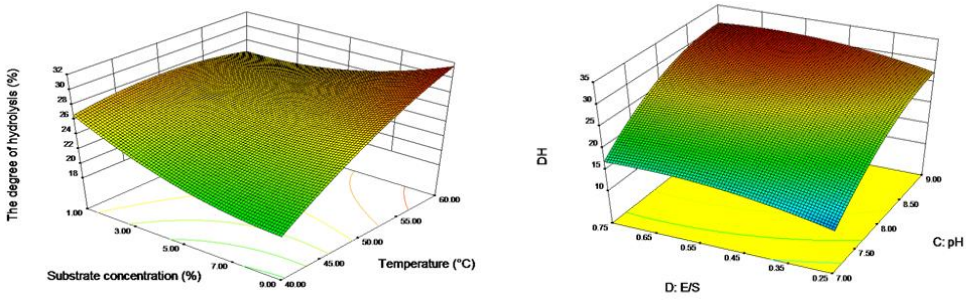

Figure 1. Response surface plots showing the effect of gluten concentration, X_1 and temperature X_2 (A) and E/S ratio, X_4 and pH, X_3 (B) on the degree of

hydrolysis.

The three-dimensional plots representing DH versus gluten concentration and temperature and *E*/*S* ratio and pH corresponding to this model are presented in Fig. 1. It was observed that an increase in temperature or pH provided increase in DH, but the gluten concentration had an opposite trend.

The effect of reaction conditions on antioxidant activity of the wheat gluten hydrolysates

The relationship between reaction conditions and antioxidant properties of the obtained hydrolysates, measured by two methods, was studied using Box-Benken experimental design and RSM. The following models were developed to describe the two response (*Y*) surfaces:

$$\boldsymbol{Y_2} = 65.42 + 13.78 \cdot x_1 - 10.75 \cdot x_2 - 18.26 \cdot x_3 + 1.45 \cdot x_4 - 3.68 x_1 \cdot x_2 - 6.88 \cdot x_1 x_3 \cdot -8.91 \cdot x_1 \cdot x_4 - 14.54 \cdot x_2 \cdot x_3 - 8.56 \cdot x_1^2 + 2.78 \cdot x_4^2$$

$$\boldsymbol{Y_3} = 98.10 + 9.65 \cdot x_1 - 1.31 \cdot x_2 - 1.84 \cdot x_3 + 4.77 \cdot x_4 + 3.54 \cdot x_1 \cdot x_2 + 2.92 \cdot x_1 \cdot x_3 - 5.41 \cdot x_1 \cdot x_4 + 6.36 \cdot x_2 \cdot x_3 + 5.38 \cdot \mathrm{x}_2 \cdot \mathrm{x}_4 - 8.39 \cdot \mathrm{x}_1^2 - 8.93 \cdot \mathrm{x}_2^2 - 1.99 \cdot \mathrm{x}_3^2 + 0.12 \cdot x_4^2$$

Regarding the DPPH activity, all of the tested variables showed significant effect. The most significant was the negative influence of the pH and its interaction with temperature, followed with the positive influence of gluten concentration. Similar to the DPPH scavenging activity, all of the tested variables showed significant effect on the $ABTS^{\bullet+}$ quenching ability. The most significant was the positive influence of the gluten concentration, followed with the quadratic term of temperature and interaction between pH and temperature. Nevertheless, there was important difference amongst the obtained responses, since with $ABTS^{\bullet+}$ radical quenching activity this interaction had positive term.
The three-dimensional response surface plots were shown in Fig. 2 to Fig. 3, which illustrate the interactive effects of the independent variables on the DPPH and ABTS scavenging capacity, respectively.

Contrary to *DH*, it seemed that the highest DPPH activity was achieved with the highest gluten concentration and the lowest value of temperature and/or pH (Fig. 2). Furthermore, the operating conditions comparably affect antioxidant activity measured by DPPH and ABTS methods (Fig 3.), suggesting that there are peptides with considerable size presenting a good antioxidant activity.

A) B)

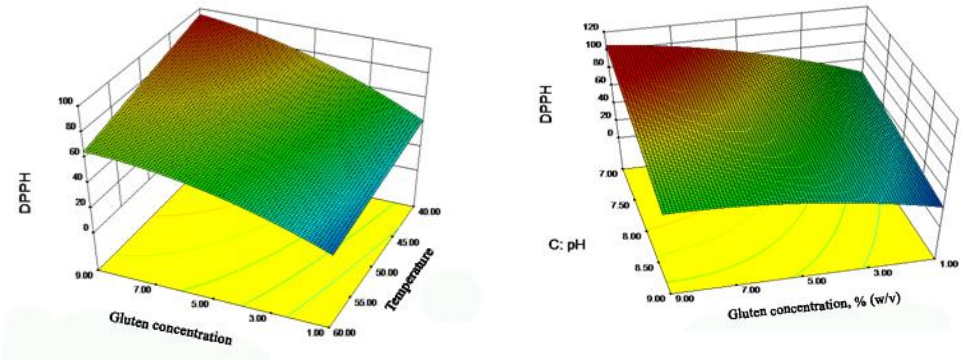

Figure 2. Response surface plots showing the effect of gluten concentration, X_1 and temperature X_2 (A) and pH, X_3 and gluten concentration, X_1 (B) on the degree of hydrolysis.

A) B)

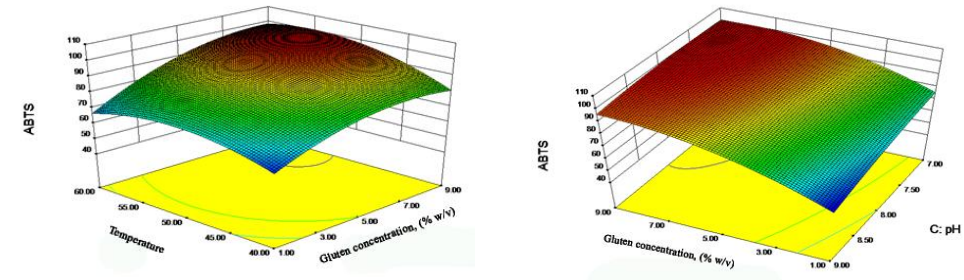

Figure 3. Response surface plots showing the effect of temperature, X_2 and gluten concentration, X_1 (A) and gluten concentration X_1 and pH, X_3 (B) on the degree of hydrolysis.

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