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ORIGINAL PAPER

Production of Antioxidant Egg White Hydrolysates in a Continuous Stirred Tank Enzyme Reactor Coupled with Membrane Separation Unit

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Abstract The objective of this research was to design an efficient continuously operated membrane reactor with a separation unit for egg white protein (EWP) hydrolysis and production of hydrolysates with improved antioxidant properties. For this purpose, a mechanically stirred tank reactor coupled with the polyethersulfone ultrafiltration module with a molecular weight cutoff of 10 kDa was employed. Several proteolytic enzymes have been tested in order to obtain the best quality of peptide-based formulations intended for human consumption. Among protease from *Bacillus licheniformis* (Alcalase), protease from *Bacillus amyloliquefaciens* (Neutrase), and protease from papaya latex (papain), the highest degree of hydrolysis (DH), as well as the best antioxidant properties of obtained hydrolysates, was achieved with Alcalase. The effects of operating variables such as enzyme/substrate ([*E*]/[*S*]) ratio, impeller speed, and permeate flow rate were further studied using response surface methodology (RSM) and Box–Behnken experimental design. Results obtained in RSM analysis confirmed that over the studied range [*E*]/[*S*] ratio, impeller speed and permeate flow rate had the significant effect on the DH and reactor capacity. The effects of different impeller geometries were also studied and four-bladed propeller stirrer enabled the highest DH. Antioxidant properties were analyzed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH), by the 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical scavenging activity, and by the linear voltammetry methods. Results show that the use of Alcalase in the membrane reactor system is of potential interest for the EWP hydrolysis and obtaining value-added egg products.

Keywords Egg white protein hydrolysis · Proteases · Continuous membrane reactor · Polyethersulfone ultrafiltration module · Response surface methodology · Antioxidant properties

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Introduction

Egg white proteins (EWPs) are well established as a valuable source of dietary nitrogen, but recently, these proteins have reclaimed scientist's interests due to a number of newly discovered biological functions (Li-Chan and Nakai 1989; Ruan et al. 2010). Additionally, EWPs possess very important functional properties, which make them very important ingredients for food products (Jing et al. 2011). In addition to providing color and aroma, these proteins are known for the improvement of foaming, emulsifying, coagulating, and gelling characteristic of foods (Van der Plancken et al. 2007). For this reason, the reciprocity of the structural and functional properties of the EWPs has been the subject of the extensive studies during the last few years (Van der Plancken et al. 2005; Chen et al. 2012). On the other hand, the main obstacles for the more extensive use of the EWPs are their high viscosity and allergenicity. The EWPs are recognized as the second most extensive source of the allergenic reactions, where the main allergens are ovomucoid (OM), ovalbumin (OVA), ovotransferrin (OVT), and lysozyme (LYS) (Martos et al. 2013).

Enzymatic hydrolysis of EWPs has shown a great potential to improve their functional properties and biological properties and to reduce the protein allergenicity, while retaining their nutrition value (Knežević-Jugović et al. 2012). The high selectivity and mild reaction conditions associated with the enzymatic process have made this approach an attractive alternative in the production of a high-value protein hydrolysate with added functional and health benefit, which are often difficult to obtain by conventional chemical (acid or alkali) route and thermal processing. Hence, egg white hydrolysates became irreplaceable ingredients of food products, from the viewpoint of their functional, nutritional, and immunological properties (Prieto et al. 2010).

Batch hydrolysis is the most common way to produce peptide-based bioactive ingredients intended for food industries. The batch hydrolysis, though easy to control, has several downsides to it, mainly concerning the economical aspects, due to the high enzyme and labor costs (Prieto et al. 2010). One of the ways to overcome these drawbacks is the usage of immobilized enzymes, which enables working in continuous systems, providing higher process productivity compared to the batch bioreactors (Jakovetić et al. 2013b). Nevertheless, working with the immobilized enzymes has several disadvantages since immobilization is a rather complex process in which the loss of the enzyme activities usually occurs and, along with the diffusional limitations, constrains more extensive applications of these systems (Prieto et al. 2010). Some of these drawbacks can be overcome by the use of free, unimmobilized enzymes confined in a membrane reactor.

The application of the membrane reactor design enables reusage of the water-soluble enzymes in the continuous protein hydrolysis by allowing lower peptides to pass through the membrane while retaining larger molecules such as enzyme intact and partially hydrolyzed substrate within the bioreactor. Hydrolysates with specific molecular weight peptides' profile and biological activity can be obtained by using membrane with proper molecular weight cutoff (Chiang et al. 2006). Enzyme inhibition by products could also be overcome in the membrane reactor, and in addition, it enables continuous production of end products of a consistent quality. Nevertheless, the application of membrane reactors is limited because of the several disadvantages, including the loss of the enzyme due to a leakage through the membrane, increased enzyme deactivation as a repercussion of the combined thermal and shear stress, and irreversible membrane fouling, which causes a decline in permeate flux and frequent elimination of nonreacted substrates (Prieto et al. 2007, 2008). Prieto et al. proposed usage of a cyclic batch enzyme membrane reactor (CBEMR) for the production of reduced-antigenicity whey protein hydrolysates (Prieto et al. 2007). CBEMR is designed to overcome the main disadvantages in the operation of both individual reactors and, at the same time, to exploit their main benefits (Prieto et al. 2007, 2008, 2010). New strategies for the improvement of proteins' hydrolysis rate and enzyme usage efficiency focus on a continuous membrane reactor regime which are suitable for reactions subject to substrate and products' inhibition, combining minimization of the substrate concentration, short-chain peptide removal, and the use of the proteolytic enzymes for long periods of time, via retention in the system. To the best of our knowledge, this system has never been applied for selective production and separation of biologically active peptides derived from EWPs.

The main objective of this study was to design an efficient, low-cost enzyme process for continuous production of egg white hydrolysates with improved antioxidant properties. For this purpose, a continuous stirred tank reactor coupled with the polyethersulfone ultrafiltration module with a molecular weight cutoff of 10 kDa was employed. The first task was to find adequate protease, which could provide not only high degree of hydrolysis (DH), but also, at the same time, products of superior quality. Further aim was to examine the effects of key reactor operational features on EWP hydrolysis efficiency, i.e., the influence of $[E]/[S]$ ratio, impeller speed and permeate flow rate on the rates, and degree of hydrolysis. In order to facilitate process optimization and to evaluate possible interactions between examined parameters, response surface methodology (RSM) and Box–Behnken experimental design were used. They 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 functional properties (de Oliveira et al. 2014; Sanhong et al. 2014). The quality of obtained hydrolysates was evaluated throughout DH, protein concentration, and antioxidant properties, measured by three methods, and compared.

Materials and Methods

The substrate, commercial pasteurized egg white was a kind gift from Jata Emona d.o.o. (Ljubljana, Slovenia), with the total protein content ($N\times6.25$) of 11.9 (*w*/*w*) as determined according to the standard Kjeldahl method (Sáez-Plaza et al. 2013). The proteases used in the hydrolysis were as follows: protease from *Bacillus licheniformis*, Alcalase® 2.4L (EC 3.4.21.14), protease from *Bacillus amyloliquefaciens*, Neutrase® 0.8L (EC 3.4.24.28), and papain from *papaya latex* (EC 3.4.22.2) with the claimed activities of 2.4 Anson unit (AU) g^{-1}, 0.8 AU g^{-1}, and 1.5–10 AU mg^{-1} solid, respectively, all purchased from Sigma-Aldrich (St. Louis, USA). One Anson unit 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, which gives the same color with Folin–Ciocalteu phenol reagent as 1 mEq of tyrosine at

25 °C and at pH 7.50 (Jin et al. 2012). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) were also purchased from Sigma-Aldrich (St. Louis, USA). Membrane module, Vivaflow 50 10000MWCO PES, was purchased from Sartorius Stedim Biotech (Goettingen, Germany). Operating conditions for Vivaflow 50 are as follows: pump flow 200–400 cm^3 min^{-1} and maximum pressure of 4 bar. Peristaltic Pump Drive 5201 was purchased from Heidolph Instruments GmbH & Co (Schwabach, Germany). Potentiostat/galvanostat Bio-Logic SP-150 was purchased from Bio-Logic Science Instruments (Claix, France).

Selection of the Membrane Modulus

The membrane was chosen based on the results obtained in a preliminary study in the batch reactor. The hydrolysate obtained by Alcalase-catalyzed EWPs (the degree of hydrolysis was 27 %) was separated by ultrafiltration using an ultrafiltration cell (Model 8050, Millipore, Billerica, MA, USA) in five ultrafiltered peptide fractions. The fractions were prepared by using sequentially four molecular weight cutoff (MWCO) membranes (1, 3, 10, and 30 kDa) including the fraction >30 kDa (30-kDa retentate), 10–30 kDa (30-kDa permeate–10-kDa retentate), 3–10 kDa (10-kDa permeate–3-kDa retentate), 1–3 kDa (3-kDa permeate–1-kDa retentate), and <1 kDa (1-kDa permeate). The protein content was determined in each of the fractions, and the antioxidant activity was measured with the DPPH assay as described below. It was shown that the DPPH activity was significantly dependent on peptide fraction molecular weight ($p<0.05$) and was the highest in the 3–10-kDa fraction for the Alcalase hydrolysate. Thus, the membrane modulus with a protein cutoff of 10 kDa has been selected for further study.

Continuous Preparation of Egg White Hydrolysates by the Means of Enzymatic Hydrolysis

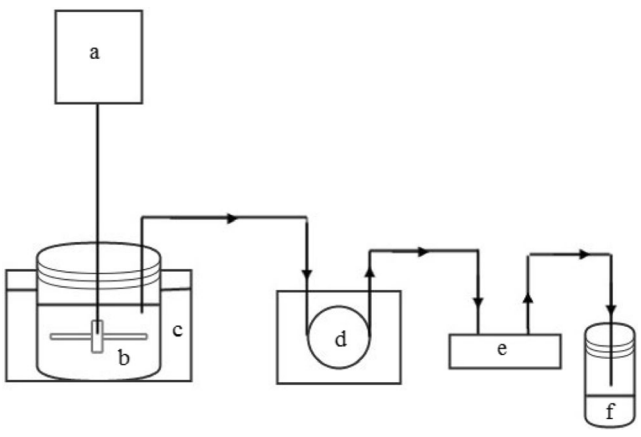
Egg white (10 % *w*/*w*) was dissolved in distilled water (10-mM cysteine solution for papain) under constant stirring and then subjected to the thermal denaturation at 75 °C for 30 min. Afterward, the sample was adjusted to pH and temperature adequate for the corresponding enzyme (Alcalase: pH 8, t= 50 °C; papain: pH 7, t=55 °C; Neutrase: pH 7, t=50 °C). Enzymatic hydrolyses with different proteases were started by adding the appropriate amount of enzyme at a ratio of 0.72 AU g^{-1} of protein. The reactions were carried out under controlled conditions in a 300-cm^3 stirred tank reactor (300 rpm, 1.9 cm^3 min^{-1}) with a water jacket equipped with a stirrer and a pH electrode and connected with the polyethersulfone membrane modulus, as shown in Scheme 1. Except otherwise stated, the four-bladed propeller has been used. The reaction mixture was continuously pumped, with peristaltic pump, from the stirred tank through a membrane modulus with a protein cutoff of 10 kDa. Larger proteins were recycled in the stirred tank, while permeate with molecules smaller than 10 kDa was collected. The volume of the reaction mixture was kept constant; hence, the feed flow was the same as the permeate flow. Progress of the hydrolysis was followed using pH stat method with automatic dosage of the base. The moment when no more base (0.2 M NaOH) was needed to keep pH at a constant value was considered as the end of hydrolysis. DH (%) was calculated according to the following equation (Guadix et al. 2006):

$$\mathrm{DH}\ (\%) = \frac{\left(M_\mathrm{B} - V_\mathrm{p} \cdot \frac{K_\mathrm{w}}{10^{-\mathrm{pH}}}\right)^{\frac{1}{\alpha}}}{\left(V_\mathrm{r} + V_\mathrm{p}\right) \cdot S_\mathrm{f} \cdot h_\mathrm{t}} \tag{1}$$

where M_B represents the amount of base necessary to keep pH constant (mol), V_P and V_R are volumes of permeate and reaction mixture, respectively (cm^3), S_F is the protein concentration in the feed (mg cm^{-3}), h_T=7.67 (mmol g^{-1} of protein) presents the number of peptide bonds in the substrate, K_w= 5.8×10^{-14} is the ionic product of water at 50 °C, and α=0.88 is the average degree of dissociation of the α-amino groups.

Further, in the selected membrane system with Alcalase, effects of different operating conditions were investigated as well as several different impeller geometries. In order to model enzymatic hydrolysis in the stirred tank reactor with membrane modulus, previously developed mathematical model, which assumes zero-order kinetics of hydrolysis and second-order kinetics of enzyme deactivation, is used (Prieto et al. 2010):

$$\frac{n \cdot S_0 \cdot x}{k_\mathrm{h}} = \frac{1}{k_\mathrm{d}} \cdot \ln\left(1 + k_\mathrm{d} \cdot e_0 \cdot t_\mathrm{n}\right) \tag{2}$$



Scheme 1 The schematic presentation of a continuous stirred tank reactor connected with a membrane modulus: *a* mechanical stirrer, *b* reactor vessel, *c* water jacket, *d* peristaltic pump, *e* membrane modulus, and *f* collected hydrolysates

where n is the number of enzyme uses and t_n (min) is the cumulated time of the consecutive hydrolysis, x is DH (%), k_h (min^{-1}) and k_d ($dm^3\ min^{-1}\ g^{-1}$) are the rate constants for hydrolysis and deactivation, respectively, e_0 (g dm^{-3}) is the enzyme concentration, and S_0 is the initial substrate concentration (g dm^{-3}). The experimental data were fitted to a proposed kinetic model using Matlab software. Overall process productivity was assessed from the values of the achieved reactor capacities expressed as milligram of obtained proteins divided by the added enzyme activity units in the system and reaction time.

Determination of Protein Concentration

Protein concentration in samples was determined using the standard Lowry method, which was previously described elsewhere (Lowry et al. 1951).

Determination of Antioxidant Activity as the Ability to Scavenge DPPH Free Radicals

The ability of the obtained hydrolysates to reduce stable DPPH radical by accepting an electron or hydrogen was used to evaluate their antioxidant efficiency. Experiments were carried out according to the method previously described elsewhere with hydrolysate samples containing 2 mg cm^{-3} of protein (Jakovetić et al. 2013a; Gorjanović et al. 2010). DPPH antiradical scavenging activity, DPPH (%), was determined by using the following equation (Gorjanović et al. 2010):

$$\mathrm{DPPH}(\%) = 100 \cdot \left[1 - \left(\frac{A_s - A_b}{A_c}\right)\right] \tag{3}$$

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.

Determination of Antioxidant Activity as the Ability to Scavenge ABTS Free Radicals

The ability of EWP hydrolysates to scavenge ABTS radical was tested according to the previously described method (Re et al. 1999). ABTS radical cation ($ABTS^{\cdot+}$) was produced in the reaction between ABTS (7 mM) and potassium persulfate (2.45-mM final concentration). The reaction mixture was left in the dark for 12–16 h. Further, $ABTS^{\cdot+}$ solution was diluted with PBS, pH 7.4, until the absorbance of 0.700 (±0.05) was reached. Diluted $ABTS^{\cdot+}$ solution (500 μl) was mixed with hydrolysates containing 2 mg cm^{-3} of protein (5 μl), and absorbance was measured at 734 nm after 5 min. ABTS antiradical scavenging activity was determined by using the following equation (Zheleva-Dimitrova et al. 2010):

$$\mathrm{ABTS}(\%) = 100 \cdot \left(\frac{A_c - A_s}{A_c}\right) \tag{4}$$

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

Determination of Antioxidant Properties via Linear Sweep Voltammetry

The linear sweep voltammetry was also used to measure antioxidant properties of the produced hydrolysates. Experiments were carried out in a three-electrode electrochemical cell, where glassy carbon electrode was used as working and Pt wire as counter, and saturated calomel electrode (SCE) was used as a reference electrode. Electrodes were connected with the potentiostat/galvanostat Bio-Logic SP-150. Experiments were conducted at ambient temperature. Prior to each experiment, the working electrode was cleaned with 0.3 M H_2SO_4, isopropyl alcohol, and finally 3 % NaCl solution. The linear sweep voltammograms were recorded at a scan rate of 10 mV s^{-1}, starting at open circuit potential (vs SCE) to 1 V. All samples were dissolved in the 3 % NaCl, and voltammograms were recorded for hydrolysates with protein concentrations in the range of 2 mg cm^{-3}. Voltammetry methods were previously used for determination of protein antioxidant properties (Chen et al. 2002).

Statistical Analysis

As Alcalase proved to be the best biocatalyst for the hydrolysis reaction, the optimization of process parameters was continued with this enzyme. The effects of some relevant process parameters for the Alcalase-catalyzed EWP hydrolysis such as $[E]/[S]$ ratio (X_1; 0.36–0.72 AU g^{-1} of protein), impeller speed (X_2; 100–500 rpm), and permeate flow rate (X_3; 1.60–2.50 $cm^3\ min^{-1}$) were investigated by the means of an experimental design. For this purpose, RSM and a three-factor Box–Behnken experimental design were used, including 17 experimental points (with five replicates at the center of the design), and experiments were carried out in a random order. The selected variables were chosen based on the results obtained in a preliminary study and are the most commonly used for modeling protein hydrolysis reactions (de Oliveira et al. 2014; Demirhan et al. 2010; Nouri et al. 1997; Cheison et al. 2007). Two major process outputs, DH and reactor capacity, were taken as the response variables. The actual and coded settings of experimental

factors as well as the design of experiments employed are presented in Table 1. The data obtained were fitted to a second-order polynomial equation:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j \quad (5)$$

where x_i, $x_j \ldots x_k$ are independent variables ([*E*]/[*S*] ratio, impeller speed, and permeate flow rate) which affect the predicted response y (y_1—DH and y_2—reactor capacity) and β_0, β_i, β_{ii}, and β_{ij} are coefficients for intercept, linear, quadratic, and interaction terms, respectively. The coefficients of the response function and their statistical significance were evaluated by the response surface regression analysis, using the statistical software Design Expert Statistical Software package 8.0.7.1 (Stat Ease Inc., Minneapolis, USA). The Fisher test (F value) was used to determine whether the constructed model was adequate to describe the obtained data. The goodness of fit of the model was evaluated by the determination coefficient (R^2).

Experiments were carried out in duplicate or triplicates and expressed as means with SD. The effects of applied protease and impeller type were analyzed for significant differences in achieved DH (%) using one-way analysis of variance (ANOVA) and Student t test. All analyses were done using Microsoft Office Excel 2007 software (Microsoft Corporation, WA, USA). Significance was determined at $p<0.05$. The experimental data were fitted to a proposed kinetic model using Matlab (MathWorks, USA) software, and the goodness of fit was evaluated through the values of R^2.

Results and Discussion

The Influence of Different Proteases on DH and Antioxidant Properties of the Obtained Hydrolysates

The progress of the EWP hydrolysis was followed by monitoring the DH using the pH stat method. The quality of the obtained products was assessed from the viewpoint of their antioxidant properties using three methods, namely, DPPH and ABTS radical scavenging activity methods and linear sweep voltammetry method. Figure 1 represents the influence of three proteases from different microbiological and plant sources on the DH. Results obtained in the proteolysis of untreated and thermally treated pasteurized EWPs implied that the thermal treatment improved their susceptibility to proteolysis by all tested enzymes (data not shown). On the other hand, DH achieved in the hydrolysis of EWPs varied from 5 to 60 % over the 120-min time period, depending on the applied protease. At the same [*E*]/[*S*] ratio (0.72 AU g^{-1} of protein), Alcalase showed higher hydrolytic activity than

Table 1 Box–Behnken design with actual/coded values for the three variables and obtained and predicted responses

Run	Variable levels			Experimental DH (%)	Predicted DH (%)	Experimental reactor capacity (mg of protein AU^{-1} min^{-1})	Predicted reactor capacity (mg of protein AU^{-1} min^{-1})
	X_1	X_2	X_3				
1	0.36 (−1)	100 (−1)	2.05 (0)	41.12	41.35	2.95	2.98
2	0.72 (1)	100 (−1)	2.05 (0)	41.67	42.37	2.80	2.75
3	0.36 (−1)	500 (1)	2.05 (0)	53.05	52.40	1.60	1.65
4	0.72 (1)	500 (1)	2.05 (0)	53.72	53.43	2.10	2.07
5	0.36 (−1)	300 (0)	1.60 (−1)	47.00	47.47	1.72	1.65
6	0.72 (1)	300 (0)	1.60 (−1)	47.60	48.50	1.70	1.75
7	0.36 (−1)	300 (0)	2.50 (1)	50.28	50.22	1.96	1.95
8	0.72 (1)	300 (0)	2.50 (1)	52.56	51.25	2.01	2.04
9	0.54 (0)	100 (−1)	1.60 (−1)	40.00	38.85	1.97	1.99
10	0.54 (0)	500 (1)	1.60 (−1)	56.38	56.16	1.26	1.26
11	0.54 (0)	100 (−1)	2.50 (1)	47.64	47.86	2.55	2.55
12	0.54 (0)	500 (1)	2.50 (1)	51.50	52.65	1.31	1.29
13	0.54 (0)	300 (0)	2.05 (0)	53.76	53.50	1.46	1.46
14	0.54 (0)	300 (0)	2.05 (0)	54.00	53.50	1.46	1.46
15	0.54 (0)	300 (0)	2.05 (0)	55.25	53.50	1.50	1.46
16	0.54 (0)	300 (0)	2.05 (0)	51.25	53.50	1.42	1.46
17	0.54 (0)	300 (0)	2.05 (0)	53.25	53.50	1.47	1.46

X_1 ([*E*]/[*S*]) ratio (AU g^{-1} of protein), X_2 impeller speed (rpm), X_3 permeate flow rate (cm^3 min^{-1})

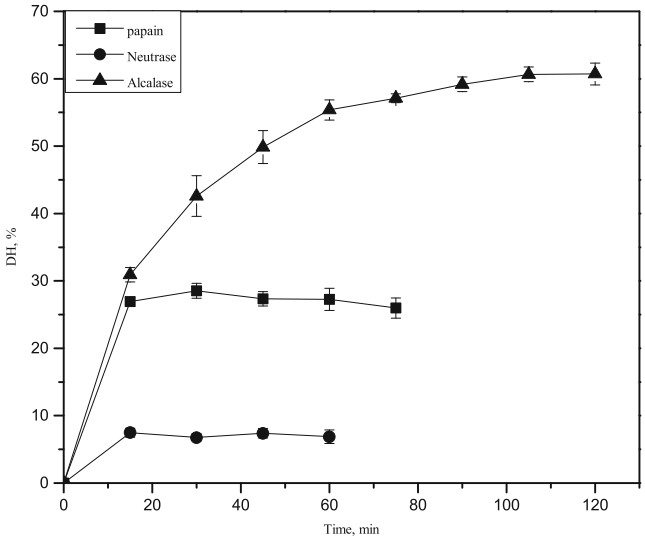


Fig. 1 The progress curves for EWP hydrolysis with different proteases. The hydrolytic reaction was performed at pH 8.0, 50 °C; pH.7, 55 °C; and pH 7.0, 50 °C for Alcalase, papain, and Neutrase, respectively. Results are the mean values of triplicate analyses with the standard errors less than 3 % of means

both, papain and Neutrase. The hydrolysis, with each tested enzyme, proceeded at a rapid rate during the initial 15 min of the reaction. Afterward, the rate of hydrolysis decreased for Neutrase and papain, while, in the reaction catalyzed by Alcalase, proceeded with a slow increase in hydrolysis rate for the next 45 min, and then entered the steady state. ANOVA test confirmed that applied protease is a highly significant factor in EWP hydrolysis ($p<0.01$).

The reason for the more extensive proteolysis achieved with the Alcalase compared to Neutrase and papain could be its broader specificity toward the substrate and therefore its higher capacity for cleavage of various peptide bonds (Ou et al. 2010; Kim et al. 2007). Alcalase is a commercial preparation of relatively crude bacterial extract comprising several proteases with different specificities (Kim et al. 2007). The DH values presented here are significantly higher than those reported for the comparable reactions in a batch reactor, where DH of only 10.0 or 19.0 % was obtained after hydrolysis of EWPs by Alcalase for 3 and 4 h, respectively (Cigić and Zelenik-Blatnik 2004; Ren et al. 2014). Higher hydrolysis efficiency obtained in current work could be explained by different reactor design used for the hydrolysis. The application of the continuous stirred tank membrane reactor system in this work allowed continuous lower-molecular peptide removal during the hydrolysis, providing the lower product inhibition and thus higher reaction rate. Qu et al. have previously reported that the continuous coupling of enzymatic hydrolysis and membrane separation enabled an increase of conversion rate of protein by 63.4 % as well as 7.1 times higher yield of peptides in comparison with traditional enzymatic hydrolysis and batch membrane separation in the hydrolysis of defatted wheat germ protein (Qu et al. 2013). Similarly, Chiang et al. reported improved productivity of soy protein hydrolysis in the continuous membrane reactor compared to the batch reactor (Chiang et al. 1999).

Changes in both biological and functional properties of egg white are expected to occur during the hydrolysis by proteases, since the molecular weight (MW) of proteins decreases, hydrophobic groups become exposed, and reactive amino acid chains are disclosed (Ou et al. 2010). Biological functions of obtained hydrolysates are dependent upon DH as well as the specificity of the applied protease.

The aim of this work was to obtain hydrolysates with the improved antioxidant properties compared to the untreated egg white. As the activities of antioxidants may vary in different biological and food systems, hydrolyzed samples and unhydrolyzed raw protein solution were tested with three methods. Specifically, DPPH and ABTS radical scavenging activity as well as linear voltammetry were used, and the results are presented in Fig. 2a.

DPPH method is widely used method for measurement of the ability of compounds to act as free radical scavengers or hydrogen donors since it is simple, accurate, economic, and rapid. The antioxidant capacity of compound is measured by their ability to reduce the odd electron of nitrogen atom in DPPH by donating a hydrogen atom to form corresponding hydrazine, what is followed with a color change from purple to yellow (Kedare and Singh 2011). Similarly, the scavenge of ABTS radical is considered to occur via an electron transfer mechanism (Li et al. 2012).

The Alcalase hydrolysate was found as the most effective antioxidant determined by all tested methods DPPH, ABTS, and voltammetry methods. The reason for such results is probably closely associated with the value of DH, i.e., MW profile of the obtained hydrolysates. It is well documented that these activities depended on the amino acid sequence and appeared to be lost if the peptides were replaced by a mixture of the corresponding free amino acids (Suetsuna et al. 2000). The highly interesting finding was the remarkable difference in the capability of the same hydrolysates to scavenge ABTS radical compared to DPPH radical. The reason for this difference is probably due to differences in solubility and diffusivity of tested radicals (Zhu et al. 2008). It appeared that Alcalase showed the highest capacity to hydrolyze EWP and correspondingly produce short-chain peptides and amino acids, which are hydrophilic, and therefore, they react more easily with water-soluble ABTS radical than with lipid-soluble DPPH radical (Zhu et al. 2008). Therefore, Neutrase hydrolysate exhibited strong antioxidant activity toward oil-soluble DPPH at a rather low DH, probably due to exposing of more hydrophobic groups. As assumed, these hydrolysates are less efficient as ABTS scavengers compared to Alcalase hydrolysates.

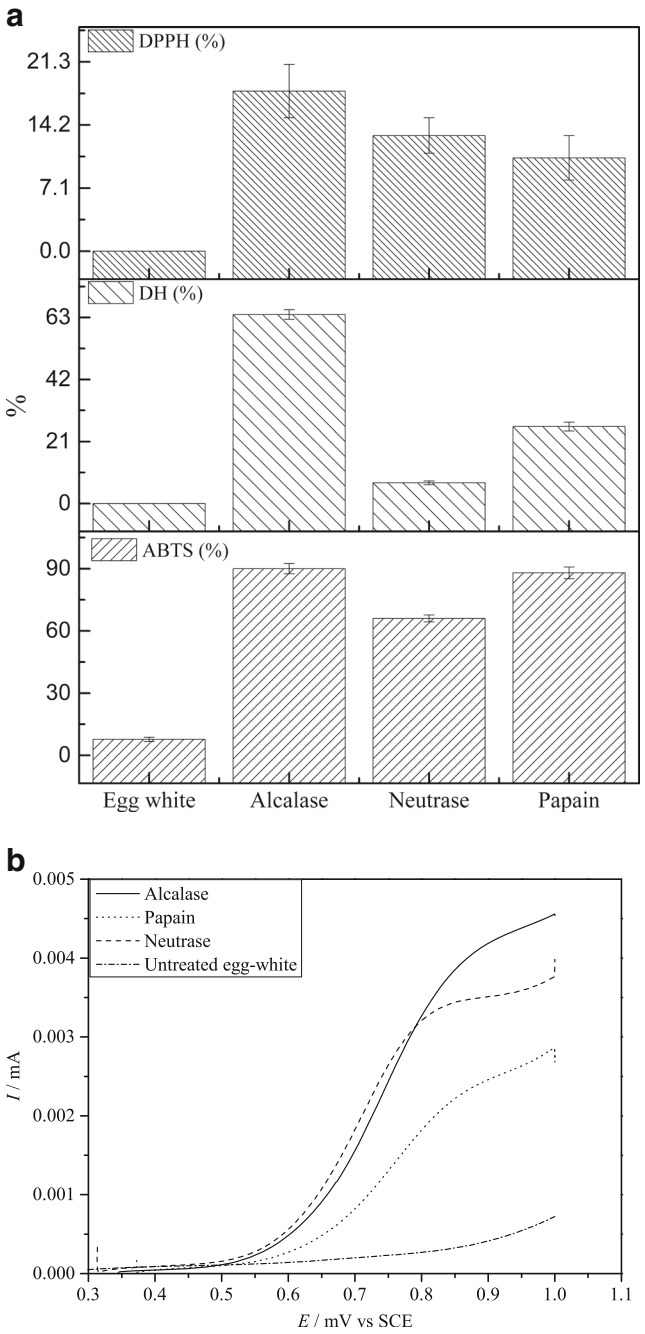


Fig. 2 Antioxidant activity of the hydrolysates (2 mg cm^{-3}) obtained with different proteases measured with **a** DPPH and ABTS methods and **b** linear voltammetry method

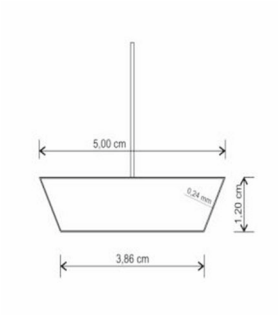
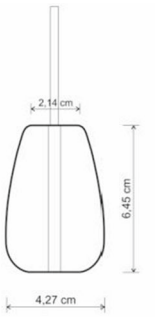
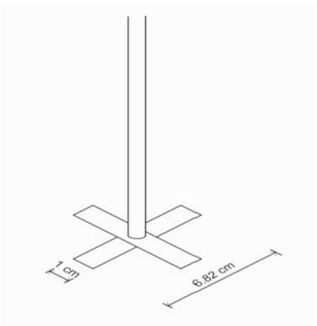
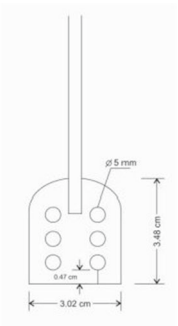
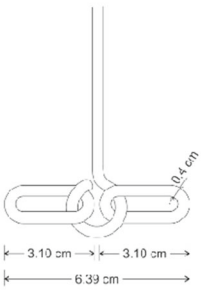
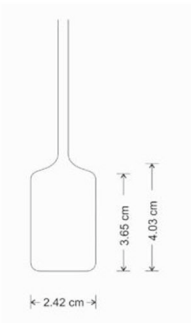
Application of EWP hydrolysates as food supplements is in direct correlation with their redox properties; hence, the reducing ability of obtained hydrolysates in current research was tested with the linear sweep voltammetry (Fig. 2b). Although a knowledge of the redox behavior of obtained EWP hydrolysates is a very important basis to obtain better explanations of their properties, their electrochemical aspects have not been investigated. Linear sweep voltammetry and cyclic voltammetry have been applied to characterize the reducing ability of several natural bioactive compounds including antioxidants in bovine milk, whey, and low-molecular-weight fractions of whey (Chen et al. 2002). It is found that compounds with strong scavenging capabilities are oxidized at low potentials. Results obtained in current research with the linear sweep voltammetry confirmed results previously obtained with DPPH and ABTS methods. Hydrolysates obtained in the Alcalase-catalyzed egg white hydrolysis have the highest antioxidant potential, what is depicted in the Fig. 2b with the highest achieved current at the same protein concentration of 2.0 mg cm^{-3}. Furthermore, the oxidation of Alcalase and Neutrase hydrolysates started at lower potential than these for papain hydrolysates or untreated egg white, revealing their higher reducing ability. The highest anodic current is also the consequence of the easiest oxidation of Alcalase obtained hydrolysates. Hydrolysate obtained with Neutrase seemed to possess higher antioxidant capacity than that with papain, even though achieved DH was lower, indicating that the Neutrase has the capacity to cleave proteins to smaller peptides. Accordingly to our results, it has been reported that despite more extensive rawhide hydrolysis by papain than that by Neutrase, the recovered protein from Neutrase hydrolysis cannot form a gel, revealing different mechanisms of papain and Neutrase hydrolysis (Damrongsakkul et al. 2008).

The Influence of Different Impeller Types on the DH of Egg White Proteins

The maximum utilization of the enzyme under favorable operating conditions in order to obtain desired DH, hence product quality, is preferable and necessary in making the hydrolysis economical. Efficient mixing in bioreactors enables uniform distribution of substrates, enzymes or other components, and temperature throughout bioreactor. It is well established that the enzyme-catalyzed hydrolysis depends in a great deal upon temperature and substrate concentration, hence finding of an adequate mixing in the stirred tank reactor becomes an imperative. On the other hand, enzymes show different levels of sensitivity to shear stress and therefore compromise between enzyme deactivation and efficient mixing; i.e., mass transfer has to be found. In order to provide an efficient system for producing antioxidant egg white hydrolysates, six impellers with different geometries (Table 2) were tested at a constant impeller speed, and their effects on the hydrolysis of EWPs were evaluated from the viewpoint of the values of the hydrolysis and deactivation rate constants (Table 2, Fig. 3).

Figure 3 displays effects of the different impeller types on the DH over reaction time. Common to all tested impellers is that hydrolysis progresses rapidly for the first 45 min, after which gradually increases, reaching a plateau after 100 min.

Table 2 Values of the kinetic constants obtained in the Alcalase-catalyzed egg white hydrolysis in the continuous stirred tank membrane reactor system with different impellers

Impeller type	k_h, (min^{-1})	k_d, (dm^3 min^{-1} g^{-1})	R^2	Impeller type	k_h, (min^{-1})	k_d, (dm^3 min^{-1} g^{-1})	R^2
Turbine				Beater paddle			
	1.90	0.041	0.9979		3.24	0.084	0.9922
4-bladedpropeller				Paddle			
	4.03	0.078	0.9961		2.16	0.037	0.9902
Chains				Curved paddle			
	2.58	0.072	0.9953		1.98	0.051	0.9973

The highest DH was achieved with the four-bladed propeller, when DH reached 65 % after 135 min.

From the presented results, conclusion ought to be made that the four-bladed propeller is the most favorable impeller for the Alcalase-catalyzed EWP hydrolysis. Such result is a repercussion of the generated axial flow, which directs liquid toward the base of the reactor, and typically exhibits low shear conditions (Radzi et al. 2010). This result is quantified throughout the values of kinetic constants, and it is shown (Table 2) that the value of k_h for this hydrolysis is by far the highest. On the other hand, the value of k_d indicated that enzyme deactivation occurred, but efficient agitation enabled high interactions between enzyme and substrate, providing the highest DH among all tested impellers. Turbine impeller also

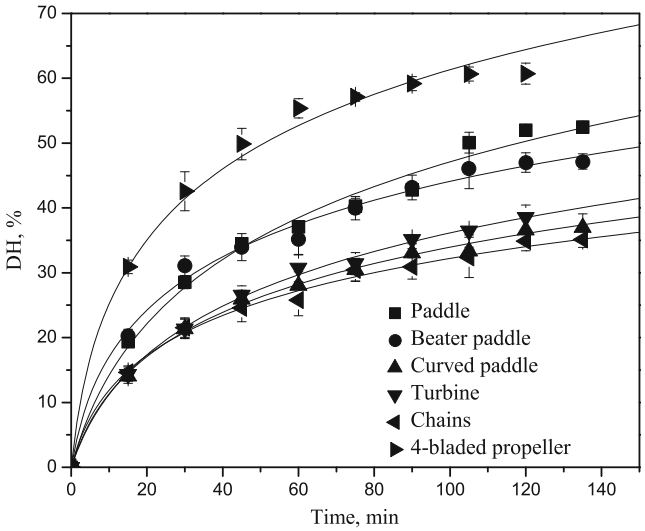


Fig. 3 The time course curves of Alcalase-catalyzed EWP hydrolysis in the continuous stirred tank reactor coupled with membrane modulus for different impeller types. Reaction conditions: pH 8.0, 50 °C, [*E*]/[*S*] ratio 0.48 AU g^{-1} of protein, and permeate flow rate 1.9 cm^3 min^{-1}

generates an axial flow, but with the minimal shear forces, what is confirmed by the obtained k_d value. However, the obtained value of k_h indicates that mass transfer was not efficient probably due to an inadequate mixing.

Beater paddle is appropriate for mixing of foaming fluids, since its design helps reducing incorporation of air into the mixture. This impeller provides radial flow and high shear conditions, which could be an explanation for the most extensive enzyme deactivation, described with the highest value of k_d. Hydrolysis constant indicated that vigorous mixing improved enzyme–substrate contact along with the considerable enzyme deactivation.

Tangential flow generated by paddle impeller enabled good heat exchange and gentle treatment of the enzyme, but with limited mixing due to the small velocity gradients. As a result enzyme deactivation was reduced, inefficient mixing resulted in the low value of hydrolysis constant (Table 2).

Curved paddle impeller did not improve process efficiency compared to the one achieved with the paddle impeller, since lower DH was achieved (Fig. 3), probably due to almost 40 % higher enzyme deactivation rate constant, while the hydrolysis constant also decreased.

Chain impeller was also proved to be inadequate for this hydrolysis, due to the high value of the deactivation rate constant and low value of the hydrolysis rate constant, resulting with the low value of achieved DH. According to ANOVA test, the impeller type is a highly significant factor in EWP hydrolysis ($p<0.01$). Further, results of the Student *t* test proved that four-bladed propeller was the best impeller for this hydrolysis, since significantly higher DH was obtained with this impeller compared to the five other tested impeller types. Statistical analysis showed that there were no significant differences in achieved DH, when the following impellers were compared: curved paddle chains, curved paddle turbine, and turbine chains (p 0.05).

Optimization by RSM

Each enzymatic reaction requires careful planning and optimization in order to achieve product of the desired quality. The RSM was used to assess the significance of each individual factor, to acknowledge their interactive effects, and to determine optimum conditions for Alcalase-catalyzed egg white hydrolysis. This statistical tool has been previously applied for optimization of protein hydrolysis catalyzed by different proteases (Laohakunjit et al. 2014; Sanhong et al. 2014). Based on the results from the RSM analysis, the empirical relationship between response variables (DH and reactor capacity) and the tested parameters was expressed by second-order polynomial equations with interaction terms:

$$Y_1 = 53.50 + 0.51X_1 - 5.53X_2 + 1.38X_3 - 3.13X_2X_3 - 2.82{X_1}^2 - 3.30{X_2}^2 - 1.33{X_3}^2 \quad (6)$$

$$Y_2 = 1.46 + 0.048X_1 - 0.50X_2 + 0.15X_3 + 0.16X_1X_2 - 0.13X_2X_3 + 0.49{X_1}^2 + 0.41{X_2}^2 - 0.10{X_3}^2 \quad (7)$$

Table 3 Analysis of variance

Coefficients	*p* value			
	Model for DH (%)		Model for reactor capacity (mg of protein AU^{-1} min^{-1})	
X_1—[*E*]/[*S*] ratio (AU g^{-1} of protein)	0.2915		0.0258	
X_2—impeller speed (rpm)	<0.0001		<0.0001	
X_3—permeate flow rate (cm^3 min^{-1})	0.0148		<0.0001	
X_1X_3			0.0002	
X_2X_3	0.0009		0.0007	
${X_1}^2$	0.0016		<0.0001	
${X_2}^2$	0.0005		<0.0001	
${X_3}^2$	0.0648		0.0027	
	F value	*p* value	*F* value	*p* value
Model	33.81	<0.0001	216.62	<0.0001
Lack of fit	0.62	0.62	4.69	0.0764
R^2	0.9634		0.9954	
Adjusted R^2	0.9349		0.9905	

where all symbols have the same meaning as in the "Statistical Analysis" section.

The results of the analysis of variance (ANOVA) for the significance of the linear, quadratic, and interaction effects of the following: [*E*]/[*S*] ratio, impeller speed and permeate flow rate on DH, and reactor capacity and for the significance of polynomial models are presented in Table 3.

It seemed that the experimental data, and the statistical models fitted to them, perfectly agreed predicting the performance of Alcalase-catalyzed EWP hydrolysis in the membrane reactor system very well. The models are statistically significant as is evident from the high *F* values of 33.81 and 216.62 for model 1 and model 2 ($p<0.0001$), respectively, and explained most of the variability with high coefficient of multiple determination ($R^2=0.9634$ and 0.9954, respectively). The lack-of-fit *F* values of 0.62 and 4.69 imply that the lack of fit is not significant in either of obtained models as they were more than 0.05, indicating that both models are valid for the present work. It was also apparent that the factor effects and their interactions were different for the two process outputs: DH and reactor capacity.

It is easily observed that the linear term of impeller speed and quadratic terms of both impeller speed and [*E*]/[*S*] ratio are highly significant, as well as the interaction between impeller speed and permeate flow rate ($p<0.01$). Such results indicated that all of the studied parameters showed significant influence on the DH within the studied experimental ranges. [*E*]/[*S*] ratio has been previously reported as a significant factor in the hydrolysis of soy proteins (de Oliveira et al. 2014) and sesame cake proteins (Demirhan et al. 2010).

The curve presenting predicted values for DH versus [*E*]/[*S*] ratio appeared to have a maximum (Fig. 4a). The highest DH of 56.37 was obtained at 0.56 AU g^{-1} of protein, and above this level, further increase did not potentiate increase in DH. Similar results have been reported in the Alcalase-catalyzed hydrolysis of sesame cake proteins, where an

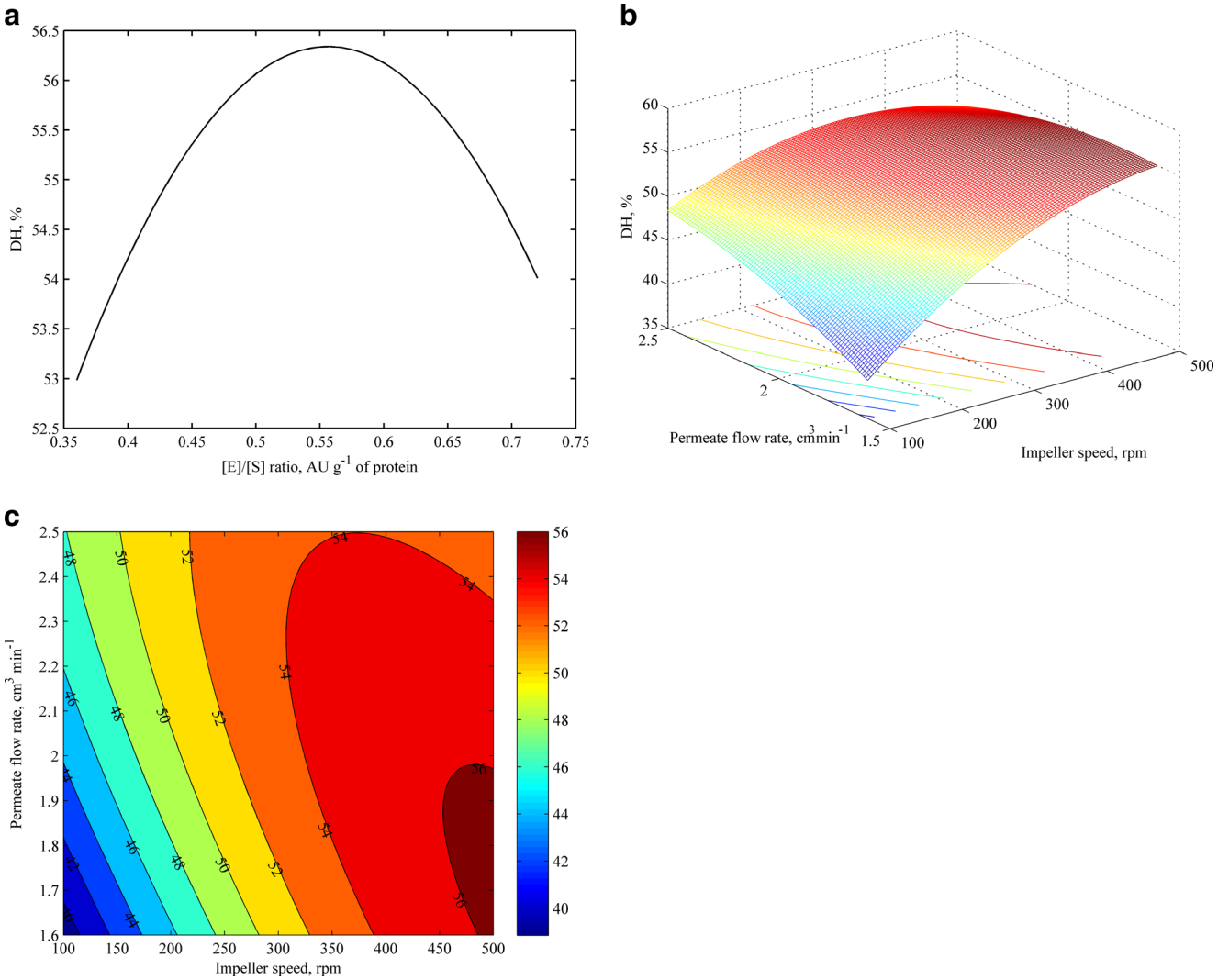


Fig. 4 **a** The curve presenting dependency of DH on [*E*]/[*S*] ratio (impeller speed 500 rpm and permeate flow rate 1.75 cm^3 min^{-1}). The effect of the impeller speed and permeate flow rate on the DH **b** surface plot and **c** contour plot at fixed [*E*]/[*S*] ratio of 0.54 AU g^{-1} of protein

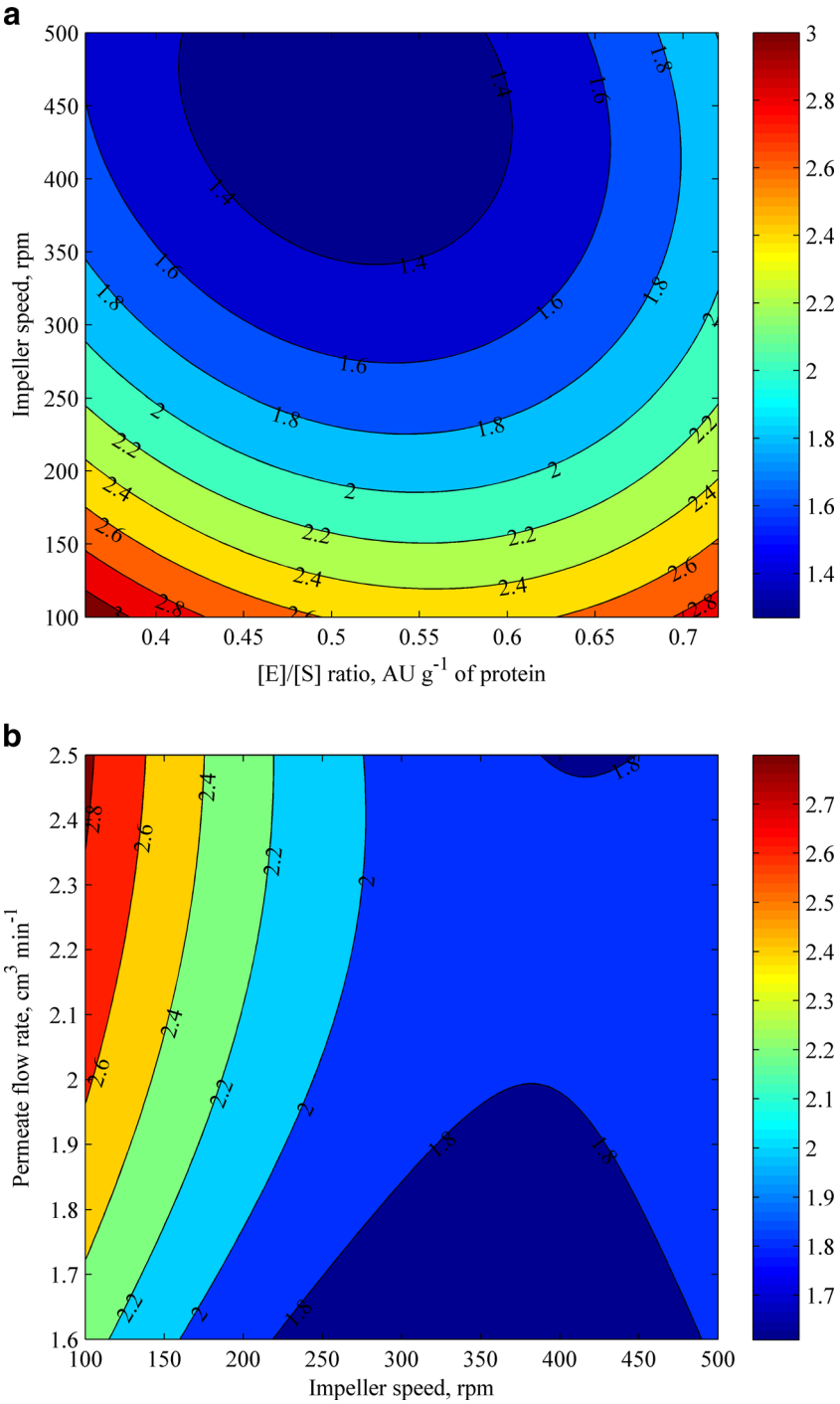


Fig. 5 The influence of impeller speed on the reactor capacity. **a** Contour plot of impeller speed and [*E*]/[*S*] ratio effects (permeate flow rate 2.44 cm^3 min^{-1}). **b** Contour plot of impeller speed and permeate flow rate effects ([*E*]/[*S*] ratio 0.70 AU g^{-1} of protein)

increase in enzyme concentration up to 3 cm^3 dm^{-3} (corresponding to 0.56 AU g^{-1} protein) led to an increase in DH, while a further increase in enzyme concentration did not improve the DH (Demirhan et al. 2010). This result is explained by the enzyme saturation with substrate at higher [*E*]/[*S*] ratios. Although accidentally the identical optimal [*E*]/[*S*] value was obtained in current and reported study (Demirhan et al. 2010), the DH of EWPs achieved in current research (56.38 %) was almost twofold higher than that obtained in hydrolysis of sesame cake proteins (26.3 %), probably due to the substrate and/or product inhibition which are indicated to occur in the batch reactor system.

Similarly, all of the studied parameters had the significant effect on the reactor capacity, where the impeller speed was the most significant variable with high linear and quadratic effects on the reactor capacity. The highest quadratic effect of

permeate flow rate on the reactor capacity was apparent as well as high interaction effects of impeller speed with both [*E*]/[*S*] ratio and permeate flow rate, revealing high impact of all variables on the process.

Figure 4b, c shows the response surface and contour plots for the predicted values for the DH vs the impeller speed and the permeate flow rate at fixed [*E*]/[*S*] ratio of 0.54 AU g^{-1} of protein. The impeller speed showed an interactive negative effect with permeate flow rate on the DH. It is easily observed that the lowest DH is achieved with the lowest impeller speed and permeate flow rate. In the stirred tank bioreactor coupled with a membrane modulus, the overall mixing process is the result of two independent operations: liquid mixing due to stirring and due to recycling of reaction mixture. At low impeller speeds and low permeate flows, both of these processes are at insufficient level, therefore limiting interactions between enzyme and substrate and resulting in low DH. By increasing the impeller speed at lower permeate flow rates, the DH gradually increased over the tested range. The highest DH achieved at the lowest permeate flow rate and the highest impeller speed. The same trend is observed in the reversed case, but the achieved DH is lower. High values of both impeller speed and permeate flow rate lead to lower values of DH probably due to the high shear stress conditions, under which enzyme deactivation suppresses the positive effects of efficient mass transfer (Goswami et al. 2012). High impeller speeds could also provide conditions for generation of foam which are additionally favored by foaming properties of produced peptides (Nouri et al. 1997). For these reasons, higher impeller speeds were not tested. Additionally, higher permeate flows reduce reaction time between enzyme and substrate, and correspondingly, lower DH is achieved, and simultaneously, lower permeate flows favor higher DH (Shi et al. 2009).

Optimum conditions for achieving the highest DH proposed by the model were at highest impeller speed, 500 rpm, permeate flow rate of 1.75 cm^3 min^{-1}, and [*E*]/[*S*] ratio of 0.56 AU g^{-1} of protein. Nouri et al. reported the same impeller speed as optimum in the hydrolysis of wheat proteins, where further increase in impeller speed reduced achieved DH (Nouri et al. 1997).

Enzyme cost often causes limitation of industrial enzymatic processes; thus, a reasonable compromise between achieved DH and reactor capacities is an absolute imperative. It seems that the most relevant variable for the process performance was the impeller speed showing the highest linear negative impact on the reactor capacity. However, its effects are rather complex with the positive quadratic effect and some interaction effects with both, [*E*]/[*S*] ratio and permeate flow rate.

Figure 5a shows the contour plot of the reactor capacity vs impeller speed and [*E*]/[*S*]. As expected, higher reactor capacities are achieved at lower studied [*E*]/[*S*] ratio. Positive interaction between [*E*]/[*S*] ratio and impeller speed is well presented in Fig. 5a, indicating that a rater high reactor capacities are possible with small amounts of Alcalase when low impeller speed is used, which is beneficial from the economic viewpoint. Interaction between impeller speed and permeate flow is depicted in Fig. 5b. In contrast to the results of the first model, the highest reactor capacity was achieved at the lowest impeller speed and the highest permeate flow. Such conditions provided efficient mass transfer and, at the same time, favored product formation. Similar results were reported in the continuous enzymatic production of 5′-nucleotides using free nuclease, where increase in permeate flow up to 2 cm^3 min^{-1} enabled significant increase in reactor productivity compared to lower values of permeate flow, which favored higher nucleotides concentrations (Shi et al. 2009).

Optimum conditions for this reaction from the economic viewpoint are as follows: [*E*]/[*S*] ratio of 0.36 AU g^{-1} of protein, impeller speed of 100 rpm, and permeate flow of 2.5 cm^3 min^{-1}. Since the found optimum values for the DH and reactor capacities are in disagreement, compromise between economic aspects and product quality should be found. According to the both obtained models, reaction conditions: [*E*]/[*S*] ratio of 0.71 AU g^{-1} of protein, impeller speed of 500 rpm, and permeate flow of 1.83 cm^3 min^{-1} would enable the production of hydrolysates of desired quality along with satisfactory reactor capacities.

Conclusion

In conclusion, the hydrolysis of EWPs was conducted in a continuous stirred tank membrane reactor system, and small peptides' fraction with increased antioxidant activity was successfully produced. The protease, which was the most promising enzyme for this catalysis, was Alcalase, enabling the highest DH and the hydrolysates with the best antioxidant properties, determined by three methods. Neutrase also expressed good potential for producing antioxidant peptides even though DH was comparable low. Response surface regression models were established to predict the process outputs such as degree of hydrolysis and reactor capacity as a function of three operating process variables (enzyme/substrate ratio, impeller speed, and permeate flow rate). RSM and statistical analysis revealed significant effects of all three parameters on the process performances and interesting interactions between them under the range studied. It seems that the high reactor capacity of .1.98 mg of protein AU^{-1} min^{-1} and DH of 54.2 % could be achieved with small amounts of Alcalase such as [*E*]/[*S*] ratio of 0.71 AU g^{-1} of protein, when impeller speed of 500 rpm and permeate flow of 1.83 cm^3 min^{-1} were used.

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