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Anti-Diabetic Properties of Hydrolysates from Egg White Proteins Using Immobilized Enzymes Followed by *in vitro* Gastrointestinal Digestion

Behzad Gazme[1], Chibuike C. Udenigwe[2,3*], Karamatollah Rezaei[1*]

1- Department of Food Science, Engineering, and Technology, University of Tehran, 31587‐77871 Karaj, Iran.

2- School of Nutrition Sciences, Faculty of Health Sciences, 415 Smyth Road, University of Ottawa, Ottawa, Ontario, K1H 8L1, Canada.

3- Department of Chemistry and Biomolecular Sciences, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada.

Abstract

Background and Objective: Enzyme-assisted hydrolysis of the proteins from food sources is an effective way to generate peptides with various bioactive properties. Furthermore, enzyme immobilization is a way to recycle enzymes for the future uses. The objective of this study was to investigate the effects of neutrase and thermolysin immobilization on the enzyme properties and *in vitro* anti-diabetic properties of intestinal digests achieved by the simulated digestion of egg white protein hydrolysates.

Material and Methods: Neutrase and thermolysin were immobilized on cellulose-coated magnetite nanoparticles. Then, enzyme activity, thermal resistance, reusability and optimum conditions of the egg white protein hydrolysis were assessed. Egg white protein hydrolysates were then digested *in vitro* and inhibitory activities of the intestinal digests against dipeptidyl peptidase IV (DPP-IV) and α-glucosidase were investigated.

Results and Conclusion: Enzymes immobilization resulted in increases in the thermal stabilities of them. Optimum temperatures for the egg white protein hydrolysis increased by 4.0 and 3.2 °C for neutrase and thermolysin, respectively. Digests from the hydrolysates of free neutrase effectively inhibited DPP-IV and α-glucosidase by 17.9 and 29.7%, respectively. These values for the hydrolysates released by the free thermolysin were higher (37.2 and 35.1%, respectively). The enzyme immobilization resulted in a 4.4% decrease in DPP-IV inhibitory activities of the digests for the hydrolysates from neutrase and a 28.6% decrease for those from thermolysin. Decreases in α-glucosidase inhibition due to the immobilization included 9.8% for neutrase and 12.2% for thermolysin for the digests from the hydrolysates. Based on the results from the current study, hydrolysates from the egg white proteins achieved by the free and immobilized neutrase and thermolysin can be used in formulations of the functional foods and nutraceuticals with multifunctional properties.

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***Corresponding authors:**
Chibuike C. Udenigwe
Department of Chemistry and Biomolecular Sciences, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada.
E-mail: cudenigw@uottawa.ca
Tel: +1-613-562-5800

Karamatollah Rezaei
Department of Food Science, Engineering, and Technology, University of Tehran, 31587‐77871 Karaj, Iran.
Tel/Fax: +98-26-32248804
E-mail: krezaee@ut.ac.ir

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1. Introduction

Enzymes are used as effective catalysts for a wide range of processes from food to pharmaceuticals. However, enzymes are sensitive to changes in the pH and temperature and also their multiple uses as free enzymes are somewhat impossible [1]. Therefore, immobilization has been used to solve these problems, especially those linked to their reusability [2]. Among the carriers used to immobilize enzymes, magnetite nanoparticles (MNPs, Fe_3O_4) have increasingly become popular due to their strong magnetic properties, low toxicities and compatibilities with most biological components [3]. Presence of the functional groups on the surfaces of MNPs is necessity to immobilize

biomolecules. Functionalization can result in increases in loading capacities of the immobilized biomolecules. Generally, natural and synthetic polymers are used for the functionalization of MNPs [4]. In addition to magnetic susceptibility, another advantage of polymer-coated magnetic nanoparticles is inked to their reactive polymer surfaces to bind with biomolecules such as enzymes. Cellulose is one of the most abundant renewable resources and is a biodegradable and eco-friendly non-toxic polymer [5]. Namdeo and Bajpai [5] used cellulose to coat MNPs for further immobilization of α-amylase [5]. Neutrase and thermolysin were successfully immobilized on cellulose-coated magnetite nanoparticles (CMNP) to remove IgE epitopes in egg white proteins by enzymatic hydrolysis [6].

Use of egg white proteins in food industries is widespread due to its functional and dietary properties [7]. Several studies have reported effects of egg white protein hydrolysates on inhibition of DPP-IV [8,9] and α-glucosidase [9], which are important targets for relieving type 2 diabetes. Neutrase and thermolysin are enzymes used for the generation of bioactive peptides from egg white proteins [7,10]. Ortega et al. [11] reported that the immobilization of neutrase on alginate-glutaraldehyde beads increased thermal stability and resistance of the enzyme to various pH levels. Thermolysin immobilization on various carriers such as mesocellular siliceous foam support [12] and activated agarose gels [13] resulted in improvements in certain enzyme properties. However, effects of neutrase and thermolysin immobilization with magnetite nanoparticles on the enzyme properties have not been investigated. More specifically, no studies have reported effects of immobilization and digestive tract conditions on inhibitory activities of the egg white protein hydrolysates released by neutrase and thermolysin against DPP-IV and α-glucosidase. Therefore, effects of neutrase and thermolysin immobilization on CMNP on enzyme properties (enzyme activity, thermostability and reusability) and optimum conditions for the hydrolysis of egg white proteins were investigated in this study. Egg white proteins were hydrolyzed at the optimum conditions for each enzyme and effects of immobilization and *in vitro* digestion on anti-diabetic properties of the egg white protein hydrolysates were investigated.

2. Materials and Methods

2.1. Materials

Pasteurized liquid egg white (Naturegg Simply Egg Whites, Burnbrae Farms, Brockville, ON, Canada) was purchased from local stores in Ottawa, Canada. Neutrase 0.8 L (P1236) as free neutrase (FN), thermolysin (T7902) as free thermolysin (FT), pepsin (P7000), pancreatin 8× USP specifications (P7545), α-glucosidase (G0660), DPP-IV (D3446), 4-nitrophenyl α-D-glucopyranoside (purity ≥ 99%) (N1377), glycine-proline-p-nitroanilide hydrochloride (purity ≥ 99%) (G0513), bovine serum albumin (BSA) and other chemicals and reagents were purchased from Sigma-Aldrich, Oakville, ON, Canada.

2.2. Enzyme immobilization

Enzyme immobilization was carried out based on a modified procedure originally reported by Namdeo and Bajpai [5]. Immobilization was carried out within four steps of a production step for MNP, a coating step with cellulose for MNP, an oxidation step of CMNP and a step for the immobilization of enzymes on CMNP.

2.2.1. Preparation of magnetite nanoparticles

Iron oxide nanoparticles (Fe_3O_4) were produced based on a procedure described by Jain et al. [14]. Briefly, 60 ml of $FeCl_3$. $6H_2O$ (0.125 M in deionized water, DW), 30 ml of $FeCl_2$. $4H_2O$ (0.125 M in DW) and 80 ml of DW were mixed together with mild agitation at 200 rpm at room temperature. Then, 50 ml of NaOH (1 M) was added drop-wise under constant stirring for 60 min and the solution was centrifuged at 40,000 ×g for 60 min (Sorvall LYNX 4000, Thermo Scientific, Waltham, MA, USA). Precipitate (magnetite particles) was washed twice with DW followed by another wash with chloroform. A solution of 3.0 g of magnetite particles in 50 ml of DW was prepared and ultrasonicated at 35 kHz (Ultrasonic Water Bath, VWR International, Radnor, PA, USA) for 60 min. Magnetite nanoparticles were freeze-dried at a surface temperature of 24 °C and a pressure of less than 0.2 mbar (Rotary Vane Vacuum Pump, Labconco, Kansas, MI, USA) using bulk tray-drier (Labconco, Kansas, MI, USA) and stored at -20 °C until use.

2.2.2. Preparation of cellulose-coated magnetite nanoparticles

To prepare CMNP, a uniform suspension was first prepared by mixing microcrystalline cellulose (2.0 g), NaOH (3.0 g) and urea (2.0 g) in 25 ml of DW. Suspension was stored at -5 °C for 12 h until a transparent solution was achieved. Then, 50 ml of MNP stock solution (8%, w v^{-1}) in DW and the prepared suspension were vigorously mixed together. Mixture was drop-wise added to the ammonium sulfate solution (8% w v^{-1}), mixed at 800 rpm (Isotemp Hotplate Stirrer 7 × 7, Fisher Scientific, Hampton, NH, USA) [5] and centrifuged at 12,000 ×g for 15 min. Sediment was washed several times with DW, freeze-dried and stored at -20 °C until use.

2.2.3. Oxidation of CMNP

To prepare oxidized CMNP (OCMNP), a mixture of 0.2 g of CMNP in 25 ml of periodic acid solution (0.03 M) was prepared and its pH was adjusted to 4.00. Mixture was incubated at 80 °C for 15 h with a constant stirring rate at 300 rpm [5]. After centrifugation, washing and freeze-

drying steps (as described in Section 2.2.2), the solution was stored at -20 °C until use.

2.2.4. Immobilization of neutrase and thermolysin on CMNP

To immobilize neutrase and thermolysin on oxidized CMNP, a mixture of the oxidized CMNP in DW (1.0 g in 50 ml) and 5.0 ml of neutrase or 100 mg of thermolysin were mixed together for 60 min at 50 °C [5] and the mixture was centrifuged at 12,000 ×g for 15 min. Precipitate was washed with DW to separate non-immobilized enzymes and then immobilized enzymes on CMNP were separated using neodymium magnets. Supernatant from the centrifugation stage and DW were analyzed for the enzyme concentration using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). BSA was used as reference standard. Enzyme load (q, U mg^{-1}) and the immobilization yield (Y) were assessed using Equations 1 and 2, respectively [15].

$$q = \frac{(Cb-Ca)V}{W} \times enzyme\ activity \quad \text{Eq. 1}$$

$$Y = \frac{(Cb-Ca)V}{Cb} \times 100 \quad \text{Eq. 2}$$

Where, C_b and C_a were concentrations of the enzyme before and after immobilization, respectively; *v* was volume of the reaction medium and *w* was weight of the CMNP.

2.3. Size measurement of MNPs and CMNP and infrared spectroscopy

Zetasizer Nano ZS Instrument (Malvern Instrument, Worcestershire, UK) was used to determine the particle size and polydispersity index (PDI) of MNPs and CMNP. Briefly, a suspension of the sample was prepared in DW (0.005% w v^{-1}) and the measurement was carried out at ambient temperature. A Nicolet iS 10 Fourier-Transform Infrared (FTIR) Spectroscopy System (Thermo Scientific, Waltham, MA, USA) was used to analyze the chemical structures of the immobilized enzymes at 4000-600 cm^{-1}.

2.4. Enzyme activity assay

A method by Ortega et al. [11] was used to assess the enzymatic activities of free and immobilized enzymes. Casein was used as substrate and the content of tyrosine released by the enzyme was assessed after reacting with Folin-Ciocalteu reagent and formation of a compound detectable at 660 nm [11]. The pH and temperature of assay were adjusted based on the optimum conditions predicted by the quadratic model for free and immobilized neutrase as well as those of free and immobilized thermolysin. Enzyme activities were assessed at three different concentrations (0.05, 0.07 and 0.10 mg ml^{-1}) of immobilized and free enzymes and the enzyme activities were reported as mean ±SD (standard deviation).

2.5. Thermal resistance of free and immobilized enzymes

Heat resistance levels for free and immobilized enzymes were assessed by exposing enzymes (60 min) to heat (40-90 °C) at the optimum pH predicted by the quadratic model for each enzyme. After heat treatment, enzyme activity was assessed based on the procedure explained in Section 2.4. The remaining activity of each enzyme after heat treatment was expressed as proportion of the enzyme activity when no heat was used [11].

2.6. Reusability assessment of the immobilized enzymes

To assess enzyme reusability, certain quantities of immobilized enzymes were mixed with the egg white protein solutions and hydrolysis was carried out under the optimum conditions of each enzyme based on a modified method originally described by Yang et al. [15]. Enzymes were separated from the reaction mixture using neodymium magnet followed by estimation of the enzymatic activity (Section 2.4) and then reused for the hydrolysis in the following cycle. This was carried out for five hydrolysis cycles. Residual activities were reported as enzyme activity in each cycle relative to the activity of the original immobilized enzyme. An overall activity (accumulated activity) was assessed by summing the residual activities over five and the original enzyme activity.

2.7. Experimental design for the egg white protein hydrolysis

Effects of reaction temperature, pH, enzyme to egg-white protein ratio and incubation time on degrees of hydrolysis of egg white proteins (section 2.9) were investigated using Box-Behnken design from response surface methodology (RSM) applying four variables at three levels each. Based on the data provided by the enzyme manufacturer, temperature levels were assigned at 40, 50 and 60 ºC for neutrase and 60, 70 and 80 ºC for thermolysin. Furthermore, pH levels at 5.50, 6.75 and 8.00 were set for neutrase and 6.50, 7.75 and 9.00 for thermolysin. The applied enzyme to protein ratios were based on the literature data [16,17] at 0.020, 0.035 and 0.050 (w w^{-1}). Three levels of incubation time (1, 2 and 3 h) were selected and a total of 29 runs including five replicates at the center point were carried out according to the experimental design (Tables 1, 2, 3 and 4). Based on the results from analysis of the data, conditions for the optimum performance of the enzymes were achieved and experiments to assess the suggested conditions were carried out in triplicates. Design Expert software v.10 (Stat-Ease, Minneapolis, MN, USA) was used for optimizing hydrolysis conditions of the egg white proteins and analysis of variance (ANOVA) at 95% confidence level.

2.8. Egg white protein hydrolysis

Protein concentration of the pasteurized liquid egg white was assessed using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) as explained in Section 2.2.4 and then diluted with water to reach a protein concentration of 0.1% (w v^{-1}). Mixture was heated at 60 °C for 5 min to achieve partial denaturation. Then, final temperature, pH, enzyme concentration and time were set based on the suggested experimental conditions (Tables 1-4) and hydrolysis was carried out for each run. After completion of the hydrolysis, enzymes were deactivated at 90 °C for 15 min and degrees of hydrolysis were assessed based on the procedure described in Section 2.9. After analyzing the results and determining the optimum conditions, samples were rehydrolyzed under the suggested conditions and centrifugation was carried out at 10,000 ×g for 10 min. Supernatant was freeze-dried and maintained at -20 °C until use.

Table 1. Experimental data for degree of hydrolysis (%) by Box-Behnken design using response surface methodology for free Neutrase. Values showing different letters are significantly different ($P < 0.05$).

Run	pH	Temperature (°C)	Time (h)	Enzyme concentration (w w^{-1})	Degree of hydrolysis (%)
1	6.75	50	1	0.050	5.2 ± 0.2^{c}
2	6.75	40	3	0.035	6.9 ± 0.5^{ab}
3	5.50	60	2	0.035	5.9 ± 0.3^{b}
4	6.75	60	2	0.050	6.7 ± 0.6^{ab}
5	6.75	40	1	0.035	2.0 ± 0.7^{ef}
6	6.75	50	2	0.035	1.7 ± 0.9^{ef}
7	6.75	50	2	0.035	2.0 ± 0.2^{e}
8	6.75	50	3	0.050	7.2 ± 0.2^{a}
9	6.75	50	2	0.035	1.5 ± 0.1^{f}
10	6.75	50	2	0.035	2.6 ± 0.4^{e}
11	8.00	50	1	0.035	3.2 ± 0.6^{de}
12	5.50	50	2	0.020	4.0 ± 0.7^{d}
13	5.50	50	1	0.035	4.6 ± 0.5^{cd}
14	5.50	50	2	0.050	6.1 ± 0.4^{b}
15	6.75	60	2	0.020	3.0 ± 0.6^{de}
16	6.75	60	3	0.035	3.8 ± 0.5^{d}
17	8.00	50	3	0.035	4.0 ± 0.9^{cde}
18	6.75	60	1	0.035	3.2 ± 0.7^{de}
19	6.75	40	2	0.020	5.0 ± 0.1^{c}
20	6.75	50	2	0.035	2.4 ± 0.4^{e}
21	6.75	40	2	0.050	6.0 ± 0.5^{b}
22	6.75	50	3	0.020	3.9 ± 0.4^{d}
23	5.50	40	2	0.035	3.5 ± 0.5^{de}
24	8.00	60	2	0.035	3.2 ± 0.4^{de}
25	5.50	50	3	0.035	4.8 ± 0.3^{cd}
26	8.00	50	2	0.020	5.2 ± 0.4^{cb}
27	8.00	40	2	0.035	4.3 ± 0.7^{cd}
28	8.00	50	2	0.050	6.6 ± 0.4^{ab}
29	6.75	50	1	0.020	2.6 ± 0.6^{e}

Table 2. Experimental data for degree of hydrolysis (%) by Box-Behnken design using response surface methodology for immobilized Neutrase. Values showing different letters are significantly different ($P < 0.05$).

Run	pH	Temperature (°C)	Time (h)	Enzyme concentration (w w^{-1})	Degree of hydrolysis (%)
1	5.50	50	1	0.035	2.4 ± 0.2^{d}
2	8.00	40	2	0.035	1.7 ± 0.1^{f}
3	6.75	50	2	0.035	0.9 ± 0.1^{h}
4	6.75	50	2	0.035	1.8 ± 0.5^{deg}
5	6.75	50	1	0.050	2.7 ± 0.1^{c}
6	8.00	50	1	0.035	1.7 ± 0.2^{f}
7	6.75	50	2	0.035	1.4 ± 0.1^{fg}
8	6.75	50	3	0.020	2.3 ± 0.2^{de}
9	6.75	40	2	0.050	3.4 ± 0.1^{ab}
10	6.75	50	1	0.020	1.3 ± 0.1^{g}
11	6.75	60	1	0.035	1.7 ± 0.1^{f}
12	5.50	50	3	0.035	2.6 ± 0.1^{cd}
13	5.50	50	2	0.020	2.1 ± 0.2^{def}
14	8.00	50	2	0.020	2.8 ± 0.3^{bcd}
15	6.75	40	3	0.035	3.9 ± 0.4^{a}
16	6.75	40	2	0.020	2.7 ± 0.1^{bcd}
17	5.50	60	2	0.035	2.8 ± 0.1^{bc}
18	6.75	50	3	0.050	3.9 ± 0.3^{a}
19	6.75	60	3	0.035	2.0 ± 0.1^{e}
20	5.50	40	2	0.035	1.9 ± 0.3^{def}
21	6.75	50	2	0.035	1.3 ± 0.4^{fgh}
22	8.00	50	2	0.050	3.3 ± 0.5^{abc}
23	6.75	60	2	0.020	1.5 ± 0.1^{f}
24	6.75	60	2	0.050	3.1 ± 0.3^{bc}
25	6.75	50	2	0.035	1.2 ± 0.1^{gh}
26	8.00	50	3	0.035	2.1 ± 0.1^{de}
27	5.50	50	2	0.050	2.8 ± 0.1^{bc}
28	8.00	60	2	0.035	1.7 ± 0.2^{f}
29	6.75	40	1	0.035	1.0 ± 0.1^{h}

Table 3. Experimental data for degree of hydrolysis (%) by Box-Behnken design using response surface methodology for free Thermolysin. Values showing different letters are significantly different ($P < 0.05$).

Run	pH	Temperature (°C)	Time (h)	Enzyme concentration (w w^{-1})	Degree of hydrolysis (%)
1	7.75	80	2	0.020	9.3 ± 0.5^{l}
2	6.50	70	2	0.050	15.8 ± 0.4^{f}
3	7.75	80	1	0.035	11.4 ± 0.7^{jk}
4	9.00	70	2	0.020	9.7 ± 0.2^{l}
5	7.75	70	2	0.035	15.0 ± 0.1^{g}
6	9.00	70	3	0.035	11.6 ± 0.6^{jk}
7	7.75	70	2	0.035	18.1 ± 0.8^{de}
8	9.00	60	2	0.035	10.5 ± 0.5^{kl}
9	9.00	70	1	0.035	7.9 ± 0.9^{m}
10	7.75	70	1	0.050	15.8 ± 0.2^{f}
11	6.50	80	2	0.035	13.5 ± 0.7^{hi}
12	9.00	80	2	0.035	11.4 ± 0.5^{jk}
13	7.75	60	2	0.020	17.7 ± 0.3^{e}
14	7.75	70	3	0.050	23.0 ± 0.3^{a}
15	7.75	80	2	0.050	20.5 ± 0.4^{cd}
16	7.75	70	2	0.035	10.9 ± 0.8^{jk}
17	7.75	70	2	0.035	12.9 ± 0.1^{i}
18	6.5	70	2	0.020	9.7 ± 0.1^{l}
19	7.75	70	1	0.020	8.1 ± 0.4^{m}
20	9.00	70	2	0.050	20.5 ± 0.2^{c}
21	6.50	70	3	0.035	15.0 ± 0.5^{fg}
22	7.75	70	2	0.035	11.8 ± 0.4^{j}
23	7.75	70	3	0.020	12.2 ± 0.8^{ij}
24	7.75	60	1	0.035	13.7 ± 0.3^{h}
25	6.50	60	2	0.035	19.2 ± 0.9^{d}
26	7.75	60	3	0.035	21.8 ± 0.2^{b}
27	7.75	60	2	0.050	20.7 ± 0.5^{c}
28	6.50	70	1	0.035	10.4 ± 0.1^{k}
29	7.75	80	3	0.035	17.5 ± 0.2^{e}

Table 4. Experimental data for degree of hydrolysis (%) by Box-Behnken design using response surface methodology for immobilized Thermolysin. Values showing different letters are significantly different ($P < 0.05$).

Run	pH	Temperature (°C)	Time (h)	Enzyme concentration (w w^{-1})	Degree of hydrolysis (%)
1	7.75	70	2	0.035	10.4 ± 0.2^{a}
2	6.50	80	2	0.035	7.4 ± 0.8^{cd}
3	9.00	60	2	0.035	9.5 ± 0.5^{b}
4	7.75	60	3	0.035	4.3 ± 0.4^{f}
5	7.75	70	1	0.020	3.3 ± 0.5^{g}
6	9.00	70	1	0.035	5.2 ± 0.8^{ef}
7	6.50	70	2	0.020	5.6 ± 0.3^{e}
8	7.75	80	2	0.050	7.1 ± 0.5^{d}
9	6.50	70	1	0.035	5.5 ± 0.3^{e}
10	7.75	70	3	0.050	10.8 ± 0.7^{a}
11	7.75	60	2	0.020	5.6 ± 0.6^{e}
12	6.50	70	3	0.035	6.6 ± 0.3^{d}
13	6.50	70	2	0.050	6.4 ± 0.2^{d}
14	7.75	70	1	0.050	6.3 ± 0.5^{de}
15	7.75	80	2	0.020	5.7 ± 0.2^{e}
16	9.00	70	3	0.035	8.5 ± 0.8^{c}
17	7.75	70	2	0.035	7.7 ± 0.6^{cd}
18	7.75	70	3	0.020	6.9 ± 0.7^{d}
19	7.75	60	2	0.050	8.5 ± 0.8^{c}
20	7.75	70	2	0.035	6.7 ± 0.2^{d}
21	7.75	80	3	0.035	8.2 ± 0.5^{c}
22	6.50	60	2	0.035	5.2 ± 0.8^{ef}
23	7.75	60	1	0.035	7.2 ± 0.2^{d}
24	7.75	80	1	0.035	6.2 ± 0.6^{de}
25	7.75	70	2	0.035	7.7 ± 0.5^{cd}
26	9.00	70	2	0.050	9.9 ± 0.1^{b}
27	9.00	80	2	0.035	7.5 ± 0.4^{cd}
28	9.00	70	2	0.020	4.1 ± 0.4^{f}
29	7.75	70	2	0.035	8.3 ± 0.9^{cd}

2.9. Degrees of hydrolysis

Degrees of hydrolysis were measured based on the chemical reaction between the amino groups of the proteins and o-phthaldialdehyde (OPA), resulting in the formation of a compound, which is detected at 340 nm [18]. For the preparation of OPA solution, disodium tetraborate deca-hydrate (762 mg), sodium dodecyl sulfate (20 mg) and OPA (16 mg dissolved in 400 µl of methanol) were added to 15 ml of DW. Then, 17.6 mg of dithiothreitol (prepared in 3 ml of DW) were added to the prepared mixture and made a final volume of 20 ml using DW. For OPA assessment, an aliquot of 30 µl of hydrolysates solution (1.00 mg ml^{-1}) and 225 µl of the OPA solution were mixed together and maintained at room temperature for 2 min. Then, the absorbances were recorded at 340 nm using microplate reader (Spark Multimode Microplate Reader, Tecan, Mannedorf, Switzerland). DW and serine (0.9516 meq l^{-1} in DW) were used as blank and reference standard, respec-tively.

2.10. Simulated digestion

To carry out simulated digestion, egg white protein hydrolysates released via hydrolysis with free and immobilized enzymes were dissolved in DW (50.0 mg ml^{-1}). *In vitro* digestion of hydrolysate samples were carried out using an adapted method described by Minekus et al. [19] for oral, gastric and intestinal phases. Solutions were preincubated at 37 °C before using at each stage. For the oral phase, equal volumes of the hydrolysate solutions and the simulated salivary fluid (SSF, 150 U ml^{-1} human salivary α-amylase, pH 7.00) were mixed and incubated at 37 °C under mechanical stirring at 95 rpm for 2 min using in a shaking water bath (Precision SWB 15S, Thermo Scientific, Waltham, MA, USA). Oral bolus from the oral phase was mixed with a simulated gastric fluid (SGF, 4000 U ml^{-1} porcine pepsin, pH 3.00) for the gastric phase. Digestion was carried out in gastric phase at pH 3.00 and incubated at 37 °C with agitation at 95 rpm for 2 h. Finally, in the intestinal digestion, gastric chyme and the simulated intestinal fluid (SIF, 0.50 mg ml^{-1} porcine pancreas, 20 mM bile salts, pH 7.00) were mixed together in equal volumes and pH of the mixture was adjusted to 7.00 and incubated under the same conditions as those explained above for the gastric phase. Digests were stored at -80 °C before freeze-drying and stored at -20 °C until use.

2.11. Dipeptidyl peptidase IV inhibition

Assessment of the inhibitory effects for the hydrolysates obtained from food proteins on DPP-IV is one of the approaches for demonstrating their anti-diabetic properties [20]. A procedure described by Lacroix and Li-Chan [21] was used to investigate DPP-IV inhibitory values of the intestinal digests from egg white protein hydrolysates. First, 10.0 mg ml^{-1} of the intestinal digests were prepared in SIF and then centrifuged at 10,000 ×g for 10 min and the clear

phase was used in the assay. Then, 25 µl of this solution and 25 µl of the substrate solution (1.60 mM glycine-proline-*p*-nitroanilide hydrochloride prepared in 0.10 M tris-HCl buffer, pH 8.00) were mixed well in a microplate and stored at 37 °C for 10 min. In the next step, 50 µl of DPP-IV solution (1852 U ml^{-1}) prepared in 0.1 M tris-HCl buffer (pH 8.00) were added to the microtube and incubated at 37 °C for 60 min. Then, 100 µl of 1.0 M sodium acetate buffer (pH 4.00) were added to stop the enzymatic reaction and the absorbance was recorded at 405 nm. A positive control containing 25 µl SIF, 25 µl substrate solution and 50 µl enzyme solution was also used to assess the maximum activity of the enzyme in absence of the intestinal digests. For the negative control, SIF was used instead of the sample and tris-HCl buffer was used instead of DPP-IV solution. The DPP-IV inhibition was assessed using Equation 3 as follows [21]:

$$\text{DPP-IV inhibition (\%)} = \left\{1 - \frac{(As - Ab)}{(Apc - Anc)}\right\} \times 100 \qquad \text{Eq. 3}$$

Where, A_s, A_b, A_{pc} and A_{nc} represented the absorbances of the sample, blank, positive control and negative control at 405 nm, respectively.

2.12. α-Glucosidase inhibition

A feature to describe the anti-diabetic properties of some compounds is the level of inhibition they can exhibit against α-glucosidase activity. In the current study, α-glucosidase inhibition values for of the intestinal digests of egg white protein hydrolysates were evaluated using a method reported by Mojica and de Mejia [22]. Briefly, 50.0 µl of the clarified intestinal digest solution (10.0 mg ml^{-1} in SIF) and 100 µl of α-glucosidase solution (1 U ml^{-1}) in 0.10 M sodium phosphate buffer (pH 6.90) were mixed well in a microplate and incubated at 25 °C for 10 min. Then, 50 µl of the substrate (5 mM 4-nitrophenyl-α-D-glucopyranoside solution in 0.10 M sodium phosphate buffer, pH 6.90) were added to the mixture. The microplate was incubated at 25 °C for 5 min and the absorbance was recorded at 405 nm. In the current assay, the positive control included 50 µl of SIF, 50 µl of the substrate solution and 100 µl of the enzyme solution. Level of α-glucosidase inhibition (in %) was determined using Equation 3 with the exception that SIF and 0.10 M sodium phosphate buffer (pH 6.90) were respectively used instead of digest and substrate solutions for negative control.

2.13. Statistical analysis

Experiments of the particle size, degree of hydrolysis, enzyme activity, DPP-IV and α-glucosidase inhibition properties were carried out in triplicates. Results were analyzed using one-way analysis of variance (ANOVA), Duncan test and SPSS software v.22 (IBM, New York, NY, USA) applying 95% confidence level.

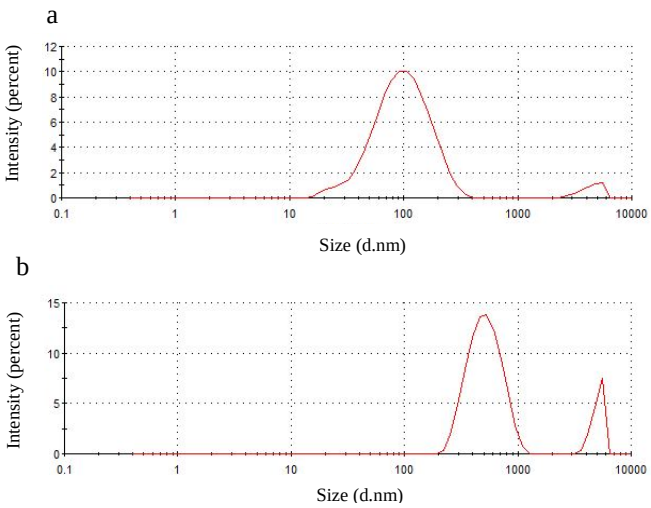

Figure 1. Dynamic light scattering size-distribution profile by intensity for magnetite nanoparticles after 60 min of ultrasonication (a) and cellulose-coated magnetite nanoparticles (b) in aqueous media at 25 °C

3. Results and Discussion

3.1. Characterization of MNPs and CMNP

In this study, MNPs were produced by adding NaOH solution to the aqueous solution of Fe^{+2} and Fe^{+3}. Size distribution profiles for the produced MNPs (60 min of ultrasonication) and CMNP are shown in Figures 1a and 1b, respectively. The particle size for MNPs decreased from 1342 ±23 to 88 nm ±12 by increasing the sonication time from 10 to 60 min. Sonication improved the uniformity of particles and increased PDI from 0.32 ±0.04 at 10 min to 0.62 ±0.02 at 60 min. Ultrasound affects formation of MNPs via cavitation. Formation, growth and collapse of the bubbles in aqueous solution lead to high temperatures and pressures, affecting formation process and properties of MNPs. Collapse of the bubbles creates microscopic agitations leading to a somewhat uniform reaction media [23]. Riva'i et al. [24] reported that an increase in the ultrasonication time from 30 to 120 min resulted in decrease in the sizes of MNPs from 14.6 to 7.3 nm. In fact, MNPs from the current study were larger than the MNPs from the studies of Namdeo and Bajpai [5] and Riva'i et al. [24], which might be due to the agglomeration of MNPs after ultrasonication. After coating MNPs with cellulose, particle size and PDI of CMNP were 710 ±48 and 0.50 ±0.10, respectively. Therefore, particle size increased and uniformity decreased by cellulose coating because there was no control over the coating process of MPNs with cellulose. FTIR spectra of cellulose, CMNP, OCMNP**,** IT and IN are shown in Figure 2. The peak at 3288 cm^{-1} was associated to -OH group on cellulose. The peak appeared at 2891 cm^{-1} was linked to the stretching vibrations of C-H groups in methyl and methylene [25,26].

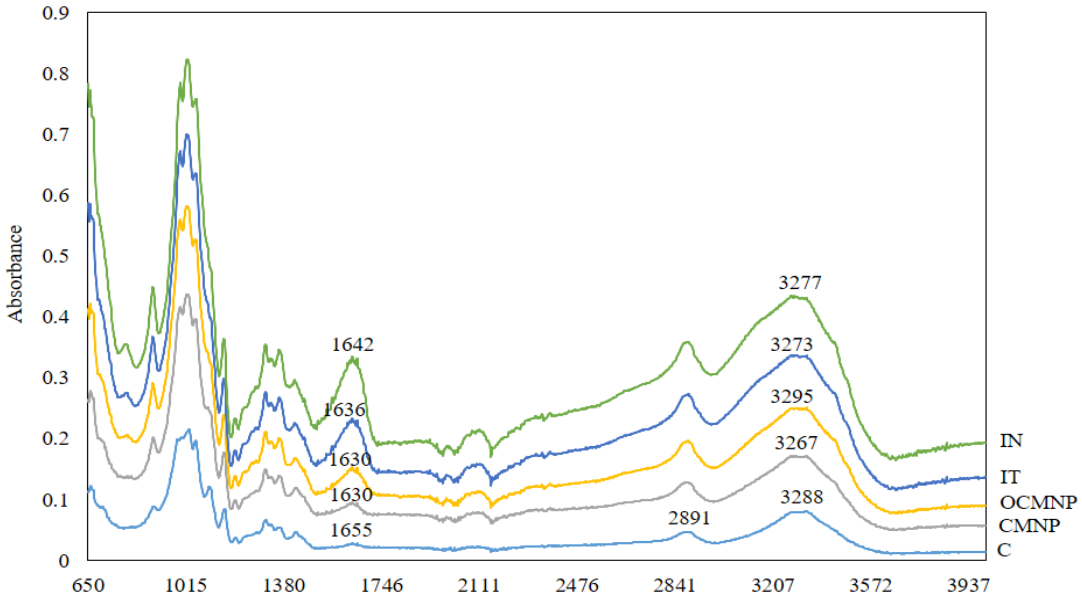

Figure 2. Fourier-transform infrared spectroscopy (FTIR) spectra of cellulose (C), cellulose-coated magnetite nanoparticle (CMNP), oxidized cellulose-coated magnetite nanoparticle (OCMNP), immobilized neutrase (IN) and immobilized thermolysin (IT) at 4000-600 cm^{-1}

The peak of C=O stretching was seen at 1655 cm^{-1}. After oxidation, the absorbance of C=O peak increased from 0.02 in CMNP to 0.05 in OCMNP, which indicates that the number of C=O groups has increased. As a result of peak overlap and low quantity of enzyme in the sample, enzyme peaks and specific peak for the cross-links between the enzyme and cellulose (for C=N bond) were not visible in the spectra of immobilized enzymes. Peaks linked to amide I and N-H in structures of the enzymes appeared at 1600-1700 cm^{-1} and 3300-3500 cm^{-1} [27], respectively. These peaks overlapped with the peaks of C=O and O-H, respectively. Thus, absorbance of these peaks in immobilized enzymes increased at 1636 and 3273 cm^{-1} for IT and 1642 and 3277 cm^{-1} for IN, compared to those for CMNP (1630 and 3267 cm^{-1}) and OCMNP (1630 and 3295 cm^{-1}).

3.2. Enzyme load and immobilization yield

Enzyme load (U mg^{-1}) and immobilization yield (%) were found at 0.03 and 25.4 for neutrase and 67.4 and 31.8 for thermolysin, respectively, which were lower than those previously reported for neutrase [11] and thermolysin [28]. Such differences could be associated with differences in the carrier type, ratio of enzyme to carrier, temperature, pH, contact time between the enzyme and the carrier, cross-linker and coupling agent [29]. Higher values of enzyme load and immobilization yield could possibly be achieved by optimizing the immobilization conditions, which was not carried out in this study.

3.3. Optimization of egg white protein hydrolysis

Degrees of hydrolysis of egg white protein hydrolysates by FN and IN under various experimental conditions in the current study are shown in Tables 1 and 2, respectively. Degrees of hydrolysis included 1.5 ±0.1 to 7.2 ±0.2% for FN and 0.9 ±0.1 to 3.9 ±0.3% for IN. Degrees of hydrolysis for the egg white protein hydrolysates by FT and IT under various experimental conditions of this study are given in Tables 3 and 4, respectively. Degrees of hydrolysis for FT (7.9 ±0.9 to 23.1 ±0.3%) and IT (3.3 ±0.5 to 10.8±0.7%) were higher than those for FN and IN, respectively. Degrees of hydrolysis achieved for FN and IN in the current study are lower than those for whey protein [30], rice dreg protein [31], corn gluten [32] and egg white protein [10]. Values reported for the degrees of hydrolysis could vary depending on the method (e.g., OPA, pH-stat, 2, 4, 6-trinitrobenzene sulfonic acid and TNBS) used for its estimation, type of the substrate, level of enzyme to substrate ratio and hydrolysis conditions (pH; temperature, time). Cho et al. [10] applied TNBS assay after 12 h of hydrolysis for egg white proteins. But, in the current study the OPA assay was used after 3 h of digestion. Even under similar conditions, whey protein, rice dreg protein and corn gluten might be better substrates than egg white proteins for neutrase and therefore include a higher degree of hydrolysis than those for egg white proteins [31].

Table 5 shows ANOVA data of the model predicted for the hydrolysis of egg white proteins by free and immobilized enzymes. Based on the ANOVA, quadratic models

For egg white protein hydrolysis by free and immobilized enzymes were significant ($p < 0.05$). Effects of enzyme concentration and incubation time in FN, IN, FT and IT on the degree of hydrolysis are illustrated in Figure 3 (a, b, c, d). Effects of temperature and time, and temperature and enzyme concentration in FT are respectively shown in Figures 4a and 4b. Results in Figure 3 (a, b, c, d) show that increases in enzyme concentration and incubation time

significantly increased degrees of hydrolysis levels for the two enzymes under free and immobilized conditions. However, based on the ANOVA Table (Table 5), changes in pH and temperature did not have considerable effects on the degree of hydrolysis related to FN, IN and IT. Immobilization of thermolysin resulted in decreased sensitivity to the temperature. Changes in the temperature resulted in significant changes in the degree of hydrolysis for FT (Figures 4a and 4b). However, such changes were not seen for IT. The optimum conditions predicted for the hydrolysis using FN included pH of 7.90, temperature of 43.2 °C, incubation time of 2.7 h and enzyme concentration of 0.05 (w w^{-1}). Slightly different conditions were obtained when using IN for the hydrolysis (pH of 8.00, temperature of47.2 °C, incubation time of 2.8 h and enzyme concentration of 0.05, w w^{-1}). Furthermore, the optimum conditions predicted by Design Expert software for the hydrolysis by FT included pH of 8.00, temperature of 76.8 °C, incubation time of 3 h and enzyme concentration of 0.05 (w w^{-1}). For IT, these conditions included pH of 9.00, temperature of 80 C, incubation time of 3 h and enzyme concentration of 0.05 (w w^{-1}). Within the ranges used in the current study, longer hydrolysis times and higher enzyme concentrations led to increases in degrees of hydrolysis. This suggested that higher levels of the two parameters could be appropriate for the experimental design.

However, the optimum temperature levels for the immobilized forms of neutrase and thermolysin respectively are 4.0 and 3.2 °C higher than those for the corresponding free enzymes. Increase in the optimum temperatures might be due to the presence of covalent bonds between the enzyme and cellulose, which result in a higher structural rigidity of the enzyme [11].

Shifts in the optimum pH from 7.90 to 8.00 for neutrase and thermolysin from 8.00 to 9.00, respectively, occurred due to the immobilization. Similar changes were reported in a study by Rao et al. [33] on the optimum pH values for α-chymotrypsin (8.50-9.00) and trypsin and papain (8.00-8.50) immobilized on tri (4-formyl phenoxy) cyanurate. Enzymatic activities for FN, IN, FT and IT under the optimum conditions included 69.1 ±6.4, 50.1 ±4.2, 5260 ±56 and 4990±69 U mg^{-1}, respectively. Therefore, immobilization decreased enzyme activities by 27.5% for neutrase and 5.1% for thermolysin. According to Sheldon [34], decreases in the enzyme activity could be due to the structural changes in active conformations of the enzymes, restriction in accessibility of the active sites for substrates and decreases in contact areas between the enzymes and substrates due to the steric hindrance of the enzyme as a result of enzyme multipoint attachment to CMNP during the immobilization.

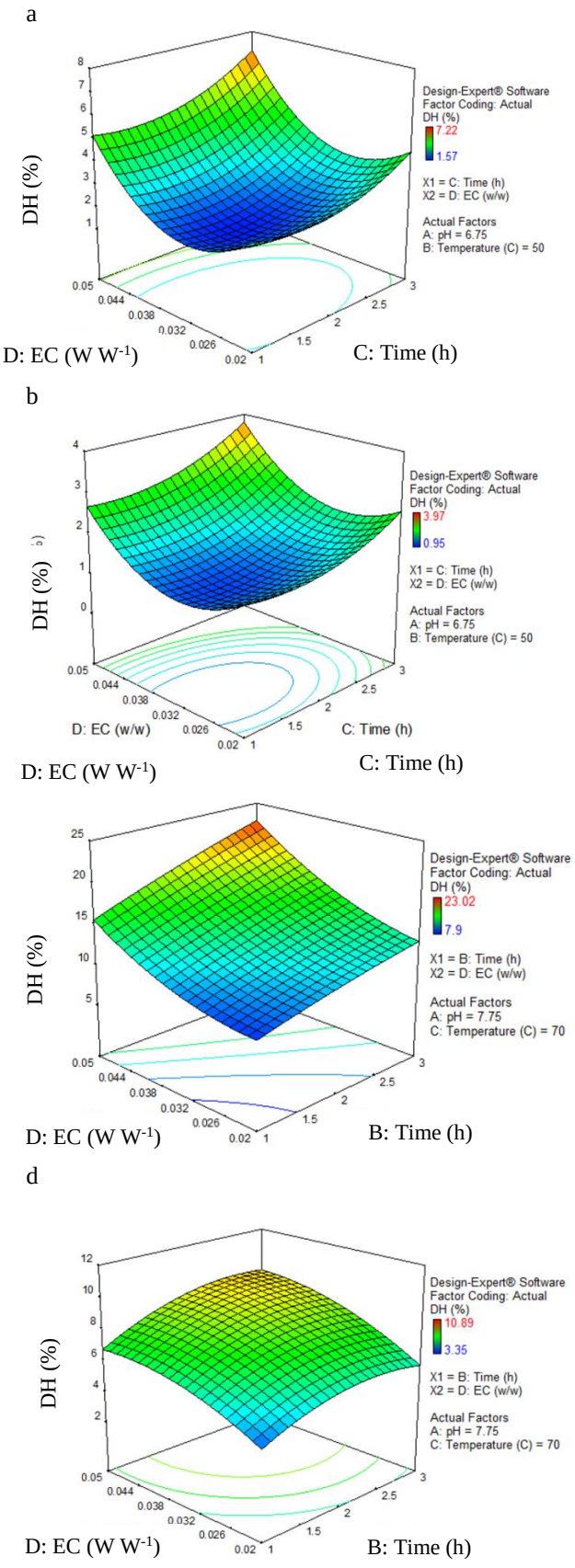

Figure 3. Response surface plots showing effects of enzyme concentration (EC) and time on the degree of hydrolysis (DH) using free neutrase (a), immobilized neutrase (b), free thermolysin (c) and immobilized thermolysin (d)

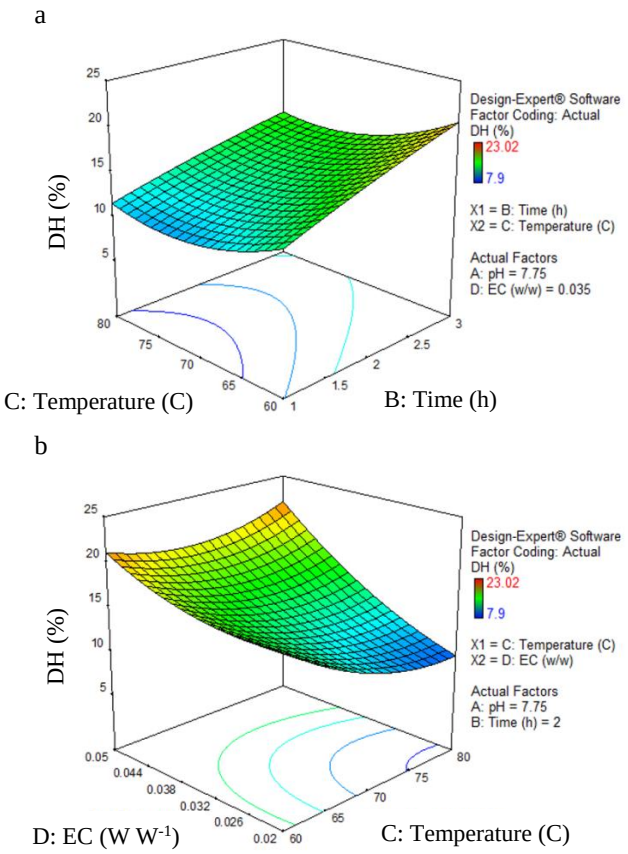

Figure 4. Response surface plots showing effects of temperature and time (a) and enzyme concentration (EC) and temperature (b) on the degree of hydrolysis (DH) using free thermolysin

3.4. Changes in the thermal resistance of free and immobilized enzymes

Briefly, FN, IN, FT and IT were heated at various temperatures for 1 h to assess the heat resistance and effects on the enzymatic activity (Figure 5). Overall, residual activities of both enzymes under free and immobilized conditions decreased at higher temperatures. However, losses in the residual activities for thermolysin were less than those for neutrase since thermolysin is considered a thermostable enzyme. FN was almost fully inactivated at 80 °C (residual activity of 1%). However, a higher residual activity (53%) was observed for IN at this temperature due to the immobilization, which decreased to 41% at 90 °C (Figure 5a). A similar trend was observed for thermolysin. While FT preserved nearly 36% of its residual activity at 90 °C, IT preserved 66% of its activity under the same conditions (Figure 5b). Considering the effects of temperature, the residual activities of FN significantly ($p < 0.05$) decreased due to an increase of 10 °C in the temperature. Therefore, FT was less sensitive due to the nature of the enzyme. For immobilized enzymes, rates of such losses in activities due to the temperature were not as significant as those of the free enzymes (Figures 5a and b). One factor that can be considered to justify the losses in the enzyme activity include unfolding in the enzyme structure [35], which was minimized due to the immobilization. The covalent bonds between the enzyme and the carrier (e.g., CMNP in the current study) could increase the enzyme resistance to such unfolding during thermal stresses [36]. According to Mansfeld et al. [37], effects of immobilization could be higher if cross-links between the enzyme and the carrier occur in a region of the enzyme (such as the region between residues 56 and 69 in thermolysin) that is most susceptible to unfolding. Reportedly, immobilization can prevent autoproteolysis of the enzymes, which is another reason for the inactivation of proteases in reaction media [11].

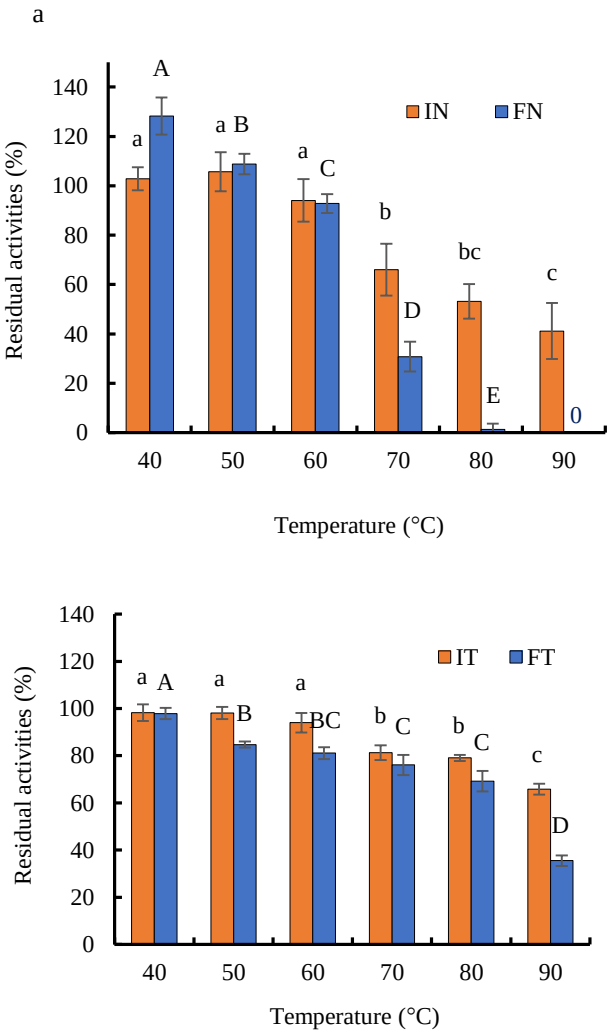

Figure 5. Thermal stability of free (FN) and immobilized (IN) forms of neutrase (a) and free (FT) and immobilized (IT) forms of thermolysin (b). Values in each sample with different letters (upper cases for free and lower cases for immobilized enzymes) are significantly different ($p < 0.05$)

3.5. Reusability levels of the enzymes

Data for the enzyme reusability of neutrase and thermolysin are shown in Figure 6 as changes in residual activities of the immobilized enzymes during five consecutive cycles. Hydrolysis conditions were set based on the optimum conditions for egg white protein hydrolysis. A 34% decrease in the enzyme activity was reported for IN during the first cycle of hydrolysis. Such a decrease for IT was 74%. Changes in residual activities of the enzymes during the subsequent cycles were not as significant as those for the first cycle in a way that no significant differences were observed ($p > 0.05$) among the residual activities during the last four cycles of IT and the last three cycles of IN. The final residual activities of IN and IT were found at 28 and 17%, respectively. Enzyme reusability is an important factor when considering cost effectiveness in food industries [38]. Leaching enzymes are described as one of the most important reasons for decreasing enzyme activities during the consecutive cycles [39].

Although enzyme multipoint attachment to CMNP is one of the major factors in decreasing the enzyme original activity, it can help decrease the leaching effects during the hydrolysis [34]. Losses in the enzyme activities due to the frequent contacts of substrates and active sites over the consecutive stages of use have been reported [39]. Similar results have been reported on the loss of enzyme activity (immobilized alcalase [15] and xylanase [39]) due to the leaching of enzymes during the consecutive cycles.

Table 5. Analysis of variance analysis of degree of hydrolysis affected by time, temperature, pH and enzyme concentration during the optimization of egg white protein hydrolysis by free and immobilized enzymes

Source	Sum of squares	Mean square	F value	P value	
Free neutrase					
Model	67.74	4.84	6.48	0.0006	Significant
pH	0.91	0.91	1.22	0.2876	Not significant
Temperature	0.094	0.094	0.13	0.7286	Not significant
Time	7.84	7.84	10.49	0.0059	Significant
Enzyme concentration	16.22	16.22	21.70	0.0004	Significant
Residual	10.46	0.75			
Lack of fit	9.68	0.97	4.97	0.0681	Not significant
Immobilized neutrase					
Model	15.24	1.09	4.19	0.0056	Significant
pH	0.099	0.099	0.38	0.5478	Not significant
Temperature	0.24	0.24	0.92	0.3526	Not significant
Time	2.82	2.82	10.85	0.0053	Significant
Enzyme concentration	3.54	3.54	13.64	0.0024	Significant
Residual	3.64	0.26			
Lack of fit	3.20	0.32	2.93	0.1557	Not significant
Free thermolysin					
Model	464.31	33.16	6.12	0.0008	Significant
pH	12.26	12.26	2.26	0.1549	Not significant
Temperature	94.98	94.98	17.51	0.0009	Significant
Time	33.73	33.73	6.22	0.0258	Significant
Enzyme concentration	204.77	204.77	37.76	0.0001	Significant
Residual	75.93	5.42			
Lack of fit	43.34	4.33	0.53	0.8093	Not significant
Immobilized thermolysin					
Model	71.14	5.08	2.97	0.0252	Significant
pH	5.41	5.41	3.16	0.0970	Not significant
Temperature	0.26	0.26	0.15	0.7026	Not significant
Time	11.31	11.31	6.62	0.0221	Significant
Enzyme concentration	26.26	26.26	15.36	0.0015	Significant
Residual	23.93	1.71			
Lack of fit	16.47	1.65	0.88	0.6048	Not significant

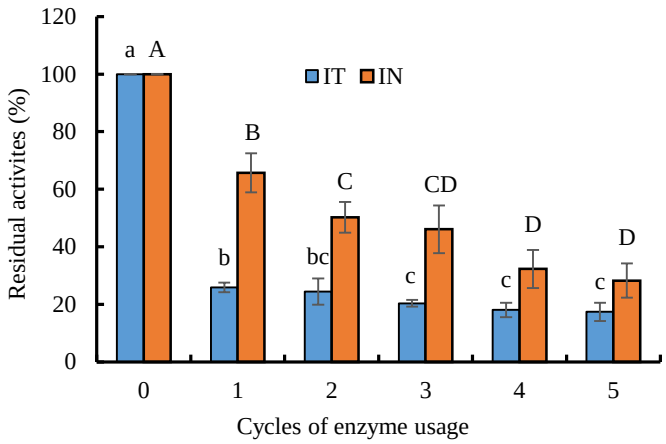

Figure 6. Reusability of immobilized neutrase (IN) and immobilized thermolysin (IT). Values in each sample with different letters (upper cases for IN and lower cases for IT) are significantly different ($p < 0.05$)

3.6. Changes in the inhibitory levels of the digests against DPP-IV and α-glucosidase

Figure 7 demonstrates changes in the inhibitory effects of egg white protein digests against DPP-IV and α-glucosidase activities. For DPP-IV, the highest and the lowest inhibitions of 37 and 4.4% were achieved with digests obtained by FT and IN, respectively. Inhibitory activities of the digests achieved by thermolysin in both free and immobilized forms were significantly higher than those achieved by neutrase ($p < 0.05$). Respectively, 14 and 8.8% decreases in the inhibitory activities of the digests from the hydrolysates of neutrase and thermolysin were observed against DPP-IV, respectively equivalent to 67 and 65% losses in inhibitory effects of the enzymes due to the immobilization. Zambrowicz et al. [8] showed that DPP-IV inhibitory activity (IC_{50}) for the peptides derived from egg yolk proteins by pepsin ranged 0.222-1.402 mg ml^{-1}. Therefore, DPP-IV inhibitory activities of the digests from the hydrolysates using FN, IN, FT and IT were lower than those for the peptides from egg yolk proteins using pepsin [8]. For α-glucosidase, free enzymes resulted in digests with significantly higher inhibitory activities than those achieved by the immobilized enzymes ($p < 0.05$), similar to DPP-IV. The highest and the lowest inhibitions included 35 and 9.8% for FT and IN-derived digests, respectively (Figure 7). Decreases of 20 and 23% in inhibitory activities of the digests from neutrase and thermolysin-derived hydrolysates against α-glucosidase (respectively equivalent to 75 and 23% losses in the original activity) were observed as a result of immobilization. Yu et al. [9] identified several alcalase-derived hydrolysates from egg white proteins with various activities against α-glucosidase. The RVPSLM (arginine-valine-proline-serine-leucine-methionine) and TPSPR (threonine-proline-serine-proline-arginine) with IC_{50} values of respectively 23 and 40 μmol L^{-1} were reported as the most potent peptides. The KLPGF (lysine-leucine-proline-glycine-phenylalanine) is a peptide derived from albumin with the potential to inhibit α-glucosidase with an IC_{50} value of 60 μmol l^{-1} [40]. Immobilization of the two enzymes resulted in hydrolysates; for which, digests showed much lower inhibitory effects against DPP-IV and α-glucosidase, compared to free forms of the enzymes (Figure 7). Studies have shown that hydrogen bonds, hydrophobic forces and polar interactions between the peptides and the active sites of DPP-IV and α-glucosidase include positive effects in decreasing activities of these enzymes [22]. Smaller peptides likely have better DPP-IV and α-glucosidase inhibitory activities than those of the larger peptides, possibly due to a better accessibility of the R-groups for binding to active sites. Mirzapour et al. [41] reported that the smaller peptides derived from wild almond proteins have better inhibitory effects against angiotensin-converting enzyme, compared to those of the larger peptides. Difference in the inhibitory potentials of FN and IN-derived hydrolysates with those of FT and IT-derived hydrolysates could be due to the differences in enzyme specificity, reactivity and substrate affinity [42].

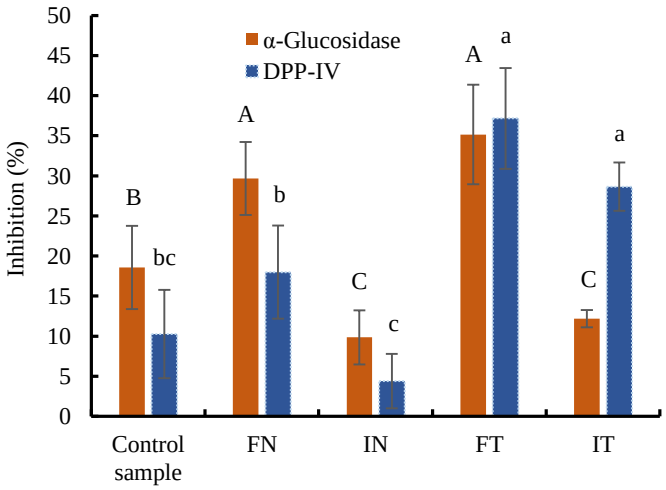

Figure 7. Dipeptidyl peptidase IV (DPP-IV) and α-glucosidase inhibition for intestinal digests of control sample and free neutrase (FN), immobilized neutrase (IN), free thermolysin (FT) and immobilized thermolysin (IT) hydrolysates. Values in each column with different letters (upper cases for α-glucosidase and lower cases for DPP-IV inhibition) are significantly different ($p < 0.05$)

4. Conclusion

In general, hydrolysis of egg white proteins were investigated to produce peptides with anti-diabetic properties after gastrointestinal digestion. Digests, which were produced after simulated digestion of the egg white protein hydrolysates prepared with the free enzymes showed

better inhibitory activities against DPP-IV and α-glucosidase than that for digests released from hydrolysates produced by their immobilized forms. Immobilization of neutrase and thermolysin were assessed as an approach to reduce the costs of enzymatic reactions and also as a means to separate the enzyme from the final products due to safety concerns. Considering the enzyme activities for the five extended cycles of the immobilized enzymes, increases in the overall usability of 223 and 106% were obtained for neutrase and thermolysin, respectively, due to the immobilization. In addition, immobilization resulted in improvements in thermal resistances of the enzymes and changed the optimum pH and temperature of egg white protein hydrolysis. It is recommended to investigate hydrolysates achieved by free and immobilized forms of neutrase and thermolysin in future studies for their functional properties. Therefore, egg white protein hydrolysates obtained by free and immobilized neutrase and thermolysin can be used in formulations of functional foods and nutraceuticals with multifunctional activities.

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6. Conflict of Interest

The authors declare no conflict of interest.

References

1. Mehta J, Bhardwaj N, Bhardwaj SK, Kim K-H, Deep A. Recent advances in enzyme immobilization techniques: Metal-organic frameworks as novel substrates. Coordin Chem Rev. 2016; 322: 30-40. doi: 10.1016/j.ccr.2016.05.007

2. Vaghari H, Jafarizadeh-Malmiri H, Mohammadlou M, Berenjian A, Anarjan N, Jafari N, Nasiri S. Application of magnetic nanoparticles in smart enzyme immobilization. Biotech Lett. 2016; 38(2): 223-233. doi: 10.1007/s10529-015-1977-z

3. Fortes CC, Daniel-da-Silva AL, Xavier AM, Tavares AP. Optimization of enzyme immobilization on functionalized magnetic nanoparticles for laccase biocatalytic reactions. Chem Eng Process. 2017; 117: 1-8. doi: 10.1016/j.cep.2017.03.009

4. Kalkan NA, Aksoy S, Aksoy EA, Hasirci N. Preparation of chitosan-coated magnetite nanoparticles and application for immobilization of laccase. J Appl Polym Sci. 2012;123(2):707-716. doi: 10.1002/app.34504

5. Namdeo M, Bajpai S. Immobilization of α-amylase onto cellulose-coated magnetite (CCM) nanoparticles and preliminary starch degradation study. J Mol Catal B-Enzym. 2009; 59(1-3): 134-139. doi: 10.1016/j.molcatb.2009.02.005

6. Gazme B, Udenigwe CC, Rezaei K. Effect of enzyme immobilization and *in Vitro* digestion on the immune-reactivity and sequence of IgE epitopes in egg white proteins. Food Funct. 2020; 11: 6632-6642 doi: 10.1039/D0FO00938E

7. Chen C, Chi Y-J, Zhao M-Y, Lv L. Purification and identification of antioxidant peptides from egg white protein hydrolysate. Amino Acids 2012; 43(1): 457-466. doi: 10.1007/s00726-011-1102-0

8. Zambrowicz A, Pokora M, Setner B, Dąbrowska A, Szołtysik M, Babij K, Szewczuk Z, Trziszka T, Lubec G, Chrzanowska J. Multifunctional peptides derived from an egg yolk protein hydrolysate: Isolation and characterization. Amino Acids 2015; 47(2): 369-380. doi: 10.1007/s00726-014-1869-x

9. Yu Z, Yin Y, Zhao W, Yu Y, Liu B, Liu J, Chen F. Novel peptides derived from egg white protein inhibiting alpha-glucosidase. Food Chem. 2011; 129(4): 1376-1382. doi: 10.1016/j.foodchem.2011.05.067

10. Cho D-Y, Jo K, Cho SY, Kim JM, Lim K, Suh HJ, Oh S. Antioxidant effect and functional properties of hydrolysates derived from egg-white protein. Korean Soc Food Sci An Resour. 2014; 34(3): 362-371. doi: 10.5851/kosfa.2014.34.3.362

11. Ortega N, Perez-Mateos M, Pilar MaC, Busto MaD. Neutrase immobilization on alginate-glutaraldehyde beads by covalent attachment. J Agr Food Chem. 2008; 57(1): 109-115. doi: 10.1021/jf8015738

12. Chen F, Zhang F, Du F, Wang A, Gao W, Wang Q, Yin X, Xie T. A novel and efficient method for the immobilization of thermolysin using sodium chloride salting-in and consecutive microwave irradiation. Bioresource Technol. 2012; 115: 158-163. doi: 10.1016/j.biortech.2011.11.059

13. Rocha-Martin J, Fernandez-Lorente G, Guisan JM. Sequential hydrolysis of commercial casein hydrolysate by immobilized trypsin and thermolysin to produce bioactive phosphopeptides. Biocatal Biotransfor. 2018; 36(2): 159-171. doi: 10.1080/10242422.2017.1308499

14. Jain TK, Morales MA, Sahoo SK, Leslie-Pelecky DL, Labhasetwar V. Iron oxide nanoparticles for sustained delivery of anticancer agents. Mol Pharm. 2005; 2(3): 194-205. doi: 10.1021/mp0500014

15. Yang A, Long C, Xia J, Tong P, Cheng Y, Wang Y, Chen H. Enzymatic characterisation of the immobilised Alcalase to hydrolyse egg white protein for potential allergenicity reduction. J Sci Food Agr. 2017; 97(1): 199-206. doi: 10.1002/jsfa.7712

16. Lin S, Guo Y, Liu J, You Q, Yin Y, Cheng S. Optimized enzymatic hydrolysis and pulsed electric field treatment for production of antioxidant peptides from egg white protein. Afr J Biotechnol. 2011; 10(55):11648-11657.

17. del Mar Contreras M, Hernandez-Ledesma B, Amigo L, Martin-Alvarez PJ, Recio I. Production of antioxidant

hydrolyzates from a whey protein concentrate with thermolysin: Optimization by response surface methodology. LWT Food Sci Technol. 2011; 44(1): 9-15.
doi: 10.1016/j.lwt.2010.06.017

18. Nielsen P, Petersen D, Dambmann C. Improved method for determining food protein degree of hydrolysis. J Food Sci. 2001; 66(5): 642-646.
doi: 10.1111/j.1365-2621.2001.tb04614.x

19. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, et al. A standardised static *in Vitro* digestion method suitable for food-an international consensus. Food Funct. 2014; 5(6): 1113-1124.
doi: 10.1039/C3FO60702J

20. Kehinde BA, Sharma P. recently isolated antidiabetic hydrolysates and peptides from multiple food sources: A review. Crit Rev Food Sci Nutr. 2020; 60(2): 322-340.
doi: 10.1080/10408398.2018.1528206

21. Lacroix IM, Li-Chan EC. Dipeptidyl peptidase-IV inhibitory activity of dairy protein hydrolysates. Int Dairy J. 2012; 25(2): 97-102.
doi: 10.1016/j.idairyj.2012.01.003

22. Mojica L, de Mejia EG. Optimization of enzymatic production of anti-diabetic peptides from black bean (*Phaseolus vulgaris L.*) proteins, their characterization and biological potential. Food Funct. 2016; 7(2): 713-727.
doi: 10.1039/C5FO01204J

23. Dang F, Kamada K, Enomoto N, Hojo J, Enpuku K. Sonochemical synthesis of the magnetite nanoparticles in aqueous solution. J Ceram Soc Japan. 2007; 115(1348): 867-872.
doi: 10.2109/jcersj2.115.867

24. Rivai I, Wulandari IO, Sulistyarti H, Sabarudin A. Ex-situ synthesis of polyvinyl alcohol (PVA)-coated Fe3O4 nanoparticles by coprecipitation-ultrasonication method. IOP Conference Series: Mat Sci Eng. 2018: 299:1-8.

25. Jia N, Li S-M, Ma M-G, Zhu J-F, Sun R-C. Synthesis and characterization of cellulose-silica composite fiber in ethanol/water mixed solvents. Bioresources 2011; 6(2): 1186-1195.

26. Kassanov B, Wang J, Fu Y, Chang J. Cellulose enzymatic saccharification and preparation of 5-hydroxymethylfurfural based on bamboo hydrolysis residue separation in ionic liquids. RSC Adv. 2017; 7(49): 30755-30762.
doi: 10.1039/C7RA05020H

27. Jensen JK, Rubio JM, Engelsen SB, van den Berg F. Protein residual fouling identification on UF membranes using ATR-FT-IR and multivariate curve resolution. Chemometr. Intell Lab. 2015; 144: 39-47.
doi: 10.1016/j.chemolab.2015.03.006

28. Xin Bj, Si Sf, Xing Gw. Protease immobilization on γ-Fe2O3/Fe3O4 magnetic nanoparticles for the synthesis of oligopeptides in organic solvents. Chem Asian J. 2010; 5(6): 1389-1394.
doi: 10.1002/asia.200900696

29. Dal Magro L, Kornecki JF, Klein MP, Rodrigues RC, Fernandez-Lafuente R. Optimized immobilization of polygalacturonase from *Aspergillus niger* following different protocols: Improved stability and activity under drastic conditions. Int J Biol Macromol. 2019; 138: 234-243.
doi: 10.1016/j.ijbiomac.2019.07.092

30. Ou K, Liu Y, Zhang L, Yang X, Huang Z, Nout MR, Liang J. Effect of neutrase, alcalase, and papain hydrolysis of whey protein concentrates on iron uptake by Caco-2 cells. J Agric Food Chem. 2010; 58(8): 4894-4900.
doi: 10.1021/jf100055y

31. Zhao Q, Xiong H, Selomulya C, Chen XD, Zhong H, Wang S, Sun W, Zhou Q. Enzymatic hydrolysis of rice dreg protein: effects of enzyme type on the functional properties and antioxidant activities of recovered proteins. Food Chem. 2012; 134(3): 1360-1367.
doi: 10.1016/j.foodchem.2012.03.033

32. Kılıc Apar D, Ozbek B. Hydrolysis and solubilization of corn gluten by neutrase. J Chem Technol Biotech. 2007; 82(12): 1107-1114.
doi: 10.1002/jctb.1740

33. Rao RS, Borkar PS, Khobragade C, Sagar A. Enzymatic activities of proteases immobilized on tri (4-formyl phenoxy) cyanurate. Enzyme Microb Technol. 2006; 39(4): 958-962.
doi: 10.1016/j.enzmictec.2006.03.020

34. Sheldon RA. Enzyme immobilization: The quest for optimum performance. Adv Synth Catal. 2007; 349(8-9): 1289-1307.
doi: 10.1002/adsc.200700082

35. Saqib AA, Siddiqui KS. How to calculate thermostability of enzymes using a simple approach. Biochem Mol Biol Educ. 2018; 46(4): 398-402.
doi: 10.1002/bmb.21127

36. Zhang Y, Tang Z, Wang J, Wu H, Lin C-T, Lin Y. Apoferritin nanoparticle: A novel and biocompatible carrier for enzyme immobilization with enhanced activity and stability. J Mater Chem. 2011; 21(43): 17468-17475.
doi: 10.1039/C1JM11598G

37. Mansfeld J, Vriend G, Van den Burg B, Eijsink VG, Ulbrich-Hofmann R. Probing the unfolding region in a thermolysin-like protease by site-specific immobilization. Biochem. 1999; 38(26): 8240-8245.
doi: 10.1021/bi990008p

38. Wu X-c, Zhang Y, Wu C-Y, Wu H-x. Preparation and characterization of magnetic Fe_3O_4/CRGO nanocomposites for enzyme immobilization. T Nonferr Metal Soc. 2012; 22: s162-s168.
doi: 10.1016/S1003-6326(12)61703-8

39. Pal A, Khanum F. Covalent immobilization of xylanase on glutaraldehyde activated alginate beads using response surface methodology: Characterization of immobilized enzyme. Process Biochem. 2011; 46(6): 1315-1322.
doi: 10.1016/j.procbio.2011.02.024

40. Yu Z, Yin Y, Zhao W, Liu J, Chen F. Anti-diabetic activity peptides from albumin against α-glucosidase and α-amylase. Food. Chem. 2012; 135(3): 2078-2085.
doi: 10.1016/j.foodchem.2012.06.088

41. Mirzapour M, Rezaei K, Sentandreu MA. Identification of potent ACE inhibitory peptides from wild almond proteins. J Food Sci. 2017; 82(10): 2421-2431.
doi: 10.1111/1750-3841.13840

42. Mudgil P, Kamal H, Yuen GC, Maqsood S. Characterization and identification of novel antidiabetic and anti-obesity peptides from camel milk protein hydrolysates. Food Chem. 2018; 259: 46-54.
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خواص ضد دیابتی هیدرولیزات‌های به‌دست آمده از پروتئین‌های سفیده تخم مرغ با استفاده از آنزیم-های تثبیت‌شده به‌دنبال هضم معده‌ای روده‌ای برون‌تنی

بهزاد گزمه[1]، چیبویکه سی آدنیگو[2و3*]، کرامت الله رضائی[1*]

۱- گروه علوم و مهندسی صنایع غذائی، دانشگاه تهران، ۷۷۸۷۱-۳۱۵۸۷ کرج، ایران.
۲- گروه تغذیه، دانشکده بهداشت، دانشگاه اوتاوا، جاده ۴۱۵ اسمیت، K1H 8L1، اوتاوا، اونتاریا، کانادا.
۳- گروه شیمی و علوم زیستی، دانشگاه اوتاوا، K1N 6N5، اوتاوا، اونتاریا، کانادا.

چکیده

سابقه و هدف: آبکافت پروتئین‌های مواد غذایی به کمک آنزیم راهکاری موثر برای تولید پپتیدهایی با خواص زیست فعال می‌باشد. به علاوه، تثبیت آنزیم روشی برای بازیابی آنزیم‌ها برای استفاده‌های بعدی به شمار می‌رود. هدف مطالعه حاضر، بررسی اثرات تثبیت بر خواص آنزیم های نوترئاز و ترمولیزین و همچنین خواص ضد دیابتی ترکیبات حاصل از هضم شبیه‌سازی شده هیدرولیزات‌های پروتئین‌های سفیده تخم مرغ در شرایط برون تنی می باشد.

مواد و روش ها: نوترئاز و ترمولیزین بر روی نانوذرات آهن پوشش داده شده با سلولز ثبیت شدند. سپس، فعالیت آنزیم، پایداری حرارتی، قابلیت استفاده مجدد و شرایط بهینه آبکافت پروتئین‌های سفیده تخم مرغ مورد بررسی قرار گرفتند. هیدرولیزات‌های پروتئین‌های سفیده تخم مرغ در شرایط برون تنی هضم و فعالیت مهارکنندگی ترکیبات حاصل از هضم روده‌ای در برابر دی‌پپتیدیل‌پپتیداز IV (DPP-IV) و آلفا-گلوکوزیداز بررسی شد.

یافته‌ها و نتیجه‌گیری: تثبیت آنزیم ها باعث افزایش پایداری حرارتی آنها گردید و ضمنا دمای بهینه برای هیدرولیز پروتئین‌های سفیده تخم مرغ برای نوترئاز و ترمولیزین به‌ترتیب تا ۴/۰ و ۳/۲ درجه سلسیوس افزایش یافت. ترکیبات حاصل از هضم هیدرولیزات‌های به دست آمده از نوترئاز آزاد به طور موثری DPP-IV و آلفا گلوکوزیداز را به ترتیب تا ۱۷/۹ و ۲۹/۷ درصد مهار کرد. این ارقام برای هیدرولیزات‌های رها شده توسط ترمولیزین بیشتر بود (به ترتیب ۳۷/۲ و ۳۵/۱ درصد). تثبیت آنزیم موجب کاهش ۴/۴ درصدی در فعالیت مهارکنندگی ترکیبات حاصل از هضم هیدرولیزات-های به دست آمده از نوترئاز و کاهش ۲۸/۶ درصدی در فعالیت مهارکنندگی ترکیبات حاصل از هضم هیدرولیزات‌های به دست آمده از ترمولیزین بر علیه DPP-IV شد. تثبیت آنزیم باعث ۹/۸ درصد کاهش در فعالیت نوترئاز و ۱۲/۲ درصد کاهش در فعالیت ترمولیزین در مهار آلفا گلوکوزیداز توسط ترکیبات حاصل از هضم هیدرولیزات‌ها شد. بر اساس نتایج تحقیق حاضر، هیدرولیزات‌های پروتئین‌های سفیده تخم مرغ حاصل از نوترئاز و ترمولیزین آزاد و تثبیت شده می‌توانند در فرموله کردن غذاهای فراسودمند[1] و غذاداروهایی[2] با خواص چندگانه مورد استفاده قرار گیرند.

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- نانوذرات آهن
- نوترئاز
- ترمولیزین

***نویسنده مسئول**

کرامت الله رضائی[1]،
گروه علوم و مهندسی صنایع غذائی، دانشگاه تهران، ۷۷۸۷۱-۳۱۵۸۷ کرج، ایران.
دورنگار: ۳۲۲۴۸۸۰۴-۲۶-۹۸+
پست الکترونیک:
krezaee@ut.ac.ir

چیبویکه سی آدنیگو[2]
گروه تغذیه، دانشکده بهداشت، دانشگاه اوتاوا، جاده ۴۱۵ اسمیت، K1H 8L1، اوتاوا، اونتاریا، کانادا.
گروه شیمی و علوم زیستی، دانشگاه اوتاوا،K1N 6N5، اوتاوا، اونتاریا، کانادا.
تلفن: ۵۸۰۰-۵۶۲-۶۱۳ - ۱+
پست الکترونیک:
cudenigw@uottawa.ca

[1] Functional foods
[2] Nutraceuticals