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Full Research Paper

Improvement of antioxidant and emulsifying properties of *Cajanus cajan*'s protein hydrolysate by glycosylation through maillard reaction

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Abstract

The aim of the present study was to use the Maillard reaction as a means to glycosylate protein hydrolysates obtained from *Cajanus cajan* and to evaluate the effects of this chemical modification on antioxidant and emulsifying properties. Chemical properties, amino acid composition, and molecular weight distribution of the hydrolysates were evaluated. Glucose, galactose, and maltodextrin in the ratios of 1:2, 1:1, and 2:1 (hydrolysate: sugar, dry weight basis) were used for glycosylation. Antioxidant activity was evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and nitric oxide scavenging. The sonication technique was used to prepare the (oil/water) emulsions. The droplet size distribution and zeta potential of the emulsions were measured during 4 days of storage. Results showed that glycosylation by glucose in the ratio of 2:1 increased DPPH scavenging activity from 37.96% to 85.53% and nitric oxide inhibition activity from 14.50% to 54.83%. Although glycosylation improved emulsifying stability of glycosylated hydrolysates compared to non-glycosylated hydrolysates, no significant difference was observed between the three examined sugars.

Key words: Glycosylation, Maillard reaction, Protein hydrolysate, Antioxidant, Emulsion

Introduction

Cajanus cajan or pigeon pea is a legume from the family *Papilionoideae* is mostly cultivated in tropical and subtropical countries. This legume is known to have a good nutritional quality (amino acid profile) and a high amount of hydrophobic amino acids (Saxena *et al*., 2010).

Consumer demands for plant-based ingredients have led the food and cosmetic industries to focus on the attempts towards the replacement of animal proteins with plant protein (Kutzli *et al.*, 2020). Although the quality of the plant proteins is lower than animal proteins, enzymatic hydrolysis can improve some of the biological and functional

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properties of the plant proteins (Wouters *et al*., 2016). On the other hand, small peptides produced due to the hydrolysis process can cause some new problems in food systems; such as instability or inducing allergenicity (Wouters *et al.*, 2016). In this regard, protein hydrolysate modification might be a proper means to improve the quality (e.g. antioxidants and emulsifying properties), and decreasing the possibility of making allergens.

Glycosylation is a chemical modification and a process of adding sugar to a protein by enzymatic or chemical reactions such as the Maillard reaction. It has been shown that glycosylation has a positive effect on antioxidant (Hou *et al*., 2013; Wang *et al*., 2020), biological (Jeewanthi *et al*., 2015), functional properties (Jongh and Broersen, 2012; Wang *et al*., 2020), and the flavor (Hong *et al*., 2016) of protein hydrolysates. However, Plant-based protein and peptide glycosylation are not fully studied in the literature (Kutzli *et al.*, 2020).

Maillard reaction is defined as a "series of non-enzymatic chemical reactions between carbonyl compounds (mainly carbohydrates) and amino compounds from natural resources". Maillard reaction is a natural and non-toxic reaction that is mostly used to improve biological and functional properties of proteins and protein hydrolysates (Karnjanapratum *et al*., 2018; Li *et al*., 2016; Lie *et al*., 2014; Liu *et al*., 2020; Zhang *et al.*, 2018). This reaction has a complicated effect on the protein surface, protein structure, and surface hydrophobicity. The type of sugar and amino acid and the reaction conditions are important factors affecting the biological and functional properties of final product (Anzani *et al*., 2019; Kutzli *et al*., 2020; Wang *et al.*, 2020).

To the best of our knowledge, the functionality of *Cajanus cajan*'s protein hydrolysate as a food emulsifier, and the modification of this protein hydrolysate has not been studied.

The aim of the present study was to use the Maillard reaction as a means to glycosylate *Cajanus cajan*'s protein hydrolysates and to evaluate the effect of this chemical modification on antioxidant and emulsifying properties.

Materials and methods

Cajanus cajan was purchased from a farm in Azarshahr, East Azerbaijan Province, Iran. Alcalase enzyme (from *Bacillus licheniformis* with proteolytic activity of 2.4 (AU/ml) was purchased from Sigma-Aldrich (Spain). Commercial cod liver oil was kindly provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway) and stored at -40°C until use. The fatty acid composition (major fatty acids only) of the fish oil used was C16:0, 9.5%; C16:1, 8.7%; C18:1, 16.3%; C20:1, 12.6%; C20:5, 9.2% and C22:6, 11.4%. The tocopherol content of the fish oil was: alpha-tocopherol, 200± 3 µg/g oil; beta-tocopherol, 5± 1 µg/g oil; gamma-tocopherol, 96± 3 µg/g oil and delta-tocopherol, 47± 1 µg/g oil. The peroxide value (PV) of the fish oil used was 0.38± 0.04 meq/kg oil. All other chemicals and solvents used were of analytical grade.

Protein extraction

Protein extraction was performed according to Akintayo et al. (1999) with some modifications. A suspension (1:10 w/v; pH 11) of *Cajanus cajan*'s sieved flour in distilled water was prepared. The suspension was stirred for 1 h at room temperature and stored at 4°C overnight, then centrifuged for 20 min at 6000 rpm at 4°C. The supernatant was collected and its pH was adjusted to 3 (by 0.1 N HCl) and centrifuged at the same conditions as the previous step. The pellet was freeze-dried (FDB-5503, Operon, South Korea) and stored at -20°C until use.

Protein hydrolysis

Protein concentrate (freeze-dried pellet from the previous section) solution (5% w/v) was hydrolyzed using Alcalase enzyme at pH 8 (adjusted by 0.1 N NaOH) by enzyme concentration of 2.47%. The reaction took place in a shaker incubator (48.35°C, 3.26 h, 200 rpm), on the next step, the solution was transferred to 85°C water bath for 10 min to stop the reaction. The solution was centrifuged

for 20 min at 6000 rpm at room temperature. The supernatant was freeze-dried and stored at -4°C for further experiments (Meshginfar *et al*., 2014).

Chemical analysis

The moisture content was measured by drying the samples at 105°C to reach the constant weight (AOAC, 2005). The protein content was measured by the Kjeldahl method and using a protein factor of 6.25 (AOAC, 2005). Ash content was measured by pre-drying the sample and using a 600°C furnace until obtaining white ash after 24 h (AOAC, 2005). The lipid content of the samples was measured by Soxhlet extraction (AOAC, 2005).

Amino acid composition of protein concentrate and hydrolysate

Cajanus protein concentrate and protein hydrolysate were completely hydrolyzed and derivatized by EZ: faast Amino Acid Kit (Phenomenex, Torrance, Ca, USA) with 6 M HCL for 1 h at 110°C. Then, the hydrolyzed samples were neutralized and purified by solid-phase extraction sorbent tip and derivatized. The samples were injected into an Agilent HPLC 1100 (Santa Clara, CA, USA) coupled to Agilent Ion Trap MS. Separation was done at 35°C on 250× 3.0 mm Zebron ZB-AAA column (Phenomenex, Torrance, CA, USA) using a gradient of 68- 83% 10 mM ammonium formate in methanol and 10 mM ammonium formate in water by 0.5 ml/min flow rate. An external standard mixture was used to identify the compounds (García-Moreno *et al*., 2016).

Chemical score of protein concentrate and hydrolysate

The chemical score is based on the essential amino acid profile (g 100 g^{-1}) in a standard protein that is described by FAO/WHO and is calculated as follows (Raftani Amiri *et al*., 2016):

$$Chemical\ score = \frac{Essential\ amino\ acid\ in\ test\ protein}{Essential\ amino\ acid\ in\ standard\ protein} \quad (1)$$

Molecular weight distribution of hydrolysate

To measure the molecular weight distribution of the hydrolysate, a size exclusion chromatography (SEC) with FPLC ÄKTA system (Amersham Biosciences, Uppsala, Sweden) and Superdex Peptide 10/300GL column (GE Healthcare, Uppsala, Sweden) was used. The temperature of the column was 25°C and the mobile phase was ammonium acetate buffer at pH 8. 100 µl of the sample (5 mg/ml, filtered by 0.2 µm filter) was injected and eluted at flow rate of 0.25 ml/min, and the absorbance was measured at 280 nm. Cytochrome c (12.3 kDa), aprotinin (6.5 kDa), Gly_3 (189 Da), and Gly (75 Da) were used as molecular weight marker (Gringer *et al*., 2016).

Glycosylation

Freeze-dried protein hydrolysates and one of the dry sugars glucose, galactose, or maltodextrin (DE 20) were mixed (1:2, 1:1, and 2:1, dry weight base), distilled water was added to the dry mixture, and the pH was adjusted to 7.5 (by adding 0.1 N NaOH or HCl). The final concentration of the hydrolysate in each solution was 20 mg/ml. One solution was prepared without sugar as control. The ten prepared solutions were shaken for 2 h at room temperature and stored at 4°C overnight without shaking to complete the hydration process. On the next day, the solutions were incubated in a 90°C water bath for 8 h. Finally, the solutions were cooled down with cold water and used for antioxidant assays (Mulcahy et al., 2016).

DPPH scavenging activity

DPPH solution (0.1 ml, 0.1 mM in 95% ethanol) was added to 0.1 ml of each sample solution (from the previous step) and incubated in the dark at room temperature for 60 min. Any particle in solutions was removed by centrifugation (10 min, 6000 rpm, room temperature) before the end of incubation time. The negative control was prepared in the same way without the test compounds, and conjugated samples were compared to the non-conjugated hydrolysate. The absorbance of the samples was measured at 517 nm and DPPH

scavenging activity was calculated as described by Farvin et al. (2014):

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (2)$$

Nitric oxide inhibition activity

60 µl of sample solution was mixed with 60 µl sodium nitroprusside in PBS buffer (0.025 M) and incubated at ambient temperature for 150 min. The negative control was prepared in the same way without the test compounds, and conjugated samples were compared to the non-conjugated hydrolysate. After incubation, the same volume of Griess reagent was added to the samples, and absorbance was measured at 546 nm. The nitric oxide inhibition activity was calculated as described by Tsai et al. (2007) using the following formula:

$$\%Inhibition = \frac{Absorbance\ of\ the\ control - Absorbance\ of\ the\ sample}{Absorbance\ of\ the\ control} \times 100 \quad (3)$$

Fourier transform infrared spectroscopy

The FTIR spectra of the freeze-dried protein hydrolysate and glycosylated protein hydrolysates were recorded by FTIR spectrophotometer (Bruker Tensor II FTIR, Bruker corporation, US) from 400- 4000 cm^{-1} at a resolution of 4 cm^{-1} (Li *et al*., 2016).

Preparation of emulsions

To prepare the 5% fish oil-in-water emulsions, the samples with the highest antioxidant activity were selected as aqueous phase and prepared as described in glycosylation section. Sodium caseinate solution (0.2% aqueous phase) was used as a control. The pH of all samples was adjusted to 7-7.5. After the addition of the oil phase to each aqueous phase, ultraturrax (Polytron, PT 1200 E, 18000 rpm, 30 sec) was used to partially emulsify the solution. The samples were vortexed to break any produced foam. A sonicator (Microson XL2000, probe P1, 75% amplitude, 30 sec, 2 passes) was used for emulsification. The vortex step was repeated after the sonication. The prepared emulsions were covered and stored at room temperature for further experiments (García-Moreno et al., 2018).

Droplet size distribution

The droplet size distribution of the samples was measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) at days 0, 1 and 3 of storage at room temperature. Emulsions were diluted in the system's circulating water (3000 rpm). The laser obscuration was 8-12%. Results were reported as volume mean diameter ($D_{4,3}$) (García-Moreno *et al*., 2016).

Zeta potential

Diluted solution (2:1000) of emulsions in distilled water was prepared at day 1 and the zeta potential of the solutions was measured by using the Zetasizer Nano ZS system (Malvern Instruments Ltd., Worcestershire, UK) at 20°C (García-Moreno *et al*., 2016).

Statistical analysis

All experiments were done in triplicate. ANOVA analysis was carried out. Differences were significant at $p<0.05$ .Results were reported as mean± standard deviation. Microsoft Office Excel (2010) was used for data management and graph generation, and SPSS (16.0) for statistical analysis.

Results and discussion

Chemical analysis

The chemical composition of *Cajanus cajan*'s flour, protein concentrate, and protein hydrolysate is shown in Table 1. The protein content of the concentrate was 73.85% that is 52.4% more than the protein content of the flour and shows the efficiency of the protein extraction process. The low amount of lipids in the flour is advantageous because no defatting step using chemical solvents is needed to remove the excess lipids. The lipid content decreased further during the protein extraction and hydrolysis processes, which increases the oxidative stability of protein concentrate and hydrolysate (Ovissipour *et al.*, 2012).

Increases in protein content of protein hydrolysate is due to the increased solubility of the proteins during the hydrolysis and the removal of the non-protein components by centrifugation. Ash content also increases due to the addition of HCl and NaOH to adjust the pH for hydrolysis (Halim and Sarbon, 2017). Water content is reduced because of freeze-drying of the protein concentrate and hydrolysate.

Table 1- Chemical composition of *Cajanus cajan*'s flour, protein concentrate and protein hydrolysate.

Sample	Protein (%)	Lipid (%)	Moisture (%)	Ash (%)
***Cajanus cajan*'s flour**	21.45±3.2[a]	4.21±0.1[a]	11.35±0.1[a]	2.60±0.0[a]
Protein concentrate	73.85±2.8[b]	1.87±0.2[b]	5.24±0.1[b]	0.94±0.1[b]
Protein hydroysate	89.41±2.4[c]	0.53±0.1[c]	2.32±0.1[c]	3.41±0.0[c]

a-c in each column indicates the significant differences ($p<0.05$)

Amino acid analysis of protein concentrate and hydrolysate

Amino acid composition of *Cajanus cajan*'s protein concentrate and protein hydrolysate is shown in Table 2. The amino acid composition is a key factor affecting the antioxidant properties of the protein hydrolysate. Histidine, tyrosine, methionine, and lysine are reported to be antioxidant amino acids (Ng *et al*., 2013), moreover, hydrophobic amino acids can improve the antioxidant activity of the hydrolysates (de Queiroz *et al*., 2017; Halim and Sarbon, 2017). The amount of hydrophobic amino acids in protein hydrolysate was 216.13 mg/g protein which helps the peptides to be solubilized in lipids and interact with hydrophobic radicals and polyunsaturated fatty acids (Yu and Tan, 2017). Aromatic amino acid content in protein hydrolysate was 68.26 mg/g protein.

Table 2- Amino acid composition of protein concentrate and protein hydrolysate.

Amino acids	Protein concentrate mg/g protein	Protein hydrolysate mg/g protein	Recommended values by FAO* mg/g protein	Chemical score of protein concentrate	Chemical score of protein hydrolysate
Arginine	73.94±13.85	67.75±20.72	-	-	-
Histidine	16.02±0.87	18.16±2.34	16	1.00±0.05	1.13±0.15
Isoleucine	24.82±0.63	25.73±6.04	30	0.82±0.02	0.85±0.20
Leucine	43.81±4.51	42.97±9.30	61	0.71±0.0.7	0.7±0.15
Lysine	38.48±4.83	53.99±22.35	48	0.80±0.10	1.12±0.46
Phenylalanine	34.91±2.64	35.58±3.34	-	-	-
Methionine	9.93±0.45	8.46±2.09	23	0.43±0.00[a]	0.36±0.09
Threonine	15.87±3.59	14.85±6.26	25	0.63±0.14	0.59±0.25
Valine	38.92±5.23	36.71±10.55	40	0.97±0.13	0.91±0.26
Alanine	21.41±2.77	22.29±6.01	-	-	-
Aspartic acid	52.86±11.68	60.60±21.93	-	-	-
Tyrosine	17.12±1.22	17.83±2.06	-	-	-
Glutamic acid	73.18±14.32	105.43±33.41	-	-	-
Glycine	20.21±4.70	20.73±0.03	-	-	-
Proline	24.41±2.86	23.66±4.62	-	-	-
Serine	25.59±4.85	28.73±13.52	-	-	-

*Recommended amino acid scoring pattern for adolescents and adults. FAO 2011. Tryptophan was not analyzed by the method applied.

Chemical scores of Histidine, Lysine and Valine had significant differences ($p< 0.05$).

These amino acids can donate proton to the radicals and stabilize the peptide molecule structure through resonance. Phenylalanine also is a radical scavenger (Sampath Kumar *et al*.,

2011). Furthermore, higher amount of histidine, as a metal chelator and hydrogen donor, can improve the antioxidant properties of the protein concentrate (Ng *et al*., 2013). Glutamic acid, arginine, aspartic acid and lysine were found in highest amount. Hydrolysis, depending on the exposure of protein to enzyme, can increase or decrease the amount of hydrophobic amino acids (Ng *et al*., 2013). Hydrolysis of the *Cajanus cajan*'s protein caused a significant ($p<0.05$) increase in lysine and glutamic acid, but did not considerably change the amount of the other amino acids.

As the antioxidant properties of the Maillard products are influenced by both amino acid and sugar, knowing the frequency of amino acids can provide us with an insight into the properties of the final products. It has been shown that a higher amount of alkaline amino acids such as Arginine and Lysine lead to Maillard products with higher free radical scavenging activities (Shen *et al*., 2018). As the *Cajanus cajan*'s protein hydrolysate is proved to be rich in the two mentioned amino acids, considerable antioxidant activity was expected

The chemical score is used to estimate the nutritive value of the examined protein in comparison to essential amino acids in a standard protein recommended by FAO. Results showed that the limiting amino acids in *Cajanus cajan*'s protein concentrate and protein hydrolysate are methionine, and threonine (Table 2). Other amino acids are almost adequate. Hydrolysis did not have significant negative effect on chemical score (except for Valine), and significantly ($p<0.05$) increased the chemical score of histidine and lysine. According to FAO (2011), both *Cajanus cajan*'s protein concentrate and protein hydrolysate met the nutritive value requirements for adults.

Molecular weight distribution of hydrolysate

Figure 1 shows the size exclusion chromatogram of *Cajanus cajan*'s protein concentrate and protein hydrolysate at 280 nm. The amount of large and small peptides in the protein concentrate is almost similar and very low because no enzyme was used during the protein extraction.

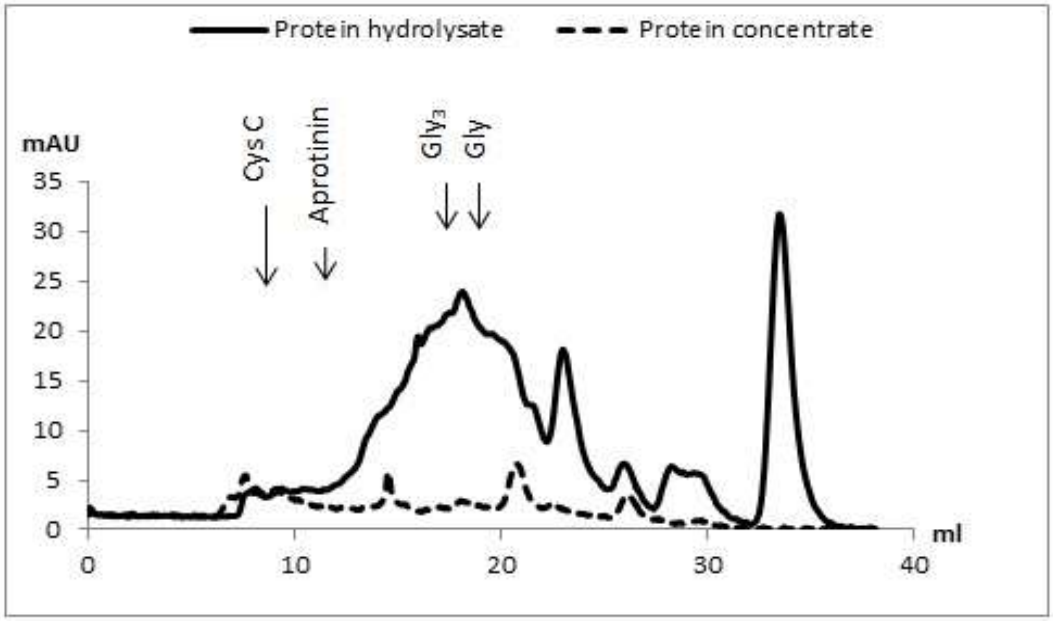

Fig. 1. Size exclusion chromatogram of protein concentrate and protein hydrolysate (by Alcalase)

These peptides were produced due to the acid and base application during the protein extraction. On the other hand, the difference in peptide sizes for protein hydrolysate was distinct. Most of the peptides in protein hydrolysate were smaller than the standards by having a molecular weight between 6.5 kDa (aprotinin) and 75 Da (Glycine). This is because

of the effect of Alcalase on peptide bonds with a wide range of specificity (García-Moreno *et al*., 2016). The peaks that are appeared beyond 20 ml are related to the retained peptides on the column because of hydrophobic interactions and higher affinity to the column and measuring their molecular weight is not feasible (Gringer *et al*., 2016).

Peptides with lower molecular weight often have higher antioxidant activities (Zou et al., 2016). Gringer et al. (2016) found that peptides with molecular weights below 10 kDa in marinated herring (*Clupea harengus*) salt brine had remarkable antiradical activity, reducing power and metal chelating activity.

Antioxidant activity

Figure 2 shows the DPPH scavenging activity and the nitric oxide inhibition activity of glycosylated and non-glycosylated hydrolysates from *Cajanus cajan*. DPPH scavenging assay is a useful method to evaluate the ability of various compounds in scavenging the free radicals (Kedare and Singh, 2011). As DPPH is a free radical, higher DPPH scavenging means the higher antioxidant potential of the sample.

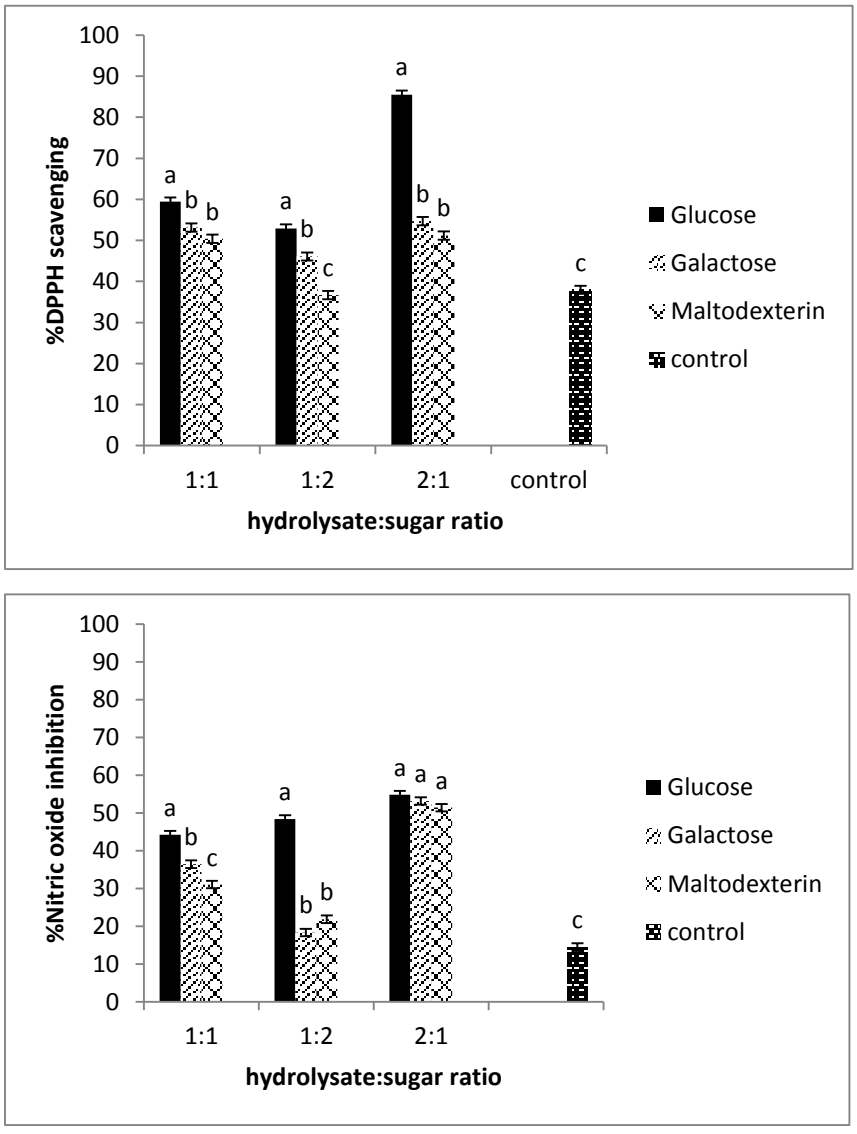

Fig. 2. Antioxidant activity of *Cajanus cajan*'s protein hydrolysate (control) and its glycosylated hydrolysates.
A-c indicates the significant difference between sugars in each ratio ($p<0.05$)

As it is shown in Figure 2, the addition of different sugars (glucose, galactose, and maltodextrin) and different hydrolysate to sugar ratios (1:2, 1:1, and 2:1) leads to different antioxidant activities for the samples. Results showed that the addition of sugars increased the DPPH scavenging activity of glycosylated protein hydrolysates compared to the non-glycosylated protein hydrolysate (except for DPPH scavenging of the hydrolysate to maltodextrin in the ratio of 1:2). The hydrolysates: glucose conjugates had higher DPPH scavenging activity in all three ratios and the ratio of 2:1 (hydrolysate: glucose), showed the highest DPPH scavenging activity. Karnjanapratum et al. (2017) reported the same ratio as the most effective ratio in the DPPH scavenging activity in glycosylated gelatin hydrolysates. Our results showed that glycosylation by glucose in the ratio of 2:1 increased the DPPH scavenging activity from 38.0% to 85.5%. This might be due to the effect of higher concentrations of sugar which provides more reducing groups and influences the degree of browning and produces Maillard products with higher antioxidant activities.

Nitric oxide is a free radical that can participate in the oxidative processes (Tsai *et al.*, 2007). The results for the nitric oxide inhibition activity was similar to the DPPH scavenging activity, where adding glucose in 2:1 ratio showed the highest nitric oxide inhibition activity (54.8%) compared to the non-glycosylated hydrolysate (14.5%).

Liu et al. (2014) reported that increasing the protein concentration against glucose, led to increase the antioxidant activity. They also stated that high molecular weight compounds produced during the Maillard reaction have a major contribution to the antioxidant activity of the product mixture. Hydroxyl groups and intermediate reductants can also possess antioxidant properties (Rao *et al*., 2011). Glycosylation, by changing the structure of the hydrolysate and addition of OH groups, have an important effect on the antioxidant activity of the glycosylates (Lie *et al*., 2014). Browning compounds produced in the Maillard reaction are the main radical scavengers (Wang *et al*., 2011). Gottardi et al. (2014) stated that DPPH scavenging activity is based on hydrogen and electron transfer that is dependent on sugar type as a hydrogen donor or acceptor. Different sugars react with different speeds, which in turn have an important effect on the production of the final products in the Maillard reaction (Oliviera *et al*., 2016). Moreover, the degree of glycosylation increases when the size of carbohydrates decreases (Niu *et al*., 2011). Zhang et al. (2018) found similar results and stated that glycosylation of *Morchella esculenta* by Maillard reaction can improve antioxidant activity and generate novel sources of antioxidant and functional foods.

Fourier transform infrared spectroscopy

Although molecular vibration of numerous atoms makes spectroscopic analysis of the polymeric molecules difficult, FTIR spectroscopy is a useful way of establishing peptide: sugar conjugate structures based on the absorption of radiation from atoms vibrations and shows the chemical composition and conformational structure of compounds (Liu *et al.*, 2014). According to the results from antioxidant properties assays, hydrolysate: glucose conjugate (2:1) had the highest antioxidant activity. Therefore, glycosylation of hydrolysate with glucose was evaluated by FTIR.

Characterization of physical stability of the emulsions

Functional properties of proteins and peptides can be altered by Maillard reaction to produce food ingredients with different applications; this is the most studied subject in the field of protein and peptide conjugates (Oliviera *et al.*, 2016). To evaluate the emulsifying properties of the glycosylated protein hydrolysates, the droplet size and zeta potential of fish oil emulsions prepared with sample solutions, as the aqueous phase, were measured (Table 4). In all emulsions, droplet size increased from day 0 to day 3. On day zero, the droplet size of the emulsions prepared with hydrolysate and its conjugates was not significantly different ($p<0.05$) compared to

emulsions produced with sodium caseinate as control; except for hydrolysate: maltodextrin conjugate which had a larger droplet size. This means that apart from hydrolysate: maltodexterin conjugate, the other emulsifiers had the same emulsifying ability. It could be as a result of the similarity of the amino acid profile of the hydrolysate and molecular weight of the glucose and galactose that makes their emulsifying ability to be similar. Furtheremore, the difference between maltodextrin conjugate emulsifying ability with other samples might be due to the difference of the sugar characteristics.

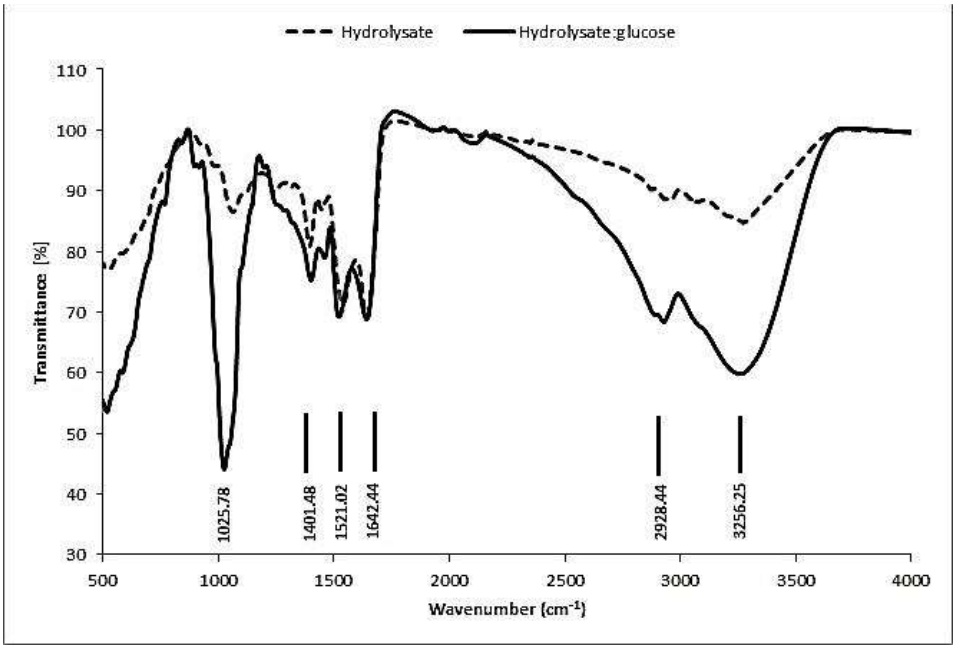

Fig. 3. FTIR spectrums of solid *Cajanus cajan*'s hydrolysate and its glucose conjugate

Table 3- Peak assignment for the FTIR spectrums

Frequency (cm^{-1})	Assignment
1025.78	C-O Stretching vibration
1404.48	C-N Stretching vibration
1521.02	N-H Plane bending and the C-N stretching vibration
1643.44	C=O Stretching vibration
2928.44	-C-H [sp^3] Antisymmetric stretching vibration
3256.25	-O-H Stretching vibration

Table 4- Droplet size and zeta potential of fish oil emulsions stabilized with different sugars

Emulsion	Droplet size [$D_{4,3}$(µm)]			Zeta potential (mV)
	Day 0	Day 1	Day 3	
Hydrolysate	1.7 ± 0.0^{a}	2.8 ± 0.4^{a}	7.8 ± 1.7^{a}	-43.4 ± 2.7^{a}
Hydrolysate: glucose	1.1 ± 0.6^{a}	2.3 ± 0.2^{a}	4.3 ± 0.2^{b}	-43.2 ± 1.6^{a}
Hydrolysate: galactose	1.5 ± 0.5^{a}	4.2 ± 0.1^{b}	5.6 ± 0.2^{b}	-44.6 ± 1.9^{a}
Hydrolysate: maltodextrin	3.7 ± 0.3^{b}	4.5 ± 0.7^{b}	5.2 ± 0.2^{b}	-44.0 ± 1.8^{a}
Sodium caseinate	0.9 ± 0.1^{a}	1.0 ± 0.1^{c}	8.5 ± 6.8^{c}	-42.2 ± 1.1^{a}

a-c in each column indicates the significant differences ($p<0.05$)

As it was mentioned, a high amount of hydrophobic amino acids in the hydrolysates (Table 2) can help the emulsifying ability and stability. Therefore, it could be anticipated that

the added sugar might make any possible difference in the emulsion stability. During the storage, the behavior of the emulsions was different. Sodium caseinate showed the smallest droplet size at day 0 but the largest droplet size at day 3. On day 1, there was no significant difference between hydrolysate and hydrolysate: glucose emulsions, and between hydrolysate: galactose and hydrolysate: maltodextrin emulsions, however, the difference between these two groups was significant ($p<0.05$) (droplet size of hydrolysate and hydrolysate: glucose< hydrolysate: galactose and hydrolysate: maltodextrin). On day 3, there was no significant difference between different conjugates, but the difference between the droplet size of emulsion stabilized with hydrolysate, and emulsions stabilized with conjugates was significant ($p<0.05$). This result shows that glycosylation has had a positive effect on the emulsion stability of the *Cajanus cajan*'s protein hydrolysate, and that glycosylation also improved the emulsion stability by preventing droplet size increase due to coalescence or flocculation. All of the emulsions had a white appearance without phase breaking or creaming (Figure 4).

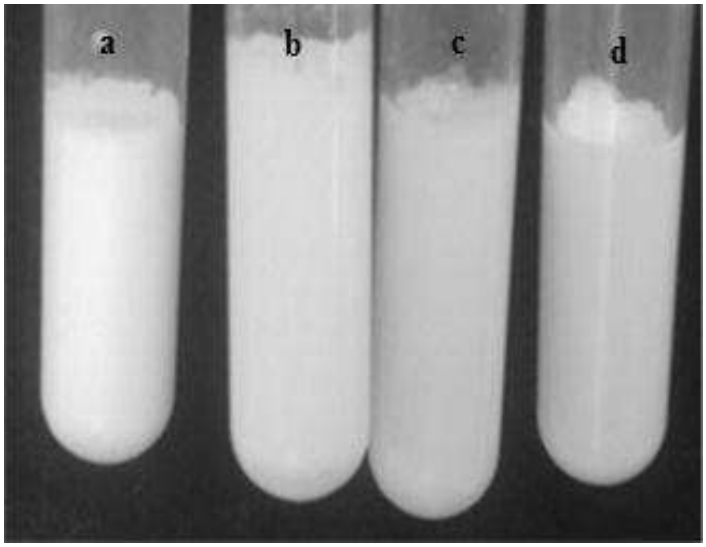

Fig. 4. The appearance of emulsions at day 3. a) Protein hydrolysate b) hydrolysate: glucose c) hydrolysate: galactose d) hydrolysate: maltodextrin

Table 5- Particle size comparison of fish oil emulsions stabilized with different sugars

	Emulsion	Particle size (µm)		
		0.1-1	1-10	10-100
Day 0	Hydrolysate	4.56 ± 0.01^{ab}	0.59 ± 0.00^{b}	0.16 ± 0.01^{a}
	Hydrolysate: glucose	4.65 ± 0.03^{a}	0.59 ± 0.02^{b}	0.08 ± 0.07^{a}
	Hydrolysate: galactose	4.43 ± 0.05^{bc}	0.83 ± 0.16^{a}	0.15 ± 0.09^{a}
	Hydrolysate: maltodextrin	4.30 ± 0.14^{c}	0.82 ± 0.11^{a}	0.22 ± 0.07^{a}
Day 1	Hydrolysate	3.97 ± 0.02^{ab}	1.22 ± 0.02^{b}	0.32 ± 0.05^{ab}
	Hydrolysate: glucose	4.07 ± 0.10^{a}	1.13 ± 0.10^{b}	0.29 ± 0.01^{b}
	Hydrolysate: galactose	3.85 ± 0.01^{bc}	1.24 ± 0.07^{ab}	0.42 ± 0.04^{a}
	Hydrolysate: maltodextrin	3.6 ± 0.15^{c}	1.36 ± 0.02^{a}	0.44 ± 0.12^{a}
Day 3	Hydrolysate	3.21 ± 0.1^{a}	1.54 ± 0.06^{c}	0.87 ± 0.11^{a}
	Hydrolysate: glucose	3.30 ± 0.07^{a}	1.74 ± 0.08^{b}	0.63 ± 0.13^{b}
	Hydrolysate: galactose	3.27 ± 0.06^{a}	1.84 ± 0.02^{ab}	0.57 ± 0.05^{b}
	Hydrolysate: maltodextrin	3.17 ± 0.05^{a}	1.91 ± 0.08^{a}	0.61 ± 0.00^{b}

a-c in each column for each day indicates the significant differences ($p<0.05$).

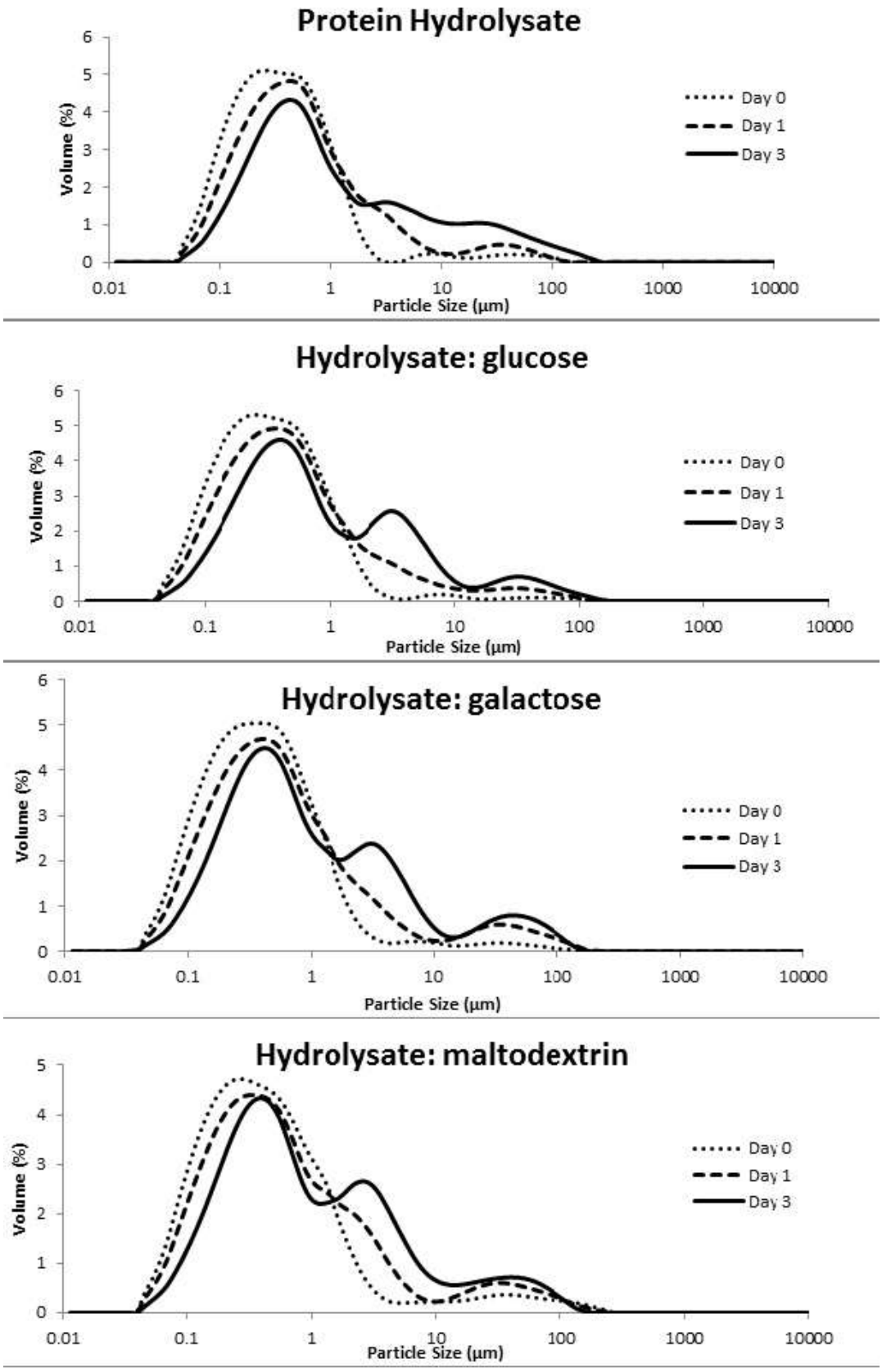

Fig. 5. Particle size distribution graphs

During the emulsification, the hydrolysate is absorbed into the interface, and the conjugated sugar helps the colloidal stability by thickening the aqueous phase (Ru *et al.*, 2009). The molecular mass, load, structure, and reaction time of the sugar and hydrolysate are important for their emulsifying properties (Kato, 2002). Moreover, the hydrophilic- hydrophobic balance of the conjugate played a critical role here (Olivier, 2006). Furthermore, glycosylation indirectly improved the emulsifying properties by increasing the solubility (Kutzli *et al*., 2020). Our results are in agreement with Li et al. (2016), Xue et al. (2017) and Kutzli et al. (2020) who reported that the glycosylation has a positive effect on emulsifying properties.

The surface charge of the emulsifier is important for stability of the emulsion (Li *et al.*, 2016), and measuring the zeta potential provides useful information about the charge of proteins and hydrolysates (Wouters *et al*., 2016). The zeta potential of the emulsions was not changed by glycosylation and all samples showed highly negative zeta potentials between -42 to -45. The induced repulsive forces can prevent the emulsions from flocculation, and maintain stability because the stability is related to the steric and electrostatic repulsions (Lam and Nikerson, 2013). There was no significant difference between zeta potential of the emulsions stabilized with different sugar conjugates ($p>0.05$). This is in agreement with the study of Li et al. (2016).

Figure 5 shows the particle size distribution graphs from mastersizer. These graphs demonstrate the changes in particle size very well. In each graph, a shift to the right during the storage indicates that the size of particles is increasing because of the flocculation and/ or coalescence during the time. All graphs had a large peak between 0.1 and 1 μm. During the storage, smaller peaks between 1 to 10 and 10 to 100 μm were observed. The number of droplets with more than 100 μm increased in the emulsions stabilized by protein hydrolysate, and on day 3, The emulsion stabilized by glycosylated hydrolysates significantly ($p<0.05$) had the lowest amount of such droplets (Table 5).

Conclusions

Evaluation of antioxidant and emulsifying properties of *Cajanus cajan*'s protein hydrolysate and effect of glycosylation by glucose, galactose, and maltodextrin showed that glycosylation has a significant positive effect on the antioxidant and emulsifying properties of the hydrolysates and glucose was the most effective sugar in the present study. FTIR results also proved glycosylation and production of Maillard reaction products. Further experiments are needed to identify the structure of active compounds. The practical application of hydrolysates and glycosylated hydrolysates in real food products should be investigated.

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نشریه پژوهشهای علوم و صنایع غذایی ایران
جلد ۱۷، شماره ۶، بهمن- اسفند ۱۴۰۰، ص. ۱۵۲-۱۳۷

بهبود ویژگی‌های آنتی‌اکسیدانی و امولسیفایری پروتئین‌های هیدرولیزشده نخود کاجان (*Cajanus cajan*) توسط گلیکوزیلاسیون با واکنش میلارد

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چکیده

هدف این مطالعه استفاده از واکنش میلارد به‌عنوان ابزاری برای گلیکوزیلاسیون پروتئین‌های هیدرولیزشده حاصل از نخود کاجان (*Cajanus cajan*) و ارزیابی این اصلاح شیمیایی بر ویژگی‌های آنتی‌اکسیدانی و امولسیفایری بود. ویژگی‌های شیمیایی، ترکیب آمینواسیدی و توزیع وزن مولکولی پروتئین‌های هیدرولیز شده مورد بررسی قرار گرفت. از گلوکز، گالاکتوز و مالتودکسترین در نسبت‌های ۱ به ۲، ۱ به ۱ و ۲ به ۱ (پروتئین هیدرولیزشده به قند، وزن خشک) برای گلیکوزیلاسیون استفاده شد. فعالیت آنتی‌اکسیدانی از طریق دو آزمون فعالیت مهار رادیکال آزاد ۱و ۱- دی‌فنیل-۲- پیکریل هیدرازیل (DPPH) و مهار نیتریک‌اکسید بررسی شد. امولسیون‌ها (روغن/ آب) توسط روش سونیفیکاسیون تهیه شدند. توزیع اندازه ذرات و پتانسیل زتا امولسیون‌ها طی ۴ روز نگهداری اندازه‌گیری شد. گلیکوزیلاسیون با گلوکز در نسبت ۲ به ۱ مهار DPPH را از ۳۷/۹۶٪ به ۸۵/۵۳٪ و مهار نیتریک‌اکسید را از ۱۴/۵۰٪ تا ۵۴/۸۳٪ افزایش داد. همچنین گلیکوزیلاسیون توسط هرکدام از قندها پایداری امولسیون‌ها را افزایش داد و تفاوت معناداری بین نوع قند مورد استفاده مشاهده نشد.

واژه‌های کلیدی: گلیکوزیلاسیون، واکنش میلارد، پروتئین هیدرولیزشده، آنتی‌اکسیدان، امولسیون

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