Journal of Food Biosciences and Technology,
Islamic Azad University, Science and Research Branch, Vol. 12, No. 3, 1-14, 2022
DOI: 10.30495/jfbt.2022.19726 http://dorl.net/dor/20.1001.1.22287086.2022.12.3.1.4

Preparation and Investigation of Bioactive Properties of Protein Hydrolysates from Yogurt Whey

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Received: 9 June 2021 Accepted: 11 July 2021

ABSTRACT: Yogurt whey contains lactose, lactic acid, soluble proteins, water-soluble vitamins, especially B vitamins, and minerals. Yogurt whey proteins can be a good source of bioactive peptides. In this study, we investigated the effect of enzyme type (pepsin and trypsin), temperature (37 and 50 °C) and time (120 and 240 min) of enzymatic hydrolysis on bioactive properties of protein hydrolysates prepared from yogurt whey. The results showed that increasing the enzymatic hydrolysis time from 120 to 240 min, increased the degree of hydrolysis, antioxidant activity (iron ion chelating activity, FRAP reducing activity and ABTS cation radical inhibitory activity) and antibacterial properties against various pathogenic bacteria (*Escherichia coli, Staphylococcus aureus, Salmonella typhimurium and Listeria monocytogenes*). Moreover, increasing the enzymatic hydrolysis temperature from 37 to 50 °C increased the degree of hydrolysis and decreased antioxidant activity and antibacterial property against *Salmonella typhimurium*. The antioxidant activity, antibacterial properties and hydrolysis degree of yogurt whey protein hydrolysates produced by trypsin were higher than those of pepsin. Two hydrolysates, were prepared with trypsin at 37 °C and pepsin at 50 °C for 240 min, were selected as the best samples in terms of bioactive properties.

Keywords: Antibacterial Properties, Antioxidant Activity, Enzymatic Hydrolysis, Protein hydrolysates, Yogurt Whey.

Introduction

Bioactive peptides are protein fragments that are released by proteolysis that are small in size and usually consist of 3-20 amino acids and their molecular weight are less than 6000 Da. Bioactive peptides are defined as specific regions that are encoded in the primary sequence of proteins and, in addition to their specific functions, also have major nutritional advantages (Udenigwe, 2014; El-Fattah *et al*., 2018). In addition, whey and whey proteins, a source of precursors to biologically active peptides (BAPs, also known as bioactive peptides or biopeptides), can have health effects (Gouda *et al*., 2021; Nguyen *et al*., 2020; Skrzypczak *et al*., 2020). The Database of biologically active peptide sequences

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(BIOPEP) reported that there are approximately 3,000 peptides with pragmatic properties such as antimicrobial, antihypertensive, antioxidant, anticancer, cholesterol-lowering, immune modulator, and anti-inflammatory activities (Ahn *et al*., 2015; Chakrabarti *et al*., 2014; Nguyen *et al*., 2020; Chai *et al*., 2020). The biological activity of peptides depends on their amino acid composition and their location in the amino acid sequence of proteins. These peptides can be formed by enzymatic hydrolysis of proteolytic enzymes during gastrointestinal digestion, fermentation by immune bacteria such as lactobacilli, and / or during food processing. The antimicrobial peptides are small, cationic and amphiphilic peptides that are produced from various resources of plant, animal, and etc. sources and have antimicrobial activity against bacteria, fungi, viruses and other pathogens (Barbosa Pelegrin *et al*., 2011). The antimicrobial peptides have 4 features in common: 1) they usually have 12-100 amino acids, 2) they are often positively charged, 3) more than 50% of them are hydrophobic amino acids and 4) they affect the function of bacterial cells in several ways. Since these peptides are often bipolar, they exhibit similar disinfectant function. These compounds inhibit the synthesis of large molecules, interact with intracellular compounds and affect microbial cell function (Jenssen *et al*., 2006). The properties of antioxidant peptides are due to metal ion chelates, inhibition of free radicals, deoxygenation and inhibition of enzymatic and non-enzymatic lipid peroxidation (El-Fattah *et al*., 2018). In different peptides with the antioxidant activity, the presence of tyrosine, tryptophan, methionine, lysine and cysteine in the peptide sequence is a good feature, because these amino acids are able to reduce Fe^{3+} to $Fe^{2+}$and chelate the metal ions $Fe^{2+}$and Cu^{2+} (Carrasco-Castilla *et al*., 2012; Huang *et al*., 2010). Currently, various commercial proteases of plant, animal and microbial origin such as trypsin, chymotrypsin, pepsin, alkalase, papain, pronase, collagenase and bromelain are used to produce bioactive peptides (de Castro and Sato, 2015; Singh *et al*., 2014; Vanderghem *et al*., 2011). The type of enzyme used in the hydrolysis process is very important because the type of enzyme determines the pattern of peptide bond breakage (Shahidi and Alasalvar, 2011). Trypsin belongs to the family of serine proteases that breaks peptide bonds from the carboxyl groups, where the amino acids lysine and arginine are located, and plays an important role in the activation of pancreatic enzyme zymogens (Ktari *et al*., 2012). Pepsin with molecular weight of 36 kDa is an important enzyme that is in the category of aspartic proteases and in the third class, hydrolase, and causes the hydrolysis of proteins. Peptide bonds are easily broken down and under acidic conditions proteins are broken down by this enzyme (Haldar and Chattopadhyay, 2010; Zhao *et al*., 2011). Yogurt contains valuable compounds, some of which enter yogurt whey and if discarded, remains unused (Varghese and Haridas, 2007). Yogurt whey is a by-product that contains lactose, lactic acid, water-soluble vitamins (especially B vitamins), minerals, water-soluble proteins and the bioactive peptides. Yogurt whey has a high nutritional value due to its high quality biological proteins and high content of essential amino acids. Yogurt whey proteins are considered as a high quality nutrient due to the high content of cysteine (Bylund, 2015; Rocha-Mendoza *et al*., 2020; Janine *et al*., 2005).

The aim of this study was to prepare the protein hydrolysates from yogurt whey and investigate the effect of three factors

of enzyme type, temperature and time of enzymatic hydrolysis on the degree of hydrolysis, antioxidant and antimicrobial activities of these hydrolysates.

Materials and Methods

- ***Preparation of protein hydrolysates from yogurt whey***

Yogurt whey was obtained from the stirred yoghurt (Taravat Dairy Company, Varamin, Iran). In order to isolate proteins, the pH of yogurt whey was adjusted to 9 by 0.1 M NaOH. After the protein precipitation, yogurt whey was centrifuged (Universal 320 R, Hettic Co, Germany) at 15,000 rpm at 4 °C for 30 min. Two types of digestive enzymes, pepsin (Sigma, USA) and trypsin (Merck, Germany) were used to hydrolyze the samples. The ratio of enzyme to substrate was 1:10. Two enzymes were added to substrate according to Mirzaei *et al*. (2015) method. Yogurt whey protein was weighed and concentration of yogurt whey protein was adjusted to about 4 mg/ml. The samples were dissolved in 50 mM of KCl-HCl at pH= 2 for pepsin enzyme activity and 50 mM of potassium phosphate buffer at pH= 7.8 for trypsin activity until the final concentration reached 6 mg/ml. After adding the enzymes to the suspension, the hydrolysis of the treatments was performed under different conditions (37 and 50 °C for 120 and 240 min). All reactions were performed in 100 ml flasks in a shaker incubator (FG Co., KM11 Model, Iran) at 200 rpm. Inorder to ensure inactivation of the enzyme, the enzymatic reaction was stopped by heating the suspension at 85 °C for 15 min as described by Corrêa *et al*. (2019). The hydrolyzed mixture was then rapidly cooled in ice bath, finally supernatant was collected in a refrigerated centrifuge (POL IDEAL TAJHIZ Company, Universal PIT 320R Model, Iran) at 6700× g at 10 °C for 20 min, placed at -18 °C and then powdered by a freeze dryer (Martin CHrist, Alpha 1-2 LD plus, Germany) at -40 °C and 0.1 mmHg.

- ***Measurement of hydrolysis degree***

In order to determine the rate of substrate proteolysis, a small amount of sample (usually 10-50 µl of sample containing 5-100 µg of protein) was added directly to 1 mL of OPA (**o**-Phthaldialdehyde) reagent in the spectrophotometer tube. The resulting solutions were mixed slowly, inverted and kept at room temperature for 2 min. The released alpha-amino groups reacted by hydrolysis with OPA and beta-mercaptoethanol, and finally, the absorption rate of the solutions was read at the wavelength of 340 nm using a spectrophotometer (Uniko, Uv / Vis 2100 Model, USA). L-serine was used to produce standard curve (Mirzaei *et al*., 2015).

- ***Measurement of iron ion chelating activity***

In order to measure this activity, 4.7 ml of hydrolyzed protein solution was mixed with 0.1 ml of 2 mM iron chloride II solution and 0.2 ml of 5 mM ferrozin and kept at room temperature for 20 min. Finally, the absorption was read at the wavelength of 562 nm (Uniko Uv / Vis 2100, USA). In the control sample, distilled water was used instead of the sample. Chelating activity was obtained from the following equation (Nalinanon *et al*., 2011):

$$\text{Chelating activity (\%)} = [(\text{control absorption} - \text{sample absorption}) / \text{control absorption}] \times 100$$

- ***Measurement of FRAP reducing activity***

In order to measure the reducing power of hydrolyzed proteins, first 900 µL of FRAP agent (300 mM of acetate buffer, pH = 3.6, 20 mM of iron chloride solution, 2, 4, 6 and 10 mM of triperidyl-S-triazine (TPTZ) in 40 mM of hydrochloric acid was freshly prepared, mixed with 100 µL of hydrolysate sample, incubated at 37 °C for 20 min and then absorption was read at a wavelength of 539 nm (Uniko model Uv/Vis 2100, USA). FRAP values were obtained from comparing the change in absorption in the test mixture with the prepared types of increasing concentrations of Fe^{3+} and reported as $FeSO_4$ mg per g of protein (Kumar *et al*., 2016a).

- ***Measurement of ABTS cation radical inhibitory activity***

First, 7 M of ABTS solution was prepared in distilled water. This solution was oxidized by potassium persulfate and then placed in the dark for 12-16 h. The cation radical solution obtained from pure ethanol was diluted 50 times and then used. Therefore, 1 ml of this solution was poured into each of the prepared test tubes and then 10 ml of the antioxidant sample was added to them. After 15 min, absorption was read at the wavelength of 734 nm (Uniko Model Uv/Vis 2100, USA). Ethanol was added to one of the test tubes instead of the sample (as the control) and its absorption was read at the first moment and after 15 min. Then, the inhibition percentage of the samples was calculated through the following equation and finally reported as an antioxidant capacity equivalent to ascorbic acid (Miliauskas *et al*., 2004).

$$I\ (\%) = [(A_{b(15)} - A_{s(15)}) / A_{b(15)}\ ] \times 100$$

$A_{b(15)}$: Control absorption rate after 15 min
$A_{s(15)}$: Sample absorption rate after 15 min
I: Inhibition percentage

- ***Evaluation of antibacterial activity***

The antimicrobial activities of the protein hydrolysates were investigated against four important pathogenic bacteria including *Staphylococcus aureus* PTCC 1112, *Escherichia coli* PTCC 1330, *Listeria monocytogenes* PTCC 1630 and *Salmonella typhimurium* PTCC 1709. These bacteria were obtained from Culture Collection of Industrial and Infectious Microorganisms (Iranian Research Organization for Science and Technology, Tehran, Iran). The concentration of 1.5 $\times 10^8$ CFU/mL of theses bacteria were prepared by McFarland method and inoculated into Brain Heart Infusion (BHI) Agar (Merck, Germany) plates. Then, 20 µl of each protein hydrolysate solution (100 mg/mL) was added to the plates and each plate was incubated at 37 °C for 24 h. After 24 h, the diameter of inhibition zone was measured (Motta and Brandelli, 2002).

- ***Statistical analysis***

For quantitative data analysis, analysis of variance (ANOVA) was used in a completely randomized design as factorial with 3 replications and the data were analyzed by SPSS 22.0 software. In order to compare the means, Duncan's multi-domain test method at 95% significance level ($p<0.05$) was used and the relevant graphs were drawn by Excel 2013 software.

Results and Discussion

- ***Degree of hydrolysis (DH) of protein hydrolysates obtained from yogurt whey***

Based on the analysis of variance (Table 1), the effect of enzyme type, temperature and time of enzymatic hydrolysis as well as their interactions on

the degree of hydrolysis of protein hydrolysates obtained from yogurt were statistically significant ($p<0.05$).The degree of hydrolysis of the obtained protein hydrolysates increased significantly ($p<0.05$) by increasing time and temperature of enzymatic hydrolysis, with the exception of treatments prepared from trypsin at constant time of 240 min, which reduced by hydrolysis temperature from 37 to 50 °C. The degree of hydrolysis of trypsin-prepared treatments was significantly ($p<0.05$) higher than pepsin. In general, among different treatments, the highest degree of hydrolysis (21.83%) was related to sample T37,240 (hydrolysate produced by trypsin at 37 °C for 240 min) and sample P37,120 (hydrolysate produced by pepsin at 37 °C for 120 min) had the lowest degree of hydrolysis (10.21%) (Figure 1). The higher DH of hydrolysates prepared with trypsin indicates the higher ability of this enzyme to break peptide bonds in yogurt whey proteins compared to pepsin. The difference between DH values of different enzymatic treatments is due to differences in catalytic sites and endopeptidases and exopeptidases (Reed *et al*., 1975). Due to the increase in the hydrolysis temperature of yogurt whey proteins from 37 to 50 °C, the amount of DH reduced slightly, which was probably due to the partial denaturation of the structure of enzymes and yogurt whey proteins. Shou *et al*. (2018) in a study on the effect of different proteases on the degree of hydrolysis of milk proteins found that in all treatments by increasing enzymatic hydrolysis time up to 50 min, the degree of hydrolysis increased rapidly, but increasing hydrolysis time above 50 min showed no significant change in the degree of hydrolysis of milk proteins. In general, increasing the degree of hydrolysis maximizes the production of hydrolysates containing free amino acids or small peptides, which is an important factor for the production of protein hydrolysates with biological activity. However, over-hydrolysis can also be harmful, as it leads to a loss of biological activity of peptides (Silva *et al*., 2006).

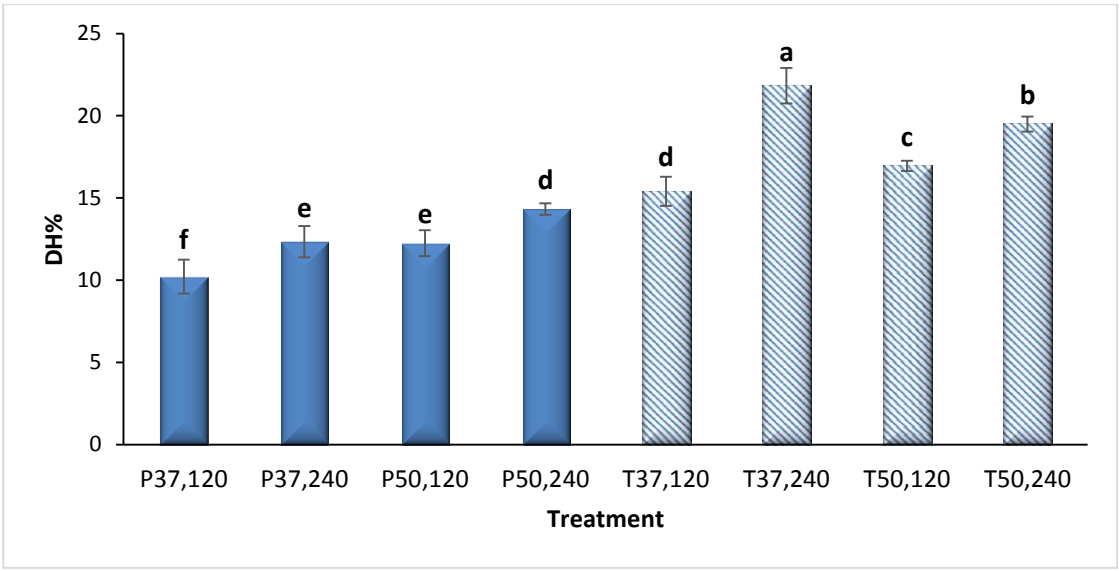

Fig. 1. Degree of hydrolysis (DH) of protein hydrolysates obtained from yogurt whey under different hydrolysis conditions

* The presence of at least one similar letter indicates that no significant difference is between the values ($p>0.05$).

- ***Iron chelating activity of protein hydrolysates derived from yogurt whey***

Based on the analysis of variance (Table 1), the effect of enzyme type, temperature and time of enzymatic hydrolysis as well as enzyme interactions in time on the chelating activity of protein hydrolysates obtained from yogurt whey were statistically significant ($p < 0.05$).), But other interactions were not significant ($p < 0.05$). According to Figure 2, by increasing enzymatic hydrolysis time, the chelating activity of the obtained protein hydrolysates significantly ($p<0.05$) increased and increasing the enzymatic hydrolysis temperature from 37 to 50 ° C, in general, caused a significant ($p<0.05$) reduction in the chelating activity of the hydrolysates obtained from yogurt whey proteins. The chelating activity of the samples prepared with trypsin was significantly ($p<0.05$) higher than that of pepsin. Among different treatments, the highest chelating activity (36.43%) was related to sample T37,240 (hydrolysate produced by trypsin at 37 °C for 240 min). Sample P50,120 (hydrolysate produced by pepsin at 50 °C for 120 min) and sample P37,120 (hydrolysate produced by pepsin at 37 °C for 120 min) had the lowest iron chelating activity (21.34 and 23.57%, respectively).

Table 1. Analysis of variance of the effect of temperature, time and enzyme on the degree of hydrolysis (DH), Iron chelating activity and FRAP of protein hydrolysates obtained from yogurt whey at 95% confidence level

		Mean Squares		
Source of changes	**DF**	**DH**	**Iron chelating**	**FRAP**
Enzyme	1	226.437**	135.850**	0.394^{ns}
Temperature	1	3.924*	23.010**	0.806*
Time	1	65.060**	396.094**	8.687**
Enzyme×Temperature	1	86.658*	0.049^{ns}	3.670**
Enzyme×Time	1	8.468*	14.602*	0.016^{ns}
Temperature×Time	1	5.828*	0.027^{ns}	0.010^{ns}
Enzyme×Temperature×Time	1	5.464*	0.150^{ns}	0.451^{ns}
Error	16	0.627	1.111	0.180
Total	24			

ns=not significant; *=significant at P≤0.05; **=significant at P≤0.01

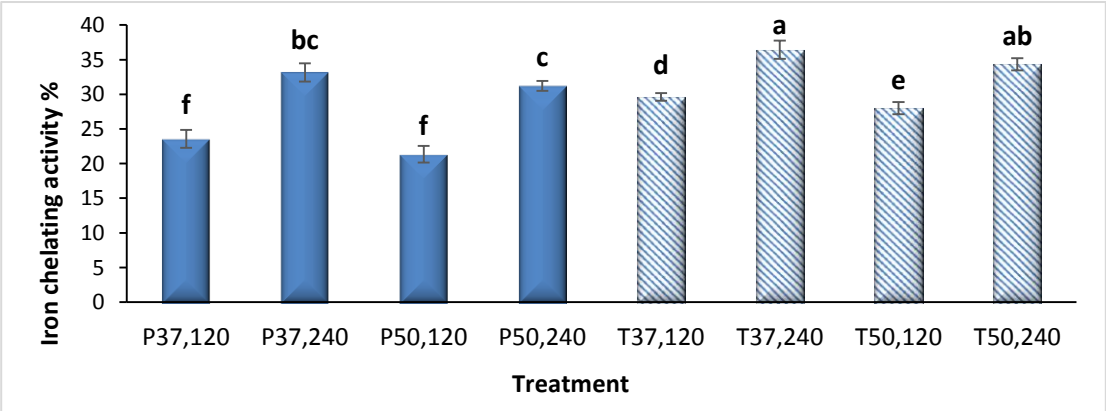

Fig. 2. Iron chelating activity of protein hydrolysates derived from yogurt whey under different hydrolysis conditions

* The presence of at least one similar letter indicates that no significant difference is between the values ($p>0.05$).

The chelating capacity of Fe^{2+} proteins hydrolyzed by various enzymes improves the bonding of metal ions by breaking peptide bonds, thereby increasing the concentration of carboxylic and amino groups in the branching of acidic and alkaline amino acids, and removing peroxidative free metal ions from the hydroxyl radical system (Liu *et al*., 2010). Trypsin and pepsin in the present study were able to produce peptides with different side chains and metal trapping. Correa *et al*. (2014) found that by increasing the hydrolysis time of sheep cheese proteins from 0 to 2 h, chelating activity of produced iron hydrolysates increased significantly, but increasing the hydrolysis time from 2 to 6 h reduced the chelating percentage.

- ***Ferric reducing antioxidant power (FRAP) of protein hydrolysates derived from yogurt whey***

Based on the analysis of variance (Table 1), the effect of temperature and time of enzymatic hydrolysis as well as the interactions of enzyme on temperature on the regenerative power of protein hydrolysates obtained from yogurt were statistically significant ($p < 0.05$), but The effect of enzyme type and other interactions was not significant ($p < 0.05$). The results showed that by increasing the time of enzymatic hydrolysis, the reducing power of the obtained protein hydrolysates increased significantly ($p<0.05$) (Figure 3). In the treatments prepared from pepsin, by increasing the enzymatic hydrolysis temperature from 37 to 50°C, the reducing power of the produced hydrolysates significantly ($p<0.05$) increased, but in the treatments prepared from trypsin, increasing the temperature reduced the reducing power of the samples. Among different treatments studied in this study, the highest reducing activity (5.67 mg $FeSO_4$/ g protein) was related to sample T37,240 (hydrolysate produced by trypsin at 37 °C for 240 min) and sample P50, 240 (hydrolysate produced by pepsin T 50 °C FOR 240 min). T50,120 (hydrolysate produced by trypsin at 50 °C for 120 min),

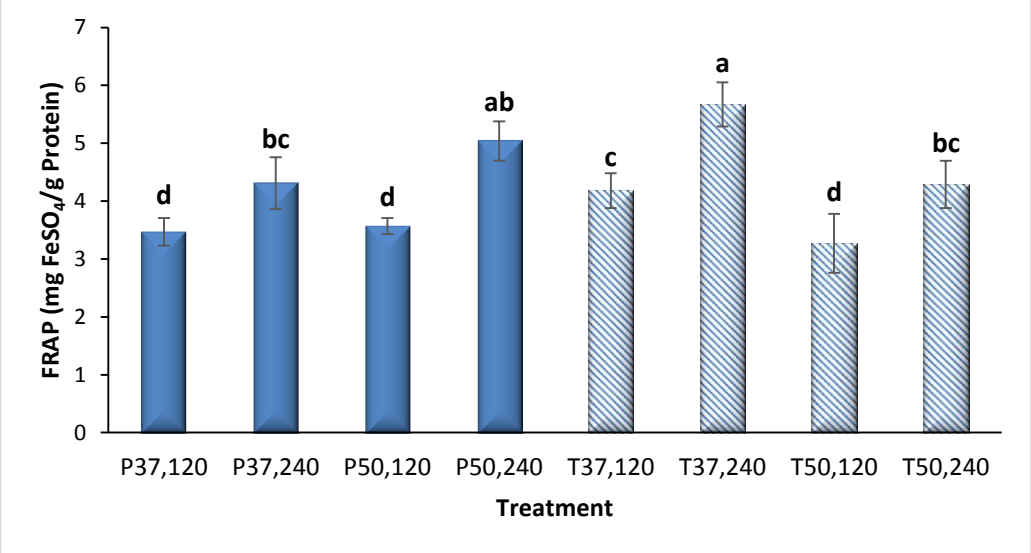

Fig. 3. Ferric ***reducing*** antioxidant ***power*** (***FRAP***) (mg $FeSO_4$/g protein) of protein hydrolysates derived from yogurt whey under different hydrolysis conditions

* The presence of at least one similar letter indicates that no significant difference is between the values ($p>0.05$).

P50,120 (hydrolysate produced by pepsin at 50°C for 120 min) and P37,120 (hydrolysate produced by pepsin at 37 °C for 120 min) samples had the lowest FRAP ($FeSO_4$/ g mg protein (3.27, 3.57 and 3.47 mg, respectively). Increasing the reducing power of iron hydrolysates from yogurt whey proteins by increasing the time of the hydrolysis process is associated with increasing the access time of protons and electrons produced by enzymatic hydrolysis (Luo *et al*., 2014). Studies have shown that reducing power is affected by the presence of peptides or amino acids with electron-donor ability that reacts with free radicals. The degree of hydrolysis and the type of enzyme play a decisive role in this index (Je *et al*., 2009). El-Fattah *et al*. (2017) reported that reducing power of iron hydrolysate milk prepared by enzymatic hydrolysis under different conditions was different. So that by increasing enzymatic hydrolysis time, as in the results of the present study, reducing power increased and increasing the enzyme concentration also increased reducing activity of iron in the produced protein hydrolysates.

- ***ABTS cation radical inhibitory activity of protein hydrolysates derived from yogurt whey***

By increasing time and temperature of enzymatic hydrolysis, ABTS radical inhibitory activity of the obtained protein hydrolysates increased significantly ($p<0.05$), except for the treatment prepared from trypsin at constant time of 240 min, which reduced by increasing temperature from 37 to 50 °C. Among different treatments studied in this study, the highest ABTS radical inhibitory activity (1768/Unit/mL) was related to sample T37,240 (hydrolysate produced by trypsin at 37 °C for 240 min) and sample P37,120 (hydrolysate produced by pepsin at 37 °C for 120 min) had the lowest inhibitory activity (9.92 Unit/mL) (Figure 4). However, the exact mechanism for the

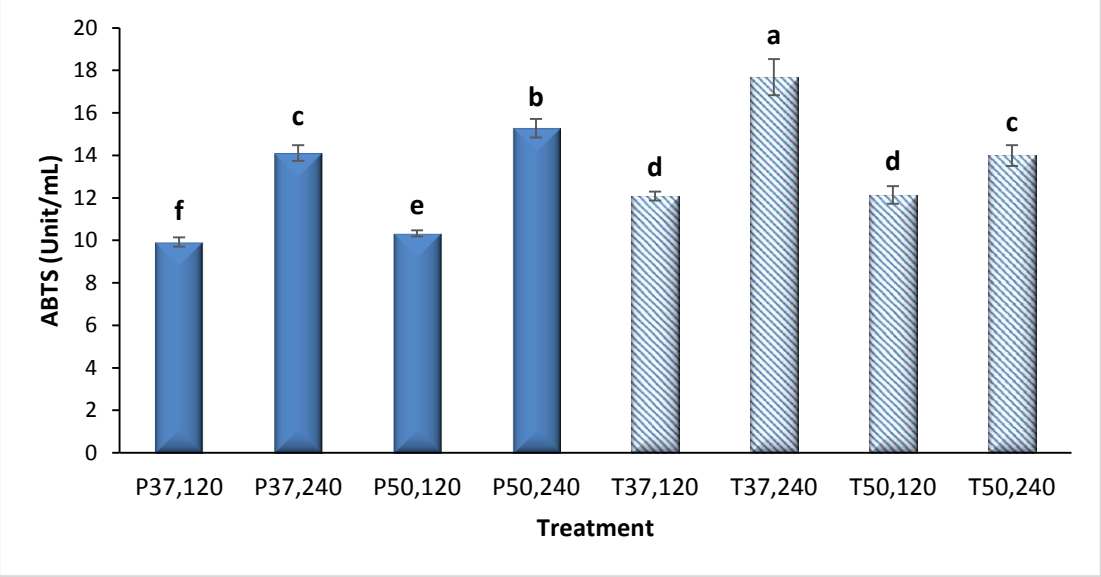

Fig. 4. ABTS cation radical inhibitory activity of protein hydrolysates derived from yogurt whey (Unit/mL) under different hydrolysis conditions

* The presence of at least one similar letter indicates that no significant difference is between the values ($p>0.05$).

antioxidant activity of peptides has not been determined. Some researchers in previous studies have concluded that peptides play an important role in inhibiting free radicals, chelating metal ions, and preventing the oxidation of fats. The specific composition, structure and bonds, as well as the hydrophobic properties of peptides, are effective on their antioxidant properties. In addition, the presence of some amino acids and their location in the structure of peptides also play an important role in the antioxidant activity of peptides (Sarmadi and Ismail, 2010). The most important amino acids with antioxidant and free radical inhibitory properties are proline, leucine, histidine, phenylalanine, methionine, tyrosine, tryptophan and cysteine. The sulfhydryl group in cysteine has a unique antioxidant activity and reacts with radicals by donating hydrogen from SH group. The imidazole-histidine group traps metals and hydrogen and is capable of trapping proxyl lipids (Elias *et al*., 2008). Kumar *et al*. (2016b) observed that by increasing enzymatic hydrolysis time of camel milk casein by catalase, chymotrypsin and papain for more than 4 h, ABTS radical inhibitory in the produced hydrolysates increased.

- ***Antimicrobial activity of protein hydrolysates derived from yogurt whey***

An example of the growth inhibition zone against pathogenic bacteria in protein hydrolysates obtained from yogurt whey is shown in Figure 5. In Table 2, the diameter of the growth inhibition zone (mm) of protein hydrolysates derived from yogurt whey under different enzymatic hydrolysis conditions against *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Listeria monocytogenes* are compared with each other. By increasing enzymatic hydrolysis time, the antibacterial activity of the obtained protein hydrolysates against *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Listeria monocytogenes* increased significantly ($p<0.05$). By increasing enzymatic hydrolysis temperature, the diameter of the growth inhibition zone against *Staphylococcus aureus Escherichia coli, Salmonella typhimurium, Listeria monocytogenes* increased. The antibacterial activity of the obtained protein hydrolysates against *Salmonella typhimurium* by increasing the time of enzymatic hydrolysis in the treatments prepared from pepsin at 50 °C and produced by trypsin at 37 °C increased significantly($p<0.05$). In the samples prepared with pepsin and trypsin at a constant time of 240 min, by increasing enzymatic hydrolysis temperature, the diameter of the growth inhibition zone against *Salmonella typhimurium* showed a significant increase and decrease, respectively. By increasing the enzymatic hydrolysis time from 120 to 240 minutes, the antibacterial activity of the obtained protein hydrolysates against *Listeria monocytogenes* increased significantly ($p<0.05$). In the treatments prepared with pepsin at a constant time of 120 min and trypsin at a constant time of 240 min, by increasing enzymatic hydrolysis temperature, the diameter of the growth inhibition zone against *Listeria monocytogenes* showed a significant increase and reduction, respectively. As the results showed that the time of enzymatic hydrolysis had a significant effect on all four bacteria studied and by increasing time, the antibacterial activity of the resulting protein hydrolysates increased, which is related to the breakdown of more proteins and production of peptides with higher antibacterial activity. The effect of

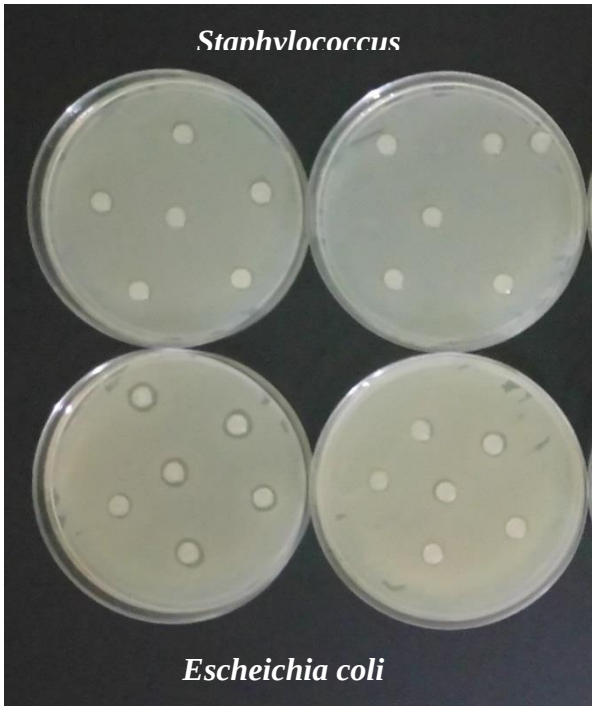

Fig. 5. An example of the growth inhibition zone against pathogenic bacteria in protein hydrolysates obtained from yogurt whey

Table 2. The diameter of growth inhibition zone (mm) against different bacteria in hydrolysates obtained from yogurt whey under different hydrolysis conditions (mean ± standard deviation)

Sample	*Listeria monocytogens*	*Salmonella typhimurium*	*Escherichia coli*	*Staphylococcus aureus*
P37,120	11.33±0.58^{c}	8.00±0.00^{b}	12.00±0.00^{c}	8.67±0.58^{de}
P37,240	12.33±0.58^{ab}	8.00±0.00^{b}	13.67±0.58^{ab}	9.33±0.58^{cde}
P50,120	12.00±0.00^{b}	8.00±0.00^{b}	12.00±0.00^{c}	8.67±0.58^{de}
P50,240	12.67±0.58^{a}	9.33±0.58^{a}	13.33±0.58^{b}	10.00±0.00^{b}
T37,120	11.67±0.58^{abc}	8.00±0.00^{b}	12.00±0.00^{c}	8.33±0.58^{e}
T37,240	12.67±0.58^{a}	9.67±0.58^{a}	14.00±0.00^{a}	11.00±0.00^{a}
T50,120	11.33±0.58^{c}	8.00±0.00^{b}	12.00±0.00^{c}	9.00±0.00^{d}
T50,240	12.00±0.00^{b}	8.00±0.00^{b}	12.00±0.00^{c}	10.33±0.58^{bc}

* The presence of at least one similar letter in each column indicates that no significant difference is between the values ($p>0.05$).

enzymatic hydrolysis temperature on the antibacterial activity of hydrolysates prepared from yogurt whey was significantl only against *Escherichia coli* gram-negative bacteria, therefore by increasing temperature, antibacterial activity against this bacterium is reduced. The type of enzyme had no significant effect on antibacterial activity against *Listeria monocytogenes* and *Salmonella typhimurium*, but the antibacterial activity of hydrolysates prepared with trypsin against *Staphylococcus aureus* and *Escherichia coli* was significantly higher than that of pepsin. The mechanism of action and effect of peptides depends on their structural properties including peptide size, amino acid composition, electric charge, hydrophobicity and the structure of the latter type. In general,

peptides derived from animal resources have a greater inhibitory effect than those derived from bacteria. The number of amino acids in many antimicrobial peptides is less than 50, and most of them have an amphipathic, hydrophobic, and cationic three-dimensional structure. The peptides that contain base amino acids such as lysine and arginine have a positive electric charge that has antibacterial properties (Shahidi and Zhong, 2008). The isolated antimicrobial peptides are often rich in leucine and glycine amino acids. The presence of arginine in the peptide sequence plays an important role in antimicrobial activity and due to its cationic properties increases the reaction with the bacterial cell wall. In fact, the cationic peptides degrade microorganisms by increasing cell membrane permeability (Tang *et al*., 2015). The death of a bacterium due to the formation of pores in its cell membrane involves three processes: 1) binding of antimicrobial peptides to the cell membrane, 2) their accumulation within the membrane and 3) formation of pores, exit of cell contents, and cell death (Yang *et al*., 2000). In general, the factors such as hydrophobicity, pH, ionic strength, temperature, surfactants, peptide origin, amino acid composition, peptide charge, size and secondary structure of the peptide can affect the antimicrobial activity (Ramos-Villarroel *et al*., 2016). Cheng *et al*. (2013) attributed the antimicrobial activity of kappa-casein against *Escherichia coli* to the high content of hydrophobic amino acids such as leucine, lysine, phenylalanine and proline. The researchers believed that antibacterial peptides can inhibit cell wall, RNA, DNA and protein synthesis by binding to intracellular molecules once they enter the cytoplasm. Sedaghati *et al*. (2016) reported that different parts of bovine beta-casein reduced the growth of *Escherichia coli* and completely stopped the growth of *Staphylococcus aureus*, *Lactobacillus casei* and *Lactobacillus acidophilus*. However, De Gobba *et al*. (2014) stated that casein hydrolysate prepared in their study lacked antimicrobial activity.

Conclusion

In recent years, the use of bioactive peptides has been considered in the field of pharmacy and production of functional foods. The biological properties of peptides in comparison with proteins have led to their consideration as functional compounds and efforts have been made to extract and use them in the food industry for production of functional products. According to the results, enzymatic hydrolysis of yogurt whey protein is an effective method on the production of protein hydrolysates with antioxidant and antimicrobial properties. The difference in the antimicrobial and antioxidant activity may depends on the enzyme properties and the degree of hydrolysis of proteins, leading to the production of hydrolysates with different amino acid compositions and can be used as an antimicrobial and antioxidant compound in food formulations. In general, the antioxidant and antibacterial activity and hydrolysis power of yogurt whey protein hydrolysate by trypsin was higher than that of pepsin, which increasing enzymatic hydrolysis time increased the degree of hydrolysis, antioxidant activity and antibacterial properties against various bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Listeria monocytogenes*). By increasing the enzymatic hydrolysis temperature from 37 to 50 °C, an increase in the degree of hydrolysis and a decrease in antioxidant activity and antibacterial property against *Salmonella typhimurium* were observed.

Two yogurt whey protein hydrolysates, were prepared with trypsin at 37 °C and pepsin at 50 °C for 240 min, were considered as the best treatments in respect of bioactive properties. These hydrolysates can be used for isolation, purification and characterization of bioactive peptides.

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