



Effect of pH, temperature and NaCl on antioxidant peptides from Iraqi buffalo whey proteins hydrolysates

على الببتيدات المضادة للأكسدة من NaCl والحرارة و pH تأثير
متحللات شرش حليب الجاموس العراق

BY

Orass Tariq Yasseen El-Ibresam

Ali khudhair Jaber AL-Rikabi

Department of Food Science, College of Agriculture, University
of Basrah, Basrah, Iraq

Doi: 10.21608/asajs.2022.246480

استلام البحث : ٢٠٢٢ / ٥ / ٩

قبول النشر : ٢٠٢٢ / ٥ / ٢٢

El-Ibresam , Orass Tariq Yasseen & AL-Rikabi, Ali khudhair Jaber. (2022). Effect of pH, temperature and NaCl on antioxidant peptides from Iraqi buffalo whey proteins hydrolysates. *Arab Journal of Agriculture Sciences (AJAS)*, The Arab Institution for Education, Sciences and Art, AIESA. Egypt, (5) 15, 68 – 98.

<http://asajs.journals.ekb.eg>

Effect of pH, temperature and NaCl on antioxidant peptides from Iraqi buffalo whey proteins hydrolysates

Abstract

The aim of this work was to isolate and characterize antioxidant peptides from Iraqi buffalo whey proteins cocentarate hydrolysates (BWCH) which were prepared 5g/10 ml by Alcalase enzyme at 240 min. and studied the effect of temperature, pH and NaCl on the antioxidant activity. Five different peptides from Alcalase hydrolysates (Alcalase1-Alcalase5) were isolated using gel filtration chromatography and showing different antioxidant activates. Two of them Alcalase1, Alcalase2 (3 mm) displayed the highest DPPH radical scavenging activity (84.31, 81.23%). The molecular mass of purified was determined using electrophoresis and amino analyzer estimated amino acids. Then tested activity of Fe⁺² chelating and reducing power. The two peptides showed stability of antioxidant activity at 60°C, pH 7 and 4% NaCl.

Keywords: Peptides, Antioxidant, Buffalo whey proteins concentrate, Proteins hydrolysates.

المستخلص

هدفت الدراسة الى عزل وتوصيف الببتيدات المضادة للأكسدة من متحللات مركز بروتينات شرش حليب الجاموس العراقي (BWCH) التي تم تحضيرها بتركيز 5 ملغم/ 10 مل ماء مقطر باستعمال إنزيم Alcalase عند 240 دقيقة ودراسة تأثير درجة الحرارة ودرجة الحموضة وكلوريد الصوديوم على النشاط مضاد للأكسدة. تم عزل خمس ببتيدات مختلفة (Alcalase1-Alcalase5) باستعمال كروماتوجرافيا الترشيح الهلامي وأظهرت فعالية مضادة للأكسدة مختلفة. أظهر اثنتان من الببتيدات المفصولة Alcalase2 و Alcalase1 بتركيز (3 ملغم) أعلى نشاط لكسح جذور DPPH (84.31 ، 81.23%). تم تقدير الوزن الجزيئي للببتيدات Alcalase1 و Alcalase2 باستعمال تقنية الترحيل الكهربائي إضافة الى تقدير نسب الاحماض الامينية. ثم تم اختبار قابلية Alcalase1 و Alcalase2 لتكبير

Fe²⁺ وسعة القوة الاختزالية كما أظهرت ثباتية عالية للنشاط المضاد للأكسدة عند ٦٠ درجة مئوية ، ودرجة الحموضة ٧ و ٤٪ كلوريد الصوديوم.
الكلمات المفتاحية: البيبتيدات، مضادات الاكسدة، مركز بروتينات شرش حليب الجاموس، المتحللات البروتينية

Introduction

Buffalo milk comprise over 12% to the milk production in the world , which is second only to bovine milk , buffaloes participate more importantly than cows to total milk production in hot and moist regions of the world (Arora et al., 2020). Southern Iraq is famous for breeding buffaloes and cheese making which leads to the production of large quantities of whey. Buffalo whey proteins are affected by the genetic context, which plays an important role in the formation of proteins (Buffoni et al., 2011) Buffalo milk proteins contain higher concentrations of beta-lactoglobulin, which is the main protein, reaching 58% of the total whey proteins. As well as the high content of lactoferrin protein, which is about 32 mg/100 ml compared to bovine milk powder (Khedkar et al., 2016).

The enzymatic hydrolysis of proteins is characterized by obtaining healthy protein hydrolyzers and does not include the breakdown of amino acids, it is a method that preserves the vital properties of the decomposition as well as the easy of controlling the conditions and degree of hydrolysis. Also enzymatic hydrolysis can be controlled by pH, temperature, time, and using low energy requirements, as well as being able to maintain functional properties such as solubility (Schaafsma, 2009). The enzymatic hydrolysis of whey has resulted in obtaining peptides with biological properties represented in the antioxidant activity that can be incorporated into the diet as a natural alternative to industrial antioxidants (Kruchinin et al., 2021). Over the past decades, studies have shown great interest in bioactive peptides derived from food for their role in disease control.

Antioxidant peptides disrupt ROS, bind oxidized metal ions, and enhance the activities of antioxidant enzymes within body cells (Manzanares et al.,2019). Changes in pH, temperature and NaCl concentrations affect the antioxidant activity of peptides that occur because of using peptides as food additives in food systems.

Materials and methods

Buffalo breeders in Al-Qurna District, Basra province, prepared the raw of Iraqi buffalo milk, Microbial rennet was used for the purpose of manufacturing cheese, which was prepared by the Italian company A.P.S clerici Coglificio, with a strength of 2040 units. Alcalase E.C 3.4.21.62 enzyme prepared from Aldrich-Sigma Company was used with specific efficiency of ≤ 5 units/gm.

Whey production by enzymatic method

The process of producing sweet whey through the manufacture of cheese by enzymatic method in the dairy factory of the College of Agriculture , University of Basrah, according to the method used by Yazdanpanh et al.(2015). The temperature was reduced to 37°C and microbial rennet was added according to the instructions of the prepared company.

Concentration of whey proteins by ultrafiltration

Whey proteins were separated using a membrane ultrafiltration (Millipore and amicon, U.S.A.) device based on the method presented in Jayaprakashs (2005) with some modifications where by the whey was passed under the pressure of 5 bar through ultrafiltration membranes with a pore size of MWCO 10 kDa .The processes occupied about 60 min. that has been separated into two-part; first (Permeate) and (Retentate) then used a rotary evaporator (Frankin electric, England) at a temperature of 40°C to concentrate the retentate which lyophilized (-54°C, 0.5 mmHg) in freeze-drying (Christ, Germany) for getting whey proteins concentrate (WPC).

Preparation of whey proteins' hydrolysate

The hydrolysates was prepared according to the Adamson and Reynolds(1996) and mentioned by Kim et al.(2007) with some modification, where the soluble protein was prepared in a ratio of 5:10 (w v⁻¹) of distilled water and the sample was mixed for 2-3 min., the solution was heated at 80°C for two min., then cooled to 25°C. Then the pH was adjusted to 8 using NaOH (0.1M). The temperature was maintained at 50°C throughout protein hydrolysis. An enzyme was added at a rate 0.1% (enzyme: substrate w v⁻¹) at (50 °C and at pH 8) after that the solution was added to (WPC). The hydrolysis continued for four hours and the enzyme was inhibited after the end of the hydrolysis period at a temperature of 90 ° C for ten min. then trichloroacetic acid (TCA) 12% (w v⁻¹) solution was added at a concentration then centrifugation (5000 × g, 10 min.) and collected the filtrate.

Gel filtration chromatography

Peptides of whey proteins hydrolysates by Alcalase separated and purified using gel filtration chromatography using Äkta pure 25 (GE Healthcar life Science, Sweden) based on the method used by Ling et al.(2018) and mentioned in Yang et al.(2020)with some modifications column (GL 10/300) Superdex G-75 (23.562 ml) filled with agarose gel and Dextran (30 × 10 mm) was used. 0.5 ml of the prepared AF sample was injected into distilled water at a concentration of 5:10 (w v⁻¹) in the column after filtering it using a filter with a diameter of (0.22 mm) and stabilizing the separation process conditions under a pressure of (1.5 MPa) and at a flow rate of (0.5 ml/min.) and equilibrating the column with the prepared potassium phosphate buffer solution (0.02 M, pH 7.2,) then the separated at (214, 220 and 280 nm)were followed up by chromatogram that appears on the computer screen. The resulting peaks were collected after each separation at an average of (2 ml) by F9-R particle

collector, then lyophilized. Two fractions (AF1, AF2) shown highest radical scavenging activity on DPPH.

SDS-PAGE

The molecular weight of the peptide (Alcalae1,Alcalase2) was estimated in a polyacrylamide gel according to the method mentioned in Blum et al.(1987) and Laemmler,(1970). Protein bands were stained with silver. The electrophoresis was conducted after preparing the gel and pouring it into the glass plates of the device and leaving it to solidify, then 30 μ M of each sample were injected and the electric power supply was (40 mA) for a period of 45, then the voltage was raised to (100 mA) to the end of the migration until the bromophenol blue dye reached the end of the gel, then the gel was removed from the glass plates to be placed in the fixing solution for one hour, after which it was washed with water to be placed in the formula for three hours. The dye removal solution was added as it was replaced several times until firmness appeared, then the gel was preserved with the preservation solution, then scanned using polypeptide of molecular weight standards from 5 to 245 kDa Cs analyzer (Atta, Japan) was used as the marker protein(MP).

Estimation of amino acids

The amino acids of the separated Alcalase1, Alcalase2 according to the method used by Clarity amino acid analyser SW company, which is equipped with an Amino Acids analyser, column (S4300) , at a flow rate of 1 ml/min., at wavelengths (440 , 570 nm).Where 10-20 mg of the lyophilized AF1,AF2 peaks were taken and 0.5 ml of hydrochloric acid (6 N) was added to it in a sealed vacuum tube at a temperature of 110 ° C for 24 hours then the sample was placed in a centrifuge (4000 \times g 10 min.), 250 μ l of the filtrate was taken and mixed with sodium hydrogen bicarbonate ,Then the solution was shaken and put in an ice bath for 15 min., then 375 μ l was withdrawn from the filtrate again and a buffer solution of lithium citrate was added

(0.12 M at pH 2.90) to make the samples ready for the determination of amino acids.

Antioxidant activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging

DPPH radical scavenging was used according to the method mentioned in Blois (1958) and described by Shah and Modi (2015). (0.5 ml) of DPPH solution (0.1mmol) prepared with methanol (98%) was mixed with (1 ml) of the samples, then the mixture was incubated in the dark for 30 minutes at room temperature, The absorbance was measured at (517 nm). DPPH radical scavenging was calculated according to the equation below:

$$\text{DPPH radical scavenging (\%)} = [A_0 - A_s / A_0] \times 100$$

A_0 = absorbance of control sample; A_s = absorbance sample

Iron chelating activity

Iron chelating activity was estimated according to the method by Dinis et al.(1994) and described by Hussein el al. (2020) Samples were prepared with different concentrations ranging from (1-3) mg/ml in test tubes, then(0.4 ml) of 8-hydroxy quinoline solution (5 mM) was mixed with each test tube. The tubes were incubated in the dark at room temperature for 10 minutes. Iron chelating activity of Ethylene diamine tetra acetic acid disodium (EDTA- Na_2) and Ascorbic acid was determined by the same method for comparison. The control sample was prepared by adding all of the above except for adding the sample where distilled water was added instead. The absorbance was measured at (562 nm), the Iron chelating activity calculated by applying the following equation:

$$\text{Iron chelating activity} = \left[\frac{\text{Absorption of the control sample} - \text{sample Absorption}}{\text{Absorption of the control sample}} \right] \times 100$$

Reducing power

The reducing power was measured according to the method of Oyaizy (1986) followed by Devi et al.(2017) prepared different concentrations ranging from (1-3) mg/ml distilled water from the samples, ascorbic acid, BHT and α -Tocopherol. Then take 1 ml of each concentration and mixed it with 2.5 ml of a phosphate buffer solution (0.2 M, pH 6.6) and (2.5 ml) of a solution of Potassium ferricyanide (1%). Then the mixture was incubated at a temperature of 50°C for 20 min., after which Trichloroacetic acid (1% TCA) was added. Then centrifugation was conducted at (3000× g, 10 min.) to separate the top layer of the solution and add (1 ml) of ferric chloride FeCl₃ (0.1%) and 5 ml of distilled water. The control sample was prepared by adding all the above-mentioned substances except for the sample with the addition of (1 ml) of distilled water instead, and the absorbance was measured at (700 nm) then the reduction power was calculated by applying the following equation based on what (Hemalatha et al., 2010)

$$\text{Reducing power} = \% 100 - \left[\frac{\text{Absorbance of sample}}{\text{Absorbance of control sample}} \right] \times 100$$

Stability of antioxidant activity

The stability of the activity of the antioxidant peptides peaks in the direction of pH values, temperatures and different concentrations of the NaCl salt was tested according to the method used by Zhu et al.(2014).

The effect of pH on antioxidant activity

The stability of the antioxidant activity of the peptides (Alcalase1, Aalcalase2) at 3 mg/ml was tested using different ranges of pH ranging from 2, 4, 7, 6, 8, 10 and 11. Then the samples were incubated for an hour at room temperature, then the pH was adjusted to 7 using (1 M) HCl or (1M) NaOH. Then the antioxidant activity was tested radical scavenging activity on DPPH.

The effect of Temperature on antioxidant activity

The temperature effect of the peptides (Alcalase1,Alcalase2) 3 mg/ml, was tested by heat treated at different temperatures 40, 60, 80, 100 °C for an hour on bath, then were cooled to room temperature .The control sample was prepared with the same concentration at 25°C, the antioxidant activity was radical scavenging activity on DPPH.

The effect of NaCl on antioxidant activity

Peptides (Alcalase1, Alcalase2) 3mg/ml were treated with NaCl solution prepared with different concentrations of 0, 2, 4, 6, 8 % and incubated at 60° C for 15 min., then cooled to room temperature. The control sample was prepared with the same concentration without adding NaCl, then the antioxidant activity was tested by DPPH scavenging radical.

Statistical Analysis

Statistical analysis of the results was conducted using a Complete Randomized Design (C.R.D.) within the statistical program (2011) GenStat and the studied factors were tested using the least significant difference L.S.D. at a significant level of 0.05.

Results and discussion**Isolation and purification Peptides**

(Figer 1.) shows isolation of five fractions of peptides from the hydrolysates of WPC prepared by Alcalase enzyme. The fractions were collected and lyophilized. The highest absorbance of the fraction (Alcalase3) was 2050 mAU at the time of appearance 32.1 min., while the absorbance of (Alcalase1) was 500 mAU at 15.2 min., (Alcalase2) was 2000 mAU at 24.4 minutes, the fraction (Alcalase4) was 1850 mAU at 39.5 min., and (Alcalase5) was 800 mAU at 45.5 min. The results agree with what was obtained by Ma et al.(2016) of five fractions obtained from isolation and purification of anti-inflammatory peptides from hydrolyzed Alcalase enzyme prepared from

bovine milk whey concentrate using gel filtration chromatography. Also with Yang et al. (2020) who isolated and purified a group of peptide fractions with antioxidant activity from hydrolyzed buffalo milk proteins prepared using gel filtration chromatography using ÄCTA pure device.

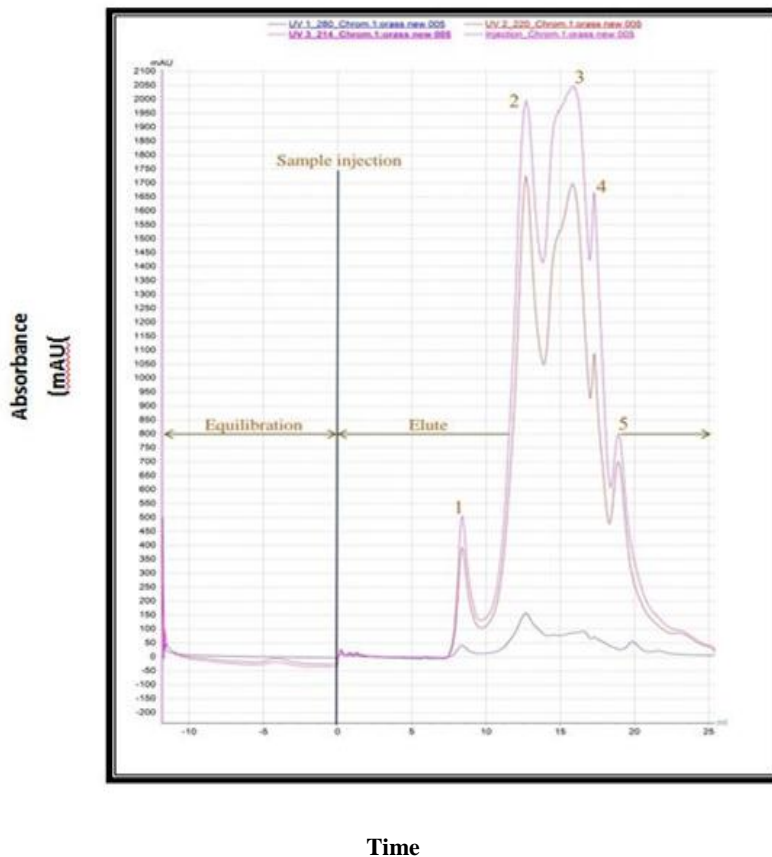


Figure 1. Fractions of peptides prepared from whey of Iraqi buffalo milk protein concentrate hydrostats using ÄKTA Pure 25 apparatus

The antioxidant activity of peptide fractions

(Figur 2.) shows the determination of the antioxidant activity of radical scavenging on DPPH for peptides fractions. The fractions showed a significant difference antioxidant activity at $P \leq 0.05$. The fractions (Alcalase1, Alcalase2) was displayed the highest DPPH radical scavenging activity (81.23, 84.31%) compared to the rest fractions. The reason for the discrepancy in the antioxidant activity of the separated peptide peaks may be due to the type and percentage of the acids present that give the ability to enhance the antioxidant activity, especially the ability of DPPH radical scavenging. The activity may also be associated with peptides of low molecular weight (Bassan et al., 2015) and (Gregory et al., 2016). As well as the mechanism of action of the enzyme may in turn affect the content of amino acids and their position in the peptide chain in addition to the molecular weight (Wali et al., 2019).

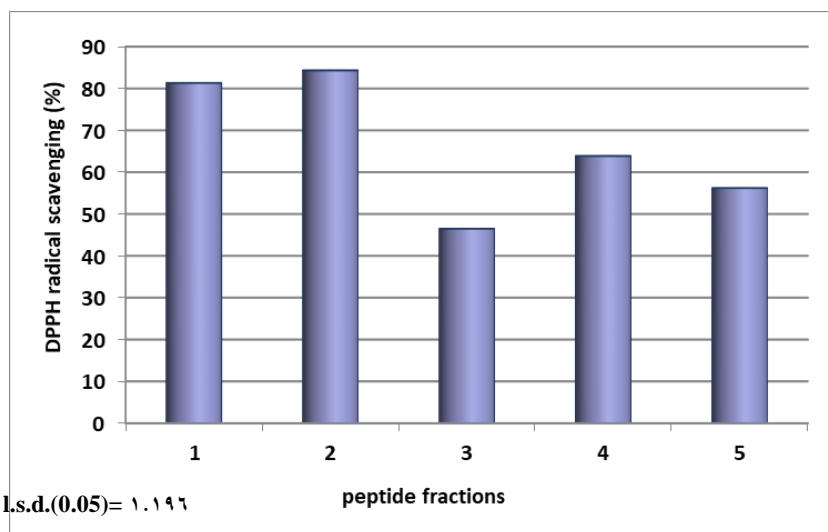


Figure 2. DPPH radical scavenging of peptides' fractions from hydrolysates of Iraqi buffalo whey proteins concentrate with Alcalase.

Molecular Weight of peptides

The molecular weight was estimated for peptides (Alcalase1, Alcalase2) by SDS-PAGE. (Figer 3.) and Table (1) indicate the appearance of 13 bunds for (Alcalase1) the molecular weights of which ranged from (9-83) kDa, and each bund represents a peptide. While five peptide bunds appeared for (Alcalase2), where each bund represented a peptide with molecular weights ranging between (7-19) kDa. The results agree to what was mentioned in Peng et al.(2009) who obtained four polypeptide separated by gel filtration with low molecular weights from the degraders of the bovine milk whey protein concentrates prepared with Alcalase enzyme, also with Bassan et al.(2015) who isolated of peptides during the hydrolysis of buffalo milk whey proteins were characterized the molecular weights ranging between 2.8-0.1 kDa, as well as with Hussein et al.(2020) who found peptides which had antioxidative activity from the hydrolyzed concentrates of bovine milk whey proteins prepared with Alcalase enzyme with molecular weights less than 10 kDa.

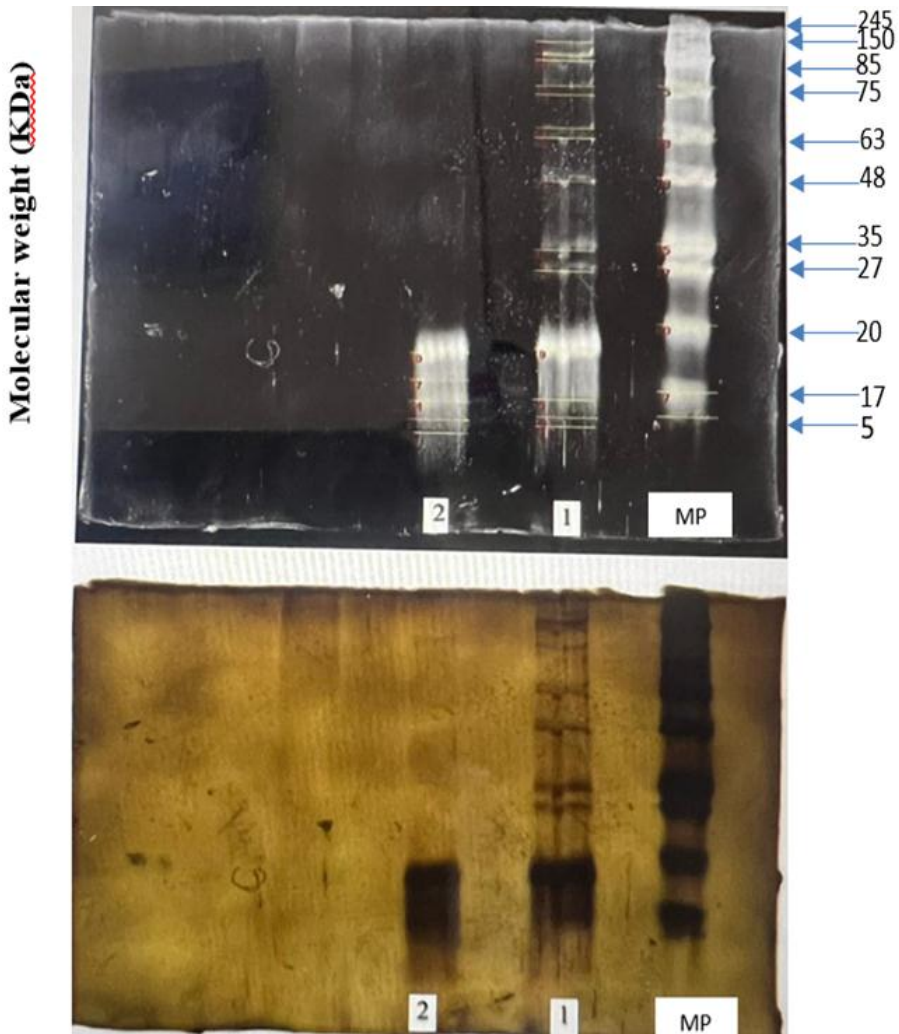


Figure 3. SDS-PAGE of peptides (Alcalase1, Alcalase2) from the hydrolysates of Iraqi buffalo whey protein concentrates with Alcalase.

Table 1. Molecular weights of peptides fractions Alcalase1 and Alaclase2 peptide from Iraqi buffalo whey hydrolysate with Alcalase.

Bands number	Alcalase1 (kDa)	Alcalase2 (kDa)
1	9	7
2	10	8
3	15	14
4	19	17
5	25	19
6	32	
7	47	
8	62	
9	65	
10	73	
11	75	
12	81	
13	83	

Estimation of amino acids (AA)

Table (2) indicates AA profile content of the peptides (Alcalase1, Alcalase2) separated and purified from the hydrolyzed Iraqi buffalo whey protein concentrate by gel filtration chromatography using Äkta pure 25 that were prepared with Alcalase. AA composition (Alcalase1, Alcalase2) reached 17 amino acids. It is noted from the results that the amino acids of Alcalase1 are Methionine, Leucine, Arginine, Threonine, Aspartic, Histidine, Glutamic and Cysteine, which formed the highest concentrations 5.45%, 5.49%, 5.65%, 5.74%, 7.20%, 7.46%, 8.64% and 9.75%, respectively, of total amino acids, While the amino acids of Alcalase2 are Aspartic acid, Lysine,

Proline, Tyrosine, Histidine, Isoleucine, Glutamic, Phenylalanine and Cysteine predominated and the concentrations of each of them were 5.32%, 5.60%, 5.80%, 5.88%, 6.0%, 6.45%, 6.67% and 7.78% and 8.98%, respectively of total amino acids. The hydrophobic amino acids was 31.85% and 37.96%, and the basic concentrations were 30.03% and 35.69%. The acidity amino acids were 16.1% and 11.99% for Alcalase1 and Alcalase2 respectively, of the total acids .The Cyclic amino acids reached 7.62% and 13.6 for Alcalase1 and Alcalase2 respectively. As shown in Table (2), Cysteine was the predominant amino acid among the total amino acids for both fractions 9.75% and 8.98%, respectively. The results converged with what found Bassan et al.(2015) who obtained 14 amino acids from hydrolysates of buffalo milk whey proteins, of which six were basic and Neto et al. (2019)who purified seven fractions of peptide from hydrolysates of bovine milk whey proteins that contained 19-10 amino acid. The reason for the variation in the concentrations of amino acids may be due to the hydrolysis conditions in terms of the specificity of the action of the enzyme used the time of hydrolysis, the type of amino acids and their position in the peptide depends on the mechanism of action of the enzyme (Wali et al., 2019).

Table 2. Amino acids composition of Alcalase1 and Alcalase2 peptides separated by gel filtration from hydrolysates of Iraqi buffalo whey proteins concentrate with Alcalase .

AA	Alcalase1 (%)	Alcalase2 (%)
L-Alanine	2.59	1.82
L-Aspartic acid	7.2	5.32
L-Arginine	5.65	1.49
L-Cysteine	9.75	8.98
L-Glutamic acid	8.64	6.67
Glysin	1.72	3.83
L-Histidine	7.46	6.00
L-Isoleucine	4.58	6.45
L-Leucine	5.49	4.55
L-Lysine	3.85	5.60
L-Methionine	5.45	2.84
L-Phenylalanine	3.78	7.78
L-Proline	3.75	5.80
L-Serine	2.49	3.79
L-Threonine	5.74	3.58
L-Tyrosine	3.84	5.88
L-Valine	1.54	4.89
Total	83.52%	85.27%

Antioxidant activity peptides

Fe⁺² chelating activity

(Figer4.) Shows the Fe⁺² chelating ability of the peptides fractions Alcalase1 and Alcalase2 and compared it with EDTA-Na₂ and Ascorbic acid at concentrations (1-3 mg/ml). It is noted from the results that AF1 has the highest activity of Fe⁺² chelating (58.30%) than Alcalase2 (49.50%) at (1mg/ml), they were significantly affected by increasing the concentration at p≤0.05, while EDTA-Na₂ and Ascorbic acid were (67.82,

62.46%) respectively, at the same concentration then the activity of Fe^{+2} chelating for Alcalase1 and Alcalase2 increased to (82.75, 73.33%) at (3 mg / ml), respectively. While EDTA- Na_2 and Ascorbic acid (96.60, 90.71%) respectively, at the same concentration. The results agree with Caetano-Silva et al. (2015) who found the peptides which isolated from the hydrolysates of WPC of bovine milk using Alcalase had a high activity of chelating Fe^{+2} and the high activity of Fe^{+2} chelating of whey peptides and the formation of an iron peptide complex is closely associated with the Glutamic and Aspartic amino acids, in addition Lysine, Isoleucine, Threonine, Histidine and Proline, which enhances the activity when they composed the peptide's structure. That what was obtained by Jiang et al. (2017) from low molecular weight peptides from hydrolysates of bovine milk whey proteins which prepared by Alcalase, which showed a high the Fe^{+2} chelating ability of the separated peptides' fractions may be due to the high concentrates of Glutamic, Aspartic and basic amino acids represented by Arginine, Lysine, Methionine and Histidine. The structure of the peptide, type of amino acid and its sequence have a major role as a promoter of the high Fe chelating activity and that the presence of Glutamic and Aspartic amino acids in the peptide is important for Fe^{+2} chelating ability also, the imidazole in Histidine supports the ability of Fe chelating of the peptides (Neto et al ., 2019).

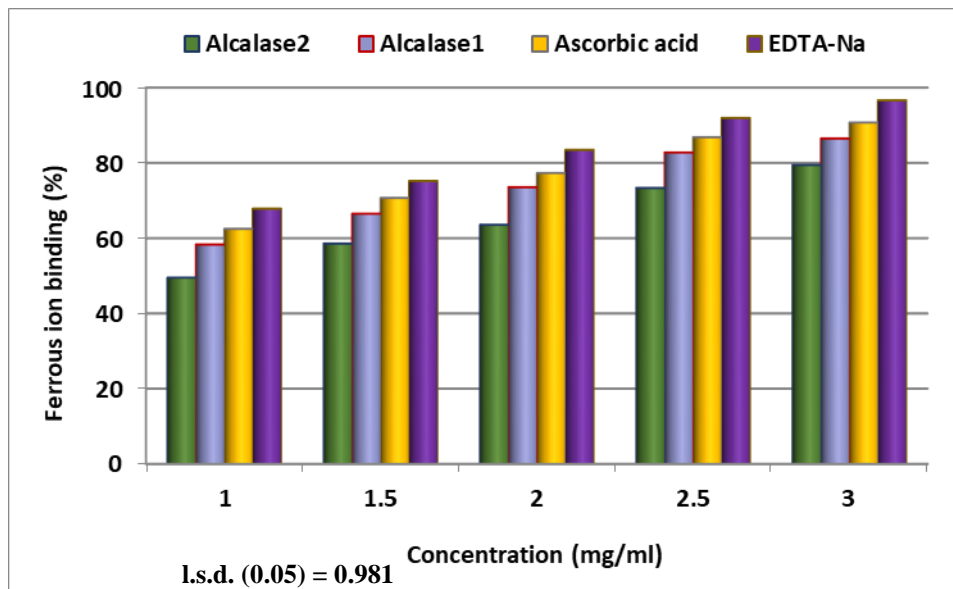


Figure 4. The Fe^{+2} chelating activity of the peptides of Iraqi buffalo whey protein concentrates prepared with Alcalase.

Reducing power

The reducing power that is depend on activity of peptide to reduce Fe^{3+} /ferricyanide complex to the Fe^{2+} form. The reducing power of peptides' fractions Alcalase1 and Alcalase2 is illustrated in (Figer5.) shows. The obtained results of peptides' ability to reduce Fe^{3+} /ferricyanide complex increases with the increasing concentration at significant effect at the level $P \leq 0.05$.for (Alcalase1,Alcalase2) at concentrations (1-3 mg/ml) and it was compared with Ascorbic acid, BHT and α -tocopherol at the same concentration. The results indicate that the peptide (Alcalase1) has highest activity than (Alcalase2) (84.44, 78.32%) respectively, at (1 mg/ml,) while the reduction activity of Ascorbic acid, BHT and α -tocopherol was 94.89%, 85.35%, and

80.75%, respectively. The ability of reducing power of (Alcalase1) continued to increase at (1.5, 2, and 2.5 mg/ml), it was (115.64, 123.60 and 130.48%) while (Alcalase2) was (108.49, 113.55 and 120.35%), respectively, to reach a maximum reduction at the highest concentration of (3 mg/ml) were (141.35 and 132.68%) for (Alcalase1, Alcalase2) respectively. It is noted that (Alcalase1) was highest activity than α -tocopherol (138.53%) at (3 mg/ml), while Ascorbic acid and BHT were more activity of reduction (158.81, 146.55%) respectively, compared with two fractions. The results agree with what was mentioned by Lin et al .(2012) who found that the amino acids Tyrosine, Histidine, Lysine and Methionine in hydrolysates of cows' milk whey proteins prepared with Alcalase contribute to enhancing the reducing power ability. The results were also agree with what was indicated by Da Silva et al .(2018). The presence of the amino acids Phenylalanine, Cysteine, Tyrosine and Arginine in the peptides from the hydrolysates of buffalo whey proteins increases the reduction reactions. Perhaps the reason for the reducing power that the containing of the amino acids Alanine, Glycine, Leucine, Isoleucine, Methionine, Phenylalanine, Proline and Valine, which have hydrophobic properties that enhance the ability of peptides reducing power, as well as a high concentrations of Histidine, Arginine and Lysine(Kong et al.,2012 ; Vavrusova et al., 2015).

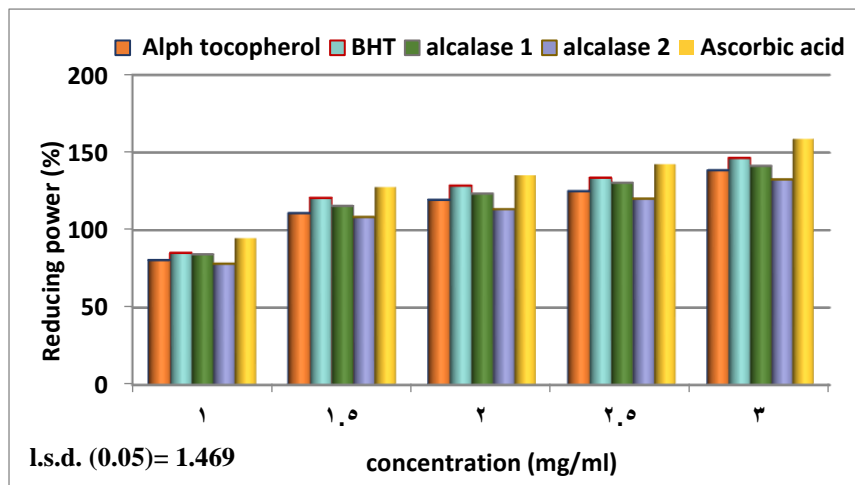


Figure 5. Reducing power of peptides from hydrolysers of Iraqi buffalo whey proteins concentrate with Alcalase.

Stability of the antioxidant activity of peptides

Effect of pH on the antioxidant activity of peptides

The antioxidant activity of peptides from hydrolysates of (WPC) with Alcalase at (3mg/ml) under different pH values is shown in (Figer6.). The results indicates that there are significant differences at the level of $P \leq 0.05$ in the antioxidant activity at different pH values. Peptides (Alcalase1,Alcalase2) at pH 7 exhibited the strongest DPPH radical scavenging activity (86.08,82.19%).While the peptides, fractions ability of radical scavenging on DPPH was less at pH 2 (74.97, 73.30%) for (Alcalase1,Alcalase2), also the DPPH radical scavenging activity of the peptides was greatly affected by the alkaline conditions. When the pH was raised to 9, the antioxidant activity decreased and at pH 11 the ability of DPPH radical scavenging sharply declined (37.14, 36.58%) for (Alcalase1, Alcalase2) .The reason for the decrease in the antioxidant activity at different pH values may be due to the fact that the pH may affect the ionization process and the ability to transfer electrons and hydrogen

donation by peptides at the carboxylic, hydroxyl and methyl groups in the peptide. In addition, the acidic medium may affect the availability of hydroxyl groups in the peptide. Which is responsible for the process of hydrogen donation and reducing free radicals, as DPPH radical SCAVENGING depends on the formation of the peptide and the number of available hydroxyl groups, in addition to the acidic medium may reduce the solubility of peptides, which hinders the reaction between peptides and free radicals (Pereira et al., 2019). The alkaline pH works to form a mixture of isomers of amino acids D and L, which differ in their biological activities, and the occurrence of the phenomenon of deaminase results in changes in the structure of the peptide, which leads to a loss of antioxidant activity in addition to that each peptide has a range of pH The peptide at which the peptide is stable and the active groups in the peptide chains are not degraded, especially small-sized peptides (Jeong et al., 2002).

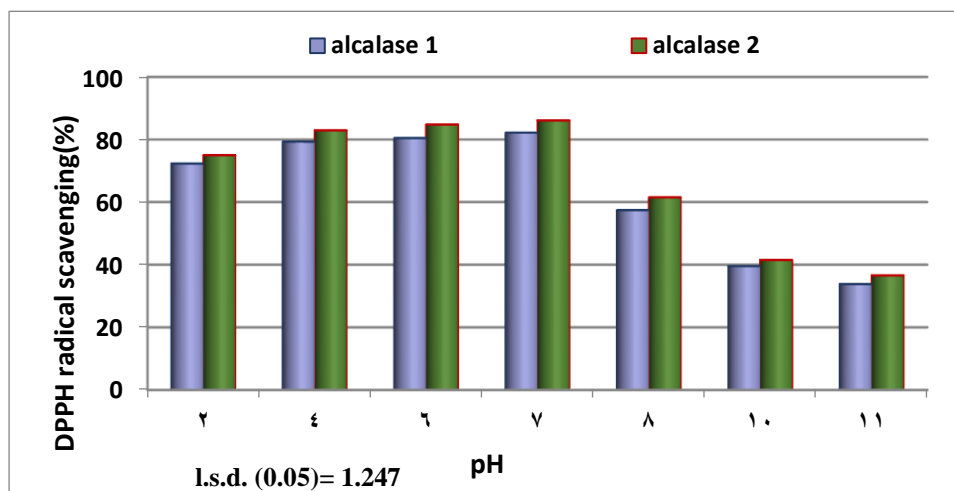


Figure 6. Effect of pH on the DPPH radical scavenging peptides from concentrated hydrolysates of Iraqi buffalo whey proteins with Alcalase.

Effect of temperature on the antioxidant activity of peptides

The results show that the DPPH radical scavenging activity of peptides (Alcalase1,Alcalase2) was significantly affected at the level of $P \leq 0.05$ with the change in temperature levels, As the increasing of temperature from 25 to 60 °C, the ability of DPPH radical scavenging increased (85.16, 87.41%) and then show a sharp decline between 60 °C and 100 °C (34.01,39.03%) (Figer7.). The reason for the decrease in the efficiency of capture at high temperatures is that the effectiveness depends mainly on the structure of the peptide in terms of the content of amino acids, their positions and molecular weight, as high temperatures affect the structure of the peptide (Pereira et al., 2019). The raising of the temperature leads to break the hydrogen bonds and the peptide bonds that play a role in the stability of the peptide towards the temperature that may explain the instability of the peptide and the denaturation (Scheraga et al.,1962).Also high temperatures may increase the hydrophobic properties of the peptides, as the hydrophobic amino acids are linked then leads to the insoluble of the peptides and denatured them (Asaduzzaman and Chun, 2013).

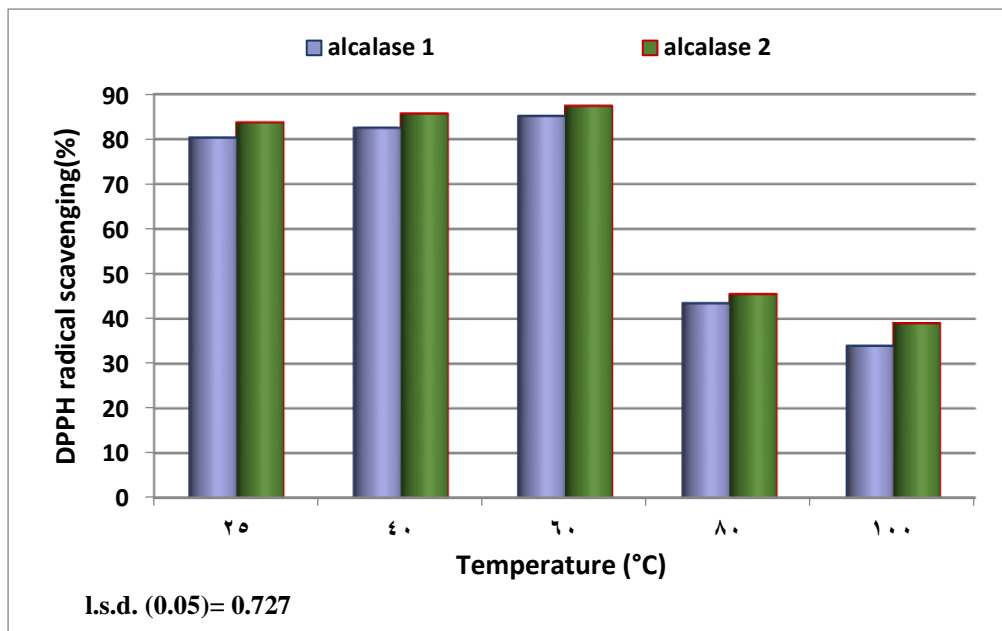


Figure7. Effect of temperature on DPPH radical scavenging a of peptides hydrolysers of Iraqi buffalo milk whey proteins concentrate with Alcalase .

Effect of NaCl on the antioxidant activity of peptides

The effect of different concentrations of NaCl on DPPH radical scavenging activity of the peptides (Alcalase1,Alcalase2) from hydrolysates of Iraqi whey proteins concentrate (Figer8.) The results showed a significant difference at the level $P \leq 0.05$ using different concentrations of NaCl. The scavenging effectiveness increased at the 2% (83.22, 85.82%) for (Alcalase1, Alcalase2). The highest activity of (Acalase1, Alcalase2) was at 4% (85.01, 86.64%) compared with activity of scavenging for the control of the two fractions (80.79, 83.91%), respectively. Then, the ability of DPPH radical scavenging of (Alcalase1, Alcalase2) declines sharply at concentration pf NaCl

at 8% to reach (38.67,41.60%).The reason for the different values of DPPH radical scavenging activity at different concentrations of NaCl may be due to the increasing of NaCl caused an increasing in the ionic strength and thus changed the structure of the peptide, which leads to its deposition, as the high salt concentrations compete with the peptides in terms of ability to bind with water, which leads to its deposition. It leads to the peptides clump together to bond with hydrogen bonds, which leads to precipitation (Zhu et al., 2014; Pereira et al., 2019).

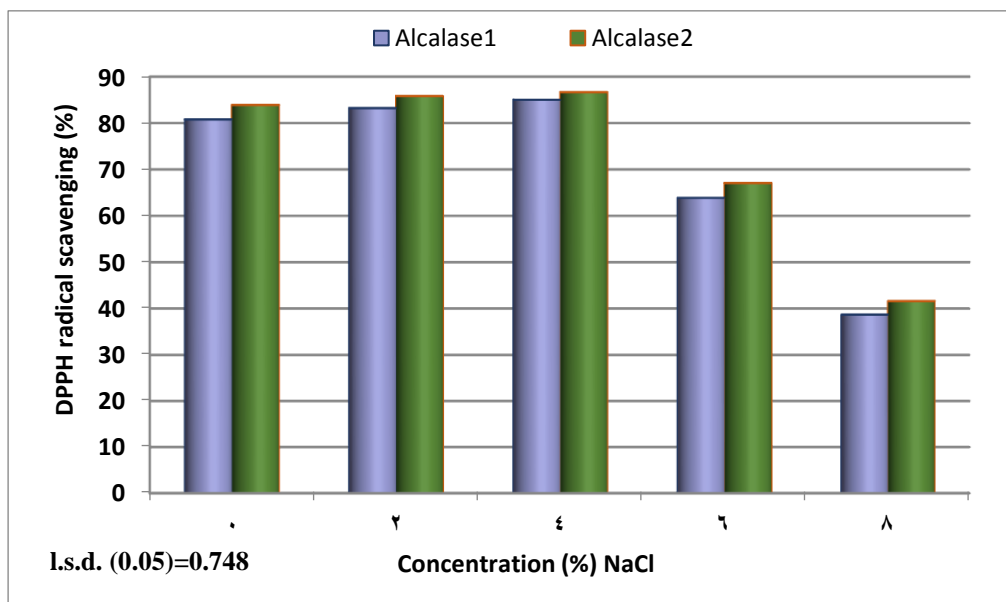


Figure 8. Effect of NaCl on DPPH radical scavenging of peptides from hydrolysates of Iraqi buffalo milk whey proteins concentrate with Alcalase.

Conclusions

The whey proteins concentrate of Iraqi buffalo milk was used in the preparation of hydrolysates using Alcalase enzyme for isolating and purifying peptides. An antioxidant activity was tested and these peptides had the ability of Fe⁺² chelating and reducing power, they had DPPH radical scavenging activity at pH 7, temperature (60 °C) and NaCl (4%).

References

- 1-Adamson, N. J. and Reynolds, E. C. (1996). Characterization of casein phosphopeptides prepared using alcalase: Determination of enzyme specificity. *Enzyme and Microbial Technology*, 19(3):202-207. [https://doi.org/10.1016/0141-0229\(95\)00232-4](https://doi.org/10.1016/0141-0229(95)00232-4)
- 2-Arora, S. ; Siondhu, J. S. and Khetra Y. (2020). Buffalo milk. *Reference Model in Food Science*, Elsevier. <https://doi.org/10.1016/B978-0-12-818766-1.00125-2>.
- 3-Asaduzzaman, A. K. M. and Chun, B. S. (2013). Recovery of functional materials with thermally stable antioxidative properties in squid muscle hydrolyzates by subcritical water. *Journal of Food Science and Technology*, 52(2):793-802.
- 4-Bassan, J. C.; Goulart, A. J.; Nasser, A. L.; Bezerra, T. M.; Garrido, S. S.; Rustiguel, C. B.; Gumiarães, L. H. S. and Monti, R. (2015). Buffalo cheese whey proteins, identification of a 24 kDa protein and characterization of their hydrolysates: *In vitro* gastrointestinal digestion. *PloS One*, 10(10): e0139550-0139568. <https://doi.org/10.1371/journal.pone.0139550>
- 5-Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, 181(4617): 1199-1200. <https://dx.doi.org/10.1038/1811199a0>
- 6-Blum, H.; Beier, H. and Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, 8(2): 93- 99.
- 7- Buffoni, J. N.; Bonizzi, I.; Pauciuolo, A.; Ramunno, L. and Feligini, M. (2011). Characterization of the major whey proteins from milk of mediterranean water buffalo (*Bubalus bubalis*). *Food Chemistry*, 127(4): 1515-1520.
- 8-Caetano-Silva, M. E.; Bertoldo-Pacheco, M. T.; Paes-Leme, A. F. and Netto, F. M. (2015). Iron-binding peptides from whey protein hydrolysates: Evaluation, isolation and

- sequencing by LC-MS/MS. *Food Research International*, (71): 132-139. <https://doi.org/10.1016/j.foodres.2015.01.008>
- 9-Da Silva, J. D. F.; Correa, A. P. F.; Kechinski, C. P. and Brandelli, A. (2018).** Buffalo cheese whey hydrolyzed with Alcalase as an antibrowning agent in minimally processed apple. *Journal of Food Science and Technology*, 55(9): 3731-3738.
- 10-Devi, K.; Haripriya, S.; Kumar, J.K. N.; Dharini, V. and Kumar, V. (2017).** Rational approach to sequential optimization of antioxidative whey protein hydrolysate production. *International Journal on Nutraceuticals, Functional Foods and Novel Foods*, 16:83-89.
- 11-Dinis, T. C.; Madeira, V. M. and Almeida, L. M. (1994).** Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxy radical scavengers. *Archives of Biochemistry and Biophysics*, 315(1):161-169.
- 12-Gregory, G.; Correa, A. P. F.; Veras, F. F. and Brandelli, A.(2016).** Bioactive peptides in buffalo whey hydrolysates. XXV Food Science and Technology Brazilian Congress (CBCTA) and the 10th CIGR Section IV International Technical Symposium, held between 24th – 27 th October 2016, at the Centro de Eventos da FAURGS, in Gramado/RS, Brazil. .
- 13-Hemalatha, S. ; Lalitha, P. and Arupriya, P. (2010).** Antioxidant activities of the extracts of aerial roots of *Pothos aurea* (Linden ex Andrel). *Der Pharma Chemica*, 2 (6): 84 – 89.
- 14-Hussein, F. A.; Chay, S. Y.; Zarei, M.; Auwal, S. M.; Hamid, A. A.; Wan Ibadullah, W. Z. and Saari, N. (2020).** Whey protein concentrate as a novel source of bifunctional peptides with angiotensin-I converting enzyme inhibitory and

- antioxidant properties: RSM study. *Foods*, 9(1):1-15. .
<https://doi.org/10.3390/foods9010064>
- 15-Jayaprakasha, H. M. and Yoon, Y. C. (2005).** Production of functional whey protein concentrate by monitoring the process of ultrafiltration. *Asian-Australasian Journal of Animal Sciences*, 18(3): 433-438.
<https://doi.org/10.5713/ajas.2005.433>
- 16-Jeong, H. J.; Lam, Y. and de Lumen, B. O. (2002).** Barley lunasin suppresses ras-induced colony formation and inhibits core histone acetylation in mammalian cells. *Journal of Agricultural and Food Chemistry*, 50(21): 5903-5908.
- 17-Jiang, B.; Zhang, X.; Yuan, Y.; Qu, Y. and Feng, Z. (2017).** Separation of antioxidant peptides from pepsin hydrolysate of whey protein isolate by ATPS of EOPO Copolymer (UCON)/Phosphate. *Scientific Reports*, 7(1): 1-12.
- 18-Khedkar, C. D.; Kalyankar, S. D. and Deosarkar, S. S. (2016).** Buffalo milk. In: *Encyclopedia of Food and Health*. Caballero, B., Finglas, P. and Toldrá, F.(eds.). 1st edn., Elsevier, Academic Press. pp: 522-528.
- 19-Kim, S. B.; Seo, I. S.; Khan, M. A.; Ki, K. S.; Lee, W. S.; Lee, H. J.; Shin, H. S. and Kim, H. S. (2007).** Enzymatic hydrolysis of heated whey: Iron-binding ability of peptides and antigenic protein fractions. *Journal of Dairy Science*, 90(9): 4033-4042. <https://doi.org/10.3168/jds.2007-0228>
- 20-Kong, B.; Peng, X.; Xiong, Y. L and Zhao, X. (2012).** Protection of lung fibroblast MRC-5 cells against hydrogen peroxide-induced oxidative damage by 0.1–2.8 kDa antioxidative peptides isolated from whey protein hydrolysate. *Food Chemistry*, 135(2): 540-547.
<https://doi.org/10.1016/j.foodchem.2012.04.122>
- 21-Kruchinin, A. G.; Savinova, O. S.; Glazunova, O. A.; Moiseenko, K. V.; Agarkova, E. Y. and Fedorova, T. V.**

- (2021). Hypotensive and hepatoprotective properties of the polysaccharide-stabilized foaming composition containing hydrolysate of whey proteins. *Nutrients*, 13(3): 2-14.
- 22-Laemmler, U. K. (1970)** . Cleavage of structural protein during the assembly of the head of bacteriophage T4 . *Nature*, 227: 680-685.
- 23-Lin, S.; Tian, W.; Li, H.; Cao, J. and Jiang, W. (2012)**. Improving antioxidant activities of whey protein hydrolysates obtained by thermal preheat treatment of pepsin, trypsin, alcalase and flavourzyme. *International Journal of Food Science and Technology*, 47(10): 2045-2051.
- 24-Ling, L.; Yang, P.; Qing-Kun, Z.; Li, H.; Yan, T.; Hao-Ru, N. and Ling, F. (2018)**. A buffalo milk antioxidant peptide and its separation and preparation method. *Patent No.* 201810310855.2. China.
- 25-Ma, Y.; Liu, J.; Shi, H. and Yu, L. L. (2016)**. Isolation and characterization of anti-inflammatory peptides derived from whey protein. *Journal of Dairy Science*, 99(9): 6902-6912.
- 26-Manzanares, P.; Gandía, M.; Garrigues, S. and Marcos, J. F. (2019)**. Improving health-promoting effects of food-derived bioactive peptides through rational design and oral delivery strategies. *Nutrients*, 11(10): 25-45.
- 27-Neto, Y. A. H.; Rosa, J. C. and Cabral, H. (2019)**. Peptides with antioxidant properties identified from casein, whey, and egg albumin hydrolysates generated by two novel fungal proteases. *Preparative Biochemistry and Biotechnology*, 49(7): 639-648.
- 28-Oyaizu, M.(1986)**. Studies on product of browning reaction prepared from glucose amine .*Japanese Journal of Nutrition*, 44(6): 307–315. <https://doi.org/10.5264/eiyogakuzashi.44.307>
- 29-Peng, X.; Xiong, Y. L. and Kong, B. (2009)**. Antioxidant activity of peptide fractions from whey protein hydrolysates

- as measured by electron spin resonance. *Food Chemistry*, 113(1): 196-201.
- 30-Pereira, A. M.; Lisboa, C. R.; Santos, T. D. and Costa, J. A. V. (2019).** Bioactive stability of microalgal protein hydrolysates under food processing and storage conditions. *Journal of Food Science and Technology*, 56(10): 4543-4551.
- 31-Schaafsma, G. (2009).** Safety of protein hydrolysates, fractions thereof and bioactive peptides in human nutrition. *European Journal of Clinical Nutrition*, 63(10): 1161-1168
- 32-Scheraga, H. A.; Némethy, G. and Steinberg, I. Z. (1962).** The contribution of hydrophobic bonds to the thermal stability of protein conformations. *Journal of Biological Chemistry*, 237(8): 2506-2508.
- 33-Shah, P. and Modi, H. A. (2015).** Comparative study of DPPH, ABTS and FRAP assays for determination of antioxidant activity. *International Journal for Research in Applied Science and Engineering Technology* 3(6): 636-641. <https://doi.org/10.1007%2Fs13197-010-0162-6>
- 34-Vavrusova, M.; Pindstrup, H.; Johansen, L. B.; Andersen, M. L.; Andersen, H. J. and Skibsted, L. H. (2015).** Characterisation of a whey protein hydrolysate as antioxidant. *International Dairy Journal*, 47: 86-93.
- 35-Wali, A.; Yanhua, G.; Ishimov, U.; Yili, A.; Aisa, H. A. and Salikhov, S. (2019).** Isolation and identification of three novel antioxidant peptides from the Bactrian camel milk hydrolysates. *International Journal of Peptide Research and Therapeutics*, 26(2):1-10.
- 36-Yang, P.; Abel-Hamid, M.; Romieh, E., Huang, L.; Zeng, Q. K.; Nong, H. R; Feng, L.; Tang, Y. and Li, L.(2020).** Effect of peptides synthesized from lactoferrin of buffalo milk

on oxidative stress in kunming mice. *Journal of Animal and Plant Sciences*, 30(1):65-71.

37-Yazdanpanah , S. ;Ehsani , M . R. and Mizani , M. (2015)

.Water mobility in accelerated ripening of UF feta cheese..

Journal of Agricultural Science Technology, 17: 663 – 674.

38-Zhu, C. Z.; Zhang, W. G.; Kang, Z. L.; Zhou, G. H. and

Xu, X. L. (2014). Stability of an antioxidant peptide extracted

from Jinhua ham. *Meat Science*, 96(2): 783-789.

