Egypt. J. Chem. **Vol. 65,** No. SI:13, pp. 1511 - 1520 (2022)

Egyptian Journal of Chemistry
http://ejchem.journals.ekb.eg/

Antibacterial and Antioxidant Activity of Sheep Whey Protein Hydrolysates and Their Fractions

Wafaa Nassar [a,b*] , Ekbal A. Ibrahim [a], Hend A. Elbarbary [a] , Hamdi A. Mohamed [a] , Håvard Jenssen [b]
[a] *Department of Food Hygiene& Control, Faculty of Veterinary medicine, Benha University*
[b] *Department of Science and Environment, Roskilde University, Denmark.*

Abstract
Sheep milk whey proteins are becoming more well-known for their bioactivity or health-promoting properties. Furthermore, the natural digestion of sheep whey proteins in the gastrointestinal tract generates peptides with a wide range of bioactivities, including antihypertensive, opioid, antibacterial, antioxidant, and immunomodulatory properties. In the current study, purified sheep whey protein has been applied by using size-exclusion chromatography (SEC), and it was hydrolyzed by pepsin and alcalase enzymes at concentration 2.5% (w/w) at different times 30, 60, 120 and 240 min. The highest active time hydrolysate was subsequently fractionated by SEC and monitored for its antibacterial and antioxidant activities and characterization using UHPLC-MS/MS. Alcalase enzyme showed a significantly ($p<0.05$) higher degree of hydrolysis reached to 30% than pepsin enzyme. While pepsin fraction (P5) characterized by significantly ($p<0.05$) higher antimicrobial activity reach to 3.18 and 2.24 log reduction against *E.coli* and *S.aureus*, respectively. For alcalase fraction (A9) exhibited significantly the highest antioxidant activity rescuing 100% of the yeast cell from Hydrogen peroxide induced oxidative stress. Proteomic analysis of the highly active fractions identified peptides from α-lactalbumin and β-Lactoglobulin from sheep whey protein with structural similarity to known antioxidant peptides and antimicrobial peptides respectively. Thus, current results supported the using food grade enzymes like alcalase and pepsin in the food industry to obtain bioactive peptides which acts as natural antimicrobial and antioxidant agent
Key words: Sheep whey protein; Size-exclusion chromatography; UHPLC-MS/MS; Antibacterial; Bioactive milk peptides; Antioxidant

1. Introduction

Whey is the primary waste product of cheese factories, accounting for 90% of milk volume and primarily consisting of 95% water, 4% lactose, 0.9% protein, and 0.8 % minerals. Because so much whey is discarded on a daily basis, it poses a serious environmental risk. Many plans have been developed to make use of whey protein due to its superior biological, nutritional, and functional properties. Whey contains β-Lactoglobulin, α-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins, lacto peroxidase and glycomacropeptides as major components [1,2].

Enzymatic hydrolysis is one of the practices that has the potential to increase whey protein revenue. This treatment, which encourages parent protein fractionation into smaller units, typically improves functional properties such as solubility, emulsifying power, and texture, potentially increasing the applicability of whey proteins in food industry. Furthermore, amino acid sequences encountered in whey proteins have the capability to enhance physiological responses after enzymatic hydrolysis, expressing antioxidant, antimicrobial, immunomodulatory, antihypertensive, angiotensin converting enzyme inhibitory, and hypocholesterolemic activities. As a result, more research was conducted on whey protein hydrolysates and bioactive peptides [3,4].

Peptic and tryptic hydrolysis of bovine whey increased the antioxidant and angiotesin- converting enzyme (ACE)-inhibitory activities of hydrolysates in vitro as discovered by [5]. Pepsin-derived caprine whey protein peptides have higher antibacterial activity against pathogens after their release [6] . Lactoferricin C (LF f 14-42) delivered by pepsin and trypsin demonstrated potent antimicrobial activity against a variety of bacteria [7] .Through the hydrolysis of β-Lactoglobulin by bacterial protease, a new peptide LAFNPTQLEGQCHV was released, which has superior antioxidant activity [2].

*Corresponding author e-mail: wafaa_nassar1991@yahoo.com.; (Wafaa Nassar).
Receive Date: 11 May 2022, **Revise Date:** 31 May 2022, **Accept Date:** 20 June 2022, **First Publish Date:** 20 June 2022
DOI: 10.21608/EJCHEM.2022.137815.6065
©2022 National Information and Documentation Center (NIDOC)

From previous reports. Most of research focused on bovine and caprine whey protein by using different commercial enzymes. On other hand sheep milk whey protein constitutes 17-22% with 70-80% β-Lactoglobulin and α-lactalbumin. Furthermore, monitoring different proteolytic enzymes derived from different sources is an intriguing approach to obtaining protein hydrolysates and bioactive peptides with desired properties [8].

Therefore, the goals of this work were to hydrolyse sheep whey protein using pepsin and alcalase enzymes under different incubation times (30, 60,120,240 min) and 2.5% (w/w) concentration and fractionate both using size exclusion chromatography. The individual fractions would be tested for antimicrobial and/or antioxidant properties, and the composition of the most active fractions would be further characterized using UHPLC-MS/MS.

2. Materials and methods

2.1 Materials

The study was carried out in Department of Science and Environment, Roskilde University, Denmark, in the time from first of February 2021 till first of August 2021.

Sheep milk was obtained from Agriculture farm (Faculty of Agriculture, Benha University, Egypt).

Alcalase enzyme 2.4 L (2.4 AU-A g-1) 112 FG (EC. 3.4.21.62 was kindly provided from Novozymes A/S Denmark. Pepsin enzyme 1:2500 (≥2500 units mg-1 protein) (EC.3.4.23.1) was purchased from Sigma (St. Louis, USA). All chemicals were of analytical grade.

2.2 Separation of whey:

Raw sheep milk (4% protein) was heated to 37 °C, then centrifuged at 2500 ×g for 30 min at 10 °C for removal of fat. Casein was separated from whey proteins by addition of 10% acetic acid to reach pH 4.6 (isoelectric point of casein), followed by centrifugation at 5000 ×g for 30 min at 4 °C). Finally, the whey was collected, dialyzed against double distilled water using porous membrane with a molecular weight cut off of 10 kDa for 48 hr at 4 °C. Then lyophilized using laboratory freeze dryer at – 55 °C, 10 Pa pressure for 48 hr and stored at -20 °C until use [9].

2.3 Purification of whey protein:

Purification of whey protein was done according to [10], using Sephadex G-25 medium grade resin on an Äkta™ pure (Uppsala, Sweden). Whey was injected at a concentration of 40 mg mL-1 and fractionated with distilled water (mobile phase) at a flow rate of 2 mL min^{-1}. All fractions were collected at regular interval using fractions collector and lyophilized, to determine protein concentration by Bradford analysis, and protein composition by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reduced condition

.

2.4 Hydrolysis of whey protein:

Hydrolysis was carried out using pepsin and alcalase, and within their optimal temperature and pH (37°C, pH 2) and (40°C and pH 8), respectively .Whey protein was dissolved in deionized water at concentration of 2.5% (w/v). The pH was maintained constant during hydrolysis either by 0.5 M NaOH or 0.5 M HCl. The degree of hydrolysis was determined at different time points (30, 60, 120 and 240 min). The hydrolysis was terminated by heat inactivation of both enzyme at 95 °C for 10 min. Hydrolysates were subjected to centrifugation at 3000 ×g for 10 min, then the supernatant was collected and lyophilized by freeze dryer, then stored at -20 °C [11].

2.5 Degree of hydrolysis (D.H):

Degree of hydrolysis was determined according to [12] , briefly the hydrolyzed mixture was mixed with 20% trichloroacetic acid at ratio(1:1), incubated at 4 °C for 30 minutes, followed by centrifugation at 3000 ×g for 10 min at 4 °C. The protein content in the supernatant was measured by Bradford analysis and expressed as mg of protein. The degree of hydrolysis was calculated as the ratio between Soluble protein content in 10% g(w/v) TCA (mg) to total protein content (mg)

$$\mathrm{D.H\%} = \frac{\text{Soluble protein content in 10\% g(w/v) TCA (mg)}}{\text{Total protein content (mg)}} \times 100$$

2.6 SDS-polyacrylamide gel electrophoresis:

The protein profile of the hydrolysates in each enzyme group was determined by using SDS-polyacrylamide gel electrophoresis under reduced conditions on 4-15% acrylamide gel, all bands appeared by using commassie brilliant blue R250 according to standard protocol of [13].

2.7 Fractionation of whey protein hydrolysates:

Highly active whey protein hydrolysates were fractionated by size exclusive chromatography using sephadex G-100 superfine grade resin on an Äkta™ pure (Cytiva, Uppsala, Sweden). Whey protein hydrolysates (20 mg mL^{-1}) were injected and eluted with distilled water at 0.8 $mLmin^{-1}$ .All fractions were collected and lyophilized to determine their antibacterial and antioxidant activities. Protein concentration in all fractions were measured by using Nano Drop (Thermo Scientific, San Jose, US) [14].

2.8 Antibacterial activity:

The antimicrobial activity of sheep whey, sheep whey hydrolysates and fractions eluted from size exclusive chromatography was determined against *Staphylococcus aureus ATCC 25923* and Escherichia *coli ATCC 25922* (Department of Science and Enviroment, Roskilde University, Denmark) as described by [15] . Test samples at concentration 10 mg mL^{-1} were dissolved in sterile distilled water, filtered with a 0.45 µm membrane filter. Single colonies of bacteria were transferred to sterile Muller Hinton broth (MHB) and grown overnight at 37 °C under constant agitation (200 rpm). The overnight cultures were diluted 1:50 in fresh growth medium and incubated with agitation at 37 °C to mid-logarithmic phase (optical density at 600nm reaching 0.4), the bacterial cultures were further diluted in fresh MHB (1:500) to obtain final inoculum concentration ranging from 2-8 $\times 10^5$ CFU mL^{-1}. Finally, 50 µL of inoculum was mixed with 50 µL of the test samples to give 10 mg mL^{-1} sample concentration, and subsequently mixed with 100 µL of MHB. The mixtures were incubated at 37 °C for 2 h, and ten-fold serially diluted in PBS before plating on Muller Hinton agar. Colony forming units were counted after incubation for 24-48 hours at 37 °C. All assays were performed in triplicates.

2.9 Minimum inhibitory concentration of highly active hydrolysates:

Minimum inhibitory concentration was measured by the microtiter broth dilution method for highly active hydrolysates as described by [16]. Briefly, 90 µL of bacterial inoculum (2-8 $\times 10^5$ CFU mL^{-1}) in mid-exponential phase were mixed with 10 µL of the tested sample at different concentrations (10, 5, 2.5, 1.25. 0.625, 0.312 and 0.156 mg ml $^{-1}$). The microtitre plates were incubated at 37 °C for 24 hours. The minimum inhibitory concentration (MIC) was determined as the lowest hydrolysates concentration resulting in no visible growth.

2.10 Yeast model for oxidative stress:

Saccharomyces cerevisiae (Department of Science and Enviroment, Roskilde University, Denmark) has been shown to parade a moderate sensitivity to H_2O_2 induced oxidative stress, so it acts as a model system for studying mitochondrial diseases, as described by [17], in brief, S. cerevisiae suspended in yeast extract peptone dextrose broth at concentration 104 CFU mL-1, were incubated with protein samples (sheep whey hydrolysates and size exclusion chromatography fractions) at concentrations of 1 mg mL^{-1}) for 1 h at 30 °C, then the oxidizing agent H_2O_2 was added (4 mmole L-1) and incubation for 48 h. After that 10 µL portion of mixture was extracted then tenth -fold serial diluted in yeast extract peptone dextrose broth containing 4 mmole L^{-1} H_2O_2 and spotted on yeast extract peptone dextrose broth agar plates containing 4 mmole L^{-1} H_2O_2. Plates were incubated at 30 °C for 48 h. Data were collected from three independent experiments and presented as survival percentage to non-treated cells.

2.11 Peptide profile by ultra-high performance liquid chromatography/tandem mass spectrometry:

Peptides were analyzed by using liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis using an ultra-high performance liquid chromatograph (UHPLC). Five µL of the size exclusion chromatography fraction of each enzyme group was injected. Peptides were trapped on a C18 column (5 µm, 5mm, 0.3mm) and separated on a 15 cm fused silica column (75 µm inner diameter) pulled and packed in-house with 1.9 µm C18 beads (Reprosil-AQ Pur, Dr. Maisch) on an Ultimate 3000 system connected to a LTQ Velos Orbitrap (Thermo Scientific, San Jose, US). The peptides were separated with a 110 min gradient with increasing buffer B (90% ACN and 0.1% formic acid), going from 5 to 30% in 70 min, 30 to 50% in 15 min, 50 to 95% in 20 min followed by a 5 min wash and re-equilibrating step. All steps were performed at a flow rate of 250 nLmin $^{-1}$. The LTQ Velos Orbitrap was operated in data-dependent top 15 mode. Full scan mass spectra were recorded in the orbitrap at a resolution of 60,000 at m/z 200 over the m/z range 375–1600 with a target value of 1 × 106 and a maximum injection time of 500 ms. CID-generated product ions were recorded in the iontrap with a maximum ion injection time set to 100 ms and a target value set to 1 × 104. Spray voltage was set to 2.2 kV, S-lens RF level at 50, and heated capillary at 300 °C. Normalized collision energy was set at 35 and the isolation window was 2 m/z [14] .

2.12 Data- and bioinformatic analysis:

LC-MS/MS data were processed using MaxQuant version 1.5.0.38 [18] with default settings. The data was searched against sheep protein sequence database from UniProt (http://www.uniprot.org). The milk bioactive peptide database(http://mbpdb.nws.oregonstate.edu) was used to identify bioactive peptides [19]. The database was searched to compare resulted peptide sequences with the identity to the database sequences.

2.13 Statistical analysis:

Data were presented as mean ± standard errors (n = 3). .Statistical analysis was performed by Graph Pad Prism 8.0. Data were analyzed by one -way ANOVA test, then multiple comparsion were made

using Tukey's test at a 95% confidence interval ($p < 0.05$) [11] .

3. Results and Discussion

3.1. Purification of sheep whey protein:

Because sheep whey protein is a more important source of protein than bovine whey protein, it is recommended that the protein be concentrated without compromising its nutritional properties [20]. In the current work, sheep whey protein was efficiently purified and concentrated using SEC (Sephadex G-25). It produced only one fractions **(Fig. 1A)** which was lyophilized and the dried whey fractions allowed for determination of protein concentration at 98% (w/w) , which corresponds to previous finding [10] . For visualization the peak eluting from the SEC was run on a SDS-PAGE gel **(Fig. 1B)**, illustrating a general desalting with all main proteins eluting in this fraction, so we complete the work on (purified whey protein).

3.2. Production of sheep whey protein hydrolysates:

The bioactivities of protein hydrolysates and peptides are heavily influenced by the enzymes used as well as the protein to be hydrolyzed. As a result, the search for suitable protein sources and proteolytic enzymes has gained traction due to the potential of hydrolysates and peptides for applications in food science, technology, nutrition, and human health [4]. In the current work, sheep whey protein (SWP) was hydrolysed by pepsin and alcalase enzyme to obtain various whey protein hydrolysates. The degree of hydrolysis was used to estimate the strength of hydrolysis, which was then visualized on SDS-PAGE [21] .

The degree of hydrolysis is an influential factor in determining the depth of whey protein fragmentation in response to enzyme exposure time [22] . In this work the degree of hydrolysis ranged from 0%- 30% (Fig 2). There is a variation in the degree of hydrolysis between both, which largely depends on the protease type, enzyme to substrate ratio and the incubation time from zero to 240 min [23] . For pepsin enzyme, there is a gradual decrease in the degree of hydrolysis after 30 min (22%) till reach significantly lower DH ($P < 0.05$) 19% after 60 min then reached the highest degree after 240 min (27%) **(Fig 2A).** This DH is higher than those reported by [24], who found that DH of sheep cheese whey after 4 hr was 15.18% . but it nearly similar to DH(25%) obtained by pepsin hydrolysis of bovine whey protein using 0.005% enzyme to substrate ratio after 4 hr [25] .

It was observed that alcalase achieved the significantly ($p < 0.05$) highest hydrolytic activity 30% after 30 min **(Fig 2B).** The superior efficacy of alcalase has been reported earlier [26]. The reasoning for its high degree of hydrolysis is its broad specificity, as it recognize more binding sites compared to the other enzymes [27]. Conversely, the decrease in degree of hydrolysis after 30 min, coincided with findings by [28] , who explained this by reduction in the number of peptides bond and inhibition of enzyme activity after intense hydrolysis.

The variation of DH can also be visualized using SDS-PAGE. in the case of pepsin **(Fig. 3A)** are clearly of the higher resistance of β-lactoglobulin (18 kDa) to be degraded by pepsin enzyme, which previously have been explained by the stabilizing effect of the intermolecular disulfide bridges [29] . On the contrary , alcalase characterized by complete hydrolysis and removal of all major protein bands **(Fig.3B),** which was explained by the low substrate specificity of this enzyme [30] .

3.3. Antibacterial activity of sheep whey protein hydrolysates:

In the current study, pepsin and alcalase enzymes were used to produce various sheep whey protein hydrolysates, each with its own specificity and cleavage site. The antibacterial activity of the hydrolysates was measured from zero to 240 minutes of hydrolysis.The best group was confirmed by using microtiter broth dilution method to measure MIC. At concentration 10 mg mL-1, unhydrolysed sheep whey protein exerts antibacterial activity against *E. coli* and *S. aureus* reached to 1.053 and 0.86 log reduction, respectively **(Fig.4).** This results contradict finding of [31] , who found that ovine scotta didn`t exert any inhibitory activity against S.aureus.

Pepsin hydrolysates exerted antibacterial activity after 30 min of hydrolysis and no significant drop in the antibacterial activity was observed as hydrolysis was allowed to continue to 240 min **(Fig.4A).** When monitoring the antibacterial potential of the hydrolysate obtained after 120 min, it was observed a non-significant ($p>0.05$) higher log reduction of 3 and 2.16, for the growth of *E. coli* and *S. aureus*, respectively. This is higher than antibacterial activity observed by [6]. Alcalase hydrolysates **(Fig.4 B)** exerted a significantly ($p< 0.05$) higher antibacterial activity against *E. coli* after 120min and against *S. aureus* after 60 min of hydrolysis. Similarly, activity appear by hydrolysis of goat whey protein by alcalase for 4 h [32]. The minimum inhibitory concentration (MIC) for pepsin and alcalase hydrolysates against *E. coli* was 0.312 and 0.625 mg mL-1 respectively. While against *S. aureus* was 0.625 and 1.25 mg mL^{-1}, respectively. This results confirmed that pepsin hydrolysates had higher bactericidal activity than alcalase hydrolysates.

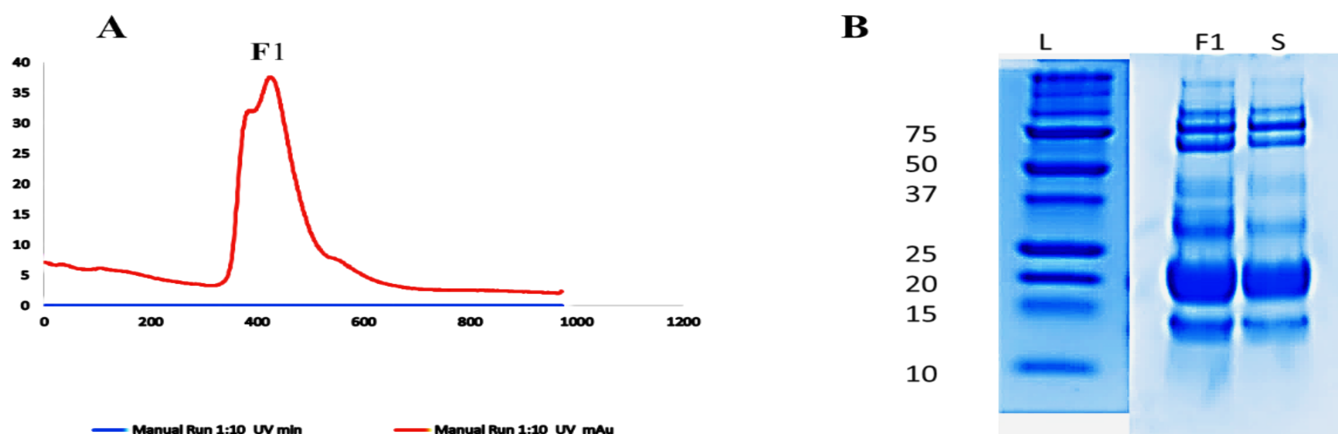

Figure 1.A: Electrophoretic patterns of sheep whey proteins on SEC G-25. **B:** SDS-PAGE of crude whey protein before purification (S), peak 1 (F1) and the molecular ladder (L).

Table (1): Sequences of bioactive peptides from Lactoferrin, α-lactalbumin and β-lactoglobulin found in active fractions of pepsin and alcalase enzyme groups.

Peptide source	Peptide sequence
Pepsin fraction 5 (P5)	
β-Lactoglobulin	**AASDISLL f(43-50)**
	AASDISLLDAQSAPLRV f(43-59)
	DTDYKKYL f(114-121)
	EELKPTPEGNLEIL f(62-75)
	FKIDALNE f(100-107)
Lactoferrin	**ALGFVRIPSKVDSAL f (324-338)**
pepsin fraction 10 (P10)	
β-Lactoglobulin	**ALEKFDKALKA f(150-157)**
	ALEKFDKALKALPMH f(150-164)
	DTDYKKYL f(114-121)
	EELKPTPEGNLEIL f(62-75)
lactoferrin	**AEIYGTEKSPQTHYYA f(97 -113)**
	ARSVDGKENL f(276 - 285)
α-lactalbumin	**QKLKDLKDYGGVSLPEW f(29-45) W5QD52|W5QD52_SHEEP Alpha-lactalbumin**
Alcalase fraction 2 (A2)	
β-Lactoglobulin	**AEKTKIPAVF f(91-99)**
	AEPEQSLA f(129-136)
	DISLLDAQSAPLRV f(46-59)
	EELKPTPEGNL f(62-72)
	IAEKTKIPA f(89-97)
Lactoferrin	**ALGFVRIPSKVDSAL f(324-338)**
	AQVPSHAVVA f(267-276)
	AQVPSHAVVAR f(267-277)
Alcalase fraction 9 (A9)	
β-Lactoglobulin Lactoferrin	**DTDYKKYLL f(114-121)**
	IAEKTKIPA f (90-97)
	DKALKALPM f (155-163)
	ALGFVRIPSKVDSAL f(324-338)
α-lactalbumin	**DDLTDDIM f(102-109)**
	EVFQKLKDL f(26- 34)
	EVFQKLKDLKDY f(26- 37)
	EVFQKLKDLKDYGGV f(26-40)

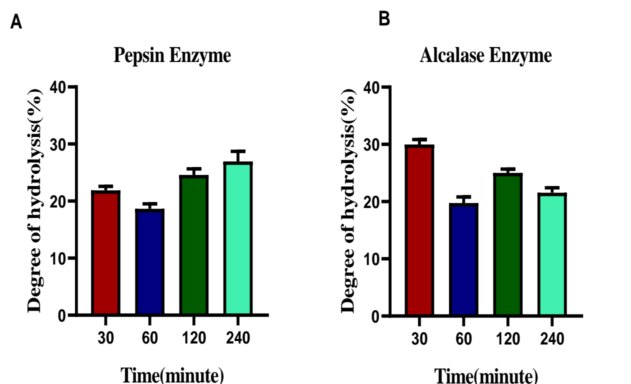

Figure 2: Degree of hydrolysis (DH) of whey protein by using different enzymes: pepsin (A); Alcalase (B). The experiment of WPC hydrolysis was carried out in triplicate (± SEM).

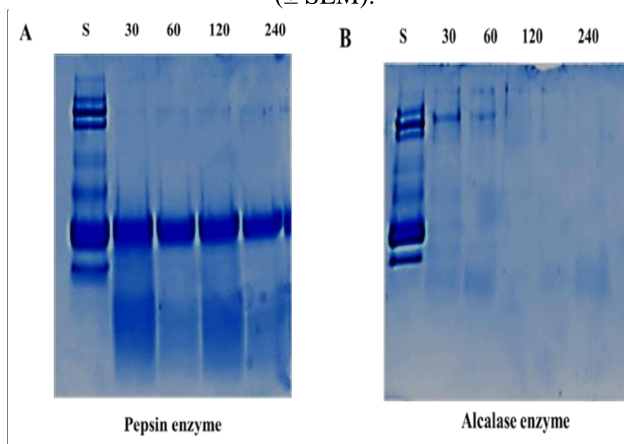

Figure 3. Sheep whey protein before and after hydrolysis, visualized on SDS-PAGE. Whey protein before hydrolysis (S), and whey protein samples digested for 30, 60, 120 and 240minutes.

A. pepsin enzyme B. alcalase enzyme

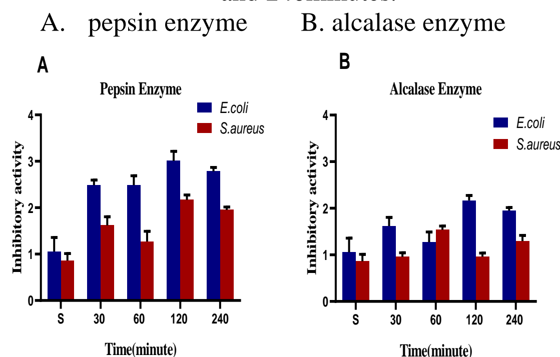

Figure 4. Antibacterial activity of whey protein hydrolysates of pepsin and alcalase enzymes against *E. coli* and *S. aureus*. Antibacterial activity is represented as log N/N1 where N refers to the control number of colonies without antibacterial material, and N1 refers to the number of colonies containing whey protein hydrolysates after an incubation period of 2 h. The assays were performed in triplicate. >1 indicate bactericidal activity, S. crude whey protein.

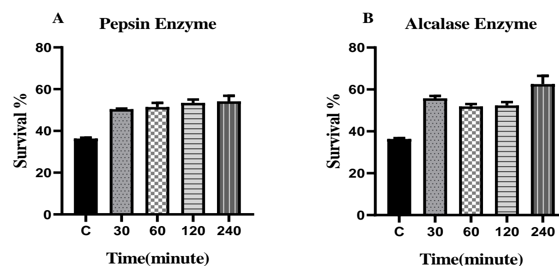

Figure 5: Viability % of yeast cell oxidized by H_2O_2 by using different whey protein hydrolysates. Viability of H_2O_2-treated yeast cells was measured by colony counting on yeast extract peptone dextrose broth agar plates. Viability (%) was represented as survival percentage to the non-induced cells.

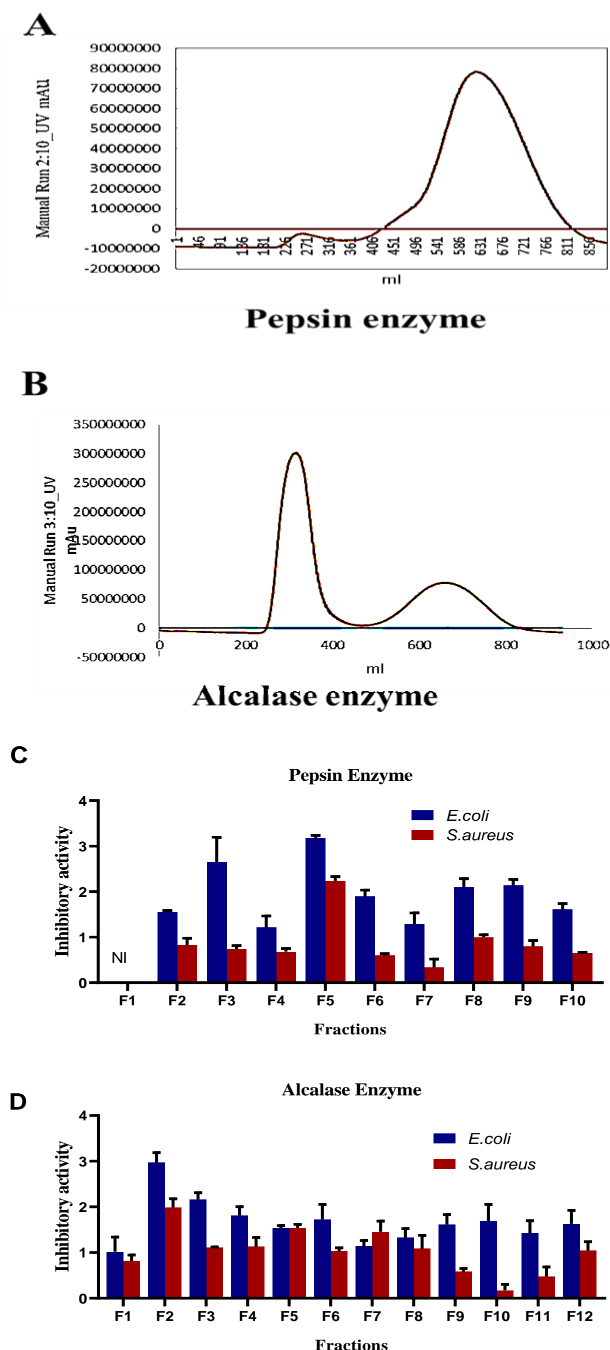

Figure 6. A, B: Size- exclusion chromatography separation of pepsin and alcalase hydrolysates, respectively. **C, D:** Antibacterial activity of whey protein peptides from pepsin and alcalase enzyme against *E. coli* and *S. aureus*. Antibacterial activity is represented as log N/N1, where N refers to the control number of colonies without antibacterial material, and N1 refers to the number of colonies containing antibacterial material (whey protein hydrolysates) after an incubation period of 2 hours. The assays were performed in triplicate. >1 indicate bactericidal activity.

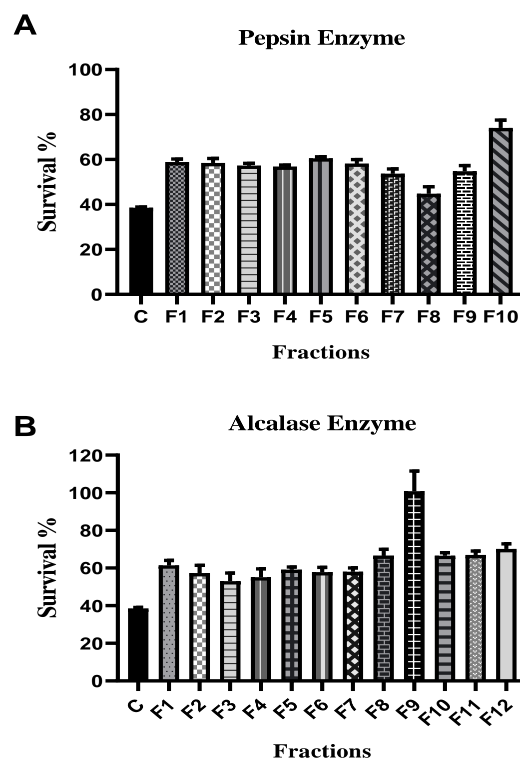

Figure 7: Viability of yeast cell oxidized by H2O2 by using pepsin (A) and alcalase (B) sheep whey protein peptides. Viability of H2O2-treated yeast cells was measured by colony counting on yeast extract peptone dextrose broth agar plates. Viability was represented as survival percentage (%) to the non-induced cells

3.4. The effect of various whey protein hydrolysates on yeast cell oxidative stress tolerance:

Oxidative stress is a condition linked to diseases such as rheumatoid arthritis and cancer that occurs as a result of damage to vital molecules such as DNA, lipids, and proteins caused by an increase in free radicals such as reactive oxygen species (ROS) and H_2O_2. As a result, natural antioxidants play an important role in preventing oxidative stress [33] . In this study, we used sheep whey protein hydrolysates from different time points of hydrolysis, and monitored how they could affect survival of yeast cell **(Fig. 5).** All whey protein hydrolysates groups exhibited a significant improvement in the survival rate of yeast cells, and increased hydrolysis time generally seemed to produce hydrolysates with higher antioxidant properties as mentioned by [2]. Alcalase hydrolysates exerted a significant ($p<0.05$) survival rate reach to 63% higher than pepsin hydrolysates (54%) **(Fig. 5 A, B)**. These results were higher than the antioxidant activity of alcalase hydrolysed sheep plasma protein hydrolysates [34].

3.5. Fractionation of hydrolysates by size- exclusion chromatography:

The most active time dependent hydrolysates of each enzyme was fractionated by SEC **(Fig. 6A,B)**, the elution profile mainly consists of two peaks and each peak was divided in multiple smaller segments. The antibacterial activity of all hydrolysate fractions were assayed against E. coli and S. aureus. The first peak from pepsin enzyme showed no antibacterial activity, which might be explained by the large size of the proteins in these fractions **(Fig. 6 C).**

In the case of pepsin the highest antibacterial activity against *E. coli* and *S. aureus* was observed in the center of the second peak (F5) **(Fig. 6C)** reaching to 3.18 and 2.24 log reduction, respectively, the activity against *E. coli* was also quite high towards the very end of peak, while for S. aureus activity then was almost lost at the end of the peak.

In contrast, the activity of the early eluting fractions (F2) from the alcalase hydrolysate was more active against *E.coli* and *S.aureus* reaching to 2.9 and 1.9 log reduction, respectively. The activity was significantly decreased till diminished at end of the second peak **(Fig. 6 D).**

The antibacterial activity of enzymatic hydrolysed sheep whey protein showed higher selectivity for *E. coli* than *S. aureus*, as drawn earlier in a study on goat whey protein hydrolysates, and the authors used transmission electron microscopy to visualize destruction of E.coli bacterial cell wall, while their hydrolysates only had bacteriostatic activity against *S.aureus* [32].

The size-exclusion chromatography fractions were also evaluated for their effect on yeast cell oxidative stress tolerance. When compared to the control group (yeast cell + 4 mmole L^{-1} H_2O_2), fractionated hydrolysates from pepsin and alcalase significantly increased S. cerevisiae survival **(Fig. 7A,B).** Late eluting fractions of the second peak had all highest antioxidant properties, ranging from 74%-100% yeast cell viability. From these results it is apparent that all highly active fractions exhibit comparable antioxidant activity, but when ranking them according to potency [alcalase (F9) > pepsin (P10)], it is also apparent that they differ slightly in their elution time. This results confirmed that alcalase sheep whey protein has a significantly ($p< 0.05$) higher antioxidant activity than pepsin ones.

The main reason for sheep whey protein's antioxidant activity may be due to its higher content of sulphur-containing amino acids cysteine and methionine, which improve antioxidant mechanisms through intracellular glutathione conversion [35].

The hydrolysed sheep whey protein is a good source for peptides which can be used to protect live eukaryotic cell against oxidative stress and prevent cell damage as in [35].

3.6. Identification of peptide sequences in highly active size- exclusion chromatography fraction:

The highly active SEC fraction from each enzyme group was subjected to a proteomic analysis, which revealed that the fractions contain peptide sequences from three major whey protein groups (Lactoferrin, α-Lactalbumin and β-Lactoglobulin). Most of the identified peptides were identical to known bioactive peptides **(Table 1).** For example from lactoferrin , the peptide AEIYGTEKSPQTHYYA and ARSVDGKENL derived through pepsin hydrolysis and separated in fraction 10, thus named P10, is nearly similar to antioxidant peptides AEIYGTKESPQTHYY and SVDGKEDLIW from bovine lactoferrin and buffalo lactoferrin ,respectively [36],While lactoferrin peptide sequence in A2 AQVPSHAVVA was nearly similar to antimicrobial peptides from kappa casein [37] .For α-lactalbumin peptides, they were mainly concentrated in fraction A9, so it mainly support the idea that fraction A9 has significantly the highest antioxidant activity. For example EVFQKLKDL was nearly similar to antioxidant peptides from bovine α-lactalbumin [38]. The peptides are also characterized as rich in hydrophobic residues, a second hallmark of antioxidant peptides [39].

Regarding β-Lactoglobulin it is mainly concentrated in fraction P5, for peptides AASDISLL , AASDISLLD and AASDISLLDAQSAPLRV are quite similar to antimicrobial peptides against E.coli derived from caprine β-Lactoglobulin as SLAMAASDISLL and DAQSAPLR [40].These findings indicate of the presence of dual activities (antibacterial and antioxidant) of sheep whey protein hydrolysates.

4. Conclusion

In the current work, the purified sheep whey protein was hydrolysed by pepsin and alcalase enzymes demonstrating different degree of hydrolysis. Alcalase was superior to pepsin enzyme, characterized by the highest degree of hydrolysis and producing a large number of peptides with significantly higher antioxidant properties, while pepsin enzyme characterized by higher antibacterial activity against E.coli and S.aureus. Proteomic analysis of the hydolysates allowed to identify peptides with antibacterial and antioxidant activites. These findings may argue the future application of sheep whey protein as bio preservatives and natural antioxidant agent.

5. Declaration of competing interests

The authors declare no competing personal or financial interests.

***6.* Acknowledgements**

The work has in part been funded by (exchange grant). The authors are also grateful for technical support with UHPLC-MS/MS by Rosa Rakownikow Jersie-Christensen.

7. References

1. Siso, M.I.G. The biotechnological utilization of cheese whey: A review. Bioresour. Technol. 1996, 57, 1–11, doi:10.1016/0960-8524(96)00036-3.

2. Corrêa, A.P.F.; Daroit, D.J.; Fontoura, R.; Meira, S.M.M.; Segalin, J.; Brandelli, A. Hydrolysates of sheep cheese whey as a source of bioactive peptides with antioxidant and angiotensin-converting enzyme inhibitory activities. Peptides 2014, 61, 48–55, doi:10.1016/j.peptides.2014.09.001.
3. Madureira, A.R.; Tavares, T.; Gomes, A.M.P.; Pintado, M.E.; Malcata, F.X. Invited review: Physiological properties of bioactive peptides obtained from whey proteins. J. Dairy Sci. 2010, 93, 437–455, doi:10.3168/jds.2009-2566.
4. Sarmadi, B.H.; Ismail, A. Antioxidative peptides from food proteins: A review. Peptides 2010, 31, 1949–1956, doi:10.1016/j.peptides.2010.06.020.
5. Adjonu, R.; Doran, G.; Torley, P.; Agboola, S. Screening of whey protein isolate hydrolysates for their dual functionality: Influence of heat pre-treatment and enzyme specificity. Food Chem. 2013, 136, 1435–1443, doi:10.1016/j.foodchem.2012.09.053.
6. Théolier, J.; Hammami, R.; Labelle, P.; Fliss, I.; Jean, J. Isolation and identification of antimicrobial peptides derived by peptic cleavage of whey protein isolate. J. Funct. Foods 2013, 5, 706–714, doi:10.1016/j.jff.2013.01.014.
7. Kimura, M.; Nam, M.-S.; Ohkouchi, Y.; Kumura, H.; Shimazaki, K.; Yu, D.-Y. Antimicrobial Peptide of Korean Native Goat Lactoferrin and Identification of the Part Essential for This Activity. Biochem. Biophys. Res. Commun. 2000, 268, 333–336, doi:10.1006/bbrc.2000.2141.
8. Park, Y.W.; Juárez, M.; Ramos, M.; Haenlein, G.F.W. Physico-chemical characteristics of goat and sheep milk. Small Rumin. Res. 2007, 68, 88–113, doi:10.1016/j.smallrumres.2006.09.013.
9. Salami, M.; Moosavi-Movahedi, A.A.; Ehsani, M.R.; Yousefi, R.; Haertlé, T.; Chobert, J.M.; Razavi, S.H.; Henrich, R.; Balalaie, S.; Ebadi, S.A.; et al. Improvement of the antimicrobial and antioxidant activities of camel and bovine whey proteins by limited proteolysis. J. Agric. Food Chem. 2010, 58, 3297–3302, doi:10.1021/jf9033283.
10. Rojas, E.E.G.; dos Reis Coimbra, J.S.; Minim, L.A.; Zuniga, A.D.G.; Saraiva, S.H.; Minim, V.P.R. Size-exclusion chromatography applied to the purification of whey proteins from the polymeric and saline phases of aqueous two-phase systems. Process Biochem. 2004, 39, 1751–1759, doi:10.1016/j.procbio.2003.07.002.
11. De Gobba, C.; Espejo-Carpio, F.J.; Skibsted, L.H.; Otte, J. Antioxidant peptides from goat milk protein fractions hydrolysed by two commercial proteases. Int. Dairy J. 2014, 39, 28–40, doi:10.1016/j.idairyj.2014.03.015.
12. Hoyle, N.T.; Merrltt, J.H. Quality of Fish Protein Hydrolysates from Herring (Clupea harengus). J. Food Sci. 1994, 59, 76–79, doi:10.1111/j.1365-2621.1994.tb06901.x.
13. Laemmli, U.K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 1970, 227, 680–685.
14. Otte, J.; Shalaby, S.M.A.; Zakora, M.; Nielsen, M.S. Fractionation and identification of ACE-inhibitory peptides from α-lactalbumin and β-casein produced by thermolysin-catalysed hydrolysis. Int. Dairy J. 2007, 17, 1460–1472, doi:10.1016/j.idairyj.2007.04.008.
15. Pellegrini, A.; Thomas, U.; Bramaz, N.; Hunziker, P.; Von Fellenberg, R. Isolation and identification of three bactericidal domains in the bovine α-lactalbumin molecule. Biochim. Biophys. Acta - Gen. Subj. 1999, doi:10.1016/S0304-4165(98)00165-2.
16. Saporito, P.; Vang Mouritzen, M.; Løbner-Olesen, A.; Jenssen, H. LL-37 fragments have antimicrobial activity against Staphylococcus epidermidis biofilms and wound healing potential in HaCaT cell line. J. Pept. Sci. 2018, 24, 1–12, doi:10.1002/psc.3080.
17. Ibrahim, H.R.; Isono, H.; Miyata, T. Potential antioxidant bioactive peptides from camel milk proteins. Anim. Nutr. 2018, 4, 273–280, doi:10.1016/j.aninu.2018.05.004.
18. Cox, J.; Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 2008, 26, 1367–1372, doi:10.1038/nbt.1511.
19. Nielsen, S.D.; Beverly, R.L.; Qu, Y.; Dallas, D.C. Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. Food Chem. 2017, 232, 673–682, doi:10.1016/j.foodchem.2017.04.056.
20. Minj, S.; Anand, S. Whey Proteins and Its Derivatives: Bioactivity, Functionality, and Current Applications. Dairy 2020, 1, 233–258, doi:10.3390/dairy1030016.
21. Wang, R.; Han, Z.; Ji, R.; Xiao, Y.; Si, R.; Guo, F.; He, J.; Hai, L.; Ming, L.; Yi, L. Antibacterial Activity of Trypsin-Hydrolyzed Camel and Cow Whey and Their Fractions. Animals 2020, 10, 337, doi:10.3390/ani10020337.
22. You, L.; Zhao, M.; Cui, C.; Zhao, H.; Yang, B. Effect of degree of hydrolysis on the antioxidant activity of loach (Misgurnus anguillicaudatus) protein hydrolysates. Innov. Food Sci. Emerg. Technol. 2009, 10, 235–240, doi:10.1016/j.ifset.2008.08.007.
23. Klompong, V.; Benjakul, S.; Kantachote, D.; Shahidi, F. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (Selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 2007, 102, 1317–1327, doi:10.1016/j.foodchem.2006.07.016.
24. Preci, D.; Fernandes, I.A.; Valduga, E.; Cansian, R.L.; Steffens, J.; Steffens, C.; Brião, V.B. Hydrolysis of sheep's milk whey concentrate by membrane and evaluation of the antioxidant and antimicrobial

properties. Sci. Plena 2021, 17, 1–9, doi:10.14808/sci.plena.2021.021501.
25. Osman, A.; Merwad, A.-R.M.; Mohamed, A.H.; Sitohy, M. Foliar Spray with Pepsin-and Papain-Whey Protein Hydrolysates Promotes the Productivity of Pea Plants Cultivated in Clay Loam Soil. Molecules 2021, 26, 2805, doi:10.3390/molecules26092805.
26. Ou, K.; Liu, Y.; Zhang, L.; Yang, X.; Huang, Z.; Nout, M.J.R.; Liang, J. Effect of Neutrase, Alcalase, and Papain Hydrolysis of Whey Protein Concentrates on Iron Uptake by Caco-2 Cells. J. Agric. Food Chem. 2010, 58, 4894–4900, doi:10.1021/jf100055y.
27. Shu, G.; Huang, J.; Bao, C.; Meng, J.; Chen, H.; Cao, J. Effect of Different Proteases on the Degree of Hydrolysis and Angiotensin I-Converting Enzyme-Inhibitory Activity in Goat and Cow Milk. Biomolecules 2018, 8, 101, doi:10.3390/biom8040101.
28. Fenoglio, C.; Vierling, N.; Manzo, R.; Ceruti, R.; Sihufe, G.; Mammarella, E. Whey Protein Hydrolysis with Free and Immobilized Alcalase®: Effects of Operating Parameters on the Modulation of Peptide Profiles Obtained. Am. J. Food Technol. 2016, 11, 152–158, doi:10.3923/ajft.2016.152.158.
29. Le Maux, S.; Bouhallab, S.; Giblin, L.; Brodkorb, A.; Croguennec, T. Bovine β-lactoglobulin/fatty acid complexes: binding, structural, and biological properties. Dairy Sci. Technol. 2014, 94, 409–426, doi:10.1007/s13594-014-0160-y.
30. Perona, J.J.; Craik, C.S. Structural basis of substrate specificity in the serine proteases. Protein Sci. 1995, 4, 337–360, doi:10.1002/pro.5560040301.
31. Cabizza, R.; Fancello, F.; Petretto, G.L.; Addis, R.; Pisanu, S.; Pagnozzi, D.; Piga, A.; Urgeghe, P.P. Exploring the DPP-IV Inhibitory, Antioxidant and Antibacterial Potential of Ovine “Scotta” Hydrolysates. Foods 2021, 10, 3137, doi:10.3390/foods10123137.
32. Osman, A.; Goda, H.A.; Abdel-Hamid, M.; Badran, S.M.; Otte, J. Antibacterial peptides generated by Alcalase hydrolysis of goat whey. LWT - Food Sci. Technol. 2016, 65, 480–486, doi:10.1016/j.lwt.2015.08.043.
33. Ibrahim, H.R.; Nanbu, F.; Miyata, T. Potent antioxidant peptides derived from honey major protein enhance tolerance of eukaryotic cells toward oxidative stress. Food Prod. Process. Nutr. 2021, 3, 11, doi:10.1186/s43014-021-00052-2.
34. Hou, C.; Wu, L.; Wang, Z.; Saguer, E.; Zhang, D. Purification and Identification of Antioxidant Alcalase-Derived Peptides from Sheep Plasma Proteins. Antioxidants 2019, 8, 592, doi:10.3390/antiox8120592.
35. Kerasioti, E.; Stagos, D.; Georgatzi, V.; Bregou, E.; Priftis, A.; Kafantaris, I.; Kouretas, D. Antioxidant Effects of Sheep Whey Protein on Endothelial Cells. Oxid. Med. Cell. Longev. 2016, 2016, 6585737, doi:10.1155/2016/6585737.
36. Hagiwara, T.; Shinoda, I.; Fukuwatari, Y.; Shimamura, S. Effects of Lactoferrin and Its Peptides on Proliferation of Rat Intestinal Epithelial Cell Line, IEC-18, in the Presence of Epidermal Growth Factor. Biosci. Biotechnol. Biochem. 1995, 59, 1875–1881, doi:10.1271/bbb.59.1875.
37. López-expósito, I.; Minervini, F.; Amigo, L.; Recio, I. Identification of Antibacterial Peptides from Bovine κ-Casein. J. Food Prot. 2006, 69, 2992–2997, doi:10.4315/0362-028X-69.12.2992.
38. Jacquot, A.; Gauthier, S.F.; Drouin, R.; Boutin, Y. Proliferative effects of synthetic peptides from β-lactoglobulin and α-lactalbumin on murine splenocytes. Int. Dairy J. 2010, 20, 514–521, doi:10.1016/j.idairyj.2010.02.013.
39. Dávalos, A.; MigueL, M.; Bartolomé, B.; López-fandiño, R. Antioxidant Activity of Peptides Derived from Egg White Proteins by Enzymatic Hydrolysis. J. Food Prot. 2004, 67, 1939–1944, doi:10.4315/0362-028X-67.9.1939.
40. Almaas, H.; Eriksen, E.; Sekse, C.; Comi, I.; Flengsrud, R.; Holm, H.; Jensen, E.; Jacobsen, M.; Langsrud, T.; Vegarud, G.E. Antibacterial peptides derived from caprine whey proteins, by digestion with human gastrointestinal juice. Br. J. Nutr. 2011, 106, 896–905, doi:10.1017/S0007114511001085.