Metal-Binding Capacity of Whey-Natural Peptides: Experimental Study and *in vitro* FRAP-Antioxidant Activity

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Abstract: Whey contains proteins and peptides that allow obtaining ion-complexes, which are bioactive and can be used in the pharmaceutical and food industries. In this paper, the complexation levels of Fe(II), Ca(II), and Mg(II) with whey peptides were evaluated, as well as the antioxidant activity. Sweet whey was treated with TCA (24%) to precipitate the high-molecular-mass proteins, and the resulting whey was dialyzed and freeze-dried. Whey powder was resuspended in an aqueous solution containing the ions separately. Metal-peptide interaction was evaluated by measurement of free ions. Besides, the molecular weight of peptides (by SDS-PAGE) and the antioxidant activity (FRAP assay) were determined. According to the results, higher metal fixation was observed to Ca(II) and Fe(II); likewise, Fe(II)-peptide fraction showed the highest antioxidant activity, increasing five-fold over sweet whey. In conclusion, whey protein hydrolysis is not essential to obtain peptides with metal binding and antioxidant activities.

Keywords: whey-peptides; metal binding; antioxidant activity.

1. Introduction

Whey is the residual fraction of milk after coagulation and casein separation obtained during cheese processing [1]. Based on the production process, whey is classified into two groups "sweet" and "acid". Sweet whey (pH 6-7) is obtained by adding proteolytic enzymes, while acid whey (pH 3.6-4.5) is the result of the acidification of milk by a fermentation process or by the addition of organic acids to coagulate the caseins [2, 3].

Differences in the chemical composition of whey depend on the origin of milk, the type of cheese manufactured, and the technological process used. However, whey components include protein, lactose, salts, vitamins, and peptides. Also, whey contains bioactive proteins and peptides, such as lactoferrin and casein macro peptide (CMP) [4].

Recently, it has been reported that peptides with common structural properties are found within whey, such as short amino acid chains (2 to 9), hydrophobic amino acid residues in addition to proline, lysine, or arginine groups, and resistance to the action of digestive

peptidases, which allows their absorption and passage into the bloodstream, without alteration [5].

Furthermore, whey proteins can interact with cations by forming intermolecular salt links, electrostatic protection (shielding), and direct linkage to protein binding sites. These interactions can change the conformation of proteins and peptides, affecting the biological functionality of industrialized whey protein powders, such as whey proteins concentrated (WPC) [6].

Protein-bound metal ions could have the advantage of forming complexes with applications in the field of health and food science and technology [7]. An example of this is the use of protein-metal systems as supplements.

In fact, it is known that the deficiency in the intake of some minerals causes a failure in the body, which leads to the suffering of diseases like osteoporosis, hypertension, and anemia [8]. Among the minerals that have received the most attention are Ca, Fe, and Mg, which perform important functions related to human health [9]. Since foods are the main source of these minerals and are not always bioavailable, alternatives have been sought to improve their systematical absorption.

Thus, considering the characteristics of whey proteins and the role played by many peptides in health [4, 5], this work aimed to evaluate the metal-binding activity of peptides from whey by complexation with Fe(II), Mg(II) and Ca(II) to consider their potential use in fields such as medicine, human health, and food science and technology.

2. Materials and Methods

2.1. Reagents.

All reagents used were analytical grade. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), and ferrozine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid, hydrochloric acid, and hydroxylamine were acquired from J.T. Baker (Center Valley, PA, USA). Solutions of arsenazo III and magnesium xylidyl were obtained from Spinreact (Barcelona, Spain). All solutions were prepared with deionized water (0.09 μS cm^{-1}). Acrylamide, bisacrylamide, cross-linker, and Coomassie Blue G-250 were purchased from Bio-Rad (Hercules, CA-USA).

2.2. Whey.

2.2.1. Sample.

Whey was obtained by enzymatic coagulation of caseins from 8 L of whole milk. Milk was skimmed and pasteurized at 90ºC for 10 min. Skim milk was heated at 35ºC, and 4 mL of renin was added, leaving for 45 min to obtain the curd, which was cut-filtered. Later, whey was pasteurized (90ºC for 10 min) and stored at -20ºC in an amber flask of 100 mL.

2.2.2. Physicochemical composition.

Physicochemical parameters were measured according to Alomirah and Alli [10]. The composition of whey was performed using Lactoscan MCC50. Total solid, protein, pH, and conductivity were determined. Whey was analyzed at room temperature without previous treatment.

2.2.3. Sample preparation.

Proteins of high molecular weight were precipitated with a 24 % trichloroacetic acid (TCA) solution, according to Mileriene *et al*. [11] methodology with some modifications. A 250-mL aliquot of whey was transferred into the beaker, then 10.5 mL of TCA solution was added, and the reaction was carried out for 3 h. After that, the mixture was centrifuged at 18 000 x g for 10 min. The supernatant was dialyzed using deionized water (0.09 µS cm^{-1}) at 4 ℃ with slow stirring until the conductivity value was constant. The conductivity was measured with a conductivity meter (ORION-130). Later, the liquid was freeze-dried (Freeze Dry System/Freezone 4.5 de Labconco), and the whey concentrate (WC) was stored in refrigeration until its use.

2.3. Evaluation of metal binding capacity.

Metal solutions (Ca^{2+}, Fe^{2+}, and Mg^{2+}) were prepared from their corresponding chlorides, according to Table 1. The recommended daily intakes (RDI) were considered for experimental conditions [9]. Samples of WC (500 mg) were resuspended in 10 mL of each metal (Table 1). Mixtures were stirred at room temperature, and a sample was taken each hour for 5 hours to measure the amount of free metal ions. All analyses were carried out in triplicate.

Table 1. Experimental conditions for the study of the metal binding capacity.

Experimental system	Metal ion	Concentration, mgL^{-1}
1	Ca(II)	700
2		800[1]
3		900
4	Fe(II)	10
5		15[1]
6		20
7	Mg(II)	250
8		350[1]
9		450

[1]These concentrations correspond to Recommended Daily Intake (RDI) for each metal.

2.3.1. Calcium.

The determination was performed according to Brown and Rydvist [12] with modifications proposed by Zhang *et al*. [13]. An aliquot of 50 µL of the sample was diluted 1:10 in a buffer solution of phosphate (1mol L^{-1}, pH 8), and 10 µL of the diluted solution was added at 1 mL of arsenzo III. The blue complex of calcium-arsenazo formed was measured at 650 nm. The concentration of free calcium ions was calculated by comparing it with a calibration curve from 0 to 50 mgL^{-1} of Ca^{2+}.

2.3.2. Iron.

The methodology proposed by Jrad *et al*. [14] was used with some modifications. An aliquot of the sample (500 µL) was added at 100 µL of hydroxylamine 1.4 mol L^{-1} in HCl 0.1 mol L^{-1}. The reaction was carried out for 10 min at room temperature, and then 100 µL of ferrocin (pH = 9.5) was added. The magenta complex formed was measured at 562 nm. The concentration of free ferrous ions was calculated by comparing it with a calibration curve from 0 to 2 mgL^{-1} of Fe^{2+}.

2.3.3. Magnesium.

A reaction between magnesium and Magon sulfonate (an alkaline solution of magnesium xylidyl) was developed. An aliquot of 50 µL of the sample was diluted 1:10 in Britton-Robinson buffer solution (0.4mol L^{-1}, pH 11.2), and 10 µL was mixed with 1 mL of xylidyl reagent. The complex formed was measured at 546 nm, and the concentration of free magnesium ion was calculated by comparing it with a calibration curve from 0 to 5 mgL^{-1} of Mg^{2+}.

2.4. Peptide separation by Tris-Tricine SDS-PAGE.

The method proposed by Schägger and Von Jagow [15] was used considering the modifications proposed by Sebastián-Nicolas *et al*. [16]. The protein concentration of the samples was analyzed with the Bradford method and standardized at 150 mg L^{-1}. This analysis was performed for the following samples: whey, WC resuspended at 5% in deionized water, WC complexed with Ca^{2+} (800 mg L^{-1}), WC complexed with Fe^{2+} (20 mg L^{-1}), and WC complexed with Mg^{2+} (350 mg L^{-1}). All samples of WC complexed with metal ions were previously freeze-dried and resuspended at 5% in deionized water. Electrophoresis was performed on a 16.5% T gel from a 30% T solution (acrylamide:bisacrylamide ratio 19:1 and 5% cross-linker). The gels were stained with Coomassie Blue G-250 and analyzed with Image J software (IJ 1.46r, v.1.8.0_112, Bethesda, MD, USA, 2004).

2.5. Antioxidant activity determination.

The antioxidant activity of the WC complexed with metal ions was measured by FRAP assay. FRAP reagent was prepared from a buffer of 300 mM acetates at pH 3.6, ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) 20 mM, and TPTZ (4,6-tripryridyl-S-triazine) 10 mM; these solutions were mixed in proportions 10:1:1. WC complexed with each metal ion was treated as described in section 2.4. A 250 µL-aliquot of resuspended was mixed with 1 mL of FRAP reagent and was diluted to 10 mL with deionized water. The solution was placed in a water bath for 10 min at 37°C. The absorbance was measured at 593 nm. The results were expressed as µmol of Trolox / g of sample.

3. Results and Discussion

3.1. Whey characterization.

The results of the whey physicochemical analysis are shown in Table 2. Water is the component that represents the highest percentage (87.09%), followed by lactose (3.33%) and proteins (2.33%). The whey composition varies considerably depending on the characteristics of the milk used to produce cheese, the type of cheese produced, and the process used to make it [17].

Table 2. Physicochemical characterization of whey.

Parameter	Value[1]	%RSD
Fat (%)	0.42±0.02	4.92
Solids (%)	6.26±0.05	0.79
Density (g/mL)	1.02±0.00	0.02
Lactose (%)	3.33±0.03	0.87
Proteins (%)	2.33±0.02	0.99
Temperature (°C)	13.15±0.64	4.84

Parameter	Value[1]	%RSD
Freezing point (°C)	-0.37±0.00	0.93
pH	6.12±0.03	0.41
Conductivity (mS cm^{-1})	3.61±0.02	0.42

[1]Values are the average of three replicates, ± is the standard deviation, and %RSD is the percentage of relative standard deviation.

The whey presented 0.57% solids, which include some minerals, such as sodium, calcium, and magnesium, mainly [18]. According to data reported by Jelen and Tossavainen [19], both sweet and acid whey present 6.3-7% solids, and phosphorus and divalent ion chlorides have been reported as the main mineral sources in whey. The results showed that the conductivity of the analyzed whey was 3.61 mS cm^{-1}; this value was lower than that reported (8 mS cm^{-1}) by Carvalho *et al*. [20], who consider that the high conductivity in whey is due to both the mineral content and the salts added to the milk during the manufacture of the cheese.

In relation to the pH detected (6.12), the whey was classified as sweet [4]. pH is a physicochemical parameter that indicates the chemical conditions within the whey. It was known that acidic pH favors calcium dissolution, while at neutral or slightly basic pH, calcium is binding as colloidal salt integrated at phosphates linked by Thr or Ser. In addition, CMP is found in whey, which has a phosphate residue derived from the breakdown of κ-casein during manufactured cheese and is capable of binding divalent ions [21].

3.2. Purification and concentration of whey peptides.

After the precipitation of whey proteins with CCl$_3$COOH, 9% of solid (fat, protein, and non-fat solids) and 91% of supernatant (water, lactose, and other dissolved solids) were obtained. The supernatant was dialyzed until obtaining a constant conductivity close to or equal to that of deionized water (0.09 μS cm^{-1}). The freeze-dried residual had a 0.45% yield of solids, corresponding to molecules with molecular weights close to 14 kDa. Because it was dialyzed with cellulose acetate films, the material's pores did not allow the passage of molecules greater than 10 kDa. This ensured both the absence of peptides less than 10 kDa and of metal ions in the freeze-dried solid after dialysis.

3.3. Evaluation of metal binding capacity.

The metal binding capacity was evaluated in the freeze-dried fraction containing molecules greater than 10 kDa. Even though most of the bioactive peptides reported are of low molecular weight, there are studies that demonstrate the binding metal capacity of whey proteins and peptides with molecular weights close to 14 kDa [3], including CMP [22].

3.3.1. Calcium-binding.

Table 3 shows the results obtained from the Ca^{2+}-peptide complexation. After the Tukey test (p=0.05), no significant differences between the concentrations evaluated were shown. In all cases, the percentages of Ca^{2+} retained were greater than 95%. The best complexation conditions were obtained at 800 mg L^{-1}, in which a fixation percentage of 98.8% was observed from the second hour, which was maintained until the end of the study.

According to the results obtained, solids present in WC, such as peptides, could be capable of complexing with calcium. This capacity could be caused by casein-derived fractions, which contain phosphate that is covalently bound as a monoester to the serine and

threonine residues, and naturally interact with calcium ions formed by colloidal calcium phosphate [23].

Table 3. Percentage of calcium fixed (%Ca_{fixed}) in the freeze-dried and dialyzed whey (containing molecules ≥10 kDa).

Time (h)	Concentration (mg L^{-1})					
	700		**800**		**900**	
	%Ca_{fixed}[1]	**Δ%Cafixed**	**%Ca_{fixed}[1]**	**Δ%Cafixed**	**%Ca_{fixed}[1]**	**Δ%Cafixed**
1	96.96±0.13		95.35±0.04		95.73±0.08	
2	97.96±0.12	1	98.82±0.08	3.47	96.50±0.12	0.77
3	99.36±0.00	0	99.15±0.06	0.33	97.64±0.08	1.13
4	99.36±0.00	0	99.31±0.06	0.16	98.69±0.01	1.01
5	99.36±0.00	0	99.58±0.06	0.27	99.11±0.01	0.42

[1]Values are the average of three replicates ± standard deviation.

3.3.2. Iron binding.

Ferrous ions at 15 mg L^{-1} showed less complexation than with the other concentrations studied (Table 4). Additionally, the statistical analysis showed that this concentration is different from each other by Tukey test (p=0.05). Likewise, Fe(II) ion had lower binding power compared to that calculated for calcium. This is also attributed to the fact that in the case of calcium the range of concentrations evaluated was up to 45 times greater than that of iron.

Table 4. Percentage of iron [as Fe(II)] fixed (%Fe_{fixed}) in the freeze-dried and dialyzed whey (containing molecules ≥10 kDa).

Time (h)	Concentration (mg L^{-1})					
	10		**15**		**20**	
	%Fe_{fixed}[1]	**Δ%Fefixed**	**%Fe_{fixed}[1]**	**Δ%Fefixed**	**%Fe_{fixed}[1]**	**Δ%Fefixed**
1	95.99±0.07[a]		93.80±0.06[b]		95.58±0.01[a]	
2	96.66±0.12[a]	0.67	93.88±0.05[b]	0.08	99.02±0.06[a]	3.44
3	96.85±0.14[a]	0.19	93.90±0.02[b]	0.02	99.11±0.01[a]	0.09
4	96.81±0.09[a]	-0.04	94.18±0.05[b]	0.28	98.34±0.00[a]	-0.77
5	99.19±0.01[a]	2.38	94.40±0.03[b]	0.22	98.34±0.00[a]	0

[1]Values are the average of three replicates ± standard deviation. Different letters between columns indicate a significant difference ($p ≤ 0.05$).

In addition, it has been shown that the affinity for the binding of calcium and iron to phosphopeptides is different for each of these metals [24]. Besides, a better result was obtained at 20 mg L^{-1} due to an increase of fixed iron by protein fraction until 99.1% during the initial three hours (Table 5). After this period, a decrease of 0.77% was observed. It is known that some casein fractions that may not be present in whey (mainly β-casein) have a higher affinity for iron than any whey protein, which could be associated with the low iron binding capacity in fractions obtained [25].

Kibangou *et al*. [26] consider that not all protein fractions are the same in their interaction with Fe(II) because their amino acid composition is diverse, especially those that have a phosphate residue (such as serine, in casein phosphopeptides), besides their electronegativity. Several studies have shown that the binding sites for the formation of iron-peptide complexes correspond mainly to the carboxylic groups, as well as to the ε-amino of lysine, the nitrogen of guanidine in arginine, and histidine imidazole nitrogen; likewise, glycine, proline, glutamic acid, and aspartic acid may be involved [27].

3.3.3. Magnesium binding.

According to the results (Table 5), the percentage of retained magnesium was higher than 94% for the three tested concentrations, and no significant differences were shown after applying a Tukey test ($p=0.05$).

Table 5. Percentage of magnesium fixed (%Mg$_{fixed}$) in the freeze-dried and dialyzed whey (containing molecules ≥10 kDa).

Time (h)	Concentration (mg L^{-1})					
	250		350		450	
	%Mg$_{fixed}$[1]	Δ%Mgfixed	%Mg$_{fixed}$[1]	Δ%Mgfixed	%Mg$_{fixed}$[1]	Δ%Mgfixed
1	97.91±0.00		98.51±0.000		94.21±0.67	
2	97.91±0.00	0	98.51±0.000	0	94.54±0.00	0.33
3	97.91±0.00	0	98.51±0.000	0	97.13±1.56	2.59
4	97.91±0.00	0	98.51±0.000	0	98.84±0.00	1.71
5	97.91±0.00	0	98.51±0.000	0	98.84±0.00	0

[1]Values are the average of three replicates ± standard deviation.

Magnesium ions showed the highest retention at 450 mg L^{-1}, and regarding results obtained at RDI of ions, magnesium had higher retention than ferrous ions. However, all magnesium and iron ions retentions obtained were lower than those shown for calcium ions. According to reports, calcium is the preferred ion linked by whey proteins special by CMP [28], in comparison with other divalent ions.

3.3.4. Comparison of metal binding.

Comparing obtained results of the ions complexation, a direct relation was observed with the affinity of each ion with peptides in the medium. Furthermore, this kind of interaction varies according to ions' coordination capacity with amino groups of free peptides. It has been demonstrated that ion affinity depends on ionic force and the peptide chain [24]. For example, α-LA hydrolysates can complex calcium, copper, iron, magnesium, manganese, phosphorus, and zinc, while β-LG hydrolysates preferentially complex calcium, cadmium, copper, iron, magnesium, manganese, and zinc.

Figure 1 shows a comparison of percentage changes in the binding ions. It was observed that although magnesium has the same coordination number as calcium, the binding percentage of magnesium does not change, and they are lower than calcium. These differences could derive from phosphate groups in the CMP structure, which has one linked to Thr161, leading to calcium fixation. In contrast, the iron fixation was higher than calcium and magnesium even though variations between concentrations had a significant difference, which was not observed in the other ions.
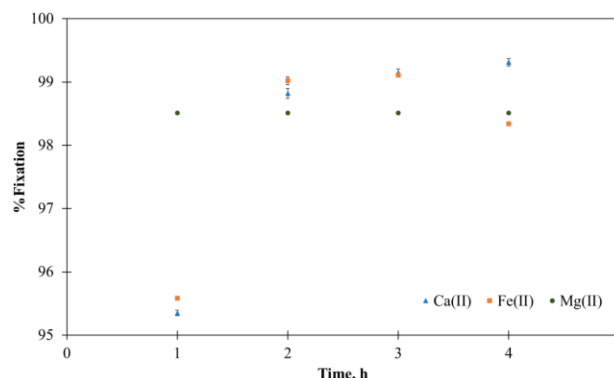

Figure 1. Comparison of percentage changes on the binding ions at the best conditions (Ca^{2+} at 800 mg L^{-1}, Fe^{2+} at 20 mg L^{-1}, and Mg^{2+} at 350 mg L^{-1}).

The interaction of metal ions with proteins affects their biological properties, which could be used both in the field of health and in industrial applications in the manufacture of functional and nutraceutical foods [27]. These interactions also lead to the formation of metalloproteins, metallo-complexes, or nanoparticles, in which the structures of proteins bind metals through carboxyl groups forming coordination bonds (metalloproteins) or by electrostatic interactions (metallo-complexes and nanoparticles) [29].

It has been observed that the factors combined, such as protein structure and pH, establish the chelating capacity of the whey proteins. Thus, this property has been used to develop peptides and proteins linked to Cu, Fe, and Zn with the possibility of application in health [30]. On the other hand, calcium, iron, and magnesium are available in their organic and inorganic forms, influencing their absorption and bioavailability. In addition, the inhibition or increase in the bioavailability of these minerals may be due to many dietary factors [31]. Phytates, oxalates, and polyphenols are examples of some inhibitors since they are chelating agents of divalent ions such as Fe(II), Ca(II), or Mg(II) [32, 33]. Due to the physiological importance of these minerals, strategies have been seeking to increase their absorption, so using peptides as metal chelators is a good alternative.

3.4. Peptides separation by SDS-PAGE: metal ions effect.

Figure 2 shows both gel and electropherograms obtained from separation of peptides before and after complexation. The complex of Ca^{2+} (lane F) and Fe^{2+} (lane E) showed a decrease in the apparent concentration of low molecular weight peptides (peptides zone) and an increase in higher molecular weight peptides concentration. This behavior was not observed in the complex with Mg^{2+} (lane D).
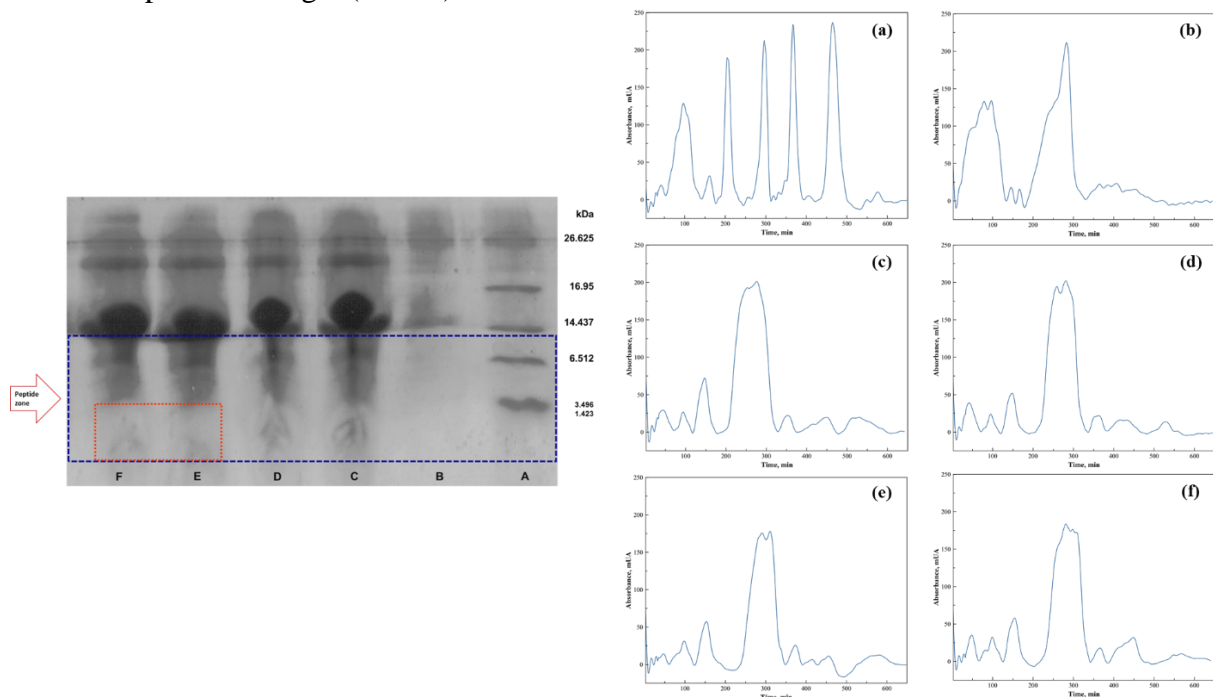

Figure 2. Peptide separation from whey, gel, and electropherograms. **(a)** peptide standard, **(b)** Whey, **(c-f)** freeze-dried whey and resuspended at 5 % in deionized water: **(c)** without complexation treatment, **(d)** complexed with Mg^{2+} (350 mg L^{-1}), **(e)** complexed with Fe^{2+} (20 mg L^{-1}) and **(f)** complexed with Ca^{2+} (800 mg L^{-1}).

Complexation reactions carried out could derive from ion metal coordination with peptides [27]. Both coordination links with nitrogen and those generated between oxygen (from phosphate) and calcium, increase the molecular weight of peptides [31].

The chelation capacity of metal ions with peptides has been associated mainly with aminoacidic composition [4]. Also, Ca(II) and Fe(II) chelation by peptide fractions derived from whey hydrolysates [30] and casein hydrolysates [34] have been evaluated. Different possibilities of ion chelating have been discussed, relating the binding with interactions with oxygen, carbonyl groups, and nitrogen of amino or imino groups. In addition, other peptides have been demonstrated to interact with the oxygen of the carboxylic group and nitrogen from the amido group, which leads to the formation of calcium coordination links through sharing an electron pair [35].

Caetano-Silva *et al*. [36] demonstrated that sequences derived from hydrolyzed whey proteins containing Glu and Asp have a high capacity to bind iron owing to their carboxylic groups. Four peptides derived from caseins hydrolysis corresponding from α-S1-casein (f99-105), α-S2-casein (f40-47), β-casein (f121-128), and β-casein (f192-198), have been studied by their iron binding capacity [37]. These reports focus on peptides obtained from protein hydrolysates of caseins or whey. However, no previous hydrolysis of whey proteins was realized in this study.

3.5. Antioxidant activity of complex ion-peptide.

The results of antioxidant activity by FRAP assay are shown in Table 6. It was observed that the highest antioxidant activity was obtained in peptide fractions linked to Fe(II). Fractions treated with calcium or magnesium did not show significant differences between them, although their antioxidant activity was higher than that of the control. Lactoferrin, as part of whey proteins, has been described as antioxidant [38]. This protein is a natural glycoprotein with different biological activities, such as: antiviral, anti-inflammatory, and anticancer, and with effects on the immune system. In some cases, whey protein hydrolysis has been used to increase the antioxidant capacity [39].

Table 6. Antioxidant capacity of the freeze-dried and dialyzed whey before and after metal complexation.

Sample	Concentration (mgL^{-1})^1	FRAP assay (as µmol Trolox/g dw)
Control	---	16.80±2.04
Ca(II)-whey	800	43.76±0.32
Fe(II)-whey	20	75.68±0.24
Mg(II)-whey	350	46.80±**0.32**

The control sample is referred to as freeze-dried and dialyzed whey without ion complex. ^{1}Corresponding to concentration for obtaining high metal fixation: Ca(II) 800 mg L^{-1}, Fe(II) 20 mg L^{-1} and Mg (II) 350 mg L^{-1}. dw: dry weight of the sample. Values are average of three replicates. ± standard deviation.

Although the antioxidant activity has been evaluated only in the whey proteins and their hydrolysates, no studies reflect the antioxidant capacity due to the transport of metal ions by specific peptide fractions. In fact, it has been shown that the whey fractions with molecular weights below 1 kDa have the best antioxidant properties compared to those with higher molecular weight [39, 40].

Recently, it has been shown that glycosylated peptide fractions of hydrolyzed whey proteins have high antioxidant activity after being thermally hydrolyzed [41]. Furthermore, antioxidant activity is also favored by enzymatic hydrolysis [42-45]. According to these studies, the antioxidant properties of whey proteins are favored by hydrolytic fractionation. In

the present work, it is demonstrated that the antioxidant activity increases with the carrying of metal ions, without the need to hydrolyze the whey proteins until obtaining peptides.

4. Conclusions

Molecular weight fractions <14.4 kDa (α-LA) could carry metal ions, with Ca(II) and Fe(II) being these metal ions with the highest affinity. The complexation of the low molecular weight fractions with the Ca(II) and the increase in the molecular weight of the peptides by the SDS-PAGE analysis verified Fe(II) ions. Through an electrophoretic study, it is possible to determine the chelating capacity of peptide fractions from food matrices, such as whey.

Complexing metal ions could increase the antioxidant capacity of the peptide fractions of whey without previous chemical or enzymatic hydrolysis. Even though calcium has been associated with milk proteins, complexation with ferrous ions seems to be the one that most favor the increase in the antioxidant activity of this type of fraction. However, deepening the structural studies of the peptide fractions capable of carrying metals is necessary to determine the incidence of certain amino acid chains or chemical factors that influence the complexation process.

Conflicts of Interest

The authors declare no conflict of interest.

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