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Original Article

IN VITRO DIGESTIBILITY OF THE DROMEDARY WHEY PROTEINS: POTENTIAL USES IN INFANT MILK ALLERGIES

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ABSTRACT

Objective: Dromedary milk has a good nutritive value and is free of β-lactoglobulin (β-Lg), which is considered one of the major antigens of cow's milk proteins responsible for the incidence of allergy in infancy. In this work, we try to assess the *in vitro* gastrointestinal digestion of dromedary's whey proteins.

Methods: The dromedary and bovine whey proteins were subjected to two successive hydrolysis: pepsic followed by the mixture of trypsin/chymotrypsin. The kinetics of degradation was determined, then the degree of hydrolysis (DH) and the peptide chains length (PCL) was calculated. SDS-PAGE was carried out to evaluate the specific protein composition before and after degradation.

Results: The determination of α-NH_2 free functions and DH shows that the dromedary's whey proteins are more susceptible to pepsin digestion (***p<0.001, *p<0.05/ **p<0.01, *p<0.05), and combined trypsin/chymotrypsin digestion (***p<0.001, **p<0.01/ **p<0.01, **p<0.01) compared to bovine whey proteins digestibility. In the other hand, the PCL of the dromedary hydrolysates obtained are shorter than those of the bovine hydrolysates. The electrophoretic profile of native dromedary's whey proteins shows the absence of the β-Lg and the presence of specific proteins (CWBP: Camel Whey Basic Protein, PGRP: Peptidoglycan Recognition Protein and WAP: Whey Acidic Protein), Also, we observed in the electrophoretic gels that a new band was present and could correspond to new peptide generated after the peptic hydrolysis of dromedary's whey proteins.

Conclusion: The gastrointestinal digestion of the two whey is important with higher DH of dromedary's whey proteins and PCL almost identical at the end of digestion.

Keywords: Dromedary's milk, Cow's milk, Whey proteins, Animal's endopeptidases, Hydrolysis, DH, PCL, Digestibility.

INTRODUCTION

Hypersensitivity to cow's milk proteins is one of the main food allergies that affects mostly but not exclusively infants. It may persist through adulthood and associated with severe symptoms. Different clinical symptoms of milk allergy have been established. The diagnosis of milk allergy differs widely due to the multiplicity and degrees of symptoms, and can be confirmed by skin prick test or blood tests. Cow's milk contains more than 20 proteins that could represent potential allergens. Casein fractions and β-Lg represent the major milk allergen [1].

Cross-reactivity between milk allergens from different mammalian species and humans occurs when they share some part of their amino acid sequence, or when they have a similar capacity to bind specific antibodies due to their molecular structures. The cross-reactivity between milk proteins from different animal species have been studied [2-8]. It was shown that IgEs (Immunoglobulins E) from sera from allergic children to cow's milk could recognize most parts of milk proteins from European mammals: sheep, goat and buffalo. A weak cross-reactivity was observed with milk proteins from mares and donkeys, but none with camel milk [9]. IgEs from sensitized infants to sheep milk did not recognize any camel proteins milk. Immunological relationships between human milk proteins and their counterparts in goat, sheep, cow, buffalo, camel, donkey, and mare milk were studied by [6]. The study performed by [10] showed that the dromedary's whey proteins have a sufficient antigenic potential to cross-reactivity with IgG anti bovine α-Lac (Alpha-Lactalbumin) and cannot bind IgG anti bovine β-Lg. As differences in protein composition between camel and bovine milk may influence their digestibility [11]. However, our work was undertaken to identify the specific composition of dromedary's whey proteins and in the other hand to study the characteristics of these proteins before and after gastrointestinal stimulating digestion.

MATERIALS AND METHODS

Milks samples collection

The dromedary's milk samples (*Camelus dromadarius*) were collected in the area of Farnaka Wilaya of Mostaganem "north of Algeria" immediately after the draft. Whereas the cow's milk used as reference was collected from a farm located in Bethioua (Elbouachria) Wilaya of Oran "north of Algeria". All the samples (dromedary and cow milk) were transported in an icebox to the laboratory.

Preparation of camel and bovine whey proteins

The milk samples were skimmed by three centrifugations at 3500 trs/ min (turns/Minute) and 10°C for 15 min. Camel and bovine whey were obtained after acid precipitation of caseins at a pH 4.4 and pH 4.6 respectively, by adding 0.1N HCl followed by centrifugation at 3500 trs/min for 15 min at +04 °C. Whey proteins were dialyzed against Distilled water before use. The samples were lyophilized and stored at -20 °C until analysis.

Gastric-pancreatic digestion

The hydrolysis was realized according to [12] and [13]. Bovine and camel whey proteins were subjected to two successive hydrolysis: pepsic followed by the mixture of trypsin/chymotrypsin. The whey samples were dissolved in 10 mM HCl pH 2.2 at the concentration of 1 mg/ ml. Pepsin « 844 E 920792, activity 10 FIP- 4/mg » prepared at 4 mg/ml in distilled water was added to whey samples at an E/S R (Enzyme/Substrate Ratio) 1:25 and incubated during 90 min at 37°C. Enzymatic kinetics was assessed by sampling 200 µL from the reactional sample at 0, 15, 30, 60 and 90 minutes. The reaction was stopped by increasing the pH at 7.8 by addition of 200 µl of phosphate buffer salin 100 mM pH 8.5. To inhibe the pepsic residual activity, phosphate buffer salin 100 mM pH 8.5 was added.

Trypsic/chymotrypsic digestion was conducted by addition of a mixture of trypsin/chymotrypsin (1:1, w/w (Weight/Weight)) to the pepsic hydrolysates. Trypsin "818 K 20146079, 404/ mg activity" and chymotrypsin "type 2 EC.3.4.21.1" prepared in HCl 10^{-3} N were added at an E/S R 1:100, W/W to pepsin whey samples and incubated at 37 °C, pH 8.2, during 90 min. The hydrolysis kinetics was investigated by sampling 200 µL aliquots of from reaction samples at 105,120,150 and 180 min. The reaction is stopped by heating of the samples in 95°C during 15 min.

***In vitro* digestibility assessment**

The kinetics of dromedary and bovine whey protein degradation was determined by a colorimetric measurement after reaction of α-NH_2 released functions with the ninhydrin according to [14].

For each time point, determine the concentration equivalents of amino groups (h') in the samples by extrapolating from each standard curve. This gives the rate of equivalents peptide bonds hydrolyzed (h'), expressed as meq/g (Mili equivalent/ Gram**)** protein from the following equation: h'= (A× b)/ m [15], Where A is the absorbance at 540 nm (in spectrophotometer visible: JASCO V-530 UV/VIS), b and m are the y intercept and slope of the calibration curve respectively.

For each time point, separately determine the amount of released amino groups (*h*) by subtracting the value of the corresponding unhydrolyzed control in the corresponding standard curve ($h=h'-h'_{control}$).

Degree of hydrolysis

The DH (**%)** was calculated from the amino acid composition of protein [16]: $DH = h/h_{tot} \times 100\%$. Where h_{tot} is the total amount of peptide bonds. When h_{tot} remains unknown, 8 amino meq/g protein is a good estimate [17].

Peptide chains length

The PCL resulting from hydrolysis can be calculated starting from the value of the DH according to the following equation: $PCL \approx 1/DH$ [17].

SDS-PAGE electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel was carried out to evaluate the protein degradation, using 18 % acrylamide gels. Samples were mixed with sample buffer: (SDS, β-mercaptoethanol, glycerol and bromophenol blue) and heated during 5 min at 90°C in a water bath. The 18% acrylamid gel slabs containing 10 % SDS were prepared and run using the discontinuous buffer system [18]. Protein bands were stained with coomassie blue R-250 and destaining in a solution of 30 % ethanol and 5 % acetic acid. two kits were used: Kit (1) containing reference proteins: the BSA (Bovine Serum Albumin (68 KDa (kilo Dalton) "A-7638 5g Lot 79H7614"), Caseines (24 KDa"Sigma, 9000-71-9, USA"), the β-Lg (18 KDa "Sigma, L-O130, USA") and the α-Lac (14,2 KDa "Sigma, L-5385, USA"); and Kit (2) containing: Triosephosphate isomerase from rabbit muscle (26.6 KDa), Myoglobin from horse heat (17 KDa), α-Lac from bovine milk (14.2 KDa), Aprotinin from bovine lung (6.5 KDa), Insulin Chain B oxidized bovine (3.496 KDa) and Bradykinin (1.06 KDa).

Statistical analysis

Results were expressed as means ± Standard Error (ES) and $p < 0.05$ were considered statistically significant. All experiments were repeated five times. Obtained data were statistically analyzed using Student's test as programmed by STATISTICA (AXXF307CO20802FA) 2006.

RESULTS AND DISCUSSION

The weak quantity of β-casein and the absence of β-Lg are linked to the hypo-allergenic properties of camel's milk and could be used in cow milk allergic children feeding. Other components such as lactoferrin, immunoglobulins, lysozyme or vitamin C were reported to play a central role in the determination of these properties [19, 20].

It's well known that pepsin activity is low in early infancy and the pH of the stomach could reach 5 until 6 during a milk meal. These physiologic conditions don't facilitate the complete hydrolysis milk proteins and in particular α-Lac by pepsin [21]. Hence, this protein may leave the stomach of infants with much lower extent of hydrolysis. As was approved by the results obtained from *in vitro* study of [16]. Thus, intestinal proteolysis of α-Lac by chymotrypsin and trypsin may play a critical role in the efficiency of digestion of this protein [16].

The present study was designed to determine the effect of *in vitro* gastrointestinal digestion of the dromedary and cow's whey proteins to verify if the dromedary's whey proteins hydrolysates can be proposed like an alternative to those of the cow's milk. Numerous reports have indicated that Camel milk has a good nutritive value, in addition to its hypoallergenic property especially after enzymatic hydrolysis.

In the hydrolyzed form, the proteins can show improved functional properties, according to the conditions of treatment used [22, 23, 24, 25, 26]. The hydrolysis can be realized under monitored conditions of the pH and temperature desired except the pHi of the substrate [27]. An allergenic residual activity of proteins was observed in the formulas resulting from partial or extensive hydrolysis of the cow's whey proteins.

Kinetics of the hydrolysis

The hydrolysis of dromedary and cow's whey proteins in two stages with the pepsin followed by trypsin-chymotrypsin, by stimulating the gastrointestinal conditions, were performed using pepsin, trypsin and chymotrypsin. These three enzymes have different specificities. Their complementary and synergetic actions allow a very effective degradation of food proteins [28].

Milk proteins consist of caseins (78.3%), whey proteins (19%) and miscellaneous proteins (2.7%). Whey proteins, which include serum albumin, immunoglobulins, α-Lac and β-Lg, are more reactive because they dissolve in the serum. β-Lg, the major protein in whey, is responsible for most of the bioactive properties of whey proteins [29], exists as a dimer at physiological pH and temperature values [30]. A disulfide bond near the C-terminus (Cys_{66}–Cys_{160}) and one in the interior of the molecule (Cys_{106}–Cys_{119}) stabilize the compact globular conformation. When exposed to higher temperatures or pH values (above 8), the dimer dissociates. Below pH 5.5, β-Lg associates to particles of up to eight units. At a pH below 3.5 no association occurs [31].

The average concentration of α-Lac in camel milk (2.2 g/ L) and human milk (2.45 g/ L) is significantly higher than that of bovine milk (0.5 g/ L) [32, 33]. It is rich in essential acids (Trp, Phe, Tyr, Leu, Ile, Thr, Met, Cys and Val) which represent a rate of 63.2 % of the whole of the amino-acids which constitute it [34]. This nutritional property as well as the similarities of human structure with the α-Lac (70 %) renders this protein ideal for the preparation of maternized milks and other infantile products [35]. The tightly bound Ca^{2+} ion of α-Lac has a large influence on its tertiary structure and molecular stability. Under a variety of conditions, e. g., removal of calcium, high temperature, strong acidic conditions, presence of denaturing agents or some food processing conditions, this protein can assume the molten globule (MG) conformation, which described as a compact state keeping the secondary structure but having a poorly defined tertiary structure [36].

The principal proteolytic enzymes used on a commercial scale are pepsin, trypsin, and the chymotrypsin of porcine origin.

Pepsin, is an endoprotease that is secreted by the gastric mucous in the form of pepsinogen and then activated by stomachal acidity, but also by autolysis [37]. The optimal pH of pepsin ranges between 1.8 and 3.5 [38] and its specificity is generally limited to the aromatic amino-acids [37]. The susceptibility of the allergens to the proteolysis strongly depends on pepsin to the relationship with the allergen [39]. For this reason we chose a pH: 2.2 and mass ratio E/S: 1/25.

Trypsin cleaves mainly the side chains charged with the arginin-X and lysin-X, with X being any amino-acid except a prolin [40]. The peptides resulting from the hydrolysis of proteins of the whey by trypsin are of great interest to food industry [41].

Chymotrypsin is a serine proteinase of the digestive system, splits peptides on the carboxylic side of aromatic amino-acids (tyrosin, tryptophan, and phenylalanin), which is insert in the hydrophobic cavity of the enzyme [42, 43].

Proportioning of the functions α-NH2

The assessment of the α-NH2 functions released during the combined pepsin and trypsin-chymotryptin hydrolysis (fig. 1) permit to evaluate the degree of the digestion of the dromedary and cow whey proteins. Our results show that the gastrointestinal digestion of the dromedary's whey proteins is more important than that of the bovine whey proteins, and the pepsic hydrolysis combined with trypsin-chymotrypsin of bovine and dromedary whey is significantly higher than the pepsic hydrolysis.

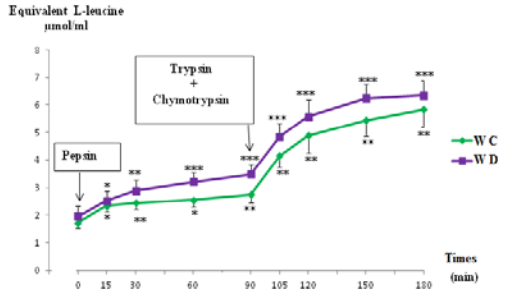

Fig. 1: Kinetics of combined hydrolysis of the wheys by pepsin (E/S R 1:25) and trypsin-chymotrypsin (E/S R 1:100), in each case for 90 min. Values are means ± SE for five determinations (* *p*<0. 05, ** *p*<0. 01 and * *p*<0. 001).W C = whey of cow's milk and W D = whey of dromedary's milk**

The β-Lg is absorbed by the intestine in a native state or slightly hydrolyzed, which explains, partially, its strong allergenicity [44]. Its primary structure is composed of 162 amino-acids, which 15 are lysins and three are residues of arginine. These amino-acids form the peptide bonds which are the preferential targets of cleavage for trypsin [43]. The analysis of the primary structure of β-Lg reveals 52 sites of theoretical cuts for trypsin and chymotrypsin, but only 30 peptides were detected in experiments performed by [42]. The tertiary structure of the β-Lg is very resistant [45], and it confers a good resistance to the acid hydrolysis [46, 47] and to pepsin digestion [46, 47, 45].

Since α-Lac is very sensitive to pH, it adopts an acidic conformation around pH 3.0, which is called the classical MG-state [48]. This conformation is a result of competition between Ca^{2+} and protons for the carboxyl side chains, which finally causes the depletion of Ca^{2+} from α-Lac protein. Because of the very low pH during hydrolysis by pepsin, α-Lac possesses a classical MG-state [16]. [49] published the primary sequence of the α-Lac of the dromedary's milk. Like the bovine α-Lac and that of several animal species, the camel α-Lac constitute of 123 amino-acids with 8 residues cystein forming 4 disulfide bridges. The analysis of the primary sequences of these two proteins bovine and cameline reveals differences in 39 residues, thus having a rate of sequential similarity of 68.29 %. On the structural level, the α-Lac is similar to the lysosyme, which shows the common ancestral origin of these two proteins [49, 50, 51].

We note a very important degradation of the two wheys (dromedary and bovine) just after the addition of the enzymatic mixture trypsin-chymotrypsin (E/S R 1/100) with substrate of gastric digestion and after 15 minutes.

The enzymatic reaction is influenced by parameters which permit to control the speed and DH: concentration of the substrate, E/S R, pH and the temperature [52]. The hydrolysis of proteins produces peptides with improved functional, immunological and bioactive properties superior to those of the native protein. Many health effects have been found and reported in bioactive peptides. For example, blood pressure-lowering ability, cholesterol-lowering effects, antioxidant activities and enhancement of mineral absorption. Many peptides, of animal protein origin, with bioactive potential have been discovered. Most of them were isolated from milk-based products or produced from milk proteins, mainly caseins and whey proteins [53].

The analysis of the hydrolysis of α-Lac using individual digestive enzymes (chymotrypsin and trypsin) revealed faster hydrolysis of the camel protein than of the bovine counterpart. Since both camel and bovine α-Lac possesses the same number of cleavage sites for chymotrypsin, then the target residues might be available in greater density on the surface of the camel α-Lac molecule. The data from intrinsic fluorescence studies suggest a different conformation with a less compact structure for native camel α-Lac compared with that of the bovine counterpart. The different conformation of camel α-Lac may explain why this protein was a better substrate than the bovine protein for hydrolysis by both trypsin and chymotrypsin [16].

The compositional and structural differences between whey proteins of camel and cow milk were established by [54, 19, 55]; these differences can influence their digestibility and consequently their DH.

Degree of protein hydrolysis

The DH is defined by the hydrolyzed peptids bound quantity (h) compared to the full number of peptide bounds (h_{tot}) per unit of mass. At the time of each cut of a peptide bound, a free amino group is formed. The DH is thus given by measuring the quantity of released amino groups [56]. It is expressed as a percentage, and permit to evaluate in globally way of the action of the enzymes on the substrate and the effectiveness of the process of hydrolysis. It thus informs in an indirect way on the length of resulting peptides and thus about their nutritional, functional and sensory properties [57, 58]. However, it was observed that as the DH of proteins is higher, as the residual allergenicity is weak [59-61].

Results of the obtained DH in fig. 2 are shown, the DH of the dromedary's whey proteins by pepsin during 90 minutes is high: 31. 6 % against: 21.37 % for the bovine whey proteins. We note a significant high rate of digestion of the two whey proteins after the addition of the trypsin-chymotrypsin mixture (W D: T_{90} = 31. 6 % and T_{105} = 60.13 %; W C: T_{90} = 21. 37 % and T_{105} = 50. 36 %), but it is less pronounced for the cow's whey proteins. The study of [16] shows that the DH of bovine and camel α-Lac was almost similar after treatment with pepsin. The assessment of *in vitro* digestibility using trypsin and chymotrypsin showed greater DH of camel α-Lac than of the bovine protein in both the native state and the MG-state; this can be explained by the different conformational and structural features of these proteins.

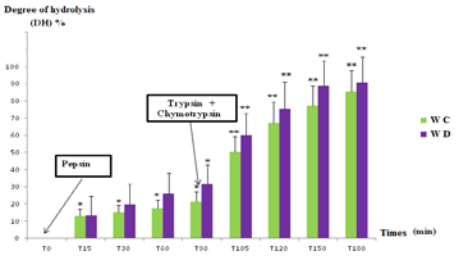

Fig. 2: Degree of hydrolysis of whey by pepsin (E/S R 1:25) and trypsin-chymotrypsin (E/S R 1:100), in each case for 90 min. Values are means ± SE for five determinations (* *p*<0. 05, ** p<0. 01 and * *p*<0. 001). W C = whey of cow's milk and W D = whey of dromedary's milk**

Peptide chains length resulting from the hydrolysis

Our results (Table 1) show that the PCL or the residues of amino-acids of peptides resulting from the gastric digestion of the

dromedary's whey proteins are lower than those resulting from the gastric digestion of the bovine's whey proteins, which show the raising in the pepsic hydrolysis of the whey proteins of the dromedary's milk compared to that of the cow's milk.

After 180 min of hydrolysis, the digestion of proteins is practically finished but it is not total (dromedary whey proteins: DH=90.84 ± 33.01and PCL=1.29 ± 0.58, bovine whey proteins: DH=85.31 ± 28.03 and PCL=1.29± 0.57).

Table 1: Peptide chains length

Times	PCL							
	T15	T30	T60	T90	T105	T120	T150	T180
W C	39,23 ± 17,51	29,52 ± 13,81	23,62 ± 10,54	7,29 ± 3,25	2,4 ± 1,07	1,76 ± 0,78	1,42 ± 0,63	1,29 ± 0,57
W D	18,74 ± 8,37	8,88 ± 3,96	6,54 ± 2,92	5,42 ± 2,42	2,36 ± 1,05	1,34 ± 0,6	1,33 ± 0,59	1,29 ± 0,58

Values are means ± SE for five determinations (W C = whey of cow's milk and W D = whey of dromedary's milk).

The functional properties of proteins and their hydrolysates depend on many physicochemical characteristics (size and structure of proteins and peptides, composition in amino-acids, ratio hydrophobicity/hydrophicity, flexibility and rigidity of the molecules…) [52]. The organoleptic quality of the oligopeptides produced during the hydrolysis is strongly dependant on the specificity of the enzymes used [17, 57].

Electrophoresis of native proteins and the hydrolysates on polyacrylamide gel in the presence of SDS in order to study the impact of hydrolysis of bovine and dromedary whey proteins, the hydrolysates and untreated protein samples were analyzed by SDS-PAGE, after 0, 90 and 180 min. It was carried out with a native protein kit. The electrophoretic profile is represented in fig. 3.

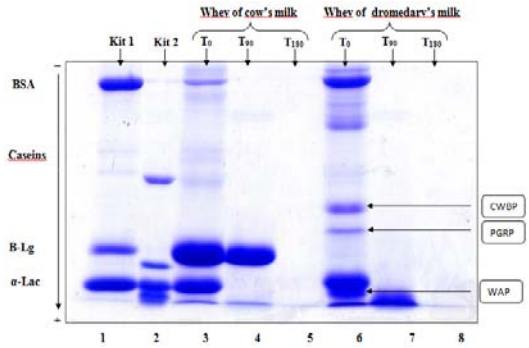

Fig. 3: SDS-PAGE Electrophoresis (18 %) of untreated and enzyme-treated protein samples (*in vitro* gastrointestinal digestion) of the wheys

Well 1and Well 2: 2 kits (proteic and peptidic, respectively)

Well 3, Well 4 and Well 5: cow's whey proteins after 0, 90 and 180 min of hydrolysis respectively.

Well 6, Well 7 and Well 8: dromedary's whey proteins after 0, 90 and 180 min of hydrolysis respectively.

Concerning the dromedary's whey proteins, we note the presence of five bands: two similar to those of the bovine whey (the BSA and the α-Lac), and three different with the absence from the band similar to the β-Lg, thus joining the results published by several authors [62-65, 10].

These same major protein entities of camel milk (BSA and α-Lac) were detected and identified by SDS-PAGE by [62, 63 and 66]. In the dromedary's milk, the α-Lac is present at the rates of 1.62 mg/ ml [66] up to 3.19 mg/ml [67].

The proteins of the camel's milk have single electrophoresis structures which are completely different with those from the cow's milk and human milk [68]. Our study also shows the presence of three specific bands, and having different molecular weights. While being based on the literature data, we can distinguish them according to the order ascending of their mobilities: the CWBP< the PGRP< the WAP.

The CWBP was identified for the first time in camel's milk by [63], then isolated and purified. Its molecular mass is evaluated by SDS-PAGE with 20 KDa by [63]. This protein was detected after only 48 hour of lactation by [32].

The PGRP isolated from camel's milk by [69], purified and crystallized by [70]. Its molecular mass is estimated at 19.1 KDa [69].

The WAP detected by [71, 72], its molecular weight is estimated by SDS-PAGE at 14 KDa [72].

On this basis, we provide that the amino-acids hydrolyzes composition will be different.

According to the results obtained by [16] the gastric digestion of the dromedary and bovine α-Lac have a similar susceptibility to pepsinolysis, and since the bovine α-Lac gives bands with molecular weights lower than 6.5 KDa" compared to the molecular weight marker. At the end of gastric digestion, we conclude that the band similar to the "Aprotinin from bovine lung" (6.5 KDa), corresponds to the hydrolyzate resulting from the gastric digestion of the one of three specific proteins to camel's milk and not to the α-Lac.

Our results obtained from the electrophoresis of the combined digestion of dromedary's whey proteins agree with those of the proportioning of the functions α-NH_2 released after the hydrolysis, and show a disappearance of all intact proteins with the appearance of a band whose molecular weight is similar to that of the "Aprotinin from bovine lung" (6.5 KDa) at the end of gastric digestion and the appearance of peptides of small sizes at the end of combined digestion, no identifiable by the electrophoretic analysis employed. These peptides have molecular weights lower than that of the "Aprotinin from bovine lung: 6.5 KDa". On the other hand the bovine whey is characterized by a certain resistance to gastric digestion due to the presence of the β-Lg with an attenuation of their intensity.

The electrophoretic analyses of camel and cow's milk revealed that whey proteins in each type of milk have their own characteristics due to their distinguished behavior in migration positions and molecular masses. On the bases of this behavior, it is expected that the amino acid and structural composition will be different. These results are in agreement with those reported by [55, 54, 73].

CONCLUSION

According to these results we confirmed that the dromedary's whey proteins exhibit better digestibility than bovine whey proteins. It reveals also that dromedary's whey proteins have a higher DH and shorter LCP. The electrophoresis gel shows the absence of the β-Lg and the presence of three specific dromedary proteins (CWBP, PGRP and WAP) and a more important degradation of these proteins compared to those of the bovine whey. These findings encourage us exploring the immunoreactivity and allergenicity of this dromedary milk products digestion.

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CONFLICT OF INTERESTS

Declared None

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