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REGULAR ARTICLE

Fast protein liquid chromatography of camel α-lactalbumin fraction with radical scavenging activity

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Abstract

The aim of this study was to investigate the radical-scavenging properties towards a stable radical cation, ABTS, of *Camelus dromedarius* whey proteins (CWP) separated onto a cation-exchanger by fast protein liquid chromatography. The highest activities were found for CWP and fraction F1 mainly composed of α-lactalbumin. Fractions F2, F3 and F4 contained a mixture of lactoferrin, immunoglobulins G and probably camel whey basic protein (CWBP). These three fractions displayed low radical-scavenging activities. Lactoferrin was eluted almost pure in the last fraction (F5) but did not possess detectable radical-scavenging activity. The present results suggested that the cation-exchange chromatography is of great interest to yield, in a single step, whey protein fractions with various biological activities, i.e. a highly-enriched α-lactalbumin fraction displaying efficient antioxidant activity, a fraction (pool of F2-F4) mainly composed of heavy-chain immunoglobulins potentially interesting for human therapy and a fraction of pure lactoferrin having numerous biological activities such as antimicrobial and immunomodulating properties.

Key words: ABTS, α-Lactalbumin, Antioxidant activity, Camel milk, Radical scavenger

Introduction

It is generally well established that the food constituents can be used to reduce the risk of developing or aggravating human disease conditions. In this regard, functional foods and nutraceuticals have emerged as adjuvant or alternative to chemotherapy especially in the prevention and management of human diseases and for maintaining optimum health state (Kris-Etherton et al., 2002). Interest in the camel milk for human nutrition is increasing due to its distinct composition and unique biofunctional properties (*e.g*. antidiabetic properties; Sboui et al., 2012).

Camel milk possesses vital role in human nutrition in hot regions and countries. It contains the essential nutrients found in bovine milk, though some of them are found in higher concentrations such as vitamin C, iron, and unsaturated fatty acids (Al Haj and Al Kanhal, 2010). Besides caseins, camel whey proteins (CWP) constitute 20–25% of the total camel milk proteins (Khaskheli et al., 2005), the majority of them having various biological activities not found or in a lesser extent in the bovine milk protein fraction. In contrats to bovine milk whey proteins, CWP contain large amounts of heavy-chain antibodies IgG2 and IgG3 which are devoided of light chains, and thus have the potential to inhibit efficiently enzymes and micro-organisms (Harmsen and De Haard, 2007; Daley-Bauer et al., 2010). Lactoferrin (Lf) is present in much larger amount in camel milk than in bovine milk (*ca*. 0.3 g L^{-1} and 0.1 g L^{-1}, respectively; El Hatmi et al., 2006; Konuspayeva et al., 2007). A number of preventive properties is attributed to Lf such as antibacterial, antiviral, fungistatic, antiparasitic, antithrombotic and immunomodulatory effects (Darewicz et al., 2011). β-Lactoglobulin known for its allergenic potential is lacking in camel whey (Elagamy et al., 2009), whereas α-lactalbumin (α-LA; SwissProt accession number P00710) constitutes the main component

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(2.2 g L^{-1} of milk; El Hatmi et al., 2006). An advantage of α-LA may be its beneficial role in the antioxidant system of the neonate (Lien, 2003). The bovine α-LA (Sadat et al., 2011) and the camel α-LA (Salami et al., 2009; 2010) are a source of free radical-scavenging peptides. Therefore, attention is being focused on producing α-LA-enriched formulae because α-LA might have an ability to attenuate oxidative stress occurring in inflammatory bowel disease after oral administration (IBD; Rezaie et al., 2007). The protein or its peptides generated by gastro-intestinal digestion might act directly on the inflammatory site in the gut without passing through the intestinal barrier.

This study was undertaken to prepare an α-lactalbumin-enriched fraction possessing a free radical-scavenging activity much better than that of CWP. This activity was investigated spectrophotometrically with the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) or ABTS method. In this work, cation-exchange chromatography performed by fast protein liquid chromatography (FPLC) revealed a single step efficient method to readily produce α-LA-enriched fraction. In addition, two other protein fractions possessing biological activities of great interest such as fractions containing IgGs with potent therapeutic applications and Lf having antimicrobial properties were also obtained.

Materials and Methods

Sample collection

Milk samples from 5 healthy camels (*Camelus dromedarius*) were collected and mixed together. The animals, all belonging to experimental herd of the Livestock and Wildlife Laboratory (Institute of Arid Land, Médenine, Tunisia) were in the third month of lactation (Atigui et al., 2013). Samples were collected manually in sterile bottles once per day usually in the morning. Three aliquots of each sample were immediately stored at –20°C until used.

Preparation of whey proteins and chromatography

The milk was firstly skimmed by centrifugation (4500 *g* at 30°C for 20 min). Then, the casein fraction was precipitated at pH 4.2 with 1 M HCl and discarded by centrifugation performed in the same conditions. The supernatant (milk whey) was neutralized with 1 M NaOH, dialyzed against distilled water at 4°C for 72 h and CWP were lyophilized.

Fractionation of CWP was performed by cation-exchange fast protein liquid chromatography (FPLC) with the ÄKTA-FPLC technology (GE Healthcare, Uppsala, Sweden) by passing sequentially through three Hitrap CM (carboxymethyl) 5/5 columns (1.5 x 2.5 cm) equilibrated in 20 mM tris(hydroxymethyl) aminomethane hydrochloride (Tris/HCl) buffer, pH 8.0 containing 0.02% sodium azide. Volumes of 10 mL of whey proteins (10 g L^{-1} of Tris/HCl buffer) were loaded onto the three columns and a 0–1 M linear gradient of NaCl in the same buffer was applied at 1 mL min^{-1}. Eluted proteins were detected at 280 nm.

Electrophoresis

Whey proteins of the different FPLC fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 1.1% SDS and 5% 2-mercaptoethanol according to the method of Laemmli and Favre (1973) with a 4.9% staking gel and a 15.4% resolving gel running in 0.125 M Tris/HCl buffer, pH 6.8 and 0.38 M Tris/HCl buffer, pH 8.8, respectively. Volumes of 20 µL of samples at 2 g L^{-1} proteins were loaded in the gel. Proteins were stained for 30 min by 0.1% Coomassie blue R250 in a mixture of 50% ethanol and 10% acetic acid followed by overnight destaining in a solution of 30% ethanol, 7.5% acetic acid and 5% trichloroacetic acid. Molecular mass standards (Precision Plus Protein All Blue Standards) were from Bio-Rad (Hercules, CA, USA).

Protein concentration determination

The protein concentration was determined by the Bradford method. The bovine serum albumin was used as standard. The results of the assay depend on the number of basic amino acid residues of each protein (Ku et al., 2013) and the data are expressed as mg L^{-1} equivalent (eq.) to BSA. Each measurement was carried out in triplicates.

$ABTS^+$ radical-scavenging assay

The radical-scavenging assay was carried out according to Sadat et al. (2011), a method adapted from that of described by Re et al. (1999). The stable radical cation $ABTS^{\bullet+}$ was produced by dissolving 7 mM $ABTS^+$ in 2.45 mM potassium persulfate and by keeping the mixture in the dark for 15 h at room temperature. The $ABTS^{\bullet+}$ radical reagent was then diluted with 5 mM sodium phosphate buffer, pH 7.4 to reach an absorbance of 0.70 ± 0.02 at 740 nm. The radical cation was stable in phosphate buffer for at least 1 h at 22°C. The decrease in absorbance in the presence of protein fractions was measured at 740 nm with an

MRX® microplate reader (ThermoLabsystems, Chantilly, VA, USA). Volumes of 150 µL of protein fractions (0-100 mg L^{-1} eq. BSA) or of Trolox or gallic acid (0-30 µM) dissolved in phosphate buffer were added to 150 µL of the $ABTS^{\bullet+}$ reagent and the mixture was incubated for 10 min at 30°C before absorbance mesurement. All the assays were carried out five times. The radical-scavenging activity was calculated as follows:

$$\text{Activity (\%)} = [1- (A_r - A_b) / (A_i - A_b)] \times 100 \qquad [1]$$

Where: A_i = the absorbance of the initial $ABTS^{\bullet+}$ radical, A_r = the absorbance of the remaining radical and A_b = the absorbance of the blank (phosphate buffer, A_b= 0.09).

The IC_{50} value is defined as the concentration of sample able to transform 50% of $ABTS^{\bullet+}$ to $ABTS^{+}$ *i.e.* when the absorbance of the remaining radical was equal to the scavenged radical. Thus, log (IC_{50}) corresponds to the x-intercept of the curve of log $[(A_r - A_b)/ (A_i - A_r)]$ *vs.* log (concentration of sample).

The Trolox-equivalent antioxidant capacity (TEAC) measures the free radical scavenging capacity of a given substance, as compared to the standard, Trolox. The TEAC (in µmol Trolox equivalent or TE per µmol of a given substance) is the ratio of the gradient of the plot of activity *vs.* concentration of the given substance over the gradient of the plot of Trolox (Re et al., 1999).

Results and Discussion

In the present study, we proposed a simple method of separation of CWP by cation-exchange chromatography with the ÄKTA-FPLC technology in order to prepare in single step different fractions containing biologically active proteins *i.e.* α-LA-, IgGs- and Lf-enriched fractions and to investigate their potential free radical-scavenging activity. Although the anion-exchange chromatography (Ochirkhuyag et al., 1998) or size-exclusion chromatography (Si Ahmed et al., 2013) allow to obtain pure α-LA, these methods have not been revealed enough suitable to recover the IgGs and Lf (Si Ahmed et al., 2013). Elagamy et al. (1996) have achieved the purification of camel milk IgGs by protein affinity chromatography. The preparation of heavy-chain antibodies (IgG2 and IgG3) from camel milk is of great interest. Indeed, after immunization of *Camelidae* species, milk instead of blood serum might be a dietary source of single-domain antibody fragments (V_HHs) able to bind therapeutic targets. For example, llama's V_HHs can specifically target the cell receptor domains of toxins of *Clostridium difficile* (Hussack et al., 2010). In addition, the V_HHs of small size (*ca.* 15 kDa) are especially suited for oral immunotherapy because of their stability against very acidic pH, proteolysis and high concentrations of denaturing agents (Harmsen and De Haard, 2007).

Fractionation of CWP by ÄKTA-FPLC chromatography

After separation of the CWP, the chromatographic fractions were analyzed by SDS-PAGE electrophoresis (Figure 1). Fraction F1 mainly contained the major soluble protein of camel whey *i.e.* α-LA and camel serum albumin (CSA). As expected, these proteins were not adsorbed onto the cation-exchanger due to their acidic isoelectric points (pHi). The theoretical pHis are 5.01 and 5.60 for the camel α-LA and bovine serum albumin (SwissProt accession number P02769), respectively (the CSA sequence is not available in the databank). The fractions F2 and F3 mainly contained heavy chains H45 and H42 of IgG2 and IgG3, respectively, these IgGs being devoided of light chains (Lauwereys et al., 1998; Daley-Bauer et al., 2010) whereas IgG1, which consisted of both heavy chains H55 and light chains L30 was recovered in fraction F4. The IgGs have generally near-neutral or basic pHis (pHis 6.5–9.5; Igawa et al., 2010) and could be adsorbed onto the cation-exchanger and then desorbed all along the ionic strength gradient. The fraction F4 might also contain the camel whey basic protein (CWBP) isolated for the first time by Ochirkhuyag et al. (1998). According to these authors, CWBP displays apparent molecular mass and pHi of 20 kDa and 9.30, respectively. The fractions F2-F4 also contained Lf at estimated molecular mass of 78 kDa by Elagamy et al. (1996). IgGs and Lf were eluted in several fractions because of their microheterogeneity of their glycan moiety (Zinger-Yosovich et al., 2011). However, fraction F5 contained almost pure Lf highly retained onto the cation-exchange column as shown by SDS-PAGE, which is in accordance to our previous work (El Hatmi et al., 2007). Like CWBP, the camel Lf is a basic protein and has a theoretical pHi of 8.63 (unglycosylated form; UniProt/SwissProt accession number Q9TUM0). As expected, these two proteins were strongly retained onto the cation-exchanger, whereas the acidic α-LA and CSA were directly eluted in the void volume of the column.

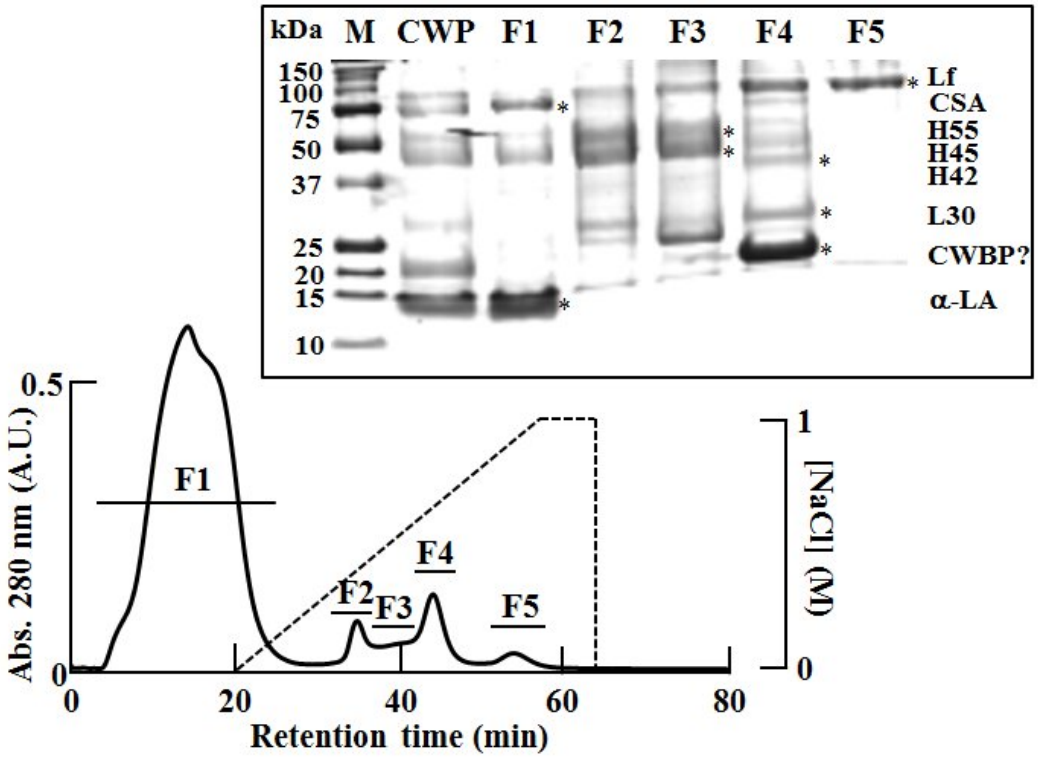

Figure 1. Cation-exchange fast protein liquid chromatography of camel whey proteins (CWP) onto three successive Hitrap CM columns connected to an ÄKTA-FPLC system and SDS-PAGE analysis of the collected fractions F1–F5. The ionic strength gradient is in dashed line and the chromatogram in solid line. Electrophoretically identified bands are indicated by an asterisk. A.U.: absorbance unit; M: molecular mass standards; Lf: lactoferrin; CSA: camel serum albumin; H55, H45, and H42: heavy-chains of immunoglobulins G of 55, 45, and 42 kDa, respectively; L30: light chains of immunoglobulins G of 30 kDa; CWBP: camel whey basic protein; α-LA: α-lactalbumin.

Investigation of radical-scavenging activity

Gallic acid and Trolox (soluble analog of vitamin E) are strong radical scavengers that were used in this study as positive controls. A linear relationship was found from the concentration response curve in the range of 0–5 µM gallic acid and 0–10 µM Trolox (Figure 2A). In the present study, the TEAC value of gallic acid was 3 µmol TE μmol^{-1} showing that gallic acid was a greater free radical scavenger than Trolox as evident from its three-fold higher antioxidant power. Its IC_{50} was 2.0 µM, close to the IC_{50} of 2.5 µM determined by Sadat et al. (2011).

Chen et al. (2003) found that the ABTS method was most suitable and sensitive to determine the antioxidant capacity of bovine milk proteins. This method was thus used in this study to assess the free radical scavenging activity of CWP. The activities of the different fractions were estimated by determination of the IC_{50} values (Table 1). The best activities were found for CWP and F1 (Figure 2B), respectively, whereas the other fractions did not display any interesting activity. The α-LA of CWP was fully recovered in F1 and was probably responsible of the respective free radical scavenging activities of CWP and F1. It was noteworthy that the basic proteins, IgGs, CWBP and Lf did not possess interesting scavenging power. Particularly, Lf did not show any detectable radical scavenging activity (Table 1). The basic amino acid residues Lys and Arg are not reported to be efficient free-radical scavengers (Hernandez-Ledesma et al., 2005) and might not confer such activity to the basic proteins containing them.

Table 1. IC_{50} and TEAC values of gallic acid, camel whey proteins (CWP) and the different chromatographic fractions. n.d.: not determined.

Sample	IC_{50} (g L^{-1} eq. BSA)	TEAC (µmol TE $µmol^{-1}$)
Gallic acid	2 µmol L^{-1}	3
CWP	0.15	n.d.
F1	0.20	1
F2	0.45	n.d.
F3	0.35	n.d.
F4	0.31	n.d.
F5	0.3 10^6	0.01

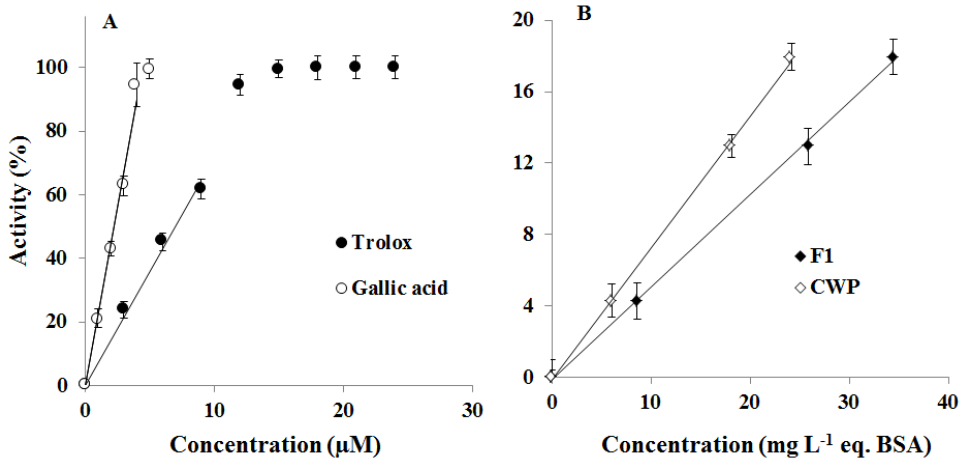

Figure 2. ABTS•+ radical scavenging activity determined at 740 nm of (A) Trolox and gallic acid, (B) the camel whey proteins (CWP) and the fraction F1 recovered from the cation-exchange chromatography separation of CWP. The equations of the curves are: (A) y = 22.24 x (R = 0.99) for gallic acid and y = 7.146 x for Trolox (R= 0.99) and (B) y = 0.737 x - 0.118 for CWP (R = 0.99) and y = 0.516 x - 0.118 for F1 (R= 0.9).

The TEAC of a protein mixture could not be compared to another one (the TEAC depends on the molecular mass of the compound tested rather than its weight expressed in g). However, the TEAC of F1 and F5 was calculated on the basis of the molecular masses of α-LA and Lf, the two proteins being considered to be the principal compounds of F1 and F5, respectively.

The TEAC value of F1 was 1 µmol TE $µmol^{-1}$, showing that the antiradical power of F1 was identical to that of Trolox. This value was, however, lower than that found by Salami et al. (2009) for the camel α-LA (3 µmol L^{-1}). This difference could be explained by the fact that F1 was a mixture of several proteins that underestimated the TEAC value of α-LA contained in F1. Recently, Sadat et al. (2011) have reported that bovine α-LA is a source of five highly antioxidant peptides and amongst them , Leu-Asp-Gln-Trp and Ile-Asn-Tyr-Trp exhibit remarkable free radical-scavenging activities towards $ABTS^{•+}$. These two peptides possess a Trp residue at their carboxy-terminal extremity. According to Tsopmo et al. (2011), in the presence of free radicals, Trp can lose the labile hydrogen linked to the nitrogen of its indole ring leading to produce a radical stabilized by electron delocalization. For these authors, Trp plays a crucial role in the ability of proteins or peptides to scavenge free radicals. The addition of an extra Trp residue at the amino-terminal extremity of peptide Ile-Ser-Glu-Leu-Gly-Trp significantly increases its antioxidant power (Tsopmo et al., 2011). The camel α-LA possesses five Trp residues on its sequence, whereas the bovine counterpart contains only four. The presence of an additional Trp residue in the case of the camel sequence might contribute to its better radical scavenging power than that of the bovine protein reported by Salami et al. (2009).

On the other hand, Salami et al. (2010) have reported that CWP are a source of hydrolysate with significantly higher free radical-scavenging properties than bovine whey protein hydrolysate. Hernandez-Ledesma et al. (2005) have reported

that the lowering of the number of peptide bonds has an increasing effect on the antioxidant activity of the constituent amino acids of small peptides (typically with molecular masses lower than 1000 Da). In the case of the bovine species, α-LA hydrolysate obtained by thermolysin action displays a high and similar free radical-scavenging power than the source protein, since no improvement of the activity has been observed after enzyme treatment (Sadat et al., 2011). By taking into consideration the results reported by Salami et al. (2010), it would be thus interesting to determine in a further work if enzyme hydrolysis of the camel proteins contained in F1, mainly α-LA, would be required to enhance the antioxidant activity of this protein fraction.

The TEAC value (0.01 µmol TE μmol^{-1}) of pure Lf eluted in F5 was very low indicating that this protein did not possess any antiradical properties. It is however reported that Lf possesses antioxidant properties. In fact, these properties are rather related to its capacity to bind iron and therefore to inhibit the Fenton reaction than to any free radical-scavenging activity (Belizy et al., 2001). The main property of Lf is that it is a source of antimicrobial peptide named lactoferricin (Lfcin; Gifford et al., 2005). The Lfcin is produced by the gastric protease pepsin and it would be thus interesting to investigate the possibility of camel Lf to be a source of Lfcin-like peptide.

Conclusion

The cation-exchange chromatography enabled us to produce an α-LA-enriched fraction that was not retained on the column. Thus, this method may be adapted for high volumes of camel whey with *e.g.* fractionation onto CM Sephadex medium to readily prepare large quantities of α-LA-enriched fraction. The latter displayed a greater antioxidant power and might therefore have capability to attenuate oxidative stress occurring in IBD after oral administration. The other fractions did not display any interesting free radical-scavenging activity and this might seem to be related to their basic property. However, the strongest adsorbed protein, Lf, was recovered almost pure and may be used for its various biological activities *i.e.* antimicrobial, antithrombotic and immunomodulatory effects, whereas the intermediate fractions containing the heavy-chain IgG2 and IgG3 may also be valorized in immunotherapy.

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