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Comprehensive characterization of bioactive peptides from Buffalo (*Bubalus bubalis*) colostrum and milk fat globule membrane proteins

N. Brijesha, H.S. Aparna *

DOS in Biotechnology, University of Mysore, Manasagangotri, Mysore 570 006, India

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ABSTRACT

Milk fat is dispersed in milk as small spherical globules stabilized in the form of emulsion by its surrounding membrane, often referred to as fat globule membrane (FGM). Buffalo, a major milking mammal of Asia and second most milking mammal across the globe presents physicochemical features different from that of other ruminant species containing higher content of lipids and proteins. The present study describes characterization of FGM proteins isolated from both buffalo milk and colostrum. A detailed proteomic analysis of peptides generated by *in vitro* gastrointestinal simulation digestion of buffalo milk and colostrum FGM fractions was performed by nLC-ESI MS/MS. The peptide based clustering of FGM proteins unravelled association of membrane proteins in fat transport, enzymatic activity, general transport, defence, cell signalling, membrane/protein trafficking protein synthesis/binding/folding including unknown functions. Gene annotation, STRING and YLoc analyses provided putative insights into major secretory pathways in milk and colostrum FGM peptides, interactive protein networks including their sub cellular localization. The peptides of milk and colostrum FGM offered cellular protection as powerful antioxidants indicated their promising perspectives in commercial formulations and nutraceuticals.

1. Introduction

Milk is a mammalian specific biological fluid while, colostrum is the early lacteal secretion produced after parturition to nourish and promote growth and development of the newborn in its new environment. Apart from various components present in milk, the milk fat synthesized in mammary epithelial cells are enveloped by cell membrane referred as milk fat globule membrane (MFGM) whose composition and properties are completely different from those of either milk or plasma (Nguyen et al., 2015; Robenek et al., 2006; Zou et al., 2015). The trilayered structure consisting of a complex mixture of proteins, glycoproteins, enzymes, phospholipids and glycosphingolipids accounts for 2–6% of the total mass of the fat globules (Singh, 2006). In milk, the MFGM enables the fat to remain dispersed and ensures structural integrity, protection and stability in the aqueous phase (Ye, Singh, Taylor, & Anema, 2002).

Extensive research has been carried out on human and bovine MFGM on the identification of proteins using proteomic techniques (El-Loly, 2011; Smoczyński, Staniewski, & Kiełczewska, 2012; Sui et al., 2014). The major MFGM proteins reported to date includes mucin 1 (MUC1), xanthine dehydrogenase/oxidase (XDH/XO), periodic acid Schiff (PAS) III, cluster of differentiation 36 (CD36), butyrophilin (BTN), PAS 6/7 or lactadherin, adipophilin (ADPH), proteose peptone 3 (PP3) and fatty acid - binding protein (FAB). Mather (2000) reported glycosylated proteins while more recent studies unravelled glycoproteomics of both human and bovine MFGM (Dallas et al., 2014; Murgiano et al., 2009; O'Riordan, Kane, Joshi, & Hickey, 2014).

More than 97 million tons of Buffalo milk produced each year represents the second largest volume of milk produced globally after cow's milk (FAOSTAT, 2012). The major constituent of the buffalo milk is fat fraction, which represents almost twice the fat content of bovine milk (Solah, Staines, Honda, & Limley, 2007). Ménard et al. (2010) reported that buffalo milk is also one of the richest milks from a compositional point of view having fat globules that are significantly larger in size compared to bovine milk fat globules. Previous authors have isolated buffalo MFGM (Abou-Dawood, Moussaa, El-Demerdash, & Ahmed, 1988; D'Ambrosio et al., 2008) and the proteomic analysis identified 50 proteins from buffalo MFGM similar to bovine MFGM proteins (D'Ambrosio et al., 2008; Fong, Norris, & MacGibbon, 2007). Recently, an insight into inter-species complexity of MFGM from Holstein, Jersey, yak, buffalo, goat, camel, horse, and human was established by an iTRAQ proteomic approach (Yang, Zheng, Zhao, Zhang, & Han, 2015). More recent studies by Nguyen et al. (2015, 2016) not only revealed the microstructure and also showed the dynamics of the biological membrane surrounding the buffalo milk fat globule as a function of temperature.

* Corresponding author at: Department of Studies in Biotechnology, University of Mysore, Mysore 570 006, Karnataka, India.
E-mail address: aparna@appbot.uni-mysore.ac.in (H.S. Aparna).

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MFGM appears to be an important source of nutraceutical components including polar lipids and membrane proteins (Spitsberg, 2005). MFGM can also be used as "natural" emulsifiers, in preventing flocculation and coalescence of fat globules in milk and protecting the fat against enzyme action (Evers et al., 2008). Human MFGM proteins such as mucins and PAS 6/7 including immunoglobulin were reported to play important role in various cell processes and defence mechanisms against bacteria and viruses in the newborn (Silvestre et al., 2005Spitsberg, 2005). Similarly, inhibitory properties of mucin 1, PAS 6/7, and PP3 during rotavirus were reported (Bojsen et al., 2007; Inagaki et al., 2010; Kvistgaard et al., 2004). In addition, XDH/XO, a major protein of MFGM plays an antimicrobial defensive role in the neonatal gut complementing endogenous enzyme of the intestinal epithelium (Harrison, 2006). Its activity is relatively high in bovine milk compared to milk from other species (*e.g.* goat, sheep, camel, donkey, mare and human milk (Uniacke-Lowe, 2011 & Gantner, Mijić, Baban, Škrtić, & Turalija, 2015). MFGM glycoproteins were also known to prevent adhesion of the colonic microbiota and resulted in increased bacterial butyrate production (Struijs et al., 2013).

The research focus on MFGM has enhanced our understanding on different proteomes of human and bovine MFGM and provided insights for their inclusion in commercial food formulations with potential health benefits. Therefore in the present study, FGM proteins isolated from buffalo milk and colostrum were subjected for *in vitro* gastrointestinal simulation digestion using hydrolytic enzymes, pepsin and pancreatin in order to generate bioactive peptides. The small peptides (<3 Da) were characterized by nLC-ESI MS/MS and their functional clustering, interaction and localization studies were carried out by gene annotation, STRING and YLoc analyses. The cellular response to oxidative stress was evaluated in the presence of FGM peptides by chemical and cell based assays.

2. Materials and methods

2.1. Chemicals

The colostrum (first day) and milk samples were collected from healthy buffaloes. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Pepsin and pancreatin, dichlorodihydro fluorescein diacetate (DCF), 4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid (HEPES), 2, 4-Dinitrophenol (DNP), homovanillic acid (HVA), o-phthaldehyde, di-thio-bis-nitrobenzoic acid (DTNB), enhanced Chemiluminescence detection reagents and hyperfilm ECL were from Sigma Chemical Co. USA. All other reagents were of HPLC or analytical grade.

2.2. Isolation of MFGM

The colostrum and milk fat globule membranes (CFGM & MFGM) were extracted from cream as described by Basch, Greenberg, and Farrell (1985) with minor modifications. Briefly, the cream was washed sequentially (4500 ×*g*, 10 min, 4 °C) in phosphate buffer saline (PBS, 10 mM, pH 7.2) and distilled water. FGM thus collected was suspended in distilled water and allowed to crystallize at 4 °C for 20 h. The separated fat and serum fractions were warmed (45 °C, 30 min) to melt the fat and washed again with distilled water to recover the residual serum. The total serum was centrifuged (5000 ×*g*, 15 min, 4 °C), washed twice in acetone (1:4, v/v) to remove fat (8000 ×*g*, 20 min, 4 °C), allowed to dry and stored at −20 °C until further analysis.

2.3. In vitro digestion

In vitro digestion of FGM was performed as described by Wu and Ding (2002). CFGM & MFGM in 3.5% KCl-HCl buffer (100 mM, pH 2) were mixed with pepsin (4%, w/w) and incubated for 4 h at 37 °C. Reaction was terminated by boiling for 10 min and neutralized using NaOH (2N). The suspension was centrifuged (10,000 ×*g*, 30 min) and digestion was continued with pancreatin (4%, w/w) at 37 °C for 4 h. The reaction was arrested by keeping in boiling water bath (10 min) followed by centrifugation (10,000 ×*g*, 30 min). Supernatants passed through Amicon filter (<3 kDa) were sequentially extracted using extraction buffer 1 (300 μl, 60% acetonitrile [ACN] containing 0.1% formic acid) and buffer 2 (150 μl, 100% ACN) respectively. Supernatant collected from both the extraction steps were pooled and dried in Speed Vac. The peptides reconstituted in 0.1% formic acid (100 μl) were desalted on C-18 solid phase extraction disks (3M Empore) (Rappsilber, Ishihama, & Mann, 2003) and quantified as described by Church, Swaisgood, Porter, and Catignani (1983).

2.4. nLC-ESI MS analysis of peptides

nLC-ESI MS separation of peptides (<3 kDa) was carried out on Agilent 6550 iFunnel QTOF mass spectrometer (Agilent Technologies) coupled to an Agilent 1260 Infinity Capillary Pump and 1260 Infinity Nano flow Pump LC system. The samples were loaded on to a Polaris - HR Chip-3 C18 reverse-phase separation column (150 mm × 75 μm, 3 μm, Agilent Technologies). The solvent system consisted of water containing 0.1% formic acid (A) and ACN: H_2O (90:10) containing 0.1% formic acid (B). The peptides were separated using a linear gradient of 3% to 97% solvent B in 90 min at a flow rate of 0.3 ul/min. Mass spectra were acquired in positive ion mode by scanning *m/z*-range from 100 to 3200. The data was analyzed using MassHunter Software LC/MS Data Acquisition; Version: B.05.01. LC was interfaced directly with a Q-TOF Mass Spectrometer and MS/MS data of the peptides was obtained in CID using Nitrogen as collision gas. The data files were processed using Spectrum Mill software (Agilent Technologies) in Swiss-Prot and peptides were identified.

2.5. Bioinformatics and statistical analysis of proteins

Protein-protein interaction for protein dataset was obtained from STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database v9.1 (www.stringdb-org).Accumulated peptide sequences of FGM proteins identified by MS/MS were uploaded into YLoc software to predict their subcellular localization. Analysis of the identified FGM-enriched proteins of colostrum and milk associated with annotated functions was performed by gene ontology (GO) annotation software (http://david.abcc.ncifcrf.gov/home.jsp). All assay methods were carried out in triplicates. Data was analyzed separately for each experiment and subjected to Arcsine transformation and analysis of variance (ANOVA). Further, the experimental results were subjected to Tukey's HSD at ($P < 0.05$) [SPSS tool (version 8)].

2.6. ABTS assay

ABTS radicals (ABTS*) were generated by reacting ABTS with potassium persulphate (2.45 mM) as described by Re et al. (1999). The absorbance of the solution was adjusted to 0.7 at 734 nm using PBS (150 mM, pH 7.5). The reaction was initiated by adding test samples (50 μl) at different concentrations to ABTS (950 μl) working solution. The reaction mixture was incubated for 5 min at room temperature and the absorbance was measured at 734 nm. Percentage inhibition was calculated and the dose response curve was plotted.

2.7. Experimental design

Venous blood was collected from healthy, non-smoking human volunteers as per the guidelines of Institutional human Ethical Committee (IHEC-UOM No. 59/PhD/2011-12) University of Mysore, Mysore. Blood samples were collected in the presence of EDTA were categorized into Group I-Control blood, Group II - blood treated with 2, 4 DNP (100 μM/ml), Group III - blood treated with 2, 4 DNP pre incubated with MFGM peptides (200 μg) for 10 min at 37 °C, Group IV - blood treated