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Research paper

The effect of proteolysis on the induction of cell death by monomeric alpha-lactalbumin

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ABSTRACT

α-Lactalbumin (α-la) is a major whey protein found in milk. Previous data suggested that α-la has antiproliferative effects in human adenocarcinoma cell lines such as Caco-2 and HT-29. However, the cell death inducing α-la was not a naturally occurring monomer but either a multimeric variant or an α-la:oleic acid complex (HAMLET/BAMLET). Proteolysis showed that both human and bovine α-la are susceptible to digestion. ELISA assays assessing cell death with the native undigested α-la fractions showed that undigested protein fractions did have a significant cell death effect on CaCo-2 cells. Bovine α-la was also more effective than human α-la. A reduction in activity corresponded with lower concentrations of the protein and partial digestion and fragmentation of the protein using trypsin and pepsin. This suggests that the tertiary structure is vital for the apoptotic effect.

1. Introduction

Numerous bioactive peptides are contained in milk and are released during protein digestion in vivo and *in vitro* [1]. Caseins and whey proteins are affected differently by proteolysis with some soluble milk proteins such as β-lactoglobulin and α-lactalbumin reportedly passing into the jejunum almost intact [2,3]. α-Lactalbumin (α-la) is a small acidic Ca^{2+} binding protein, which is very important from several points of view. In mammary epithelial cells, α-la binds to β1,4-galactosyltransferase promoting the conversion of galactose into N-acetylglucosamine resulting in the efficient synthesis of lactose from UDP-galactose and glucose in the golgi complex [4]. Analysis with 1.7 Å resolution crystallography further suggested that it's three dimensional structure is similar to that of a hen egg-white (type-c) lysozyme. This suggests that α-la may have originated from a duplication of an ancestral gene that possessed both functions [5,6].

Sternhagen and Allen (2001) showed that α-la inhibits the proliferation of mammary epithelial cells and rat kidney cells as well as having antiproliferative effects in human adenocarcinoma cell lines such as Caco-2 and HT-29 [7]. The occurrence of SDS-stable, higher M_r (30–20 kDa) multimeric forms of α-la, however, were indispensable for cell death. The Ca^{2+}-concentration elevation activity of multimeric α-la was shown to be important for causing apoptosis [8–12]. A complex of either human or bovine apo-α-la and oleic acid (BAMLET or HAMLET, respectively) is also well known for its antitumoral activity via a mechanism that may involve lysosomal membrane permeabilisation, histone binding, autophagic cell death and histone binding [8,13,14]. Native monomeric α-la from human milk whey thus far showed no activity in cell death assays [8,12,15]. Instead, the 14 kDa α-la monomer initially stimulated the proliferation of cultured IEC-6 cell lines [9,15]. However, after treatment with trifluoroethanol (TFE) monomeric α-la became cytotoxic even though no structural changes could be observed [15].

Since most of bioactive milk peptides including the α-la variants with antiproliferative effects in humans were produced and tested *in vitro*, their behavior after human in vivo proteolytic digestion is lacking [16]. In fact, it has been shown that HAMLET and apo α-la are accessible to proteases in the β-domain [17]. Thus it is important to see if functionality of the α-la variants is maintained after proteolysis by major human proteases such as trypsin and pepsin. As its antiproliferative effect is to date uncertain, native α-la especially needs to be further examined [15].

The present study was performed to assess the effect of proteolysis on the potential to cause of cell death of CaCo-2 adenocarcinoma cell lines by bioactive milk proteins, in particular α-lactalbumin from bovine and human sources of various purity.

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Residual peptides were identified using nano Liquid Chromatography Electrospray Quadrupole – Time of Flight Tandem Mass Spectrometry (nanoLC-ESI-Q-TOF-MS/MS).

2. Materials and methods

2.1. Milk protein samples

Human α-lactalbumin with <95% purity (α-la (h)) and bovine α-lactalbumin with <85% purity (α-la (b)) were obtained from Sigma (St. Louis, Missouri) unless stated otherwise. A bovine milk protein fraction containing 55% α-lactalbumin (a-la55) was obtained from Arla Foods Ingredients (Viby, Denmark, Table 1).

2.2. SDS page analysis of milk protein preparations

To assess and compare the molecular complexity of protein samples obtained, we have conducted sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the bovine milk protein fraction containing 55% α-lactalbumin (a-la55) and the two commercial controls of bovine and human α-la respectively. Protein concentrations were determined using a commercial Bradford assay kit (BioRad). For mass calibration of protein samples a precision plus all blue protein ladder (Bio Rad cat no.: 161-0373) was applied. SDS-Page was conducted using commercial 10 well PAGE Bis-Tris mini gels with a 4–12% gradient (NuPAGE®, Life technologies). Protein samples (75 μg) were mixed with SDS containing sample buffer, heat denatured and reduced with DTT (10 mM) according to manufacturer protocols. Gels were run at 120 V (T: 20 °C) for 1 h using a commercial 2-(N-morpholino) ethanesulfonic acid (MES) buffer system (Life technologies cat no.: NP0002), which allowed a wide range protein separation from 3.5 kDa to 160 kDa. Gels were fixed in Acetic acid:methanol:water mixture and stained with colloidal Coomassie Blue staining kit (Life technologies cat no.: LC6025) according to manufacturer protocols. Application of the colloidal staining methodologies allowed enhanced visualization of sample protein with concentration <10 ng. Banding patters were analyzed using a Gel Doc™ XR+ System (BioRad). Peptide fragments identified during Liquid chromatography–tandem mass spectrometry (LC–MS/MS) were used to match banding patterns to a particular sample proteins.

2.3. Peptidomics of in vitro digested lactalbumin preparations by nanoLC-ESI-Q-TOF-MS/MS

Native samples (150 μg) of a-la55 and the commercial control samples of bovine and human α-lactalbumin were subjected to sequential *in-solution* (400 μL) digestion with sequencing grade procine trypsin (Promega) and pepsin (Promega) according to manufacturer protocols and was similar to the digestion used for the cell death assay below. Therefore, no protein denaturation protocols (i.e. with urea) were applied prior to enzymatic digestion. The aim of this procedure was to mimic *in-vivo* digestion of native lactalbumin samples, which prevents exposure of protease cleavage sites, thereby allowing only partial proteolysis. Initial protein digests were dried in 96 deep well plates (Greiner) using a Micro-Modulyo freeze drier (Thermo electron Corp.) with a 96 MTP adapter. The resulting solids were resuspended in a 5 μL solution of 0.1% formic acid–acetonitrile (19:1, v/v) and filtered through 0.2 μm Minisart SRP15 (diameter: 15 mm) syringe filter (Satorius cat no: 17558Q; Polytetrafluorethylene (PTFE) membrane) to remove any remaining solid matter.

Soluble peptides resulting from sequential sample digestion were separated using an Ultimate nano HPLC system (Dionex, Sunnyvale, CA) equipped with an auto sampler. MS/MS data was collected on an Applied Biosystems QSTAR XL (Applied Biosystems, Foster City, CA) hybrid quadrupole time-of-flight mass spectrometer. In protein digests (5 μL) were automatically desalted on a C18 trap cartridge for 3 min using 0.1% formic acid–acetonitrile, 98:2 (v/v), and then applied to an RP-C18 column (Vydac Everest, 75 μm × 250 mm, 3 μm, 100 Å, Hesperia, CA). Sample separation was accomplished using a linear binary gradient system [(solvent A: 0.1% formic acid–acetonitrile, 95:5 (v/v); solvent B: 0.1% formic acid–acetonitrile, 5:95 (v/v)]. Peptides were eluted using a linear elution profile starting at 5% B (0 min), which was ramped to 40% B over 33 min. The column was then washed by an additional solvent ramping step to 95% B until 42 min. Finally, the column was allowed to re-equilibrate to 5% B at 60 min [18]. Nanoscale electrospray ionization (ESI) was performed using a Protana interface fitted with a silver coated spray tip (FS360-20-10-CE, New Objective, Woburn, MA). The mass spectrometer was calibrated daily and operated above a resolution of 8000 with a mass accuracy of 10–50 ppm with external calibration using standards supplied by the manufacturer. The information dependent acquisition (IDA) cycle time of 10 s consisted of a single time-of-flight mass spectrometry (TOF MS) "parent ion survey" scan for 1 s followed by three 3 s MS/MS scans on the three most intense peptide signals. The dynamic exclusion window was set to exclude previously fragmented masses for 60 s. The doubly and triply charged ions with intensities greater than 10 counts in the TOF MS "survey" scan were selected for fragmentation. Collision energy optimized for charge state and m/z was determined by the Analyst QS software (Applied Biosystems). Nitrogen was used for the collision gas and the pressure in the collision cell ranged between 3 and 6×10^{-6} torr.

2.4. Database search

For identification of proteolytic cleavage peptide, MS/MS data sets of individual protein digests were compared with sequence information deposited in the National Center for Biotechnology Information (NCBInr; release May 2011) database [18]. Database searches were submitted with taxonomic restrictions (*Bos Taurus*, *Homo sapiens*) using an on-site permit of the Mascot search engine (Matrix Science Ltd., UK). Database searches were performed using a 2 Da mass accuracy for peptide masses and 0.8 Da mass tolerance for MS/MS spectra. The Mascot search parameters were enzymatic cleavage with trypsin and pepsin with each one possible missed cleavage, while carbamidomethylation and carbamylation of Cys residues were set as variable modifications to account for possible amino acid modifications due to the presence of urea in the experimental procedure. In addition, methionine oxidation was set as a variable modification. Proteins were assigned a definitive identification based on the search results from NCBInr database matching. Protein function was only assigned for proteins that had an assigned name after database searching, which therefore excluded hypothetical protein hits. If a particular gel spot resulted in multiple protein database hits due to lack of gel resolution, only those proteins that showed at least 2 unique peptides at or above the 95% confidence level for each NCBI database search have been considered [16,19] in the final data

Table 1
Protein composition (%) of bovine milk protein fraction containing 55% α-lactalbumin.

Product name	*a-la55*
alpha-Lactalbumin	55
beta-Lactoglobulin	18
CGMP	23
Other	4