The formation of an anti-cancer complex under simulated gastric conditions

Louise Sullivan[1,2], K. H. Mok[2,3] and André Brodkorb[1*].

[1]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.

[2] Trinity College Dublin, School of Biochemistry & Immunology, Trinity Biomedical Sciences Institute (TBSI), Pearse Street, Dublin 2.

[3] Trinity College Dublin, Centre for Research on Adaptive Nanostructures and Nanodevices (CRANN), Dublin 2.

*Corresponding author: andre.brodkorb@teagasc.ie

Telephone +353 25 42 222

Fax +353 25 42 340

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Abstract

A potent anti-cancer complex has previously been formed from two major components of milk. Human/Bovine α-Lactalbumin made lethal to tumour cells (H/BAMLET) is a protein-fatty acid complex that has been produced using the whey protein α-lactalbumin (α-LA), and the fatty acid oleic acid (OA). It was shown that it possesses selective anti-tumour and anti-microbial activity, which was first identified in acidic fractions of human breast milk. The aim of this study was to determine whether the two components would form a bioactive complex during simulated gastric (GI) transit. Results showed that a complex consisting of α-LA and OA is formed as the protein unfolds under acidic conditions and subsequently refolds upon pH increase. Analysis of this complex using Nuclear Magnetic Resonance (NMR) and Fourier Transform Infra-Red (FTIR) spectroscopies estimated a stoichiometry of 4.1 and 4.4 oleic acids per mole of protein respectively. FTIR and fluorescence spectroscopies showed that the structure was similar to that of BAMLET. Cytotoxicity testing against cancer cell line U937 cells showed that the complex had an LC_{50} value of 14.08 μM, compared to 9.15 μM for BAMLET. These findings suggest that a BAMLET-like complex may be formed under the tested *in vitro* GI conditions.

Abbreviations:

BAMLET: BAMLET produced through chromatography

α-LA: Holo α-LA at pH 7

Gastric α-LA: Partially digested α-LA in the gastric phase at pH 2.5

Post-gastric α-LA: Partially digested α-LA after increase to pH 7

OA: Oleic acid

FTIR: Fourier Transform Infra-red Spectroscopy

NMR: Nuclear Magnetic Resonance

ANS: 8-anilino-1-naphthanene sulfonate

BSSL: Bile Salt Stimulated Lipase

Introduction

HAMLET, Human Alpha-Lactalbumin Made LEthal to Tumour cells is a protein-fatty acid complex between partially unfolded α-LA and OA. HAMLET was discovered by serendipity in 1995 when screening human milk for its anti-infective properties (Hakansson *et al*. 1995). Since then the field of HAMLET has expanded greatly to include many structural (Gustafsson *et al*. 2005; Pettersson-Kastberg *et al*. 2009; Pettersson-Kastberg *et al*. 2009) and functional (Duringer *et al*. 2003; Hallgren *et al*. 2006; Aits *et al*. 2009) studies of the complex. To date it is known that HAMLET can kill a range of over 40 different cancer cell lines with some specificity, while having little or no detrimental effect on healthy, differentiated cells (Mok *et al*. 2007). HAMLET acts with an apoptosis-like mechanism. It induces microautophagy in cells (Aits *et al*. 2009) and activates caspases (Duringer *et al*. 2003). However it has also been shown that cell death is independent of caspase activation (Hallgren *et al*. 2006). HAML*ET al*so has anti-microbial activity against *Streptococcus pneumonia* (Hakansson *et al*. 2000). The activity of HAMLET was tested in several *in vivo* clinical trials and it was observed that through application of HAMLET to bladder cancer (Mossberg *et al*. 2007), (Mossberg *et al*. 2010), skin pappilomas (Gustafsson *et al*. 2004) and also brain glioblastoma xenografts (Fischer *et al*. 2004), all of which a decrease in tumour size was observed with no effect on the healthy surrounding tissues.

HAMLET-like complexes have also been previously formed from the α-LA of milk from other mammals including caprine and ovine, with the most notable species variation being bovine α-LA, denoted BAMLET (Pettersson *et al*. 2006), which is

used by a number of different research groups (Liskova *et al*. 2010; Rammer *et al*. 2010; Spolaore *et al*. 2010).

α-LA is a small (M_w 14,200 Da), acidic (pI ~4.8), globular protein found in the whey fraction of the milk of all mammals. α-LA contains four disulphide bridges cross-linking the polypeptide chain. One of the disulphide bonds bridges the large α-helical domain and the smaller β-sheet domains thereby forming the calcium binding loop. The calcium is bound to the protein through aspartic acid (Asp) residues. However when the pH of the protein decreases below its isoelectric point these Asp residues start to become protonated and release the calcium thus leaving the protein in a partially unfolded state (Kuwajima et al, 1980). This unfolding also leaves the hydrophobic core exposed and this was confirmed through fluorescence studies by (Rösner and Redfield, 2009). This partially unfolded α-LA is structurally similar to the apo (calcium free form) and is dubbed the A-state or acid-state (Kuwajima, 1996).

The main biological function of α-LA is to specify the substrate for lactose synthesis in the mammary gland (Brew *et al*. 1968). A follow-up clinical trial with mice where the gene that expressed α-LA was removed, the mice did not produce any α-LA or lactose in their milk (Stinnakre *et al*. 1994). A second and equally important function of α-LA is that it is nutritionally significant for nourishment, growth and development, particularly in humans. The amino acid profile of α-LA delivers most of the essential amino acids in the required concentrations needed for neonatal development.

The protein composition of human milk is also of significant importance. α-LA is the predominant protein in human milk, present at concentrations of 2-3 gL^{-1} (Lönnerdal 2003). Similarly oleic acid is the most abundant fatty acid (as glycerides) in human milk and accounts for 34% of all fat (Gibson and Kneebone, 1981). Indigenously present in human milk is bile salt stimulated lipase (BSSL), which has lipolytic activity in the stomach, increasing lipolysis levels in both the stomach and intestines of infants (Jensen, 1995).

Monitoring the pH profile in early infants stomachs shows that the pH remains elevated (Mitchell *et al*. 2001) and above the required pH levels for proteolysis with pepsin (Favilla *et al*. 1997), thus there is a relatively low rate of proteolysis in the stomach of infants. Low proteolysis levels results in polypeptide chain stability and presence of significant amounts of native α-LA as milk passes through the stomach phase of gastric digestion.

The conditions under which HAMLET was initially discovered were similar to those in the stomach (Svensson *et al*. 2000; Barbana *et al*. 2006). Given that the composition of human milk may lend itself to the formation of HAMLET – high α-LA levels, high OA levels and high gastric lipolysis – there has been speculation that a HAMLET-like complex may be formed in the gastric tract of breast-fed infants.

The aim of this study was to determine if a BAMLET-like complex could be formed under simulated gastric conditions. Fractions were produced and analysed for structural and biological similarities to characterised BAMLET – monomeric with

118 approximately 5 times molar excess of oleic acid, produced using the previously
119 published chromatographical method as a reference.
120
121 **Materials and methods**
122
123 ***Materials***
124 BioPURE-Alphalactalbumin™ (95% protein; 5% β-lactoglobulin) was purchased
125 from Davisco Foods International Inc. (Minnesota, USA); Diethylaminoethyl (DEAE)
126 Trisacryl ion exchange matrix was purchased from Pall Corp. (France); alamarBlue
127 assay was purchased from Invitrogen (California, USA); Bio-Rad Protein Assay and
128 molecular weight markers for SDS-PAGE from Bio-Rad Laboratories (Hertfordshire,
129 UK). Unless otherwise stated all other chemicals and reagents were purchased from
130 Sigma-Aldrich.
131
132 ***Methods***
133 BAMLET Production
134 A stock of BAMLET was produced using the previously published chromatography
135 method of (Svensson *et al*. 2000). Briefly, apo α-LA was injected on a DEAE-
136 Trisacryl ion exchange column that was preconditioned with oleic acid. BAMLET
137 fractions were eluted using 1M NaCl, dialysed against distilled and deionised H_2O,
138 lyophilised and stored at -20°C before analysis.
139
140 Simulated digestion
141 An *in vitro* gastric model was used. Simulated digestion experiments were performed
142 at 37°C. Porcine pepsin (P7000, Sigma-Aldrich, Ireland), activity 653 U/mg protein

using haemoglobin as a substrate (Sigma Aldrich, 10-30-8378), was used at an E:S ratio of 1:100. The pH of the digest was controlled as per Figure 1 using a computer controlled Tiamo 842 pH monitoring and titration system (Metrohm) equipped with a pH probe for protein analysis. pH was maintained using 1M HCl and 1M NaOH. The pH gradient was used to effectively mimic the pH of the stomach of infants during and after feeding (Mitchell, McClure *et al*. 2001). Homogeneity of the digest was maintained through the use of a submergible stirrer at a rate of 80 rpm. Digestions were monitored for 2 hours with samples taken at regular time points for analysis.

To formulate the test milk, α-LA was dissolved at a concentration of 700 μM. $CaCl_2$ was added at a concentration of 0.986 μM to ensure α-LA was in the holo conformation. OA was dissolved in ethanol to solubilise the fatty acid and the FA-ethanol emulsion was added to the α-LA to give a final OA concentration of 70 mM. Samples were taken at T0 (α-LA), in the gastric phase at pH 2.5 (α-LA pH 2.5) and when the pH of the mixture was increased to pH 7 (post-gastric α-LA pH 7). Each sample was dialysed against distilled and deionised water at 4°C, lyophilised and stored at -20°C prior to resolubilisation at differing concentrations prior to analysis.

Fluorescence Spectroscopy

Fluorescence measurements were performed using a Cary Eclipse Fluorescence Spectrophotometer equipped with a multicell holder, peltier unit and temperature controller. Protein concentrations were determined by the method of Bradford and the protein concentration of the samples was diluted to 5 μM in the appropriate buffer. Spectra were recorded at 25°C and at a scanning speed of 80 nm/min. The excitation and emission slits were set to 10 mm.

Intrinsic fluorescence

Samples were dissolved in 10 mM Tris.HCl buffer, pH 8.5. Samples were excited at 280 nm and emissions were recorded between 300 and 420 nm.

ANS fluorescence

Samples were dissolved in 10 mM Tris.HCl buffer, pH 8.5 and ANS dye was added to samples at a concentration of 10 μM and left to incubate at room temperature for 30 minutes. The samples were then excited at 390 nm and emissions were recorded between 410 and 600 nm.

Thioflavin T (ThT) fluorescence

ThT was dissolved in 10 mL phosphate buffer (10 mM Na_2HPO_4; 150 mM NaCl), filtered through a 0.2 μM syringe filter and stored in the dark. On the day of testing 1 mL of this stock was added to 50 mL phosphate buffer. The fluorescence of the working buffer was measured with an excitation wavelength of 440 nm and emissions were recorded between 470 and 550 nm. 15 μL of the native protein solution was added to the working buffer and the spectra were recorded. This procedure was repeated for protein complexes. An increase in intensity between the control and test sample is indicative of amyloid formation.

Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR measurements were performed using a Bruker Tensor 27 instrument equipped with a thermally controlled BioATR Cell™ II (Bruker Optik, Germany) which was designed for analysis of protein in solution. Measurements were performed at 20°C and an average of 180 scans at a resolution of 4 cm^{-1} were recorded. Protein samples were diluted to a concentration of 700 μM and filtered through a 0.1 μM syringe filter. Data was processed by performing atmospheric compensation and then vector

normalisation at 1600-1720 cm^{-1} for the Amide I region, and 2800-2900 cm^{-1} for the oleic acid region. Spectra capturing software Opus (version 5.5) was used for data processing. The molar ratios of oleic acid to α-LA were determined using a multivariate calibration method that was developed using OPUS/QUANT software.

Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H NMR spectra were obtained using a 600 MHz Ultrashield NMR Spectrophotometer (14.1 Tesla magnetic field strength) with an indirect detection cryoprobe (Bruker BioSpin, Germany). Samples were prepared to a concentration of 1 mM in 10% D_2O. 1,4-Dioxane was used as an internal standard. Spectra were obtained at 25°C. The stoichiometry of the protein-fatty acid complexes was determined using integration of the peak areas for the aromatic region of the protein and the region where oleic acid binds.

High-Pressure Liquid Chromatography (HPLC)

All HPLC experiments were performed on a Waters 2695 Separations Module equipped with a dual wavelength detector set at 214 nm and 280 nm. Where applicable the column-oven was kept at 28°C. The sample chamber was set at 10°C. A sample volume of 20 µL of a 20 µM protein solution was injected onto the column.

Reversed-phase HPLC (RP-HPLC)

The two solvents used for RP experiments were 0.1% TFA in MilliQ™ (A) and 90% MeCN, 0.1% TFA in MilliQ™ (B).

For native protein analysis RP-C5 was performed using a SourceRPC5 column. The starting conditions of solvents were 60:40 A:B with a runtime of 34.1 minutes.

For peptide analysis a µSymmetry C18 column was used. The starting conditions of solvents were 100:0 A:B with a runtime of 30 minutes.

Size Exclusion Chromatography (SEC)

Digestion progression was monitored through SEC-HPLC using a TSK G2000 column. The flow was isocratic and the solvent used was 30% MeCN with 0.1% TFA. There was a flowrate of 0.5 mlmin-1 and a runtime of 60 minutes.

Electrophoresis

SDS-PAGE was performed as per the method of (Laemmli 1970). The gels were prepared to a concentration of 20% acrylamide. Samples were dissolved at a concentration of 700 μM and diluted 1:8 with reducing SDS-PAGE buffer. Samples were heated at 95°C for 5 minutes and cooled at room temperature. Molecular weight markers (BioRad, 161-0373, MW range 250 kDa – 10 kDa) were used as a reference for molecular weights.

Cell viability assay

Cytotoxicity measurements were performed using U937 cells – a suspension cell line extracted from a human diffuse histiocytic lymphoma. Cell were grown at a density of 1×10^5 in RPMI 1640 supplemented with 10% fetal bovine serum and incubated at 5% CO_2 at 37°C. For cytotoxicity assays cells were subcultured at a density of 2×10^5. Samples for cytotoxicity analysis were dissolved in RPMI 1640 and sterile filtered with a 0.1 μM syringe filtered and diluted to a concentration of 352 μM. Samples were then diluted to desired concentrations between 0 and 176 μM with RPMI to give a final volume of 50 μL. Cells at a density of 2×10^5 were then added to the samples in the well to give a final volume of 100 μL with final FBS concentration at 5%. Plates were incubated for 24 hours and 10 μL of alamarBlue reagent was added to each well and the plate was incubated for 4 hours. The fluorescence intensities of

243 the samples were measured with an excitation wavelength of 530 nm and emissions
244 were recorded at 590 nm. For samples with digestive enzymes present, pepstatin, a
245 potent pepsin inhibitor was used to inhibit enzyme activity during bioactivity testing.
246
247 Apoptosis testing
248 The type of cell death occurring after treatment with samples was analyzed using a
249 Cell Death Detection ELISA^{PLUS} kit from Roche Applied Science. Cells were seeded
250 and supplemented with different samples in the same manner as for cytotoxicity
251 assay. After 4 h of incubation at 37 °C and 5% CO_2, the cell viability was determined
252 with a Trypan blue assay. Cells with 50%viability were used for the apoptosis assay.
253
254 Ultrafiltration
255 A stirred-cell dead-end ultrafiltration unit (Millipore) equipped with a 10 kDa
256 membrane was used to separate native protein from low molecular weight compounds
257 such as peptides and free oleic acid. The pressure and flowrate were controlled using
258 nitrogen gas at a pressure of 1 psi, which gave a flowrate of 1 $mLmin^{-1}$. A diafiltration
259 step was performed by flushing the cell with MilliQ™ water. Fractions were collected
260 and freeze-dried for analysis.
261
262 Gas Chromatography
263 The OA content of the complexes were determined as described by Palmquist and
264 Jenkins, 2003. An OA standard curve was prepared by adding 0, 10, 25 and 50 µL OA
265 dissolved in dichloromethane (11.7 mM) to separate tubes. As an internal standard 50
266 µL palmitic acid dissolved in dichloromethane (23.5 mM) were added to each tube
267 used for the standard curve and to the tubes used for test samples. After evaporation

of dichloromethane in a fume cupboard for 30 min, 100 µL PBS was added to the OA tubes and 100 µL of the protein complexes (BAMLET and digested complex) were added to the test samples tubes. To prepare derivatives of OA and palmitic acid for gas chromatography (GC) methyl esters were formed by the addition of 1.5 mL 10% methanolic HCl (prepared by slowly adding 20 mL acetyl chloride to 100 mL of dry methanol while stirring), and 1 mL heptane to each tube followed by heating to 90°C for 2 hours. After cooling on ice, 1 mL heptane and 3 mL 10% K2CO3 were added to each tube followed by vortexing and centrifugation (500*g*, 5 min). The heptane phase (upper phase) containing the fatty acid methyl esters was transferred to GC vials. Samples were analysed on a HP 6890 GC-system (Hewlett Packard Co., Palo Alto, CA, USA) with a flame-ionisation detector and a Restek Rt 2560 column (100 m x 0.25 mm x 0.2 µM, Restek U.S., Bellefonte, PA, USA). The inlet temperature was 275°C with a split ratio 40:1, and the carrier gas helium with a constant flow of 1.5 mL per minute. The starting temperature of 140°C was held for 5 minutes and increased by 4°C per minute to an end temperature of °C. The detector temperature was 300°C. Results were displayed as mg oleic acid per mg of powder thus to quantitate the oleic acid content in the samples the mg protein per mg of powder was determined using the Bradford method and molarities were determined on this basis.

Results

A simulated gastric model was used for simulated digestion. *In vitro* models such as the US Pharmacopeia use static models with a set incubation time at pH 2. However *in vivo* data from neonates show a more gradual decrease in gastric pH (Mitchell, McClure *et al*. 2001). Therefore a model which employed a pH curve was used in

order to allow sufficient time for indigenous lipase to act, protein to unfold and a small amount of proteolysis to occur.

Simulated digestion

The pH of the stomach of infants was mimicked according to the pH gradient in Figure 1A, as per the study of Mitchell *et al*. This gradient represents several different studies where gastric pH levels were monitored during feeding. Pepsin has an optimum activity of between pH 1.8 and pH 2. While these pH conditions are not favourable for proteolytic hydrolysis they are ideal for gastric lipolysis. Gastric lipase enzymes are active between pH 4 and pH, 6 thus high levels of lipolysis occurs in the stomach of breast-fed infants due to the presence of BSSL.

Structural analysis of the complex

Fluorescence Studies

It was previously shown by Svensson *et al* (2003) that α-LA unfolding is a pre-requisite for the conversion of α-LA to its bioactive form. Typically Ca^{2+} chelators are used to induce the partially unfolded apo form of α-LA. However in this study the A-state of α-LA, a structurally similar form of α-LA, was induced under these acidic conditions. Unfolding was observed after acidification of α-LA during the simulated gastric phase below pH 4. This was confirmed through the comparison of the fluorescence spectra for holo α-LA and apo α-LA (Figure 2A). When the gastric pH model experienced a pH increase, this resulted in a reverse conformational change towards a partially re-folded structure (Figure 2B).

316 Using ANS it is possible to detect changes in the surface hydrophobicity of the
317 protein (Pettersson-Kastberg, Mossberg *et al*. 2009). ANS binds to holo α-LA in
318 limited amounts, however there is an increase in hydrophobicity as the molecular state
319 of the protein changes from holo to apo, and a further increase in hydrophobicity
320 between apo α-LA and α-LA in the A-state (Figure 2C). This suggests a higher level
321 of hydrophobic residues exposed thus a marked increase in surface hydrophobicity.
322
323 ThioflavinT (ThT) fluorescence is a rapid screening method for the detection of
324 extended β-sheets such as those seen in amyloid fibrils. Any treatment of proteins that
325 changes their structure or functionality can also increase the risk of amyloid-like
326 formation, which would be a negative characteristic of proteins due to the association
327 of fibrils with degenerative diseases such as Alzheimer's and dementia (Dobson
328 1999). For both the BAMLET produced through chromatography and the complex
329 extracted from the simulated gastric conditions (data not shown) there was no increase
330 in intensity at 490 nm, which is indicative that OA does not promote amyloid fibril
331 formation under these conditions.
332
333 Through intrinsic fluorescence it was shown that BAMLET and α-LA:pH 7 have a
334 similar tertiary structure. Their secondary structure was also deemed to be similar thus
335 it is a fair assumption that their respective spectra for ANS fluorescence would be
336 alike. However this was not the case. This would suggest that ANS not only binds to
337 the hydrophobic regions of the protein, but also the hydrophobic fatty acid oleic acid.
338 This is further insinuated when the oleic acid stoichiometries for the respective
339 complexes are examined as this appears to be the sole difference in the two

complexes. BAMLET has a higher molar ratio of OA bound (see OA quantification data, Figure 4, Figure 5) and also a higher ANS fluorescence intensity.

FTIR

FTIR detects changes in the secondary structure of proteins by measuring changes in the Amide I band (1600-1720 cm^{-1}) which measure C=O stretching which gives information on the conformation of the protein. α-LA has a mainly α-helical structure thus has a peak at 1652 in the Amide I region of the FTIR spectra. Under acidic conditions there is a shift in the absorbance maximum of α-LA. A random coil formation, as would be observed when the protein is in its molten globule state and lacks 2°- or 3°- structure, has a maximum intensity at 1645 cm^{-1}. α-LA at pH 2.5 under simulated gastric conditions has a maximum intensity at 1647 cm^{-1} suggesting that it is in the molten globule-like form, which is in agreement with both intrinsic and ANS fluorescence. These secondary structure results are in agreement with the tertiary structures measured with intrinsic fluorescence, and as with intrinsic fluorescence, the digested complex and BAMLET have a similar secondary structure. This corroborates the hypothesis that a BAMLET-like complex is formed under simulated gastric conditions (Figure 3).

NMR

The aliphatic region of the spectra, from 0 to -1 ppm, corresponding to the **δ**CH3 protons of Ile and the **γ**CH3 protons of Val residues, provides information on the structure and folding of the protein (Wijesinha-Bettoni, Dobson *et al*. 2001; Pettersson-Kastberg, Aits *et al*. 2009). Comparing spectra (a) from holo α-LA to α-LA in its molten globule form at pH 2.5 (spectra d) shows that the molten globule form

lacks any well-defined and 3° structure, which is characterised by the smooth line, whereas the spectra for holo α-LA, which has a series of peaks corresponding to its defined tertiary structure. The BAMLET complex that was produced through chromatography exhibits native like-structure compared to the molten globule form, although it is possible that there exists a partitioning of the population of protein molecules into native and molten globule states(Pettersson-Kastberg, Aits *et al*. 2009). It should be noted that column-BAMLET possesses cytotoxic activity, and the same was observed for the digested complex after the pH increase post-gastric phase of digestion, (Figure 4). This would suggest that structurally, it is possible that the partially unfolded state of the protein may coexist in equilibrium with the native state of molecules within the ensemble of the protein-fatty acid complex.

Oleic acid content

FTIR

Analysis of the FTIR spectra for α-LA, OA and BAMLET shows that a band at wave number 2854 cm^{-1} which corresponds to OA that is not present in α-LA but is present in BAMLET. Thus FTIR can detect the presence of oleic acid, and subsequently quantify the oleic acid content within the samples.

When α-LA is in its molten globule or apo form it is in the required conformation to bind to oleic acid and form a BAMLET-like complex. Under simulated gastric conditions at pH 2.5 there minimal levels of oleic acid were present. This suggests that hydrophobic interactions alone are not enough to bind oleic acid to α-LA. When the pH of the digest increased, α-LA refolded (Figure 5A). FTIR data suggests that there is a higher quantity of oleic acid present. Quantification of this oleic acid peak

gives an oleic acid stoichiometry of 4.4 moles of oleic acid per mole of α-LA. Quantification of the BAMLET sample produced through chromatography yields an oleic acid stoichiometry of 5.8, which is in line with the previously published literature of 5.4 with GC (Svensson *et al*, 2003) and 5.1 with NMR (Fast *et al*, 2005).

Evaluation of the ultrafiltration permeate and retentate showed that there was oleic acid present in fractions of the ultrafiltrate. This would suggest that oleic acid that is present in the retentate is bound to the protein as free oleic acid has been removed through the diafiltration step. The permeate contained the highest amount of oleic acid and also the low molecular weight fractions of the digest. (Figure 5B).

NMR

Nuclear Magnetic Resonance has previously been used to quantify oleic acid content in HAMLET and HAMLET-like complexes. By integrating the peak area of both the aromatic region of the protein (6-10 ppm) and the peak area of the olefinic oleic acid region (5-5.5 ppm) it is possible to quantify the amount of oleic acid present in samples. What distinguishes NMR from techniques such as GC and FTIR for OA quantification is that it can differentiate between bound and unbound oleic acid in samples rather than just determining if it is present in the samples. Oleic acid that is bound to protein has a chemical shift of 5.3 ppm whereas free, unbound oleic acid has a chemical shift of 5.4 ppm (Fast *et al*, 2007).

Analysis of both the column BAMLET and the digested complex show that the OA peak has a chemical shift of 5.3 ppm, verifying that the oleic acid is bound to the protein and not just in solution. Quantification of the oleic acid region of the digested

complex shows that there is a 4.1 molar excess of oleic acid bound per mole of protein, as compared to 5.6 for BAMLET produced through chromatography.

Gas Chromatography

Use of gas chromatography for quantification of oleic acid content in samples yielded results of BAMLET 11.54 mole OA/mole protein, and for α-LA-pH 7 6.84 mole OA/mole protein. This was extensively tested and it is thought that there is a lack of sensitivity for testing what is nanogram quantities of OA on a standard curve.

Poly-peptide chain composition of the complex

Ultrafiltration

In order to determine if BAMLET-like complexes can be formed with the peptides produced during *in vitro* simulated digestion an ultrafiltration step was employed using a 10 kDa UF membrane. Using size exclusion chromatography it was possible to determine that peptides were all of molecular weight less than 6.5 kDa, therefore the BAMLET-like complex (MW ~14,200 Da protein) is retained by the membrane while peptides and oleic acid were in the permeate (Figure 1B; Figure 5B). Analysis of the retentate of the UF sample of post-gastric a-LA shows that the OA present in the sample has a chemical shift of 5.3 which shows that oleic acid is bound to the protein (data not shown).

HPLC

Reversed-phase chromatography was used for both native protein analysis (C5), and peptide analysis (C18). C5 chromatography detects the loss in native protein and analysis of the samples showed that the peak obtained for α-LA had a retention time

of 11 minutes, which reduced slightly as digestion time progressed, suggesting that a slight decrease in hydrophobicity of the protein due to the loss of hydrophobic amino acids during hydrolysis. Quantification of the peak areas for α-LA before and after digestion shows that under the tested conditions ~60% of the native protein remains undigested after 30 minutes of digestion. (Figure 6A).

SEC showed that there was a breakdown of α-LA into smaller peptides. After the addition of the digestive enzyme, proteolysis began to occur. The initial sample of α-LA had a retention time of 28 minutes, and the intensity of the peak for native α-LA decreased as digestion time progressed, suggesting that there was still native protein present. There was an increase in the amount of breakdown products obtained during digestion (Figure 6B).

C-18 chromatography was used for peptide analysis. There was a decrease in native protein content and a subsequent increase in peptide formation as digestion progressed. (Figure 6C).

Electrophoresis

SDS-PAGE analysis (Figure 1B) confirmed that the digest was composed of protein and peptides. UF with a 10 kDa membrane removed some peptide material however some remained in the retentate. The permeate consisted of peptides.

Activity of the fractions

All fractions that were isolated throughout the process were tested against lymphoma cell line U937 for their cytotoxic activity and their activity was compared to the

activity of BAMLET produced through chromatography. BAMLET had an LC_{50} value of 9.15 μM. The digested complex had an LC_{50} value of 14.08 μM (Figure 7). The difference in the activity of the two samples can be related back to the oleic acid content within the samples. Testing of the fractions showed an enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm of the treated cells, indicating that the cell death was as a result of apoptosis (data not shown).

The UF permeate which contained the low molecular weight fractions from the digest was shown to contain the highest amount of oleic acid (Figure 3b) however bioactivity testing showed that this complex was not toxic to the cells in the tested concentrations.

Discussion

There has been speculation regarding the potential formation of HAMLET in the stomach of breast-fed infants (Svensson *et al*. 2003, Barbana *et al*. 2006). A recent study on the formation of BAMLET from denatured α-LA also alluded to the potential formation from α-LA from infant formula (Liskova *et al*. 2010). The data presented in this study indicates that a BAMLET-like complex, which is structurally and actively similar to BAMLET, can be formed under simulated gastric conditions.

Recently, Tolin *et al*., 2010, published results that suggest that α-LA fragments can bind to oleic acid and induce apoptosis in cancer cells with comparative activity to BAMLET. These complexes are produced through mixing oleic acid with the fragments of α-LA to produce the complexes, suggesting that should α-LA undergo digestion it may bind oleic acid and be active. However these fragments are produced

under limited proteolysis and thus may not be representative of fragments that may be produced through *in vitro* and subsequently *in vivo* digestion. Comparatively speaking, there were in excess of 20 peptides formed with the conditions tested in this study, which is considerably greater than the 3 fragments produced through limited proteolysis. Thus while bioactive complexes can be formed through α-LA fragments, this was not observed with the fragments produced in this study.

It was previously shown that unfolding is a pre-requisite for the conversion of α-LA to BAMLET; however unfolding alone is not enough to convert α-LA to its bioactive form (Svensson *et al*. 2003). The unfolding of α-LA results in an increase in its hydrophobicity (Pettersson-Kastberg *et al*. 2009) thus the current consensus is that it is the hydrophobic regions of α-LA act as binding sites for oleic acid, and that the formation of BAMLET is based on hydrophobic interactions
(Tolin, *et al*. 2010). Sequencing of peptides derived from α-LA could therefore result in the identification of hydrophobic peptides, which may form HAMLET-like complexes. Other forces, such as electrostatic charge, may also have an impact on the formation of complexes, thus the negative charge of apo α-LA and α-LA in its A-state may impact the formation of the complex. The effect of the pH conditions on both the solubility and the charge of the oleic acid may also therefore have an affect on the formation of the complex. Hydrophobic interactions may be the initial cause of interaction, however this hydrophobic interaction alone is not enough to produce the complex as at pH 2.5 the oleic acid present was not enough to have a cytotoxic effect against the cells. A secondary effect is needed to partially refold the α-LA in order to convert it to its bioactive form. Thus either the structural change in the protein (partial

refolding) or the change in the charge on the protein had an impact on the complex formation.

Another important factor to consider when determining the formation of the complex is the molecular state of oleic acid. Oleic acid has no charge below its pKa, and while the pKa of oleic acid is approximately pH 4.8, and this can change depending on osmotic conditions. Thus should the electrostatic force between the protein and the fatty acid could be a factor in the production of the complex, the oleic acid will not be in a suitable state to bind to the protein. Oleic acid is also pH dependant in that it can interchange between its acid state and its oleate (salt form) state. Therefore when looking at the complex formation it is necessary to not only look at the conformation of the protein, but also of the fatty acid state.

The structure of the complex remains a point of interest. There have been many structural studies on HAMLET and HAMLET-like complexes, the most recent by Pettersson-Kastberg *et al* in 2009 where they produced a perpetually molten-globule form of α-LA (rHLA$^{\text{all-Ala}}$-OA) and used it to produce a HAMLET-like complex. NMR spectroscopy of this complex and HAMLET produced through chromatography showed there were no chemical shifts in the upfield region of the spectra, suggesting a lack of tertiary structure within the protein. A similar structure has also previously been observed in other studies (Casbarra *et al*. 2004). However as shown in Figure 4, there are many peaks seen in the upfield region of the spectra, which corresponds to a native-like structure. We speculate that the reason lies in the fact that the alpha-lactalbumin-fatty acid complex forms a continuum of various partially-unfolded states

of varying degrees that make up the entire population (Pettersson-Kastberg *et al*. 2009).

The indigenous properties of human milk are also important for the formation of the complex. The presence of gastric lipase in the milk means that there will be elevated levels of lipolysis in breast-fed infants (Jensen 1995). The second important factor in the formation of the complex is the digestive tract of infants. The conditions are favourable for complex formation as the pH decreases over time (Mitchell *et al*. 2001), which are unfavourable for proteolysis, thus less proteolysis will occur. The pH decrease also promotes lipolysis prior to protein unfolding thus free fat will be present prior to protein refolding.

The resistance of α-LA to proteolysis in the presence of oleic acid (Casberra *et al*, 2004) is another interesting factor. This study has shown that as α-LA is converted to its bioactive form, 60% of the native protein remains. This is a significant finding, as conversion to the bioactive form is not seen with α-LA peptide fragments. It is also an inherent characteristic of α-LA that it is more resistant to tryptic hydrolysis than other whey proteins (Polverino de Laureto *et al*. 1995). Thus once peptic hydrolysis is complete, α-LA will remain more stable in the GI tract.

The binding of OA to α-LA also results in the stabilisation of α-LA. α-LA in its apo/a-state is inherently unstable as it does not have a co-factor bound (Halskau *et al*. 2002). Thus the binding of OA to the protein may also stabilise it to intestinal digestion.

Activity analysis of the digested complex compared to BAMLET yielded interesting results. Samples were solubilised in RPMI media with regards to protein content. Thus their oleic acid content is different based on oleic acid molar ratio estimation. BAMLET produced through chromatography has a lower LC_{50} value than the BAMLET-like complex produced through digestion. As the samples are structurally homologous it can be concluded that the difference in activity may be as a result of the difference in oleic acid levels, thus suggesting that OA is the active component of H/BAMLET and α-LA acts as a mule to deliver OA to the cells. This confirms previous results including (Wilhelm, Darinskas *et al*. 2009) and Permyakov *et al*, 2012.

This study also raises the potential for the production of BAMLET without chromatography. Many methods have been suggested for the formation of H/BAMLET without the use of chromatography, including mixing at room temperature and titrating with OA to its critical micelle concentration (Knyazeva *et al*. 2008), mixing at elevated temperatures (Zhang *et al*. 2009), and simple mixing in solution (Spolaore *et al*. 2010, Brodkorb & Liskova, 2009).

Thus it can be concluded from this study that under the tested simulated gastric conditions, a complex that is structurally and actively homologous to that of BAMLET can be produced.

584

	Abbreviation	Symbol	Analyses	
Stage 1: Formulation of milk			**Structural Analysis**	
α -Lactalbumin	α -LA	◆	Fluorescence	
Oleic acid dissolved in ethanol	OA	●	FTIR	
Apo α -Lactalbumin	Apo	○	NMR	Compared to BAMLET produced through chromatography (BAMLET ◇)
Stage 2: Gastric phase – pH 2.5				
Addition of HCl to mimic pH conditions (as per figure 1A)	Gastric α -LA	□	**OA Content**	
Addition of pepsin			FTIR	
Stage 3: Post-gastric phase – pH 7			NMR	
Addition of NaOH to bring pH to neutral conditions	Post-gastric α -LA	■	GC	
Stage 4: Ultrafiltration				
Ultrafiltration of digest through a 10 kDa membrane at pH 7	Retentate	△	**Bioactivity**	
	Permeate	—	Testing on U937 cells	

585 Table 1: Outline of different stages before, during and after *in vitro* gastric digestion, with corresponding samples and abbreviations used
586 throughout the study
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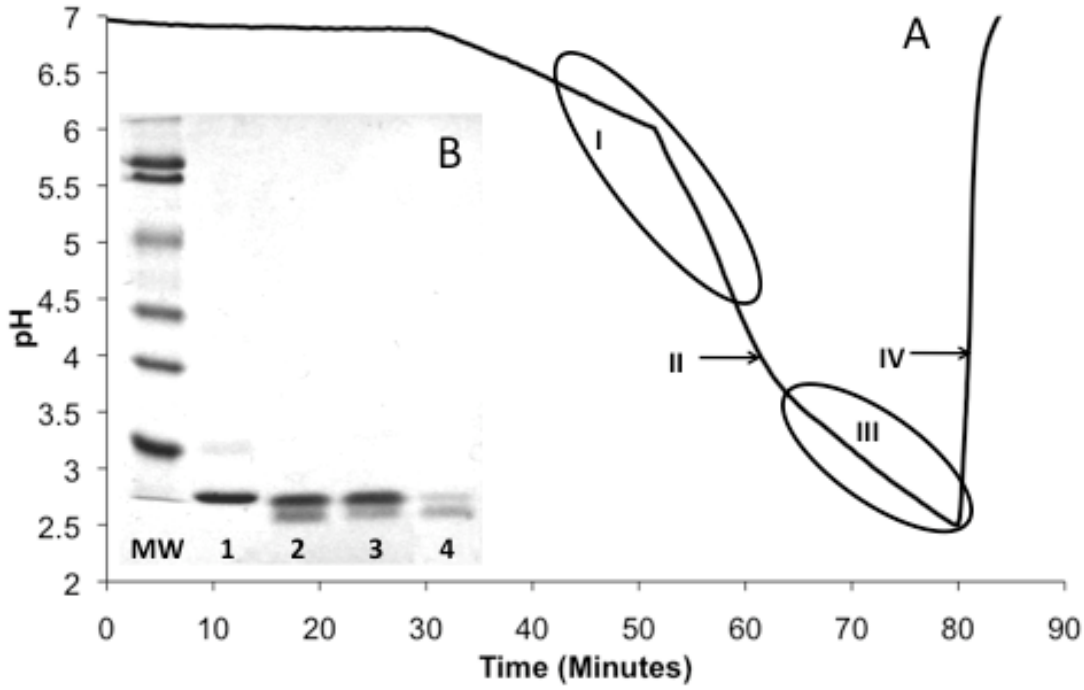

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590 Figure 1: A) pH gradient used during simulated gastric digestion with highlighted
591 areas: I) the optimum pH range for gastric lipolysis; II) the point at which α-LA
592 unfolding commences, III) the pH range of highest gastric proteolysis and IV) the pH
593 for α-lactalbumin re-folding. B) SDS-PAGE of 1- α-LA 2- Post-gastric α -LA 3- UF
594 Retentate 4- UF Permeate. MW – protein molecular weight markers 250 – 10 kDa.

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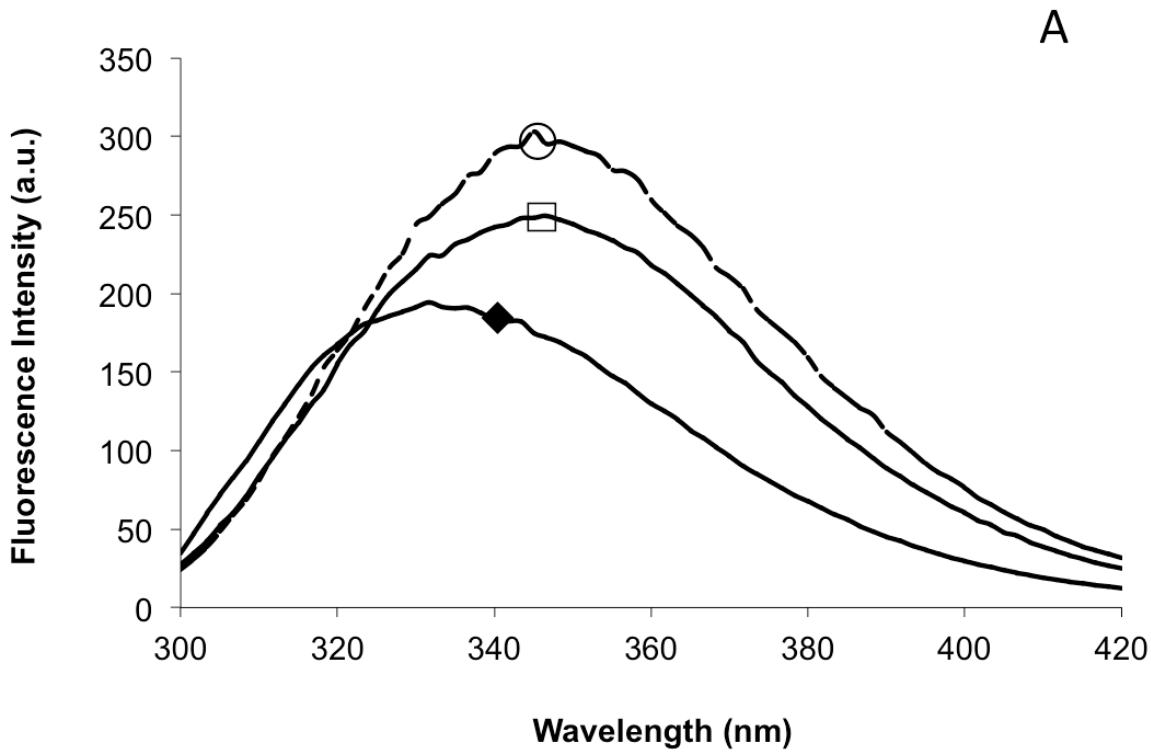

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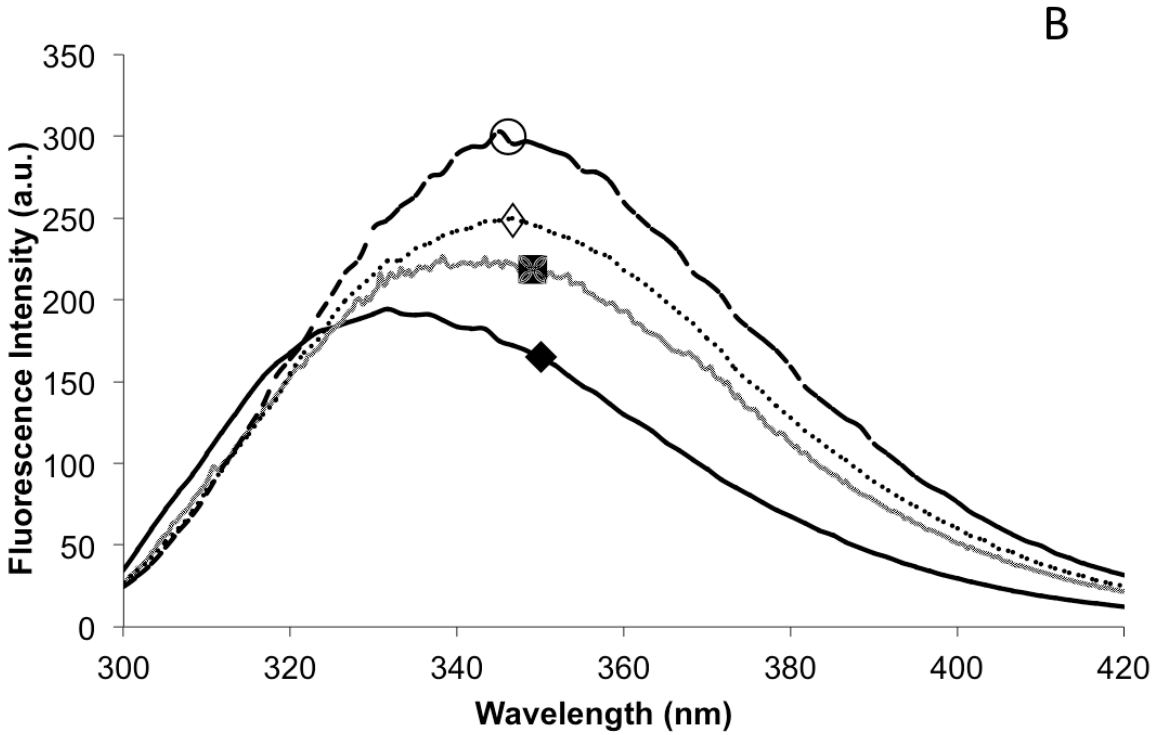

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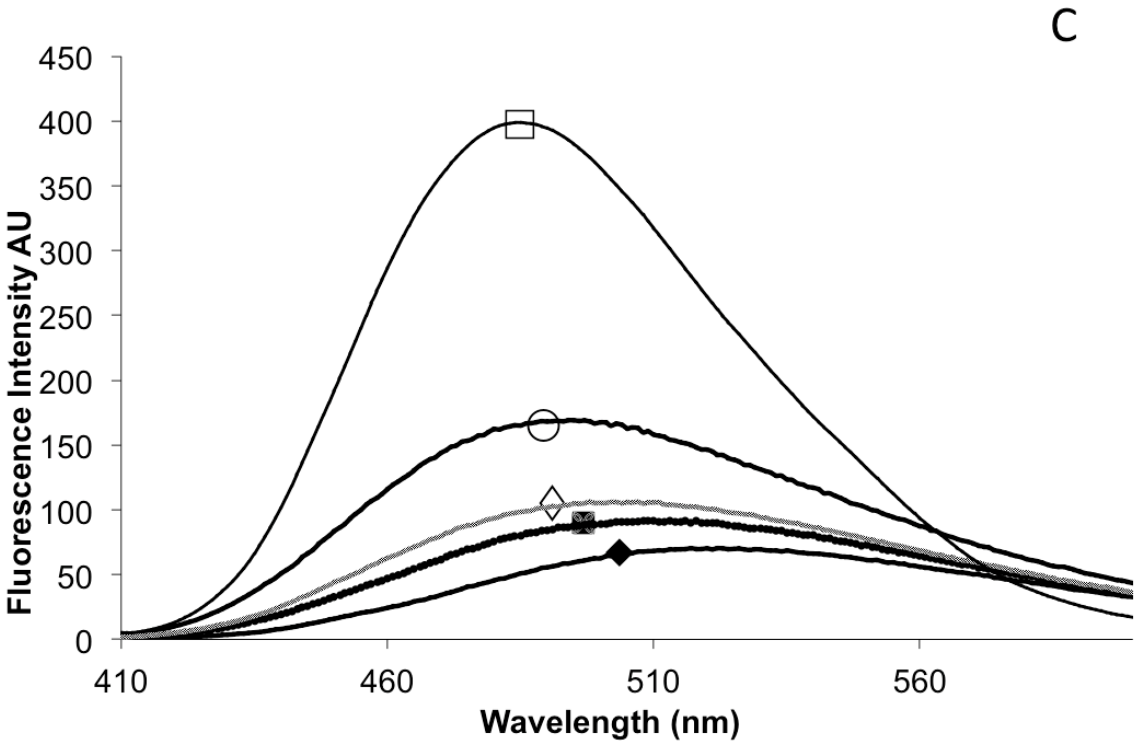
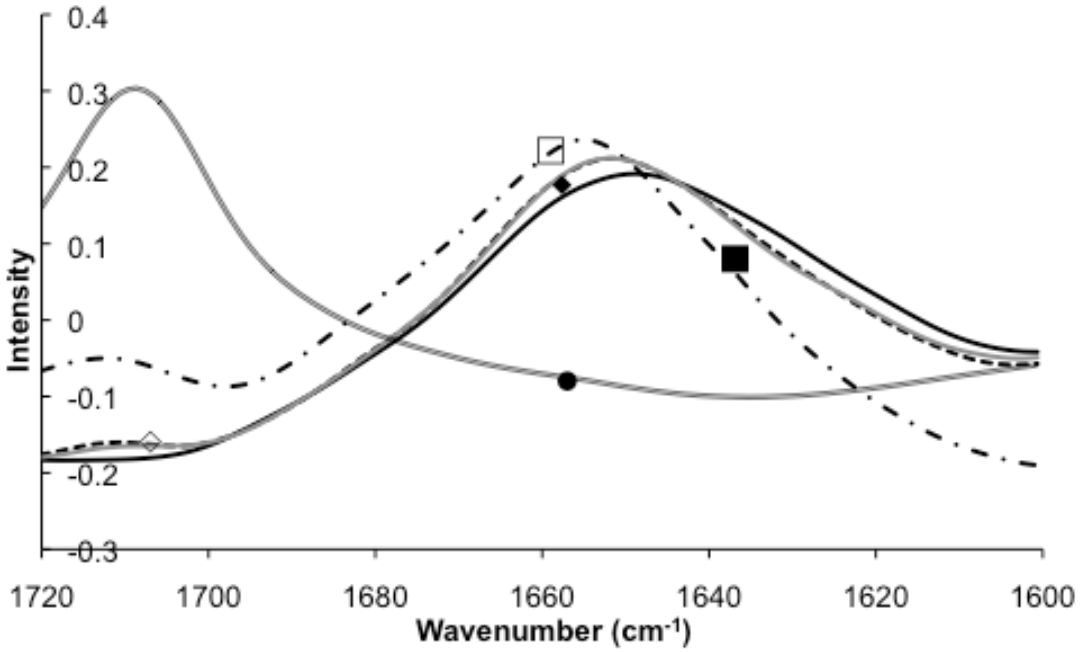


Figure 2: A) Intrinsic fluorescence spectrum of Gastric α-LA (□); for comparison, spectra of holo (◆) and apo (○) α-LA are also shown. The fluorescence maximum for Gastric α-LA was 349nm, for holo α-LA 327 nm and for apo α-LA 349 nm,

B) Intrinsic fluorescence spectrum of Post-Gastric α-LA at pH 7 (■); for comparison, spectra of holo α-LA (◆), and apo α-LA (○) and BAMLET (◇) are also shown.

C) ANS fluorescence spectra of Gastric α-LA (□); Post-Gastric α-LA (■); for comparison, spectra of apo α-LA (○); BAMLET (◇) and holo α-LA (◆) are also shown.



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613 Figure 3: Amide I region of vector-normalised FTIR of Gastric α-LA at pH 2.5 (□)

614 and Post-Gastric α-la at pH 7 (■) ; native α-LA (◆); BAMLET (◇) and oleic acid

615 (●) shown for comparison.

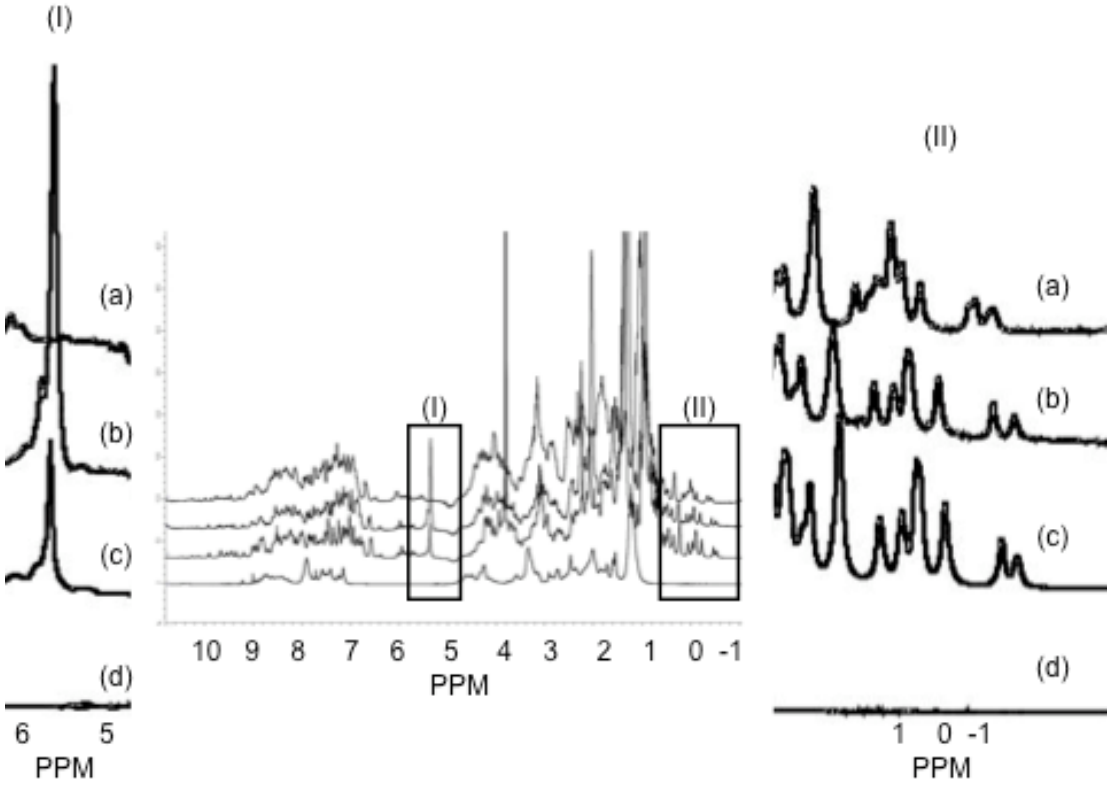
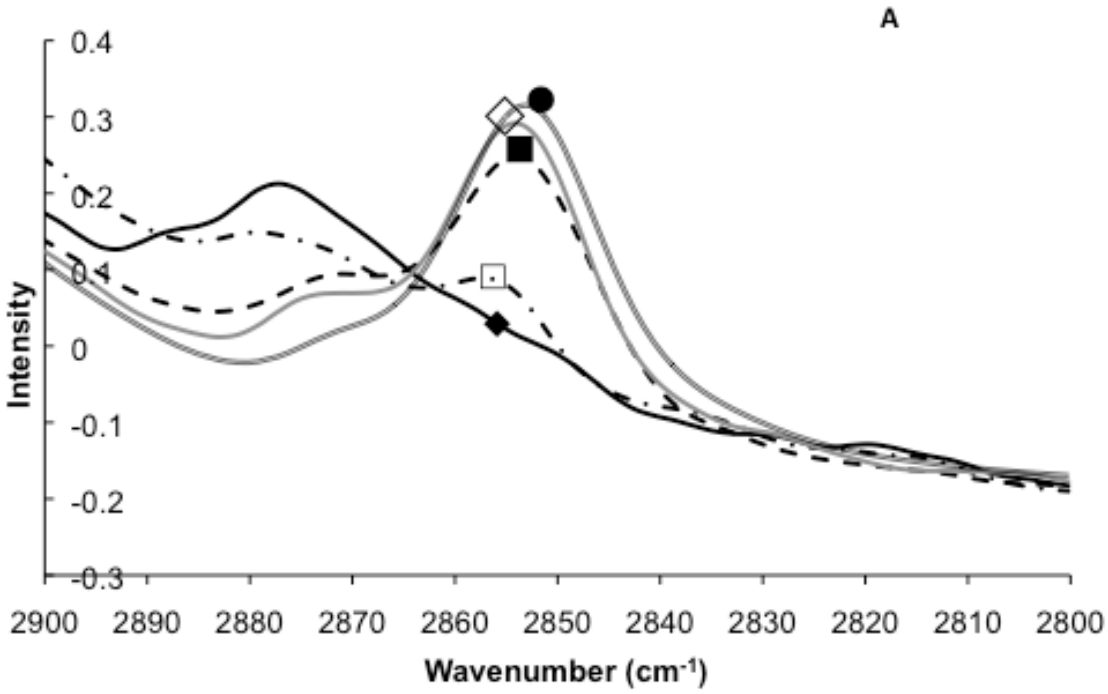


Figure 4: 1H-NMR spectra of (a) α-LA; (b) BAMLET produced through chromatography; (c) Post-gastric α -LA; (d) Gastric α-LA. I and II are magnified section of the spectra corresponding to the oleic acid and aliphatic protein region of the spectra, respectively.



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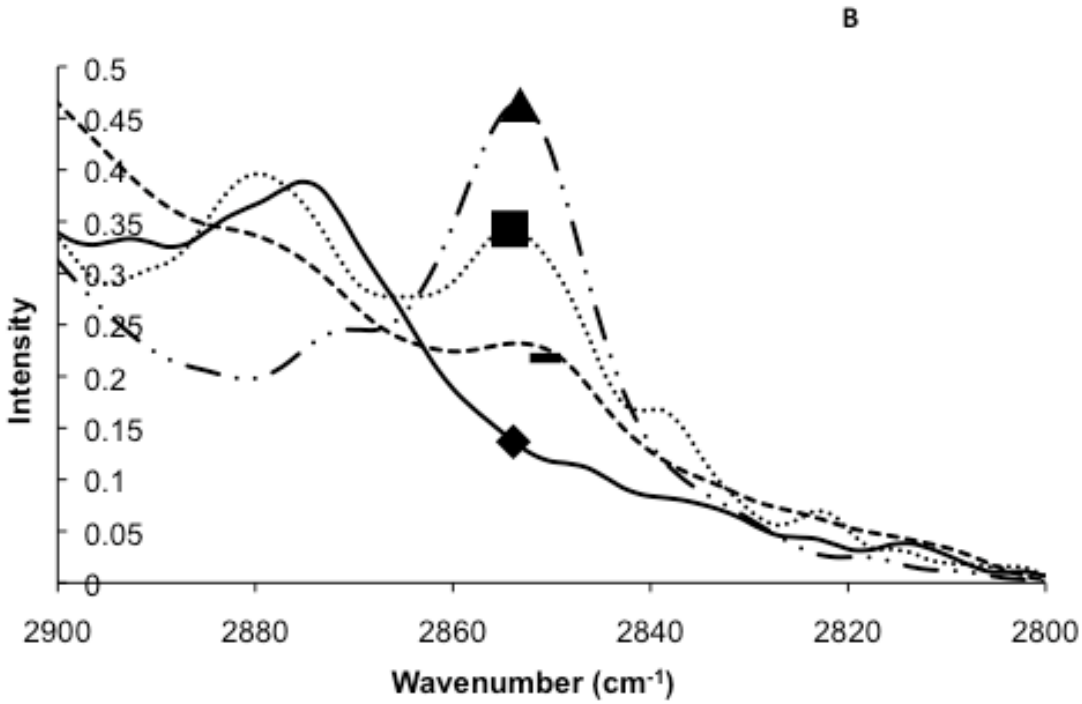

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629 Figure 5: Vector-normalised region of FTIR spectra corresponding to oleic acid
630 during (A) simulated gastric digestion and (B) subsequent fractionation by ultra-
631 filtration. (A): Gastric α-LA (□), Post-Gastric α-LA (■); for comparison OA alone
632 (●); α-LA (◆) and BAMLET (◇) are also shown.
633 (B): OA spectra for the different stages during ultra-filtration after digestion showing
634 the permeate with an increased OA content (-) and the retentate (▲), along with Post-
635 Gastric α-LA prior ultra-filtration (■), holo α-LA (◆).

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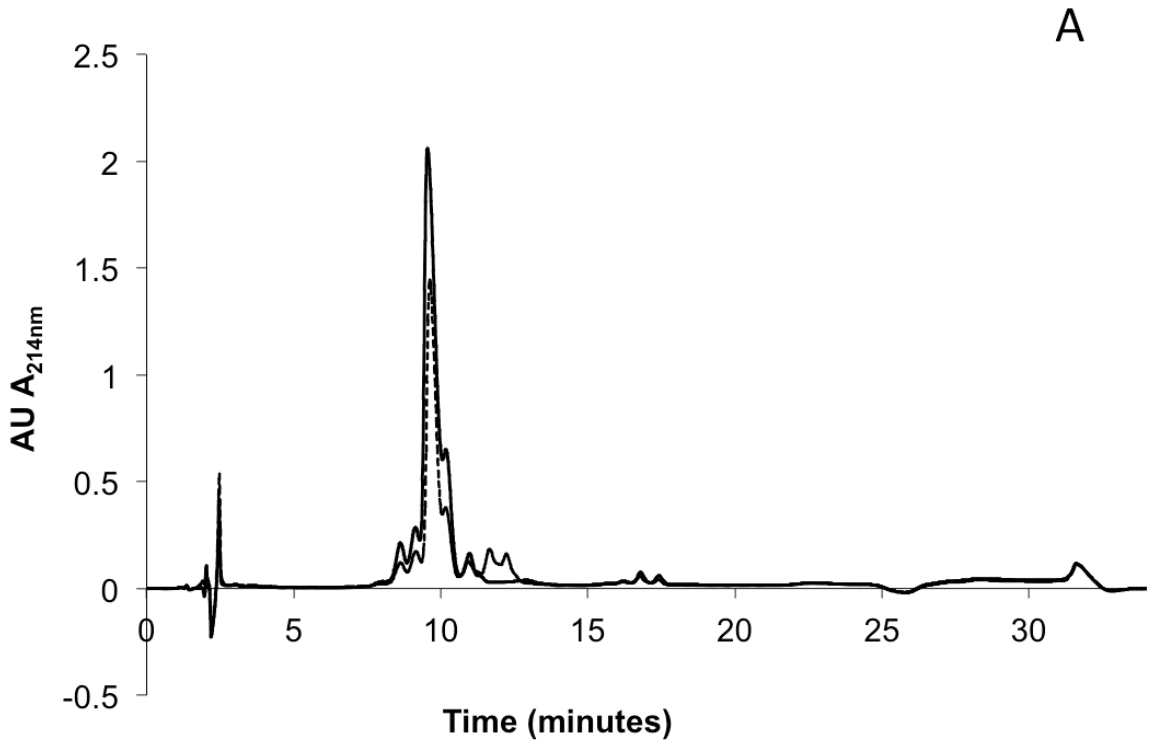
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2.5
2
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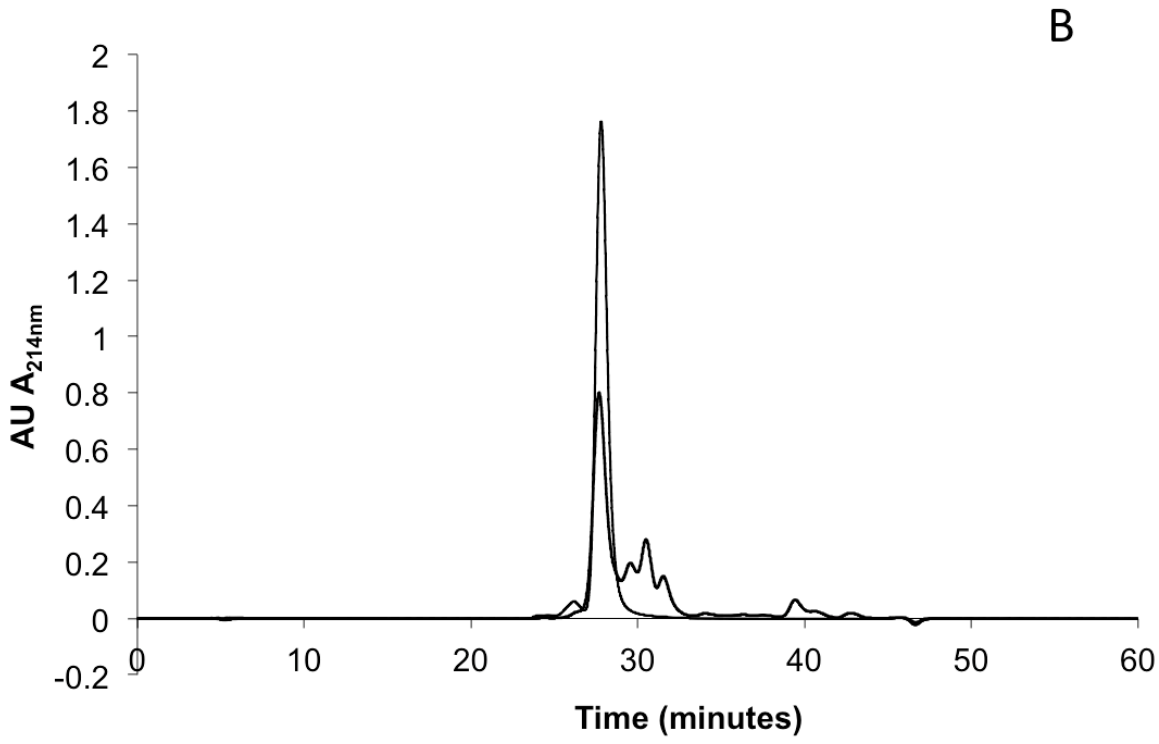
AU A_{214nm}
0
5
10
15
20
25
30
Time (minutes)

637


B
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Time (minutes)

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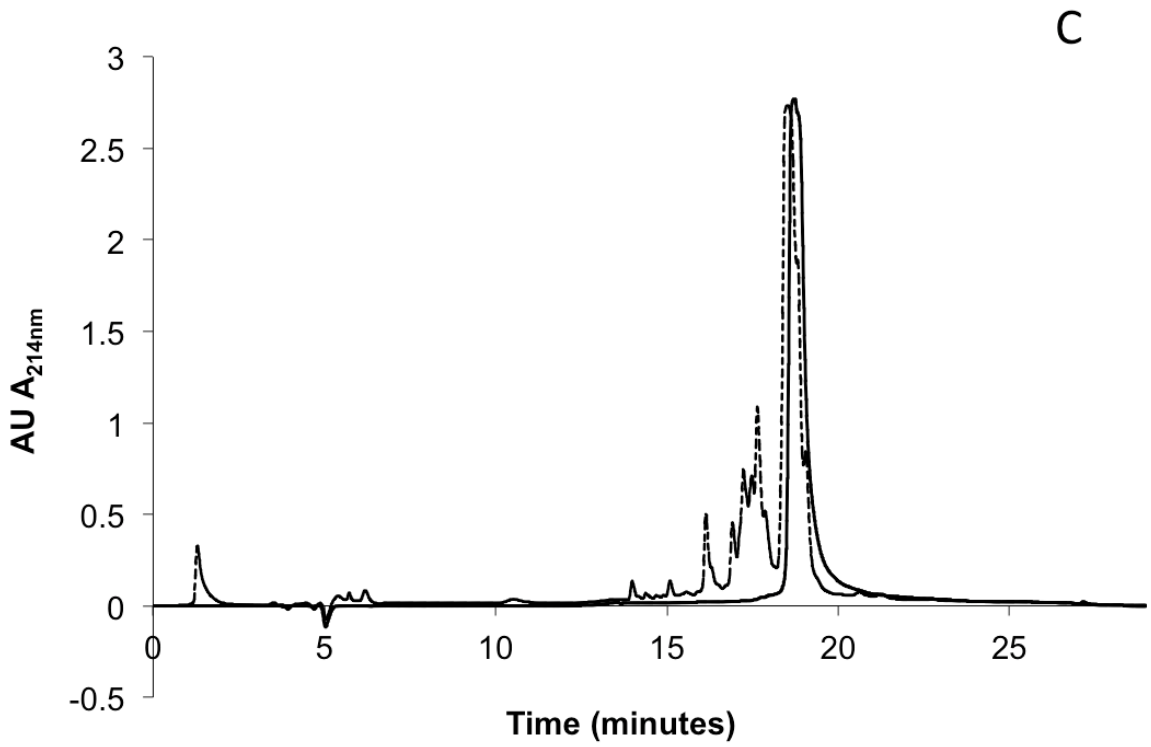

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642 Figure 6: HPLC chromatographs of α-LA (solid line) and post-gastricd α-LA (dashed
643 line) using (a) Native protein-reversed phase ; (b) size exclusion chromatography; (c)
644 C18 reversed phase. In each case a decrease in native protein content can be observed,
645 and a subsequent increase in peptide formation.

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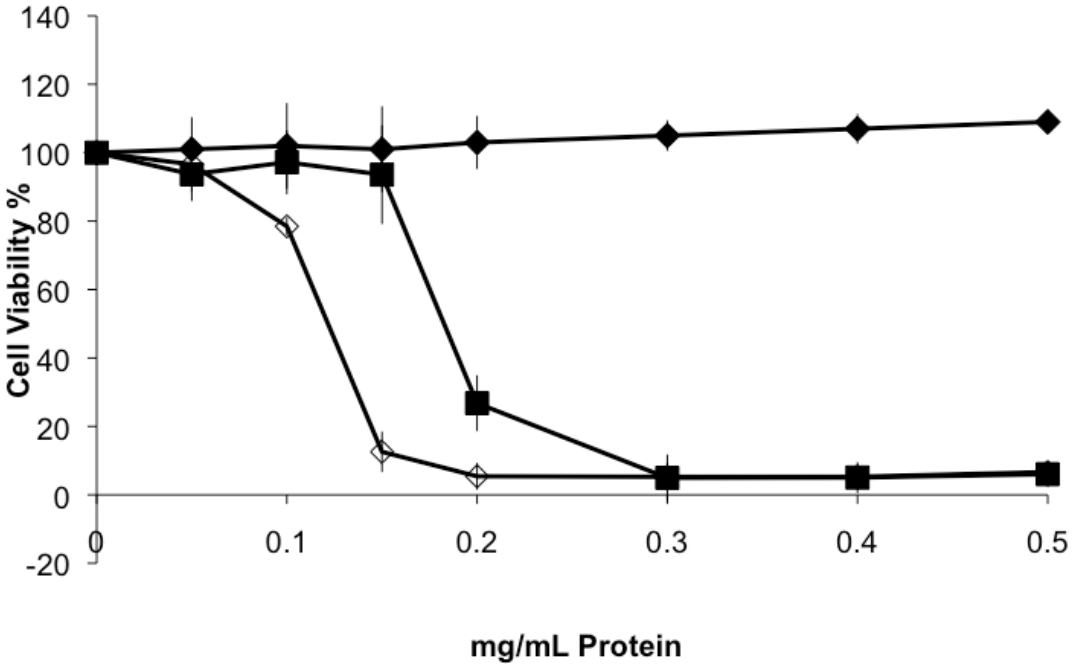
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Cell Viability %
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mg/mL Protein

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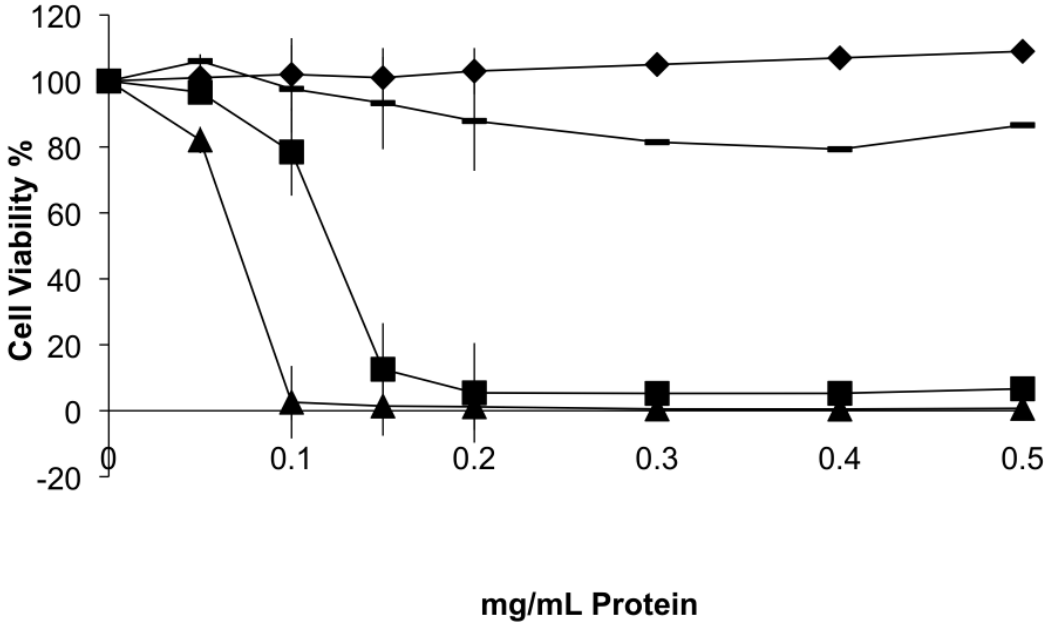
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Cell Viability %
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0.2
0.3
0.4
0.5
mg/mL Protein

672 Figure 7: Cytotoxicity against U937 cells tested with alamarBlue after a 24 hour
673 incubation with of A) the digested complex (■), BAMLET (℘), and α-LA (♦); and B)
674 the UF retentate (▲), the UF permeate (-), BAMLET (■) and α-LA (♦). Data are the
675 means of three independent measurements with SDs represented by vertical bars.

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683 **References**

684

685 Aits, S., L. Gustafsson, *et al*. (2009). "HAMLET (human alpha-lactalbumin made
686 lethal to tumor cells) triggers autophagic tumor cell death." International
687 Journal of Cancer **124**(5): 1008-1019.
688 Barbana, C., M.-D. Pérez, *et al*. (2006). "Interaction of bovine α-lactalbumin with
689 fatty acids as determined by partition equilibrium and flourescence
690 spectroscopy." International Dairy Journal **16**: 18-25.
691 Brew, K., T. C. Vanaman, *et al*. (1968). "ROLE OF ALPHA-LACTALBUMIN AND
692 A PROTEIN IN LACTOSE SYNTHETASE - A UNIQUE MECHANISM
693 FOR CONTROL OF A BIOLOGICAL REACTION." Proceedings of the
694 National Academy of Sciences of the United States of America **59**(2): 491-&.
695 Brodkorb A. and Lišková K., (2010), A process for producing a biologically active
696 globular protein complex, European patent office, Application number
697 PCT/IE2010/000034, Publication number WO2010131237.
698 Casbarra, A., L. Birolo, *et al*. (2004). "Conformation analysis of HAMLET, the
699 folding variant of human α-lactalbumin associated with apoptosis." Protein
700 Science **13**: 1322-1330.
701 Dobson, C. M. (1999). "Protein misfolding, evolution and disease." Trends in
702 Biochemical Sciences **24**(9): 329-332.
703 Duringer, C., A. Hamiche, *et al*. (2003). "HAMLET interacts with Histones and
704 Chromatin in Tumor Cell Nuclei." The Journal of Biological Chemistry
705 **278**(43): 42131-42135.
706 Fast, J., Mossberg, AK., *et al.* (2005). "Stability of HAMLET – a kinetically trapped
707 alpha-lactalbumin oleic acid complex." Protein Science **14**(2): 329-340.
708 Fast, J., Mossberg, AK, *et al.* (2007). "Compact oleic acid in HAMLET". FEBS
709 Letters. **579**(27): 6905-6100.
710 Favilla, R., A. Parisoli, *et al*. (1997). "Alkaline denaturation and partial refolding of
711 pepsin investigated with DAPI as an extrinsic probe." Biophysical Chemistry
712 **67**(1-3): 75-83.
713 Fischer, W., L. Gustafsson, *et al*. (2004). "Human α-lactalbumin made lethal to tumor
714 cells (HAMLET) kills human glioblastoma cells in brain xenografts by an
715 apoptosis-like mechanism and prolongs survival." Cancer Research **64**: 2105-
716 2112.
717 Fox, P. F. and P. L. H. McSweeney (2003). Advanced Dairy Chemistry, Proteins. Part
718 A. New York, Academic Plenum.
719 Gibson, R. and G. Kneebone (1981). "Fatty acid composition of human colostrum and
720 mature breast milk." The American Journal of Clinical Nutrition **34**(2): 252-
721 257.
722 Gustafsson, L., S. Hallgren, *et al*. (2005). "HAMLET kills tumor cells by apoptosis:
723 Structure, Cellular Mechanisms, and therapy." The Journal of Nutrition
724 **135**(5): 1299-1303.
725 Gustafsson, L., I. Leijonhufvud, *et al*. (2004). "Treatment of Skin Papillomas with
726 topical α-lactalbumin-oleic acid." The New England Journal of Medicine
727 **350**(26): 2663-2672.
728 Hakansson, P. A., M. W. Svensson, *et al*. (2000). "A folding variant of α-lactalbumin
729 with bactericidal activity against Streptococcus pneumoniae." Molecular
730 Microbiology **35**(3): 589.

Hakansson, P. A., B. Zhivotovsky, *et al*. (1995). "Apoptosis induced by a human milk protein." The Proceedings of the National Acadamy of Sciences USA **92**: 8064-8068.

Hallgren, O., L. Gustafsson, *et al*. (2006). "HAMLET triggers apoptosis but tumor cell death is independent of capases, Bcl-2 and *p53*." Apoptosis **11**(2): 221-233.

Halskau, N. A. Frystein, *et al*. (2002). "The membrane-bound conformation of alpha-lactalbumin studied by NMR-monitored 1H exchange." Journal of Molecular Biology **321**(1): 99-110.

Jensen, R. G., Ed. (1995). Handbook of Milk Composition. London, Academic Press.

Knyazeva, E. L., V. M. Grishchenko, *et al*. (2008). "Who is Mr. HAMLET? Interaction of human α-lactalbumin with monomeric oleic acid." Biochemistry **47**(49): 13127-13137.

Kuwajima, K, Nitta K and Sugai, S (1980). "Intramoleculat perturbation of tryptophans induced by the protonation of ionizable groups in goat α-lactalbumin." Biochemica et Biophysica Acta **623**(12): 389-401.

Kuwajima, K. (1996). "The molten globule state of alpha-lactalbumin." FASEB J. **10**(1): 102-109.

Laemmli, U. K. (1970). "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4." Nature **227**(5259): 680-685.

Liskova, K., A. L. Kelly, *et al*. (2010). "Effect of Denaturation of alpha-Lactalbumin on the Formation of BAMLET (Bovine alpha-Lactalbumin Made Lethal to Tumor Cells)." Journal of Agricultural and Food Chemistry **58**(7): 4421-4427.

Lönnerdal, B. (2003). "Nutritional and physiologic significance of human milk proteins." The American Journal of Clinical Nutrition **77**(Supplement): 1537S-1543S.

Mitchell, D. J., B. G. McClure, *et al*. (2001). "Simultaneous monitoring of gastric and oesophogeal pH reveals limitations of conventional oesophogeal pH monitoring in milk fed infants." Archives of Disease in Childhood **84**: 273-276.

Mok, K. H., J. Pettersson, *et al*. (2007). "HAMLET, protein folding, and tumor cell death." Biochemical and Biophysical Research Communications **354**: 1-7.

Mossberg, A.-K., B. Wullt, *et al*. (2007). "Bladder cancers respond to intravesical instillation of HAMLET (Human α-lactalbumin made lethal to tumors)." International Journal of Cancer **121**: 1352-1359.

Mossberg, A. K., Y. C. Hou, *et al*. (2010). "HAMLET Treatment Delays Bladder Cancer Development." Journal of Urology **183**(4): 1590-1597.

Pettersson-Kastberg, J., S. Aits, *et al*. (2009). "Can misfolded proteins be beneficial? The HAMLET case." Annals of Medicine **41**(3): 162-176.

Pettersson-Kastberg, J., A.-K. Mossberg, *et al*. (2009). "[alpha]-Lactalbumin, Engineered to be Nonnative and Inactive, Kills Tumor Cells when in Complex with Oleic Acid: A New Biological Function Resulting from Partial Unfolding." Journal of Molecular Biology **394**(5): 994-1010.

Pettersson, J., A.-K. Mossberg, *et al*. (2006). "α-lactalbumin species variation, HAMLET formation, and tumor cell death." Biochemical and Biophysical Research Communications **345**: 260-270.

Permyakov, SE., Knyazeva, EL., *et al.* (2012). "Oleic acid is a key cytotoxic component of HAMLET-like complexes". Biological Chemistry. **393**(1-2): 85-92.

780 Polverino de Laureto, P., V. De Filippis, *et al*. (1995). "Probing the molten globule
781 state of .alpha.-lactalbumin by limited proteolysis." Biochemistry **34**(39):
782 12596-12604.
783 Rammer, P., L. Groth-Pedersen, *et al*. (2010). "BAMLET Activates a Lysosomal Cell
784 Death Program in Cancer Cells." Molecular Cancer Therapeutics **9**(1): 24-32.
785 Rösner, H. I. and C. Redfield (2009). "The Human [alpha]-Lactalbumin Molten
786 Globule: Comparison of Structural Preferences at pH 2 and pH 7." Journal of
787 Molecular Biology **394**(2): 351-362.
788 Spolaore, B., O. Pinato, *et al*. (2010). "Î±-Lactalbumin Forms with Oleic Acid a High
789 Molecular Weight Complex Displaying Cytotoxic Activity." Biochemistry
790 **49**(39): 8658-8667.
791 Stinnakre, M. G., J. L. Vilotte, *et al*. (1994). "Creation And Phenotypic Analysis Of
792 Alpha-Lactalbumin-Deficient Mice." Proceedings of the National Academy of
793 Sciences of the United States of America **91**(14): 6544-6548.
794 Svensson, M. W., J. Fast, *et al*. (2003). "α-lactalbumin unfolding is not sufficient to
795 cause apoptosis, but is required for the conversion to HAMLET (human α-
796 lactalbumin made lethal to tumor cells)." Protein Science **12**(12): 2794-2804.
797 Svensson, M. W., P. A. Hakansson, *et al*. (2000). "Conversion of α-lactalbumin to a
798 protein inducing apoptosis." Proceedings of the National Acadamy of Sciences
799 of the United States of America **97**(8): 4221-4226.
800 Tolin, S., G. De Franceschi, *et al*. (2010). "The oleic acid complexes of proteolytic
801 fragments of alpha-lactalbumin display apoptotic activity." FEBS Journal
802 **277**(1): 163-173.
803 Wijesinha-Bettoni, R., C. M. Dobson, *et al*. (2001). "Comparison of the structural and
804 dynamical properties of holo and apo bovine [alpha]-lactalbumin by NMR
805 spectroscopy." Journal of Molecular Biology **307**(3): 885-898.
806 Wilhelm, K., A. Darinskas, *et al*. (2009). "Protein oligomerization induced by oleic
807 acid at the solid-liquid interface - equine lysozyme cytotoxic complexes."
808 FEBS Journal **276**(15): 3975-3989.
809 Zhang, M., F. Yang Jr, *et al*. (2009). "Cytotoxic aggregates of alpha-lactalbumin
810 induced by unsaturated fatty acid induce apoptosis in tumor cells." Chemico-
811 Biological Interactions **180**: 131-142.
812
813