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Cytotoxic Complexes of Sodium Oleate with β -Lactoglobulin

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Running title:

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Keywords:

β -lactoglobulin, oleic acid/oleate; HAMLET/BAMLET; denaturation and aggregation; cytotoxicity; apoptosis

Abbreviations:

α -la, α -lactalbumin; **β -lg**, β -lactoglobulin, **HAMLET/BAMLET**, Human/Bovine Alpha-Lactalbumin Made LEthal to Tumour cells, **NaOle**, sodium oleate; **OA**, oleic acid; **ELOA**, equine lysozyme with and oleic acid

Practical applications:

Globular proteins such as the milk proteins α -lactalbumin and β -lactoglobulin can bind oleic acid, thereby converting into a complex with high biological activity against tumour cells. Its cytotoxicity is strongly dependent on the binding stoichiometry, indicating the importance of oleic acid in the cell death.

1. Abstract

A complex of α -lactalbumin and oleic acid has previously been shown to induce apoptosis in cancer cells in a number of *in vitro* and *in vivo* trials. This complex is called HAMLET or BAMLET, depending on the origin of α -la (Human/Bovine Alpha-Lactalbumin Made LEthal to Tumour cells). In the current study, it was shown that bovine β -lactoglobulin (β -lg), upon binding sodium oleate (NaOle), the salt of oleic acid, also acquires cytotoxicity towards cancer cells, analogously to HAMLET/BAMLET complexes. The properties of the complex were characterized using Fourier-Transform Infrared Spectroscopy, HPLC and SDS-PAGE. It was shown that the level of covalent oligomerisation (dimers and trimers) of β -lg increased with increasing the molar ratio of sodium oleate NaOle: β -lg in the preparation procedure. At the same time, increasing the molar ratio of NaOle: β -lg increased the cytotoxicity of the complex. The increase in cytotoxicity appeared to be dependent on the amount of bound NaOle in the complex, but not on the content of multimeric forms of β -lg. The NaOle/ β -lg complex also showed similarity with BAMLET in penetrating the cell membrane and co-localizing with the cell nucleus. Furthermore, tumour cells (U937) treated with the complex died by apoptosis, as in the case of BAMLET, and healthy cells appeared to be less affected by treatment, as shown with model cells PC-12. In conclusion, β -lg and NaOle can form complexes with apoptosis-inducing qualities comparable to those of BAMLET.

2. Introduction

β -lactoglobulin (β -lg) is the major whey protein secreted in the milk of ruminants like the cow or sheep, but it is absent from the milk of humans. It is a small globular protein, with a monomeric molecular mass of about 18 kDa, consisting of 162 amino acids [1]. The quaternary structure of β -lg depends on pH, with a dimer being prevalent at physiological pH (5.5 - 7.5) [2]. Although β -lg exists in a large number of genetically determined variants, it is mostly present in milk as either the A or B variant, which only differ by two amino acids at positions 64 (Asp/Gly) and 118 (Val/Ala). The amino acid sequence of β -lg revealed some homology with serum retinol-binding protein [3] but it was the similarity in tertiary structure [4] which placed β -lg into a family of lipocalins [5]. All members of this family, for which the structure is known, contain a β -barrel, shaped into a flattened calyx, composed of eight antiparallel β -strands. Most lipocalins are able to bind small hydrophobic molecules in the internal binding cavity of the calyx [5]. Studies carried out on bovine β -lg have shown that it can bind a variety of small hydrophobic ligands, such as fatty acids or retinol [6-8], but it has not been reported whether, by binding these ligands, β -lg acquired new biological properties.

Another globular whey protein, α -lactalbumin (α -la) has been shown to bind oleic acid (OA). Interestingly, this complex has been shown to induce apoptosis-like death in cancer cells, but has little effect on healthy differentiated cells; the complex has been named HAMLET (Human α -lactalbumin made lethal to tumour cells) or BAMLET for the bovine equivalent [9]. The biological activity of HAMLET was established in a number of *in vitro* [10-14] and *in vivo* [15-17] studies. The complex was originally isolated from acid-precipitated casein, but a two-step chromatographic method was later developed that allowed preparation of HAMLET from whey-derived α -la [18]. The first step involved conditioning of a chromatographic matrix by OA while, in the second step, apo (calcium-depleted) protein was loaded onto the column. Once formed, HAMLET is eluted by a salt gradient. Bovine α -la, having about 85% sequence homology with its human counterpart, was also capable of chromatographic conversion into the cytotoxic complex with oleic acid, named in this case BAMLET (Bovine Alpha-lactalbumin Made LEthal to Tumour cells) [19]. Subsequently, homologous complexes were prepared from equine, porcine and caprine α -la, all with cytotoxicity comparable to that of HAMLET, but lower yields from the chromatographic conversion [20].

In a study by Wilhelm *et al.* [21], a complex from equine lysozyme, closest structural homologue to α -la, and OA was produced using a chromatographic matrix in a similar manner as for the preparation of HAMLET. The authors reported that lysozyme assembled with OA into multimeric complexes (ELOA), which exhibited similarities with both HAMLET and amyloid oligomers, and, as in the case of HAMLET, ELOA showed cytotoxicity towards various cancerous cell lines. The results suggested that cytotoxic complexes similar to HAMLET could be formed from other

proteins, although the choice of equine lysozyme, a protein structurally related to α -la [22-25], did not exclude the possible importance of certain structural features for the formation of these proteinaceous cytotoxic complexes. On the other hand, it has been previously shown that denaturation of α -la did not alter the formation or cytotoxic activity of BAMLET [26]. As the unique conformation and features of the protein are lost during its denaturation, a question arises whether this cytotoxic complex is exclusively formed from α -la (or related lysozyme), or whether any globular protein would be capable of formation of a homological complex.

The aim of the current study was to examine whether cytotoxic, HAMLET-like, complexes can be formed from bovine β -lg, and to characterize these potentially formed complexes.

3. Materials and Methods

3.1 Materials

α -la (95% purity, approx. 3% β -lg content) and β -lg (96% purity, approx. 4% α -la content) were purchased from Davisco Foods International, Inc. (Eden Prairie, Minnesota, USA), DEAE Trisacryl[®] M matrix from Pall Corporation (Cergy-Saint-Christophe, France), the CellTiter 96 Aqueous One Solution Cell Proliferation Assay from Promega Corporation (Madison, Wisconsin, USA), Cell Death Detection ELISAPLUS from Roche Applied Science (West Sussex, UK), Bio-Rad Protein Assay from Bio-Rad Laboratories GmbH (Hertfordshire, UK), and molecular weight markers for polyacrylamide gel electrophoresis from GE Healthcare (Uppsala, Sweden). All other chemicals and solutions were purchased from Sigma-Aldrich (Dublin, Ireland).

3.2 Methods

3.2.1 Preparation of BAMLET on Chromatographic Column

Column XK 50 (GE Healthcare, Uppsala, Sweden) was packed with 185 mL of DEAE Trisacryl M chromatographic matrix. Chromatographic runs were performed at a flow rate of 30.6 cmh⁻¹, with buffer A being 10 mM Tris-HCl, pH 8.5, and buffer B being 10 mM Tris-HCl, 1 M NaCl, pH 8.5. Conditioning of the chromatographic column with OA and chromatographic preparations of BAMLET were performed according to a previously published method [18]. Collected BAMLET fractions were pooled and extensively dialyzed against distilled water prior to freeze-drying.

3.2.2 Preparation of BAMLET and NaOle/ β -lg Complexes in solution

Samples of NaOle/ β -lg complex were prepared by heating a mixture of β -lg and NaOle according to a modification of a previously developed method [27, 28] for NaOle/ α -la complexes where it was demonstrated that α -la can be converted into BAMLET by either chromatographic conversion, see above, or heating of α -la in the presence of NaOle in solution. For NaOle/ β -lg complexes, β -lg was

reconstituted in phosphate buffered saline (PBS; 0.01 M phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) at 0.27 and 2.7 mM. Solutions were split into 5 aliquots and NaOle was added in final molar ratios to β -lg of 2.5 \times , 5 \times , 7.5 \times , 10 \times and 15 \times . Solutions were heated for 30 min at 60°C, then immediately cooled in ice bath. In the preparation of BAMLET, α -la was reconstituted in PBS at 0.21 mM and NaOle was added in 10-molar excess to α -la. The solution was heated for 1 h at 60°C. Samples were extensively dialysed prior to freeze-drying.

3.2.3 Gel Filtration and Reversed-phase HPLC

The concentration of native protein was determined by reversed-phase HPLC, using a SourceTM 5RPC column (GE Healthcare) according to a previously published method [29]. The method was calibrated using β -lg (Sigma-Aldrich). For quantification of aggregates, the native and heated protein solutions (20 μ L of 0.5 mg mL⁻¹ protein) were injected onto a TSK G3,000 column (TosoHaas, Montgomeryville, PA, USA), using a solution of 30% acetonitrile (v/v) and 0.1% (w/v) trifluoroacetic acid in Milli-Q[®] grade water as an eluent, at a flow rate of 0.5 mL min⁻¹. The method was calibrated using a set of protein molecular-weight standards (Sigma-Aldrich). The composition of the eluting solution used for the gel-permeation HPLC (GP-HPLC) did not allow noncovalent association of β -lg ; therefore, native β -lg (present in solution mostly as a dimer) was eluted in monomeric form. The data were acquired and processed using Waters Empower[®] software.

3.2.4 Calculation of Levels of Native, Unfolded and Aggregated β -Lactoglobulin

The concentrations of native β -lg were directly obtained using RP-HPLC. The concentrations of monomeric (including native and unfolded) β -lg were directly obtained using GP-HPLC. The concentrations of unfolded monomeric β -lg were calculated by subtraction of the level of native β -lg (RP-HPLC) from that of monomeric β -lg (GP-HPLC). The amount of aggregation was calculated by subtraction of the amount of monomer (GP-HPLC) from the initial protein concentration.

3.2.5 Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy (ATR - FTIR)

FTIR measurements were carried out using a Bruker Tensor 27 instrument (Bruker Optik, GmbH, Ettlingen, Germany), equipped with a thermally controlled BioATR CellTM II. Spectra were obtained at 20°C as an average of 180 scans at a resolution of 4 cm⁻¹. For determination of the molar ratios of NaOle: β -lg , a multivariate calibration method was constructed in OPUS/QUANT software applying a Partial Least Square (PLS) method.

Calibration for the amount of NaOle bound per molecule of β -lg was performed with mixtures of β -lg with NaOle. β -lg was dissolved in Milli-Q[®] water and its concentration was determined by

measuring absorbance at 280 nm, using $\frac{1\%}{A_{280}}$ of 20.1. NaOle was added at increasing ratios to β -lg (1 - 14), samples were heated for 30 min, and, after cooling, the FTIR spectra of samples were measured. The omission of the dialysis step after mixing and heating ensured that all added NaOle was still present in the sample; therefore, the exact molar ratios of NaOle: β -lg were known for all calibration standards. After atmospheric compensation for CO₂, H₂O (g) and aqueous solution, all spectra were vector-normalized between 3,000 and 2,800 cm⁻¹ and used for construction of the PLS calibration method. The region between 3,000 and 2,800 cm⁻¹ was selected due to the presence of a band of -CH₂- stretching vibrations at 2,854 cm⁻¹, which is present in the spectrum of NaOle (and subsequently NaOle/ β -lg complexes) but is missing from the spectrum of pure β -lg .

The same procedure, using α -la, was applied for constructing calibration method for quantification of NaOle or OA in BAMLET samples.

The changes in the secondary structure of β -lg upon binding of NaOle were observed in spectra after processing in software package OPUS 5.5. After atmospheric compensation for CO₂, H₂O (g) and aqueous solution, all spectra were vector-normalized between 1,700 and 1,600 cm⁻¹. The spectrum of native β -lg was then subtracted from the spectra of NaOle/ β -lg complexes.

3.2.6 Cytotoxicity Testing

Freeze-dried samples of BAMLET and NaOle/ β -lg complexes were reconstituted in the cell culture medium - RPMI 1640 at a concentration of 10 mg mL⁻¹ for 4 h, all solutions were filtered with 0.1- μ m sterile filters, and aliquots were made and stored at -80°C. For each assay, a new aliquot was used.

Human monocytic cells U937 were obtained from the European Collection of Cell Cultures, ECACC No. 85011440. Cells were subcultured every 48 h at a density of 1 \times 10⁵ cells mL⁻¹ in 10% (v/v) Fetal Bovine Serum (FBS) -supplemented RPMI 1640 medium. Cells were cultured in an atmosphere of CO₂:air (5:95, v/v) at 37°C and maintained in the absence of antibiotics. For cell viability assays, cells were incubated for 24 h in 96-well plates with increasing concentrations of NaOle/ β -lg complexes or BAMLET as reported previously [26]. Assays were performed in triplicate and three independent measurements were made.

Rat adrenal pheochromocytoma cells PC12 were kindly donated by Dr. Ken H. Mok (Trinity College Dublin, Ireland). Cells were maintained in non-differentiated state in an atmosphere of CO₂:air (5:95, v/v). Cells were subcultured every 3 days at a density of 2.5 \times 10⁵ cells mL⁻¹ in RPMI-1640 medium supplemented with 10% FBS, 100 U mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin. For viability assays performed on cells in a non-differentiated state, PC12 cells were centrifuged at 300 \times g, washed with RPMI medium and resuspended in RPMI medium supplemented with 1% FBS, 200 U mL⁻¹ penicillin and 200 μ g mL⁻¹ streptomycin at a density of 4 \times 10⁵ cells mL⁻¹. Cells were

then seeded in 96-well plates in a volume of 50 μL per well, and increasing volumes (0 – 50 μL) of tested samples (BAMLET or NaOle/ β -Ig complexes) were immediately added to the seeded cells, with pure RPMI being added to a final volume of 100 μL per well. After 24 h incubation, the MTS viability assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) was performed according to instructions of the manufacturer. Assays were performed in duplicate and three independent measurements were made.

For viability assays performed on cells in differentiated state, PC 12 cells were declumped by incubating in RPMI cell culture medium supplemented with 1 mM EDTA for 6 h. Afterwards, cells were centrifuged at 300 \times g, washed with RPMI medium and resuspended in RPMI medium supplemented with 1% FBS, 100 U mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin and 50 $\mu\text{g mL}^{-1}$ nerve growth factor (NGF, Invitrogen, Bio-Sciences, Dun Laoghaire, Ireland) at a density of 1.5×10^5 cells mL^{-1} . Cells (100 μL per well) were then seeded in 96-well plate pre-coated with type IV collagen from human placenta (Invitrogen, Bio-Sciences, Dun Laoghaire, Ireland) and cultured in an atmosphere of CO_2 :air (5:95, v/v) for 6 days prior to assays. The NGF-supplemented medium was replaced every 48 h. On the day of the assay, media was removed from the wells, cells were washed with RPMI and 50 μL of fresh RPMI medium supplemented with 1% FBS, 200 U mL^{-1} penicillin, 200 $\mu\text{g mL}^{-1}$ streptomycin and 100 $\mu\text{g mL}^{-1}$ NGF was added to each well. Increasing volumes (0 to 50 μL) of tested samples were added to the wells, with pure RPMI being added to the final volume of 100 μL per well. After 24 h incubation, the MTS viability assay was performed.

Assays were again performed in duplicate and three independent measurements were made.

3.2.7 Testing of Apoptotic Activity

The type of cell death occurring after treatment with NaOle/ β -Ig complex or BAMLET was analyzed using a Cell Death Detection ELISA^{PLUS} kit from Roche Applied Science. U937 cells were seeded in the same manner as for the cytotoxicity assay, and supplemented with BAMLET samples at the concentrations of LD_{50} (previously determined by MTS assays). The assay was performed after 4 h incubation at 37°C and 5% CO_2 .

To calculate the specific enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm (enrichment factor) of the treated cells, the following formula was used:

$$\text{Enrichment factor} = \frac{\text{Absorbance of sample at } 405 \text{ nm}}{\text{Absorbance of negative control at } 405 \text{ nm}}$$

where non-treated cells were used as a negative control.

3.2.8 Fluorescent Labelling of NaOle/ β -Ig Complex or BAMLET

BAMLET prepared with OA on a chromatographic column and NaOle/ β -lg complex prepared from 5% β -lg with 10-molar excess of NaOle were labelled with Alexa Fluor[®] 633 dye, using the protein labelling protocol provided by the producer (Invitrogen, Paisley, UK). Excess free dye was removed using centrifugal ultrafiltration tubes Vivaspin 4 (Sartorius Stedim, Aubagne, France) with a membrane of a molecular weight cut-off of 5,000 Da.

3.2.9 Treatment of Cells with Labelled NaOle/ β -lg Complex or BAMLET

U937 cells were seeded in the same manner as for the cytotoxicity assay, and supplemented with labelled samples of BAMLET or NaOle/ β -lg complex, at the concentrations of LD50 (previously determined by MTS assays for unlabelled proteins). After 4 h incubation at 37°C and 5% CO₂, cells were harvested into microtubes and confocal laser scanning microscopy was performed.

3.2.10 Confocal Laser Scanning Microscopy (CLSM)

The suspensions of cells treated with BAMLET or NaOle/ β -lg complex were split into two aliquots prior to the microscopic examination. For visualization of cells' nuclei, one aliquot was stained with Syto-9 dye (Invitrogen, Paisley, UK) which emits green fluorescence upon binding to nucleic acids (when excited with an argon laser at 488 nm). Mixing of Syto-9 dye with cells was performed according to manufacturer's protocol. The other aliquot of cell suspension was left unstained as a control. This allowed unambiguous assignment of the red fluorescence detected in dying cells to the presence of BAMLET or NaOle/ β -lg complex labelled with Alexa Fluor[®] 633 dye in the cells, and excluded an artifactual spill-over of fluorescence of Syto-9 into the red channel, when excited at 633 nm.

Transmitted light differential interference contrast (DIC) was used in combination with CLSM to visualize the cells. Imaging was performed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany) using a 63x oil immersion objective ($AN = 1.4$). RGB colour images (24 bit) 512 x 512 pixels in size were acquired. A minimum of 4 z-stacks were taken per sample, with representative cross sections of micrographs shown.

4. Results and Discussion

4.1 Preparation of NaOle/ β -lg Complexes

Denaturation characteristics of β -lg were measured prior to the preparation of the cytotoxic complexes. Samples of 0.5, 2 and 5% protein (w/v) in PBS, pH 7.4, were heated for up to 60 min at 60°C. The results confirmed that, at the low temperature used in this experiment, β -lg denatured

very slowly at all concentrations examined with the formation of some non-native monomeric structures [30].

Protein complexes were then prepared by heating β -lg at two different concentrations (0.5% and 5%, w/v, β -lg) with NaOle at increasing molar ratios (from 2.5 to 15, Figure 1). The higher concentration of protein (5% w/v) was used to examine the possibility of scale-up of the preparation. Although samples were heated at the same temperature as in the preliminary denaturation measurements (60°C) and only for 30 min, the degree of denaturation increased significantly with the increasing amount of NaOle added to protein solutions. Samples were analyzed by gel-permeation chromatography (GP-HPLC) to determine whether aggregation of the protein occurred; the loss of native β -lg was parallel with the increase of aggregated protein, as shown in Figure 2. Almost none of the non-native protein remained monomeric.

These results do not agree with the findings of Puyol *et al.* [31, 32]. These authors reported that binding of palmitic acid to β -lg increased the resistance of β -lg to thermal denaturation. However, very different ratios of fatty acid: β -lg were used in both studies. While, in the current study, ratios from 2.5 to 15 were used, the authors in the cited papers used mixtures of ratio NaOle: β -lg (monomeric) of 0.5. While it is known that binding of some ligands or coenzymes to proteins can increase the heat stability of proteins, the relatively high stoichiometric ratio used in our study probably caused NaOle to lose the protective character of a natural ligand, and, on the contrary, destabilized the protein. Salts of fatty acid are ionic detergents, and, as such, they cause unfolding of proteins even at ambient temperature. This would also explain why all of the non-native β -lg was in the form of aggregates. The most widely accepted model of denaturation and aggregation of β -lg proposes that β -lg has to be unfolded prior to its aggregation [33]. This unfolding exposes its free sulphhydryl group on Cys121, making it available for aggregation *via* disulphide bridges. In pure protein solutions, the unfolding can be facilitated by heating, but in our experiments the unfolding was probably already caused by the presence of NaOle.

The results from GP-HPLC showed a decreasing peak for monomeric β -lg , and two peaks, corresponding to a dimer and trimer of β -lg , whose areas increased with the molar ratios of NaOle: β -lg (Figure 3). These observations were also confirmed by performing non-reducing SDS PAGE (data not shown). On the other hand, under reducing conditions, the electrophoreograms showed only bands of monomeric β -lg and minute amounts of α -la and bovine serum albumin (BSA). The fact that the bands of dimers and trimers were stable during SD S-PAGE indicates that these oligomers were linked by disulphide bonds, confirming the theory that the presence of NaOle enabled unfolding of β -lg and its consequent aggregation, despite the relatively low heating temperature. In conclusion, the samples of β -lg , after heating with increasing amounts of NaOle,

were mostly composed of monomeric β -lg, with proportions of covalently linked dimers and trimers that increased with the molar ratio of NaOle: β -lg.

The number of molecules of NaOle bound to β -lg in the formed complexes was determined by FTIR. Samples were extensively dialysed prior to the measurement, to remove any excess of unbound (or loosely bound) NaOle. In both sets of samples (0.5 and 5% β -lg), the number of molecules of NaOle bound to β -lg increased correspondingly with increasing addition of NaOle to the starting mixture up to a molar ratio of 10, when the curves started to plateau (Figure 4). The solution of 5% β -lg bound NaOle with almost 100% efficiency up to a molar ratio of 7.5, while the binding efficiency for 0.5% β -lg fluctuated between 50 and 80%. This observation is in agreement with the results of Nielsen *et al.* [34], who described that, with decreasing concentration of ELOA (equine lysozyme – oleic acid complex), an increasing fraction of OA dissociated from the complex. The resulting molar ratios converged for both solutions with the addition of 10-fold and 15-fold molar excess of NaOle, indicating that the binding capacity of β -lg was reaching its limit. It should be pointed out that there are numerous reports of the binding stoichiometry and sites of β -lg with small ligands, namely the interior calyx or β -barrel, the surface hydrophobic pocket in a groove between the α -helix and the β -barrel and one additional surface site [35, 36]. Given the relatively high number of bound NaOle, it could be assumed that the binding mode may be non-specific, most likely to the surface of the protein. However, as pointed out before, the relatively high binding stoichiometry is not out of line compared to the sister-complexes BAMLET and ELOA.

4.2 Changes in Secondary Structure of β -lg upon Formation of Complexes with NaOle

Infrared spectroscopy is recognized as a major tool for analysis of secondary structure of proteins [37]. Most information is obtained from the amide I band (1600 to 1700 cm^{-1}), which is a composite of a number of bands representing various structural features of proteins (α -helix, β -structure, turn and random conformation), which results in a distorted band shape. In the analysis of secondary structure, the amide I band can be separated into its underlying structural components, providing information on quality and quantity of present motifs.

In the current study, the spectral changes of β -lg upon binding NaOle were visualized by means of difference spectra, which were obtained after the spectrum of β -lg was subtracted from the spectra of the complexes formed with NaOle. That way, structures that emerged upon formation of the complex were represented by positive bands, and conversely, disappearing structures by negative bands. The difference spectra of the samples prepared with both 0.5 and 5% β -lg showed four main bands (Figure 5A and B) corresponding to a decrease in turns (bands centred around 1,665 cm^{-1}) [38] and α -helix (bands at 1,650 cm^{-1}) [39] and an increase in β -sheets (band between 1,635 and

1,622 cm^{-1}) [40, 41]. The changes that occurred in the samples of NaOle/ β -lg complexes during their preparation were significantly higher in intensity but not fundamentally different from changes taking place during heating of pure β -lg (Figure 4B). On the other hand, additional components appeared in the spectrum of heated pure β -lg ; one band centred around 1,643 cm^{-1} , corresponding to an increase in the content of random coil, and another two bands at around 1,685 and 1,615 cm^{-1} . Appearance of the latter bands during heat-induced denaturation of β -lg has been reported in a number of studies [41-43] and the bands have been attributed to intermolecular β -sheets resulting from aggregation [42]. Intermolecular β -sheets are also often associated with the formation of amyloid fibrils [44-46]. The fact that these components were not observed in the spectra of complexes examined in the current study suggested that the presence of NaOle interfered with the formation of intermolecular β -sheets in the heated solution of β -lg . In this sense, the complexes formed from β -lg are therefore structurally very different from the cytotoxic complex of ELOA, which showed characteristics of amyloids [21].

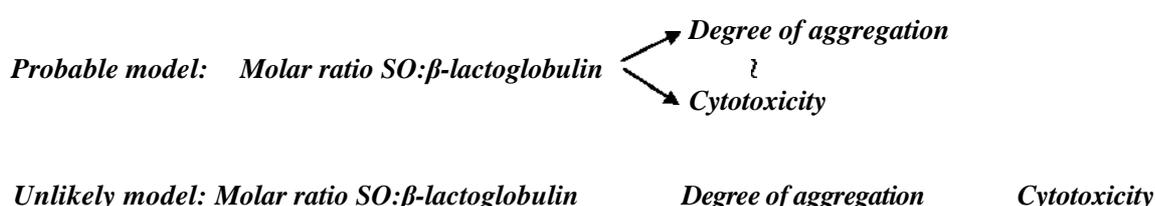
The thermal denaturation of β -lg has been extensively studied by FTIR [43, 47, 48], with most authors reporting a loss of α -helix and intramolecular β -sheets and an increase in intermolecular β -sheets upon denaturation. In the current study, a loss in α -helix was observed, but this was offset by an increase in intramolecular β -sheet, while no increase in intermolecular β -sheet was detected. The different results could, however, arise from the two main differences between the current study and previous studies. Most importantly, the presence of NaOle in the heated solutions of β -lg could have an effect on the structural changes associated with unfolding of the protein. Secondly, in the above studies, FTIR spectra of samples were measured during the heat treatment while, in the current study, spectra were measured after cooling of samples back to room temperature. This may also have played a role in the observed differences, as rearrangement of secondary structure upon cooling is very likely. Boye *et al.* [49] measured FTIR spectra of α -la during heating (to 95°C) and cooling (25°C) cycles and reported that, during heating, the β -sheet structure (present at 1,628 cm^{-1}) was transformed to turns and unordered structures. Upon cooling, these structures realigned to form β -sheets again, which could be observed in the resulting spectra as an increase in the band at 1,628 cm^{-1} .

In comparison, the changes associated with the formation of BAMLET (and molten globule-like form of α -la) were characterized by a loss of ordered structures and increase in turns [28], while in this case the content of ordered structures (β -sheets) increased. It may be of relevance that a molten globule-like conformation has also been reported for β -lg , and that this conformation was defined by a highly ordered β -sheet core, forming the lipocalin calyx, and less ordered structure in other regions [48, 50].

4.3 Cytotoxicity of NaOle/ β -Ig Complexes

The cytotoxicity of the prepared complexes was tested on U937 cells, together with samples of BAMLET, one prepared on a chromatographic column, and the other by heating α -Ia in a solution with 10-molar excess of NaOle. Apart from samples prepared with a 2.5-molar excess of NaOle, all complexes were cytotoxic. It can be seen in Figure 6 that the cytotoxicity of NaOle/ β -Ig complexes increased with increasing molar ratio of NaOle: β -Ig. On the other hand, the cytotoxicity did not appear to depend on the concentration of β -Ig at which complexes were prepared, as both sets of samples showed comparable, overlapping pattern. Also the cytotoxicity of BAMLET samples expressed similar dependence on the molar ratio of NaOle: α -Ia, as previously observed [27, 28, 51]. It was interesting that molar ratio of OA: α -Ia in BAMLET produced by chromatographic conversion was 12 (Figure 6). This was quite high compared to results obtained in two previous studies (8.2 determined by ^1H NMR and 6.4 by FTIR [28]). It should therefore be stressed again that the chromatographic method may not necessarily result in complexes of reproducible, uniform qualities. In contrast, this comparison reinforces the observation that complexes produced by this method may vary in the molar ratio of OA: α -Ia, and consequently in their cytotoxicity.

A question that repeatedly emerges in the field of HAMLET-like cytotoxic complexes is whether the cytotoxicity of a newly prepared complex arises from its similarity to HAMLET (monomeric protein) or to amyloids [21]. We have shown that the NaOle/ β -Ig complexes were only monomers, dimers and trimers of covalently-linked β -Ig (Figure 3), and lacked the intermolecular β -sheet structures (Figure 4), typical for amyloids; therefore, there was no structural similarity between amyloid structures and NaOle/ β -Ig complexes that would imply similar cytotoxic effect. However, the correlation of the degree of aggregation and cytotoxicity of NaOle/ β -Ig complexes needs to be addressed. In the insert of Figure 6, the cytotoxicity of NaOle/ β -Ig complexes was plotted against the amount of aggregated protein. Although it appears on first sight that there could be linear dependence between them, curves for 0.5 and 5% β -Ig showed diverging slopes and, furthermore, large differences in cytotoxicity were apparent in samples with similar amounts of aggregated protein. Therefore, it appeared that both the cytotoxicity of NaOle/ β -Ig complexes and their degree of aggregation were dependent (to different levels) on the ratio of NaOle: β -Ig, and their interrelation was only due to this parallel dependence. The dependence is schematically expressed below:



These results may suggest that the cytotoxic activity of complexes such as BAMLET or the NaOle/ β -lg complex was independent of the nature of the protein component of the complex and originated from the content of OA/NaOle. The conclusions are also supported by the study of Lišková *et al.* [26], in which BAMLET prepared from denatured (highly aggregated) and native α -la showed equal cytotoxicity.

The measured cytotoxicity of pure NaOle was several orders of magnitude lower (LD_{50} of $>1\text{mM}$) than that of NaOle/ β -lg complexes or BAMLET. The interaction of OA or NaOle with the protein is likely to have increased the stability of the fatty acid/salt in aqueous solution and, at the same time, the protein acted as a carrier of the fatty acid/salt to the cell membrane as suggested previously [27, 28]. Other recent paper [51, 52] further confirm the hypothesis that α -la merely serves as a carrier of the otherwise poorly soluble oleic acid. This theory was also proposed by Nielsen *et al.* [34], who examined the interaction of ELOA with lipid membrane vesicles. This seems to be a plausible hypothesis given the analogous process found in the transport of fatty acids in the blood stream, where fatty acids are transported bound to serum albumin and released based on their higher affinity for the cell membranes [53].

As both sets of samples (0.5 and 5% β -lg) appeared to be equivalent in cytotoxicity, further testing was performed only with one set of NaOle/ β -lg complexes, i.e. those prepared by heating 5% β -lg with increasing amounts of NaOle. A photometric enzyme-immunoassay showed that all NaOle/ β lg complexes caused detectable DNA fragmentation, proving that the mechanism of the cell death was apoptosis; the enrichments of nucleosomes in the cytoplasm of U937 cells treated with LD_{50} concentration of each sample are shown in Figure 7. Unlike the results of the cytotoxic assays, the increasing molar ratios of NaOle: β -lg in samples did not seem to affect the degree of apoptotic response (enrichment factor). This indicated that, even though the cytotoxicity of samples differed, from a qualitative point of view they stimulated the same cellular response. No significant difference was observed between the enrichment factor caused by NaOle/ β -lg complexes or BAMLET samples, further supporting the hypothesis that the protein component may not play as crucial role in the mechanism of the cell death as previously thought.

The death of U937 cells after treatment with BAMLET or the NaOle/ β -lg complex was examined by confocal microscopy. After 4 h of incubation with either BAMLET or the NaOle/ β -lg complex, the treated cells showed signs of undergoing apoptosis, such as membrane blebbing and pyknotic nuclei, or even fragmentation of the cells into apoptotic bodies. Both complexes, BAMLET or NaOle/ β -lg complex, were detected inside the cells independently of the stage of apoptosis. In the initial stages, the cytotoxic complexes were present inside the cell in relatively small amounts, but already appeared to be co-localized with the nucleus. The amount of BAMLET or NaOle/ β -lg complex internalized within a cell appeared to increase with the progress of apoptotic death, as did

the co-localization of both complexes with the chromatin. It can be seen in Figure 8 that, in the final stage of apoptosis, the complexes (red fluorescence) were accumulated together with the fragmented chromatin (green fluorescence) in the apoptotic bodies formed. These results are in agreement with the findings of Düringer *et al.* [11], that HAMLET entered the cell cytoplasm and accumulated over time in the cell nucleus. This similarity suggests that HAMLET, BAMLET and the NaOle/ β -Ig complex may share the same mechanism of their cytotoxic activity; therefore, β -Ig seems to be able to form a complex with apoptosis-inducing activity equivalent to that of HAMLET.

The pheochromocytoma (PC 12) cell line was originally derived from a rat adrenal medullary tumour in 1976 [54]. When grown in serum-containing medium, the PC12 cells divide and resemble precursors of adrenal chromaffin cells but, upon addition of nerve growth factor (NGF), these 'naïve' cells cease to multiply and assume a neurite-bearing phenotype that, by a number of criteria, resembles mature sympathetic neurons [55]. It is therefore advantageous to use this cell line for cytotoxicity assessments as, depending on culture methods, the same cell line can show phenotypic features of cancerous (in undifferentiated state) or mature normal cells (in differentiated state), providing information on cytotoxicity of samples towards both.

In the present study, cytotoxicity of the prepared NaOle/ β -Ig complexes was tested on the cells in both the undifferentiated and differentiated state. Samples expressed cytotoxicity which increased with the molar ratio of NaOle: β -Ig (Figure 9), similar to that seen for the U937 cell line. Cells in the differentiated state showed higher resistance towards the toxicity of NaOle/ β -Ig complexes, which, based on their biochemical resemblance to mature neurons, may imply that the complexes would be less cytotoxic towards mature healthy cells than to undifferentiated or cancerous cells. Interestingly, the cytotoxicity of BAMLET samples was significantly lower than that of NaOle/ β -Ig complexes, while the difference between cytotoxicity towards undifferentiated and differentiated cells was still present. This could indicate that, in the case of PC 12, cells the cytotoxicity of the complexes was not solely dependent on the molar ratio NaOle:protein but also on the nature of the protein (β -Ig or α -Ia), suggesting that different mechanisms of cell death might have been involved in both cases. These results were in direct contrast with observations from testing on the U937 cell line, where cytotoxicity appeared to be dependent only on the molar ratio of NaOle:protein. However, it is not uncommon to see differences in the cytotoxicity of compounds towards different cell lines [51], depending on the cell metabolism, membrane receptors expressed, and many other factors. In the case of comparing biological activity of BAMLET with that of the NaOle/ β -Ig complexes, the discrepancy between the results for both cell lines stressed the complexity of this issue and the need for studies including testing on a number of different cell lines, together with detailed examination of the mechanism of cytotoxic action for each proteinaceous complex.

5. Conclusions

Complexes of β -lg with NaOle expressed biological activity similar to that of BAMLET. The type of cell death occurring in U937 cells after treatment with NaOle/ β -lg complexes was identified as apoptosis induced upon internalization of the complex within the cell and, similarly to BAMLET, the extent of cytotoxic response was dependent on the molar ratio of NaOle:protein. Testing on PC12 cell line, however, showed different trends for cytotoxicity of BAMLET and NaOle/ β -lg complexes, indicating that a variety of cell lines need to be used for better assessment of its activity. The similar properties of NaOle/ β -lg and BAMLET complexes suggest that the protein component of the complex may not be the one responsible for the cytotoxicity of the complex, but is rather a delivering vehicle for the cytotoxic component – OA/NaOle – to the tumour cells. The obvious question would be: *Is HAMLET/BAMLET a unique case?* Moreover, are α -lactalbumin or oleic acid unique? The result presented in this manuscript point towards the possibility that other proteins than α -la would be able to form fulfil this function and form cytotoxic complexes with fatty acids. However, the different cytotoxicity of BAMLET and NaOle/ β -lg complexes towards PC-12 cells indicated that the relationship between content of NaOle/OA and cytotoxicity may not be as simple as suggested above, and that the protein may be in a certain way involved in determining the cytotoxic effect of the complex. Therefore, these conclusions will ultimately be validated only by elucidating the mechanism by which the complexes cross the cell membrane and induce an apoptotic response in cell, which is for now not fully understood.

6. Acknowledgements

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7. References

- [1] Braunitzer, G., Chen, R., Schrank, B., Stangl, A. Automatic sequence analysis of a protein beta-lactoglobulin AB, *Hoppe-Seyler's Zeitschrift fur physiologische Chemie*. 1972, 353, 832-834.
- [2] Creamer, L.K., Sawyer, L. Beta-Lactoglobulin, in: *Encyclopedia of Dairy Sciences* Eds. H. Roginski, J.W. Fuquay, P.F. Fox, Academic Press 2003.
- [3] Pervaiz, S., Brew, K. Homology of beta-lactoglobulin, serum retinol-binding protein, and protein HC, *Science (New York, N.Y.)*. 1985, 228, 335-337.
- [4] Papiz, M.Z., Sawyer, L., Eliopoulos, E.E., North, A.C., Findlay, J.B., Sivaprasadarao, R., Jones, T.A., Newcomer, M.E., Kraulis, P.J. The structure of beta-lactoglobulin and its similarity to plasma retinol-binding protein, *Nature*. 1986, 324, 383-385.
- [5] Flower, D.R. The lipocalin protein family: structure and function, *The Biochemical journal*. 1996, 318 (Pt 1), 1-14.
- [6] Spector, A.A., Fletcher, J.E. Binding of long chain fatty acids to beta-lactoglobulin, *Lipids*. 1970, 5, 403-411.
- [7] Pérez, M.D., de Villegas, C.D., Sánchez, L., Aranda, P., Ena, J.M., Calvo, M. Interaction of Fatty Acids with beta-Lactoglobulin and Albumin from Ruminant Milk, *J Biochem*. 1989, 106, 1094-1097.
- [8] Puyol, P., Perez, M.D., Ena, J.M., Calvo, M. Interaction of bovine -lactoglobulin and other bovine and human whey proteins with retinol and fatty acids, *Agricultural and Biological Chemistry*. 1991, 55, 2515-2520.
- [9] Hakansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H., Svanborg, C. Apoptosis Induced by a Human-Milk Protein, *Proc. Natl. Acad. Sci. U. S. A.* 1995, 92, 8064-8068.
- [10] Kohler, C., Gogvadze, V., Hakansson, A., Svanborg, C., Orrenius, S., Zhivotovsky, B. A folding variant of human alpha-lactalbumin induces mitochondrial permeability transition in isolated mitochondria, *Eur. J. Biochem*. 2001, 268, 186-191.
- [11] Düringer, C., Hamiche, A., Gustafsson, L., Kimura, H., Svanborg, C. HAMLET interacts with histones and chromatin in tumor cell nuclei, *Journal of Biological Chemistry*. 2003, 278, 42131-42135.
- [12] Gustafsson, L., Hallgren, O., Mossberg, A.K., Pettersson, J., Fischer, W., Aronsson, A., Svanborg, C. HAMLET kills tumor cells by apoptosis: structure, cellular mechanisms, and therapy, *J. Nutr*. 2005, 135, 1299-1303.

- [13] Hallgren, O., Gustafsson, L., Irjala, H., Selivanova, G., Orrenius, S., Svanborg, C. HAMLET triggers apoptosis but tumor cell death is independent of caspases, Bcl-2 and p53, *Apoptosis : an international journal on programmed cell death*. 2006, *11*, 221-233.
- [14] Aits, S., Gustafsson, L., Hallgren, O., Brest, P., Gustafsson, M., Trulsson, M., Mossberg, A.-K., Uwe, H., Mograbi, S.B., Svanborg, C. HAMLET (human alpha-lactalbumin made lethal to tumor cells) triggers autophagic tumor cell death, *Int. J. Cancer*. 2009, *124*, 1008-1019.
- [15] Gustafsson, L., Leijonhufvud, I., Aronsson, A., Mossberg, A.K., Svanborg, C. Treatment of skin papillomas with topical alpha -lactalbumin-oleic acid., *N. Engl. J. Med*. 2004, *350*, 2663-2672.
- [16] Fischer, W., Gustafsson, L., Mossberg, A.K., Gronli, J., Mork, S., Bjerkvig, R., Svanborg, C. Human alpha-lactalbumin made lethal to tumor cells (HAMLET) kills human glioblastoma cells in brain xenografts by an apoptosis-like mechanism and prolongs survival, *Cancer Res*. 2004, *64*, 2105-2112.
- [17] Mossberg, A.K., Wullt, B., Gustafsson, L., Mansson, W., Ljunggren, E., Svanborg, C. Bladder cancers respond to intravesical instillation of HAMLET (human alpha-lactalbumin made lethal to tumor cells), *Int. J. Cancer*. 2007, *121*, 1352-1359.
- [18] Svensson, M., Hakansson, A., Mossberg, A.K., Linse, S., Svanborg, C. Conversion of alpha-lactalbumin to a protein inducing apoptosis, *Proc. Natl. Acad. Sci. U. S. A*. 2000, *97*, 422 1-4226.
- [19] Svensson, M., Fast, J., Mossberg, A., Kristin, Durringer, C., Gustafsson, L., Hallgren, O., Brooks, C.L., Berliner, L., Linse, S., Svanborg, C. alpha-Lactalbumin unfolding is not sufficient to cause apoptosis, but is required for the conversion to HAMLET (human alpha-lactalbumin made lethal to tumor cells). *Protein Sci*. 2003, *12*, 2794-2804.
- [20] Pettersson, J., Mossberg, A.-K., Svanborg, C. Alpha-Lactalbumin species variation, HAMLET formation, and tumor cell death, *Biochem. Biophys. Res. Commun*. 2006, *345*, 260-270.
- [21] Wilhelm, K., Darinskas, A., Noppe, W., Duchardt, E., Mok, K.H., Vukojevic, V., Schleucher, J., Morozova-Roche, L.A. Protein oligomerization induced by oleic acid at the solid-liquid interface - equine lysozyme cytotoxic complexes, *Febs J*. 2009, *276*, 3975-3989.
- [22] Acharya, K.R., Stuart, D.I., Walker, N.P., Lewis, M., Phillips, D.C. Refined structure of baboon alpha-lactalbumin at 1.7 Å resolution. Comparison with C-type lysozyme, *J. Mol. Biol*. 1989, *208*, 99-127.
- [23] McKenzie, H.A., White, F.H., Jr. Lysozyme and alpha-lactalbumin: structure, function, and interrelationships, *Adv. Protein Chem*. 1991, *41*, 173-315.

- [24] Qasba, P.K., Kumar, S. Molecular divergence of lysozymes and alpha-lactalbumin, *Crit. Rev. Biochem. Mol. Biol.* 1997, 32, 255-306.
- [25] Polverino de Laureto, P., Frare, E., Gottardo, R., Van Dael, H., Fontana, A. Partly folded states of members of the lysozyme/lactalbumin superfamily: a comparative study by circular dichroism spectroscopy and limited proteolysis, *Protein Sci.* 2002, 11, 2932-2946.
- [26] Liskova, K., Kelly, A.L., O'Brien, N., Brodkorb, A. Effect of denaturation of alpha-lactalbumin on the formation of BAMLET (bovine alpha-lactalbumin made lethal to tumor cells), *J. Agric. Food Chem.* 2010, 58, 4421-4427.
- [27] Brodkorb, A., Lišková, K. Ireland Patent (2010).
- [28] Lišková, K. Biologically Active Complexes of Oleic Acid and Bovine Whey Proteins, University College Cork, Cork 2011.
- [29] Croguennec, T., Molle, D., Mehra, R., Bouhallab, S. Spectroscopic characterization of heat-induced nonnative beta-lactoglobulin monomers, *Protein Sci.* 2004, 13, 1340-1346.
- [30] Kehoe, J.J., Wang, L., Morris, E.R., Brodkorb, A. Formation of non-native β -lactoglobulin during heat-induced denaturation *Food Biophysics.* 2011, 10.1007/s11483-11011-19230-11483.
- [31] Puyol, P., Perez, M.D., Peiro, J.M., Calvo, M. Effect of Binding of Retinol and Palmitic Acid to Bovine β -Lactoglobulin on Its Resistance to Thermal Denaturation, *J. Dairy Sci.* 1994, 77, 1494-1502.
- [32] Puyol, P., Pérez, M.D., Burgos, J., Calvo, M. Effect of the Binding of Palmitic Acid to β -Lactoglobulin on Its Gelation Properties, *International Dairy Journal.* 1998, 8, 119-123.
- [33] Roefs, S.P., De Kruif, K.G. A model for the denaturation and aggregation of beta-lactoglobulin, *European journal of biochemistry /FEBS.* 1994, 226, 883-889.
- [34] Nielsen, S.B., Wilhelm, K., Vad, B., Schleucher, J., Morozova-Roche, L.A., Otzen, D. The interaction of equine lysozyme:oleic acid complexes with lipid membranes suggests a cargo off-loading mechanism, *Journal of molecular biology.* 2010, 398, 351-361.
- [35] Dufour, E., Bertrand-Harb, C., Haertle, T. Reversible effects of medium dielectric constant on structural transformation of beta-lactoglobulin and its retinol binding, *Biopolymers.* 1993, 33, 589-598.
- [36] Collini, M., D'Alfonso, L., Baldini, G. New insight on beta-lactoglobulin binding sites by 1-anilinoanthracene-8-sulfonate fluorescence decay, *Protein Sci.* 2000, 9, 1968-1974.
- [37] Elliott, A., Ambrose, E.J. Structure of synthetic polypeptides, *Nature.* 1950, 165, 921-922.
- [38] Krimm, S., Bandekar, J. Vibrational Spectroscopy and Conformation of Peptides, Polypeptides, and Proteins, *Adv. Protein Chem.* 1986, 38, 181-364.

- [39] Holloway, P.W., Mantsch, H.H. Structure of cytochrome b5 in solution by Fourier-transform infrared spectroscopy, *Biochemistry*. 1989, 28, 93 1-935.
- [40] Fang, Y., Dalglish, D.G. The conformation of alpha-lactalbumin as a function of pH, heat treatment and adsorption at hydrophobic surfaces studied by FTIR, *Food Hydrocolloids*. 1998, 12, 121-126.
- [41] Lefèvre, T., Subirade, M. Structural and interaction properties of beta-lactoglobulin as studied by FTIR spectroscopy, *International Journal of Food Science and Technology*. 1999, 34, 419-428.
- [42] Clark, A., Saunderson, D., Suggett, A. Infrared and laser-Raman spectroscopic studies of thermally-induced globular protein gels, *International Journal of Peptide and Protein Research*. 1981, 17, 353.
- [43] Fang, Y., Dalglish, D. Conformation of beta-lactoglobulin studied by FTIR: effect of pH, temperature, and adsorption to the oil-water interface, *J. Colloid Interface Sci.* 1997, 196, 292.
- [44] Benseny-Cases, N., Cócera, M., Cladera, J. Conversion of non-fibrillar beta-sheet oligomers into amyloid fibrils in Alzheimer's disease amyloid peptide aggregation, *Biochem. Biophys. Res. Commun.* 2007, 361, 916-921.
- [45] Lansbury, P., Costa, P., Griffiths, J., Simon, E., Auger, M., Halverson, K., Kocisko, D., Hensch, Z., Ashburn, T., Spencer, R. Structural model for the beta-amyloid fibril based on interstrand alignment of an antiparallel-sheet comprising a C-terminal peptide, *Nat. Struct. Mol. Biol.* 1995, 2, 990-998.
- [46] Balbach, J., Petkova, A., Oyler, N., Antzutkin, O., Gordon, D., Meredith, S., Tycko, R. Supramolecular structure in full-length Alzheimer's beta-amyloid fibrils: evidence for a parallel beta-sheet organization from solid-state nuclear magnetic resonance, *Biophys. J.* 2002, 83, 1205-1216.
- [47] Boye, J., Ismail, A., Alli, I. Effects of physicochemical factors on the secondary structure of beta-lactoglobulin, *J. Dairy Res.* 1996, 63, 97-109.
- [48] Qi, X., Holt, C., McNulty, D., Clarke, D., Brownlow, S., Jones, G. Effect of temperature on the secondary structure of beta-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: a test of the molten globule hypothesis, *Biochem. J.* 1997, 324, 341.
- [49] Boye, J.I., Alli, I., Ismail, A.A. Use of differential scanning calorimetry and infrared spectroscopy in the study of thermal and structural stability of alpha-lactalbumin, *J. Agric. Food Chem.* 1997, 45, 1116-1125.

- [50] Molinari, H., Ragona, L., Varani, L., Musco, G., Consonni, R., Zetta, L., Monaco, H. Partially folded structure of monomeric bovine beta-lactoglobulin, *FEBS Lett.* 1996, 381, 237.
- [51] Brinkmann, C.R., Heegaard, C.W., Petersen, T.E., Jensenius, J.C., Thiel, S. The toxicity of bovine α -lactalbumin made lethal to tumor cells is highly dependent on oleic acid and induces killing in cancer cell lines and noncancer-derived primary cells, *FEBS Journal.* 2011, 278, 1955-1967.
- [52] Permyakov, S.E., Knyazeva, E.L., Leonteva, M.V., Fadeev, R.S., Chekanov, A.V., Zhadan, A.P., Hakansson, A.P., Akatov, V.S., Permyakov, E.A. A novel method for preparation of HAMLET-like protein complexes, *Biochimie.* 2011, doi: 10.1016/j.biochi.2011.1005.1002.
- [53] Hamilton, J.A. Fatty acid transport: difficult or easy?, *J. Lipid Res.* 1998, 39, 467-481.
- [54] Greene, L.A., Tischler, A. S. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor, *Proc. Natl. Acad. Sci. U. S. A.* 1976, 73, 2424-2428.
- [55] Fujita, K., Lazarovici, P., Guroff, G. Regulation of the differentiation of PC12 pheochromocytoma cells, *Environ. Health Perspect.* 1989, 80, 127.

Figure 1

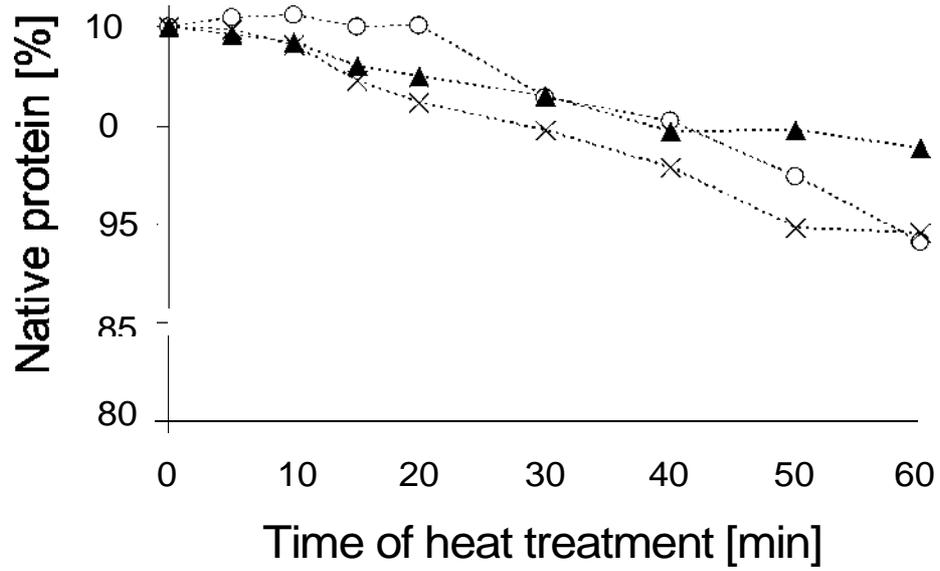


Figure 1: Denaturation curves of β -lg heated at 60°C at concentrations of: 0.5% w/v (___), 2% w/v (___) and 5% w/v (___) in PBS, pH 7.4.

Figure 2

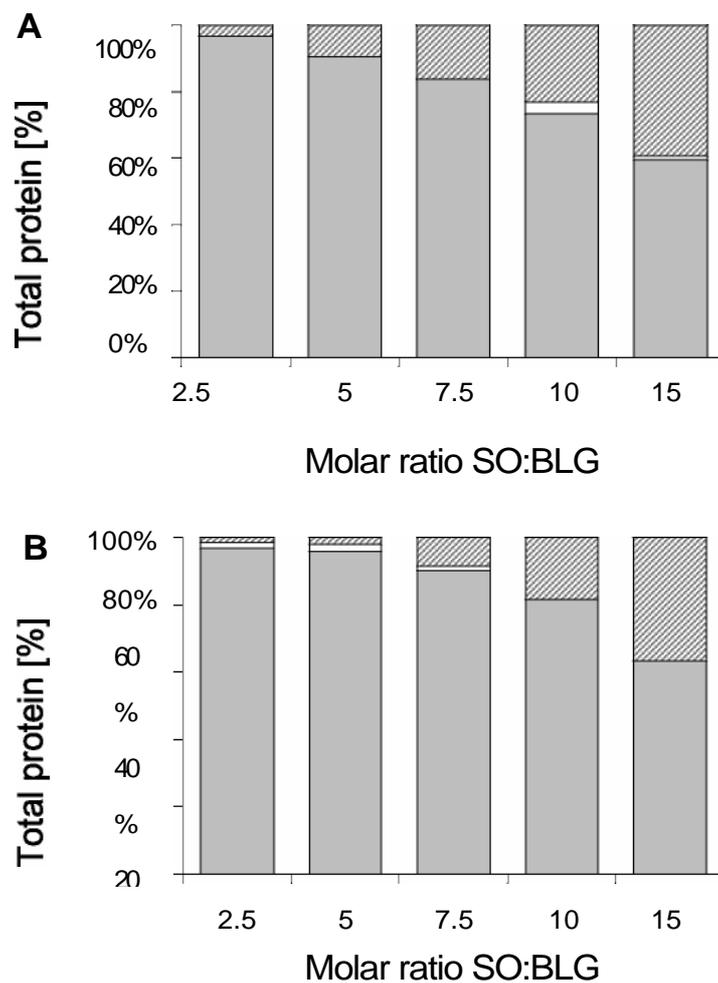


Figure 2: Composition of 0.5 (1A) and 5% w/v (1B) solutions of β -lg after heat treatment at 60°C for 30 min. The molar ratio of NaOle: β -lg in heated solutions is indicated on the x-axis, the total protein content on the y-axis as follows: grey area, native protein; white area, non-native monomeric protein; striped area, aggregated protein.

Figure 3

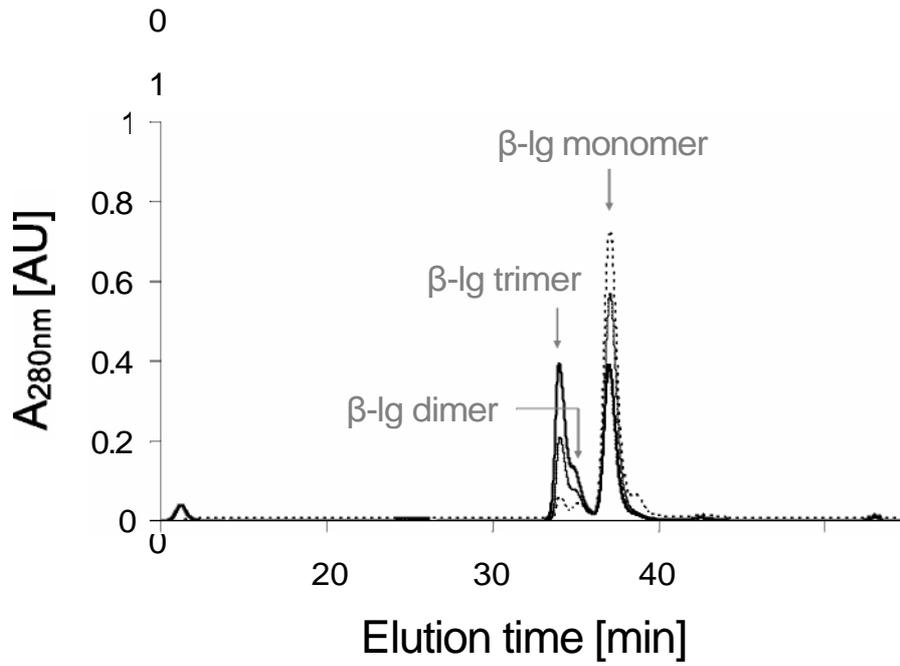


Figure 3: GP-HPLC chromatograms of solutions of native β -lg _____, β -lg (5% w/v) heated in the presence of $7.5 \times \text{NaOle}$ _____ and β -lg (5% w/v) heated in the presence of $15 \times \text{NaOle}$ _____.

Figure 4

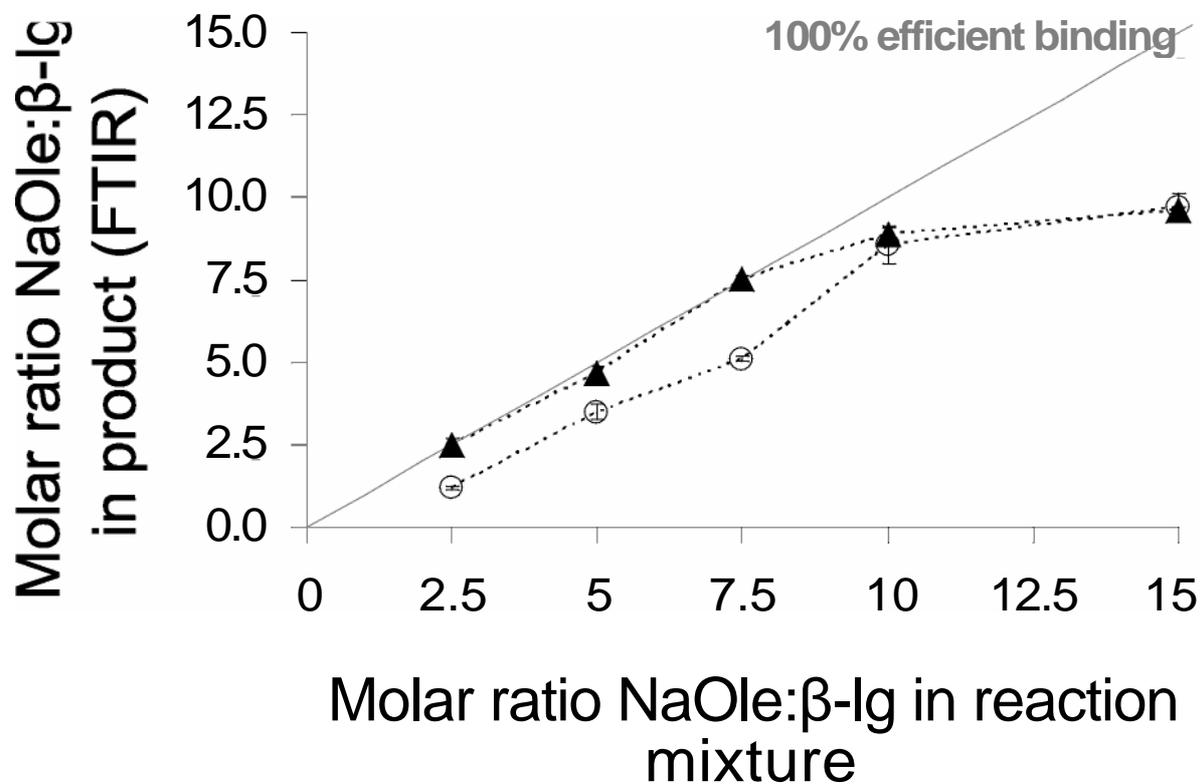


Figure 4: Correlation of the molar ratios of NaOle:β-Ig present in the starting solutions (reaction mixtures) with the molar ratios of NaOle:β-Ig detected by FTIR of the complexes after extensive dialysis and freeze-drying. Complexes of β-Ig with NaOle were prepared in one-phase solution at: 0.5% (○) and 5% (▲) protein.

Figure 5

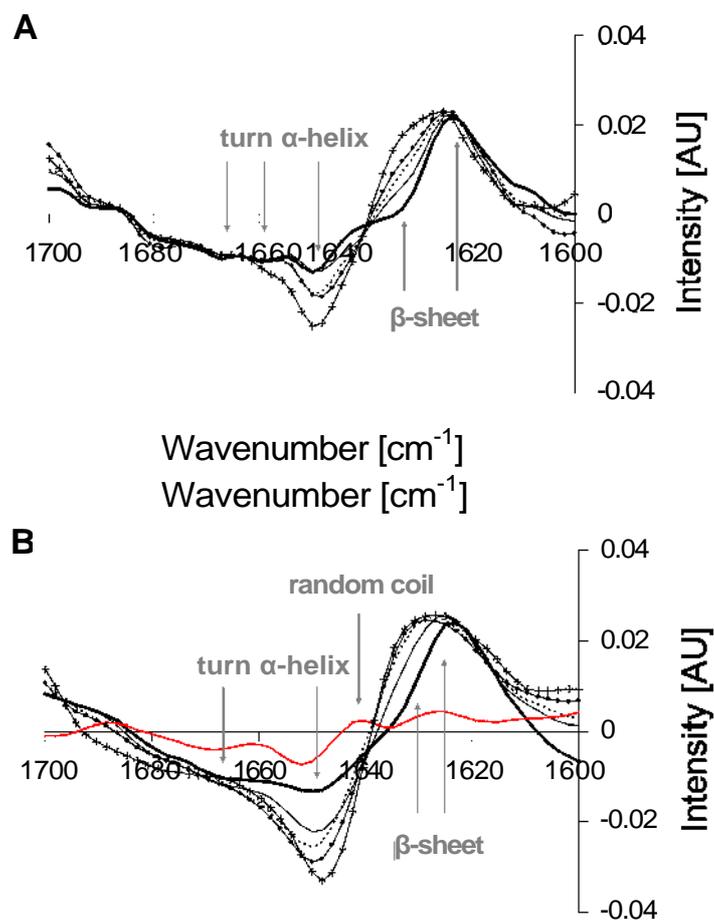


Figure 5: Difference FTIR spectra of (A) 0.5% β -Ig and (B) 5% β -Ig upon forming a complex with NaOle as a function of molar ratio of NaOle: β -Ig : () molar ratio of 2.5, () molar ratio of 5, () molar ratio of 7.5, () molar ratio of 10, () molar ratio of 15, () difference spectrum of pure β -Ig (β -Ig heated for 30 min at 60°C minus native β -Ig). Difference spectra were obtained by subtracting the spectrum of native β -Ig from the sample spectra. All spectra were vector-normalized in the region 1,700 – 1,600 cm⁻¹ prior to the subtraction.

Figure 6

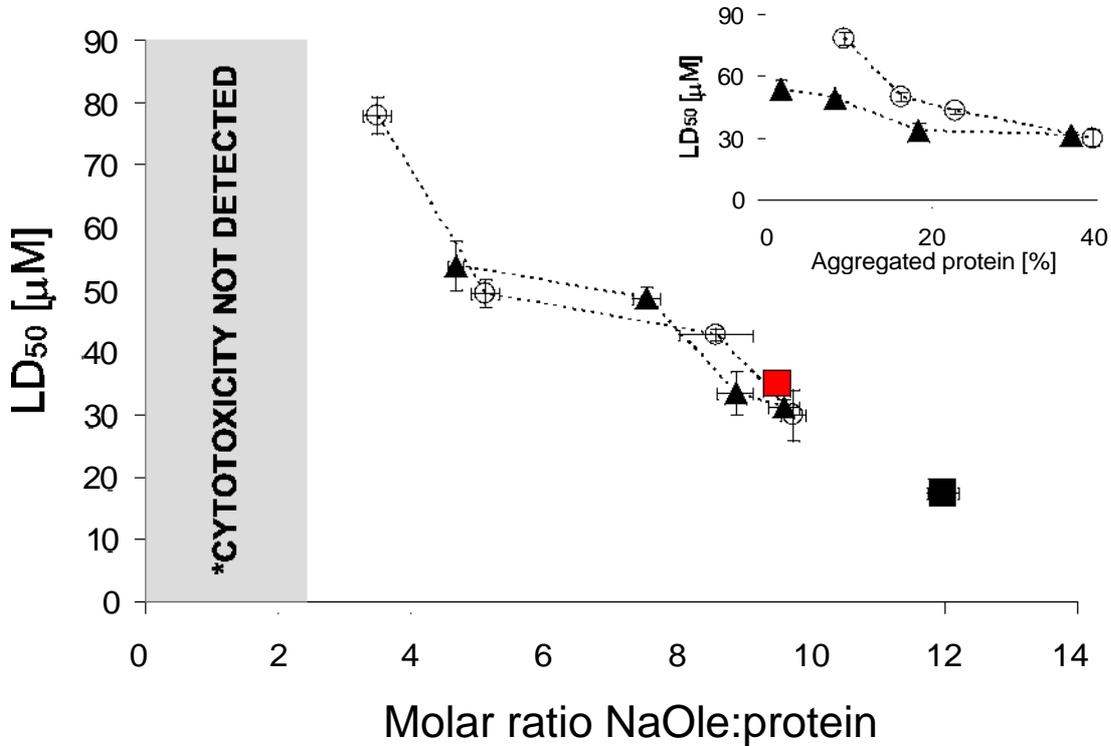


Figure 6: LD₅₀ values of NaOle/β-Ig complexes calculated from viability of U937 cells following supplementation with increasing concentrations of tested samples. Viability was determined after 24 h incubation, using the MTS assay. Data are the means of three independent measurements, with standard deviations represented by vertical bars. Horizontal bars represent standard deviations in the molar ratios NaOle:β-Ig determined by FTIR. NaOle/β-Ig complexes were prepared with: 0.5% β-Ig (○); 5% β-Ig (△); BAMLET produced on chromatographic column (□); BAMLET produced in solution with 10-molar excess of NaOle (■).

* The highest concentration tested (250 μM) did not cause a detectable decrease in cell viability. Insert: The cytotoxicity of NaOle/β-Ig complexes plotted against the content (in %) of aggregated protein, with legend as above.

Figure 7

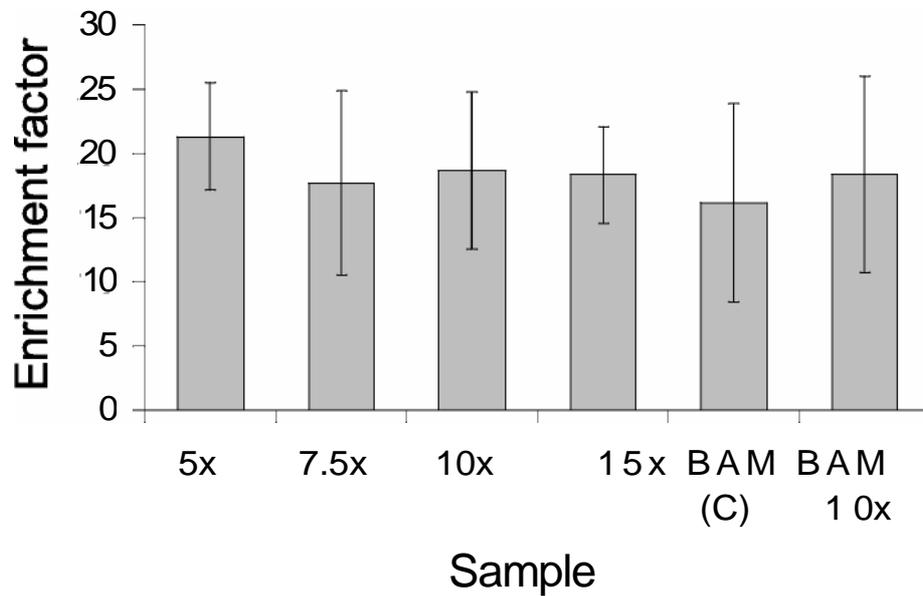
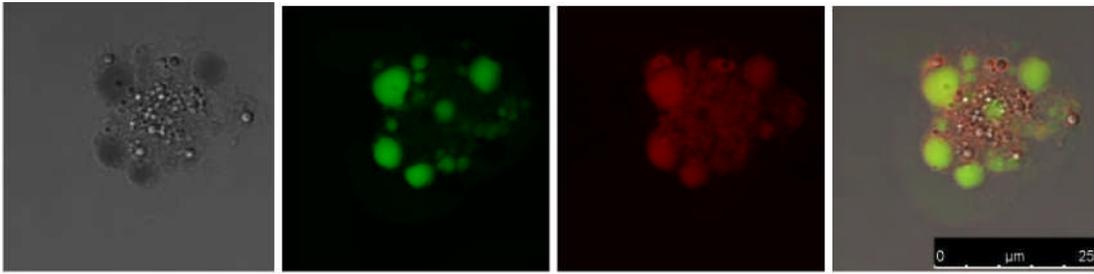
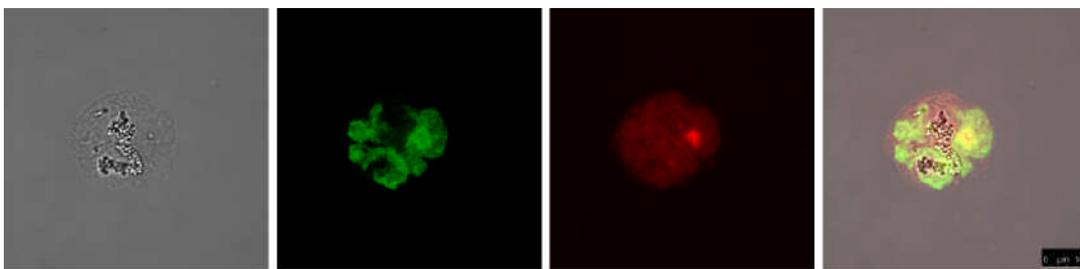


Figure 7: Specific enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm of U937 cells treated with NaOle/ β -lg complexes produced with increasing molar ratio of NaOle: β -lg (during preparation), or BAMLET samples produced by chromatography (BAM (C)), or in solution with 10-molar excess of NaOle (BAM 10 \times). Non-treated cells were used as a negative control. Data are the means of two independent measurements (performed in duplicate) with standard deviations represented by vertical bars.

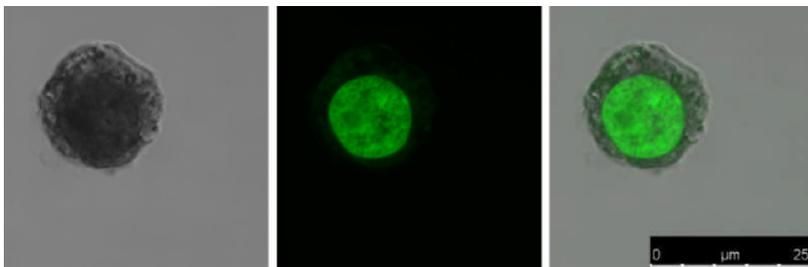
Figure 8



(A)



(B)



(C)

Figure 8: Confocal microscopic images of U937 cells incubated for 4 h with LD_{50} concentration of (A) NaOle/ β -Ig complex of molar ratio NaOle: β -Ig of 10, (B) BAMLET produced on chromatographic column and (C) control cells. Cells were stained with Syto-9 dye (green fluorescence), which visualizes the cells' chromatin, protein complexes (NaOle/ β -Ig complex and BAMLET) were labelled with fluorescent dye Alexa-fluor 633 (red fluorescence). It can be seen that both protein complexes penetrated the cell membrane and co-localized with the chromatin. Apoptotic changes, such as condensation of chromatin and formation of presence of apoptotic bodies can be seen in both treated cells, while control cells showed healthy diffuse nuclei. Images shown are representative of cells detected in the samples analysed.

Figure 9

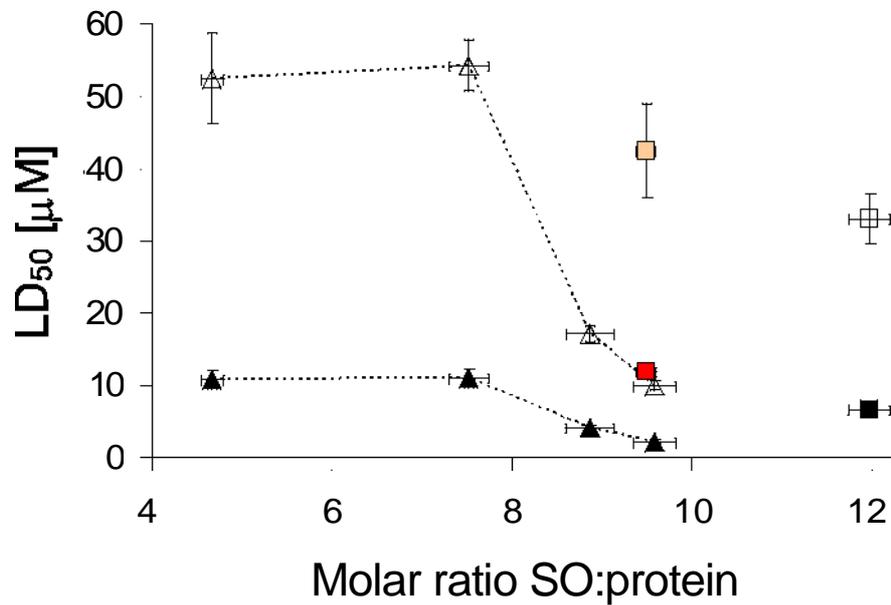


Figure 9: LD_{50} values of NaOle/ β -Ilg complexes and BAMLET samples calculated from viability of PC-12 cells in undifferentiated and differentiated state following supplementation with increasing concentrations of NaOle/ β -Ilg or BAMLET complexes. NaOle/ β -Ilg complexes are represented by triangles, BAMLET samples by squares: BAMLET produced on chromatographic column (); BAMLET produced in solution with 10-molar excess of NaOle (red square). Symbols are open for LD_{50} measured on differentiated cells, and filled for LD_{50} measured on undifferentiated cells. Viability was determined after 24 h incubation, using the MTS assay. Data are the means of three independent measurements, with standard deviations represented by vertical bars. Horizontal bars represent standard deviations for the molar ratios of NaOle: β -Ilg determined by FTIR. NaOle/ β -Ilg complexes were prepared with increasing molar ratios of NaOle:protein using 5% β -Ilg .