JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 4421–4427 4421
DOI:10.1021/jf903901j

Effect of Denaturation of α-Lactalbumin on the Formation of BAMLET (Bovine α-Lactalbumin Made Lethal to Tumor Cells)

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A complex of α-lactalbumin with oleic acid, also known as HAMLET/BAMLET (human/bovine α-lactalbumin made lethal to tumor cells), causes apoptosis-like death in tumor cells but has little effect on healthy differentiated cells. The aim of this study was to examine whether irreversible denaturation of α-lactalbumin is detrimental to the formation and cytotoxicity of BAMLET. Commercial bovine holo α-lactalbumin (1−4% w/v) was heated at 80 °C for up to 100 min. With an increasing concentration of protein, the denaturation of α-lactalbumin proceeded faster, and aggregation became more extensive. Native and sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that a high proportion of the aggregates was linked by disulfide bonds. BAMLET was prepared from native and heat-treated α-lactalbumin according to a previously described chromatographic method. Despite the high content of denatured and aggregated α-lactalbumin in the heat-treated samples, their conversion into BAMLET was not negatively affected, resulting in BAMLET complexes partly composed of covalently linked aggregates of α-lactalbumin. The cytotoxicity of all prepared BAMLET samples was comparable to that of the control sample prepared from native α-lactalbumin (LD_{50} = 34.6 ± 2.7 μmol L^{-1}). It was concluded that α-lactalbumin is not required to be in its native conformation for the conversion into its biologically active BAMLET complex.

KEYWORDS: α-Lactalbumin; HAMLET; BAMLET; denaturation; aggregation; cytotoxicity; apoptosis

INTRODUCTION

α-Lactalbumin is a whey protein present in milk of all species investigated to date. It is the predominant protein in human milk (concentration in mature milk about 2.5 g L^{-1}) (*1*) and the second most abundant whey protein in bovine milk, with a concentration of 1−1.5 g L^{-1}(*2*). It is a calcium-binding (*3*) globular protein with an approximate molecular mass 14.2 kDa, consisting of 123 amino acid residues in most species (*4*). It contains eight cysteine (Cys) residues, forming four disulfide bridges (6−120, 61−77, 73−91, and 28−111). The three-dimensional (3D) structure of α-lactalbumin consists of two domains divided by a deep cleft. A large α-domain is composed of three major α-helices and two short 3_{10} helices, and a small β-domain includes a short β-sheet, one short α-helix, and a series of loops; the two domains are connected by a calcium-binding loop (*5*). The presence of calcium increases the stability of α-lactalbumin, allowing it to refold after the protein has been heated (*6*). If the pH of the protein solution is reduced or calcium chelators [such as ethylenediaminetetraacetic acid (EDTA)] are added, the Ca^{2+} ion is released from α-lactalbumin. This results in a transition of the native state to a stable transient "molten globule" state that is different from the unfolded state (*7*). The molten globule state has been described as a slightly "swollen" conformation with a secondary structure like that of the native state but with a fluctuating tertiary structure (*5*, *8*).

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A complex with antitumor activity consisting of α-lactalbumin and oleic acid was discovered in casein coprecipitate in 1995 (*9*). This complex, called HAMLET (human α-lactalbumin made lethal to tumor cells), specifically kills cancer and immature cells, but healthy differentiated cells are unaffected by it. The preparation of HAMLET was later further developed; the original isolation from casein coprecipitate was replaced by a two-step chromatographic method (*10*), where the first step involved conditioning of a chromatographic matrix by oleic acid, while in the second step, the apo (calcium-depleted) protein was loaded onto the column. HAMLET-like complexes have been prepared from α-lactalbumin of various species [including bovine milk, bovine α-lactalbumin made lethal to tumor cells (BAMLET)], all of them with comparable cytotoxicity to HAMLET (*11*).

It has been suggested that HAMLET-like complexes could be naturally formed in the stomach of breast-fed infants (*10*), which could also apply to bottle-fed infants in the case of bovine α-lactalbumin-enriched infant formula. However, industrial methods used for the enrichment of α-lactalbumin and subsequent processing of infant formula can irreversibly alter its 3D structure and consequently affect the potential formation of BAMLET. To date, there has been no published study about the influence of irreversible denaturation of α-lactalbumin on the conversion to HAMLET/BAMLET and its subsequent properties. These issues were addressed in the present study; changes occurring during heat treatment of α-lactalbumin were

Published on Web 03/16/2010

characterized, as well as their influence on the formation and cytotoxicity of BAMLET.

The denaturation of α-lactalbumin has been extensively studied in buffer solutions or skim milk. Numerous reactions can take place during heat treatment, and the occurrence and extent of these reactions depend on heating conditions (temperature and duration of heat treatment) as well as on factors such as concentration, pH, the presence of calcium, and ionic strength. It has been shown that calcium binding increases the stability of α-lactalbumin toward denaturation (*6*). When α-lactalbumin binds less than 1 mol of calcium per mol of protein, the transition temperature of this apo α-lactalbumin is about 38 °C, but the addition of calcium increases the transition temperature to about 66 °C (*12*). The extent of renaturation is also greatly increased by the presence of calcium, which helps holo α-lactalbumin (calcium-bound) to refold into its native conformation (*12*−*15*). When pure α-lactalbumin is heated under milder conditions (about 80 °C, pH 6.7), it does not form aggregates (*16*, *17*), which is partly explained by the lack of free thiol groups. However, heating under more severe conditions (100 °C, 10−30 min) resulted in oligomers of α-lactalbumin connected by disulfide bonds and some non-native monomers of α-lactalbumin (*14*). The degree of thermal denaturation and aggregation of α-lactalbumin, however, rapidly increases when heated in the presence of other whey proteins, such as *β*-lactoglobulin or bovine serum albumin (BSA) (*18*). Seven percent (w/v) α-lactalbumin solutions heated at 80 °C required 120 min for gelation to occur, whereas BSA or *β*-lactoglobulin solutions of the same concentration gelled by the time the solution temperature reached 80 °C. The aggregation rate of α-lactalbumin in the presence of *β*-lactoglobulin increased 14-fold as compared to the pure protein (*16*). According to Gezimati et al., the free sulfhydryl group in *β*-lactoglobulin induces thiol−disulfide interchange reactions between the unfolded α-lactalbumin and the *β*-lactoglobulin, which contributes to the rapid aggregation (*19*). Heated mixtures of α-lactalbumin and *β*-lactoglobulin contained large and intermediate-sized aggregates, which were held together by disulfide bonds and, to a lesser extent, by hydrophobic interactions (*17*, *20*). In 2000, it was shown that heating α-lactalbumin in the presence of *β*-lactoglobulin (*21*) or BSA (*22*) can induce the formation of α-lactalbumin dimers and 1:1 aggregates of α-lactalbumin with *β*-lactoglobulin and possibly BSA.

As described above, heat treatment of α-lactalbumin in the presence of other whey proteins leads to a broad spectrum of structural changes, including aggregation. Establishing whether these changes, occurring during industrial processing, are detrimental to the formation of BAMLET is crucial for its potential food applications, but it can also shed light on the mechanism of action of this cytotoxic complex.

MATERIALS AND METHODS

Materials. α-Lactalbumin (95% purity, approximately 3% *β*-lactoglobulin content) was purchased from Davisco Foods International, Inc. (Eden Prairie, MN), diethylaminoethyl (DEAE) Trisacryl M matrix from Pall Corp. (Cergy-Saint-Christophe, France), the CellTiter 96 Aqueous One Solution Cell Proliferation Assay from Promega Corp. (Madison, WI), Cell Death Detection ELISA^{PLUS} from Roche Applied Science (West Sussex, United Kingdom), Bio-Rad Protein Assay from Bio-Rad Laboratories GmbH (Hertfordshire, United Kingdom), and molecular weight markers for polyacrylamide gel electrophoresis (PAGE) from GE Healthcare (Uppsala, Sweden). All other chemicals and solutions were purchased from Sigma-Aldrich (Dublin, Ireland).

Denaturation of α-Lactalbumin. α-Lactalbumin was reconstituted overnight at 4 °C in Milli-Q water at concentrations of 1−4% (w/v). $CaCl_2$ was added to a final ratio 1:1 with α-lactalbumin. Any denatured protein present in the commercial material was removed by isoelectric precipitation at pH 4.6. After filtration with 0.45 *μ*m syringe filters (Sartorius), the pH was adjusted to 7.0. To measure the denaturation kinetics, 2 mL aliquots were heated in a water bath at 80 °C for defined periods of time, after which they were immediately submerged in ice water. The degrees of denaturation (DDs) and aggregation were determined using reversed-phase and gel filtration chromatography. The DD is defined as a ratio of denatured protein to total protein.

Gel Filtration and Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). The concentration of native protein was determined by reversed-phase HPLC, using a Source 5RPC column (GE Healthcare) according to a previously published method (*23*). The method was calibrated using α-lactalbumin (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 G3000 column (TosoHaas, Montogomeryville, PA), using a 50 mM Tris-HCl buffer (pH 7.2) at a flow rate of 0.8 mL min^{-1} as the eluent. The data were acquired and processed using Waters Empower software.

Calculation of Levels of Native, Unfolded, and Aggregated α-Lactalbumin. The concentrations of native α-lactalbumin were directly obtained using RP-HPLC. The concentrations of monomeric (including native and unfolded) α-lactalbumin were directly obtained using gel permeation high-performance liquid chromatography (GP-HPLC). The concentrations of unfolded monomeric α-lactalbumin were calculated by subtraction of the level of native α-lactalbumin (RP-HPLC) from that of monomeric α-lactalbumin (GP-HPLC). The amount of aggregation was calculated by subtraction of the amount of monomer (GP-HPLC) from the initial protein concentration.

Preparation of BAMLET. α-Lactalbumin solutions (4% w/v) for preparation of BAMLET were heated in 40 mL aliquots (in 50 mL plastic tubes; Sarstedt, Wexford, Ireland). The heating times needed to reach various DDs were estimated from the denaturation curve of the low-volume measurements, and a heating delay of 7 min was added due to the increased volume of samples. Heating at 80 °C for 0, 22, 37, 52, and 97 min resulted in samples with DDs of 0, 30, 55, 72, and 95%, respectively.

Tris-HCl solution (1 M) and solid EDTA were added to heat-treated samples to final concentrations of 10 and 2 mM, respectively. BAMLET was prepared on a fast protein liquid chromatography (FPLC) instrument (ÄKTA Purifier, GE Healthcare) using a chromatographic column according to the method of Svensson et al. (*10*). Collected BAMLET fractions were pooled and dialyzed against distilled water prior to freeze drying.

Cytotoxicity Testing. Cells used for cytotoxicity assays were human monocytic cells U937 (obtained from European Collection of Cell Cultures, ECACC 85011440). Cells were subcultured every 48 h at a density of 1×10^5 cells mL^{-1} in 10% fetal bovine serum (FBS)-supplemented Roswell Park Memorial Institute (RPMI) 1640 media. Cells were cultured in an atmosphere of CO_2−air (5:95, v/v) at 37 °C and were maintained in the absence of antibiotics. Freeze-dried BAMLET samples were reconstituted in RPMI 1640 at a concentration of 7 mg mL^{-1} and filtered with 0.1 *μ*m sterile filters. Aliquots were made and stored at −80 °C. For each assay, a new aliquot was used.

U937 cells were seeded in 10% FBS-supplemented RPMI media at a density of 2×10^5 cells mL^{-1} in a volume of 50 *μ*L. An increasing volume of BAMLET samples was added to the seeded cells, with pure RPMI 1640 being added to make the final volume up to 100 *μ*L per well. This way, the cells were seeded at a density of 1×10^5 cells mL^{-1}, the final concentration of FBS was 5%, and the cells were supplemented with an increasing concentration of BAMLET (0−50% v/v) without any dilution of growing media. Cells were incubated for 24 h in 96-well plates. The cell viability was assessed using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega). Absorbance at 490 nm was recorded after 3 h of incubation with MTS reagent. Assays were performed in triplicates, and three independent measurements were made. The concentration of BAMLET in RPMI media was determined by the Bradford protein assay (*24*), using a molecular mass of 14.2 kDa .

Statistical Analysis. The acquired data were analyzed using GraphPad Prism program. The sigmoidal dose−response with variable slope was used to fit the measured curves and calculate LD_{50}. These values were then compared by one-way analysis of variance (ANOVA) using SigmaStat 3.1 software.

Testing of Apoptotic Activity. The type of cell death occurring after treatment with BAMLET was analyzed using a Cell Death Detection ELISA^{PLUS} kit from Roche Applied Science. Cells were seeded and supplemented with BAMLET samples in the same manner as for cytotoxicity assay. After 4 h of incubation at 37 °C and 5% CO_2, the cell viability was determined with a Trypan blue assay. Cells with 50% viability were used for the apoptosis assay. The concentrations of BAMLET inducing 50% cell death after 4 h of incubation were 38.8, 28.1, 31.8, 46.2, and 35.0 μM (for samples 0% DD, 30% DD, 55% DD, 72% DD, and 95% DD). To calculate the specific enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm (enrichment factor) of the treated cells, the following formula was used

$$\text{enrichment factor} = \frac{\text{absorbance of sample at 405 nm}}{\text{absorbance of negative control at 405 nm}}$$

where cells not treated with BAMLET were used as a negative control.

Fluorescence Spectroscopy. Intrinsic tryptophan fluorescence was measured using a Cary Eclipse Fluorescence Spectrometer (Varian, Palo Alto, CA). α-Lactalbumin and BAMLET samples were prepared in Milli-Q water, and the absorbance was adjusted to 0.7 at 280 nm for each sample. The excitation wavelength was 280 nm, and the emission was scanned from 300 to 400 nm. Both excitation and emission slits were set to 5 nm, the temperature was 20 °C, and the scan rate was 120 nm min^{-1}.

Electrophoresis. BAMLET samples in RPMI solution were analyzed by native and sodium dodecyl sulfate (SDS)-PAGE. Electrophoresis was performed according to the method of Laemmli (*25*), except that for native PAGE, SDS was omitted from the gel and running buffer, and loading buffer contained neither SDS nor mercaptoethanol. Samples were not heated prior to analysis. Protein was visualized by staining with Coomassie blue. In the case of SDS-PAGE, samples were prepared under both reducing (with mercaptoethanol) and nonreducing conditions.

RESULTS AND DISCUSSION

Denaturation of α-Lactalbumin. The loss of native α-lactalbumin on heating was determined by RP-HPLC. Heat-induced unfolding causes changes in hydrophobicity of α-lactalbumin and subsequently a shift in its retention time; the level of native α-lactalbumin can thus be accurately measured. GP-HPLC provided information about the amounts of monomeric and aggregated α-lactalbumin. Through a combination of the two techniques, the quantities of native and non-native monomers and aggregated protein were measured as a function of heating time.

GP-HPLC revealed the presence of aggregated α-lactalbumin in all heated samples. It is known that pure α-lactalbumin does not form covalently linked aggregates under these conditions, which is generally explained by the lack of free thiol groups in the protein (*16*, *17*). The observed aggregation can be explained by the low content of β-lactoglobulin (about 3%) in the commercial α-lactalbumin product. The free thiol group of Cys_{121} in unfolded β-lactoglobulin can act as a catalyst to break the disulfide bonds in α-lactalbumin, thus enabling the formation of intermolecular disulfide bonds in homo- and hetero-oligomers and large aggregates (*19*, *26*). Denaturation proceeded noticeably faster at higher concentrations of α-lactalbumin. Also, the extent of aggregation was correlated with the concentration of α-lactalbumin. While at low concentrations of α-lactalbumin, the majority of the denatured protein was unfolded monomer, increasing the concentration of α-lactalbumin resulted in higher aggregation. The denaturation and aggregation extent of heated solutions are shown in **Figure 1**.

The measurement of denaturation was performed in a minimum of three independent experiments. The coefficient of variation (CV) among the single runs was calculated according to the formula CV = 100% × (standard deviation/mean), and it was found to be lower than 10% for each measured data point.

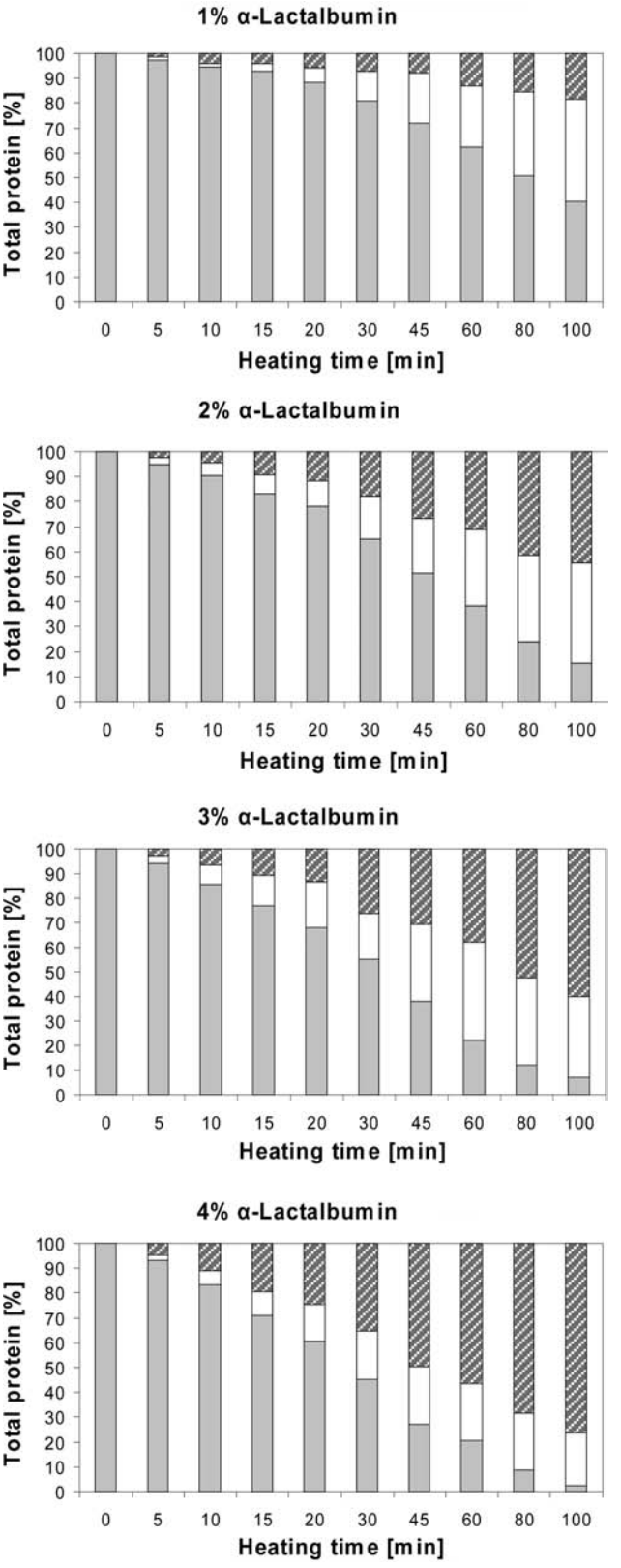

Figure 1. Compositions of 1−4% (w/v) solutions of holo α-lactalbumin after heat treatment at 80 °C for various times. Gray area, native protein; white area, non-native protein; and striped area, aggregated protein.

Changes caused by heating of α-lactalbumin were also followed by measuring fluorescence spectra of the individual samples. Unfolding of protein resulted in a significant red shift of the emission maximum from 325 nm for native holo α-lactalbumin to 345 nm for completely denatured α-lactalbumin (**Figure 2**).

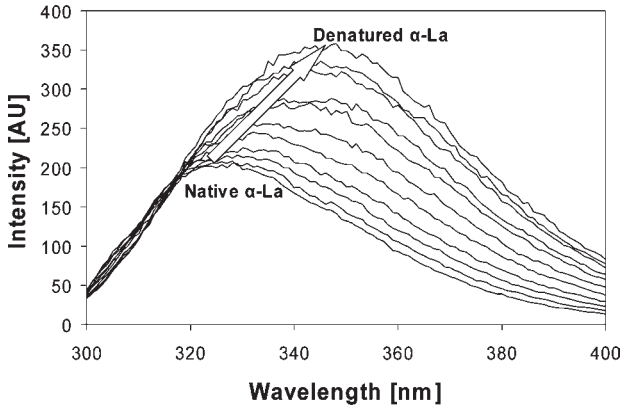

Figure 2. Intrinsic fluorescence of 1% α-lactalbumin in Milli-Q water after heat treatment at 80 °C for times of 0, 10, 20, 30, 45, 60, 80, 100, 130, 150, and 180 min. Spectra follow in the above order in the direction indicated by the arrow.

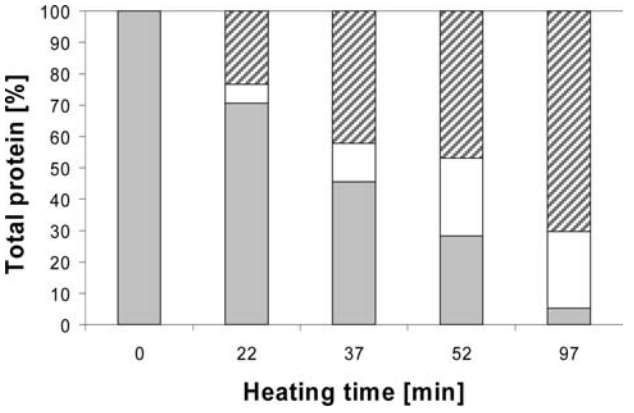

Figure 3. Compositions of five heat-treated (80 °C) α-lactalbumin solutions (4% w/v), which were used as starting material for the preparation of five BAMLET samples. Samples were subsequently referred to according to their DD: 0, 30, 55, 72, and 95%. Gray area, native protein; white area, non-native protein; and striped area, aggregated protein.

The relative fluorescence quantum yield increased by 100% for the heated protein. As previously mentioned, heating more concentrated samples produced substantially more aggregates. However, fluorescence spectra of samples (1−4% w/v) with the same DD were practically identical, despite the different degrees of aggregation of these samples (data not shown). It can be concluded that, under these conditions, intrinsic fluorescence measurement was a good technique for detection of irreversible denaturation but failed to distinguish between unfolded and aggregated protein.

Preparation of BAMLET from Heat-Treated α-Lactalbumin. The objective was to establish the impact of irreversible denaturation of α-lactalbumin on the formation and properties of BAMLET. The most pronounced changes (with prevalent aggregation) occurred in 4% protein solution; thus, the experiments were carried out with this concentration.

Aliquots of α-lactalbumin (40 mL) were heated at 80 °C for 0, 22, 37, 52, and 97 min, resulting in DDs of 0, 30, 55, 72, and 95%. The compositions after heat treatment (**Figure 3**) corresponded well to the values obtained from the low-volume measurement of denaturation curves.

BAMLET samples were then produced from the heat-treated α-lactalbumin solutions according to a previously described chromatographic method (*10*). Chromatograms of all heat-treated

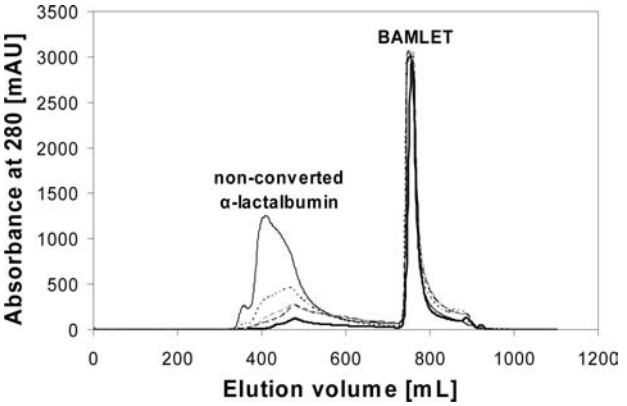

Figure 4. FPLC chromatograms of BAMLET preparations on the DEAE column. Solutions of α-lactalbumin heated at 80 °C for increasing times were used as starting material, which was loaded onto a chromatographic column preconditioned with oleic acid. Native protein (thin black line) (0% DD), 30% DD (dashed line), 55% DD (long dashed line), 72% DD (think, long dashed line), and 95% DD (thick black line).

samples (**Figure 4**) closely resembled the chromatogram of BAMLET produced from native α-lactalbumin, with two characteristic peaks, the first peak being nonconverted α-lactalbumin and second sharp peak being BAMLET. The amounts of eluted protein are summarized in **Table 1**. It is noticeable that extending the heating time of the starting material reduced the amount of protein eluted in the first peak (nonconverted α-lactalbumin). On the other hand, the yield of BAMLET increased for the sample of 30% DD and then, with progressing heat treatment, decreased stepwise. Overall, the prolonged time of heat treatment apparently caused a higher proportion of the starting material to be permanently retained on the column. It may be assumed that reversible unfolding of α-lactalbumin (loosening of the structure) occurred during heat treatment, and this increased its ability to form BAMLET, but with progressive denaturation and aggregation, this ability receded. Such changes in structure and properties of denatured α-lactalbumin could also be the reason for its increased retention on the chromatographic column. In summary, a mild heat treatment of α-lactalbumin can apparently substantially increase its conversion to BAMLET in the chromatographic preparation.

All samples of heat-treated α-lactalbumin and their corresponding BAMLET samples were analyzed by native and SDS-PAGE. It can be seen from the native PAGE gels of heat-treated α-lactalbumin samples (**Figure 5A**) that the amount of aggregates increased with the prolonging time of heat treatment, which is in agreement with the results obtained by HPLC. The nature of the interaction in formed aggregates was assessed by performing nonreducing SDS-PAGE (**Figure 5B**). The noncovalent interactions within aggregates are disrupted in the presence of SDS, so only particles connected by disulfide bridges appear on the electrophoretic gel. Comparing the amounts of aggregate on native and nonreducing SDS-PAGE, it was estimated that a large proportion of aggregates were held together by disulfide bridges, the noncovalent interactions being only a minor contributor to aggregation in the samples. The reducing SDS-PAGE gels showed bands of monomeric α-lactalbumin, β-lactoglobulin, and a faint band of approximate molecular mass of 30 kDa. The latter may correspond to a light chain of immunoglobulin present in the commercial product. However, the protein was not identified, as the amount was very low, and its incorporation into aggregates was not expected to affect properties of the samples.

Table 1. Characteristics of Individual FPLC Runs[a]

native α-lactalbumin, oleic acid-free					nontoxic up to 250 μM[b]
DD of starting material for BAMLET (%)	total protein eluted (mg)	α-La eluted (first peak) (mg)	BAMLET eluted (mg)	yield (%)	LD_{50} (μM)
0	160	88	72	36	34.6 ± 2.7 a
30	152	51	101	50.5	26.5 ± 1.7 b
55	133	40	93	46.5	29.4 ± 1.5 ab
72	123	38	85	42.5	41.9 ± 3.2 c
95	90	17	73	36.5	31.7 ± 1.9 ab

[a] The amounts of protein eluted in separate peaks during chromatographic preparations of BAMLET samples from heat-treated α-lactalbumin solutions. The amount of α-lactalbumin injected onto DEAE column was 200 mg for each chromatographic run. The calculations of yield of BAMLET are based on this amount. LD_{50} values of samples with different letter are significantly different ($P \leq 0.05$) when compared by one-way ANOVA. [b] The highest tested concentration of native α-lactalbumin (250 μM) did not cause a detectable decrease in cell viability.

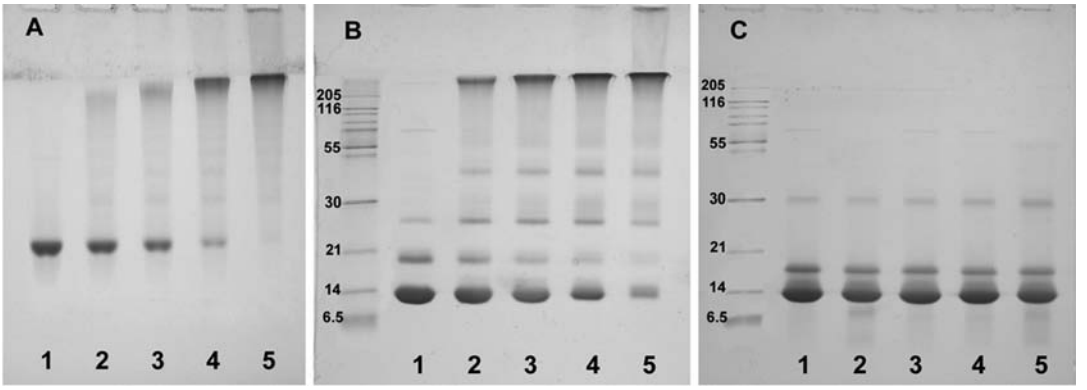

Figure 5. Polyacrylamide gels electrophoretograms of α-lactalbumin heat-treated at 80 °C for increasing times. (**A**) Native PAGE, (**B**) SDS nonreducing PAGE, and (**C**) SDS reducing PAGE. Lanes 1−5 correspond to α-lactalbumin samples of DD of 0, 30, 55, 72, and 95%.

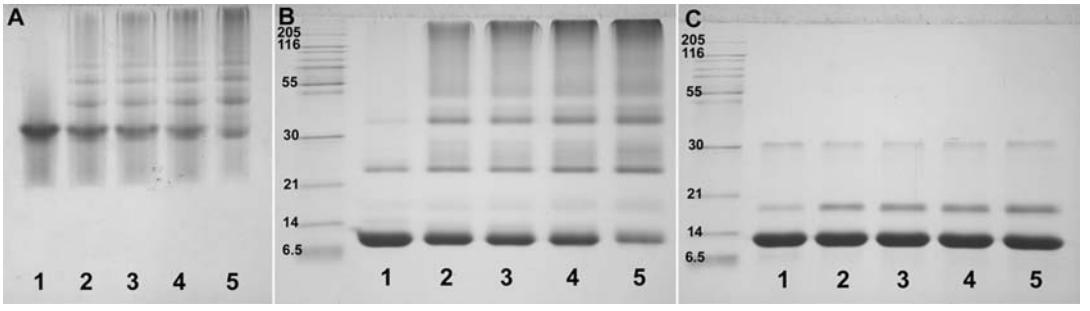

Figure 6. Polyacrylamide gels electrophoretograms of BAMLET samples analyzed by (**A**) native PAGE, (**B**) nonreducing SDS PAGE, and (**C**) reducing SDS PAGE. Lanes 1−5 correspond to BAMLET samples prepared from α-lactalbumin of DD of 0, 30, 55, 72, and 95%.

BAMLET samples prepared from heat-treated and control α-lactalbumin were also analyzed using electrophoresis. It was apparent that BAMLET samples prepared from heated α-lactalbumin contained a high level of aggregates (**Figure 6A**). Nonreducing SDS-PAGE (**Figure 6B**) revealed that these were in a large part (if not entirely) composed of covalently linked aggregates. Reducing SDS-PAGE (**Figure 6C**) showed that BAMLET samples prepared from heat-treated protein were enriched in β-lactoglobulin, as compared to the control BAMLET. This can be explained by the high content of aggregates present in the BAMLET fractions from heat-treated α-lactalbumin. In an unheated mixture of β-lactoglobulin and α-lactalbumin, the β-lactoglobulin was eluted in the first peak with nonconverted α-lactalbumin. The control BAMLET fractions were therefore almost free of β-lactoglobulin. However, when incorporated in hetero-oligomers with α-lactalbumin, the retention time of these shifted toward the peak of BAMLET. In summary, BAMLET from native α-lactalbumin consisted of monomeric protein−oleic acid complex, while BAMLET samples produced from heat-treated material also contained increasing amounts of covalently linked aggregates, some of which were hetero-oligomers of α-lactalbumin and β-lactoglobulin.

Cytotoxicity and Apoptotic Activity of BAMLET Samples. All BAMLET samples proved to be cytotoxic (**Table 1**). Despite the increasing DD and aggregation in the tested samples of BAMLET, the cytotoxicity of the samples did not show a corresponding trend. Although some differences in the cytotoxicity were observed, there was not a correlation between the cytotoxicity of BAMLET sample and its DD. For instance, the cytotoxicity of samples BAMLET 0% DD and BAMLET 95% DD was not significantly different ($P = 0.15$), despite the fact that BAMLET 95% DD was prepared from almost completely denatured (and highly aggregated) protein and BAMLET 0% DD was prepared from native monomer. In addition, all samples caused detectable DNA fragmentation (**Figure 7**), proving that the mechanism of cell death induced by all BAMLET samples was apoptosis. The mean values of enrichment factor increased for some samples, indicating that some samples may have caused higher apoptotic response. However, one-way ANOVA did not prove a significant difference

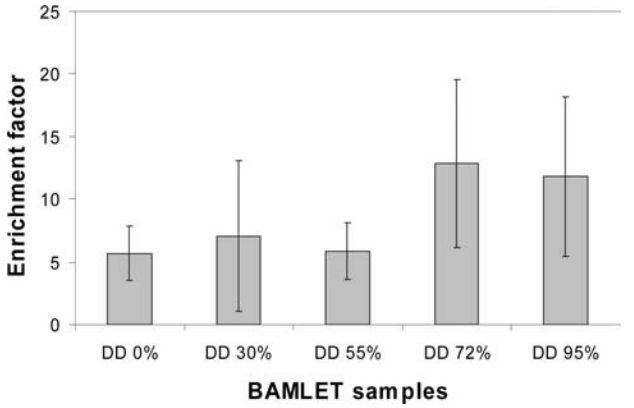

Figure 7. Effect of BAMLET samples on induction of apoptosis in U937 cells after 4 h of treatment. A Cell Death Detection ELISA kit was used to measure DNA fragmentation as a marker of apoptosis. The specific enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm of the treated cells was also calculated (enrichment factor). Data are the means of three independent experiments, with standard deviations represented by vertical bars.

between any of the measured values ($P = 0.257$) due to inter-experiment variability. The effect of DD on the level and mechanism of apoptotic response triggered by BAMLET requires further studies.

Until now, the published literature always referred to BAMLET prepared from native α-lactalbumin, and it was suggested that any alteration to its structure would impair its ability to form BAMLET (*27*); therefore, our results, suggesting otherwise, were most surprising.

On an industrial scale, solutions of α-lactalbumin are generally highly concentrated, and other components (i.e., proteins, sugars, polysaccharides, lipids, minerals, etc.) are present in significant quantities. For this reason, it could be presumed that much broader and more extensive changes occur during industrial processing. More research will need to be carried out to determine whether BAMLET could be efficiently formed from, for example, highly processed infant formula. However, in this study, it was suggested that the native conformation of α-lactalbumin is not required for the conversion into BAMLET. Neither unfolding nor covalent aggregation seemed to hinder the transformation of α-lactalbumin into the apoptosis-inducing complex BAMLET. This indicates that α-lactalbumin from highly processed dairy products, such as infant formula, may still retain its potential to form this cytotoxic complex.

ABBREVIATIONS USED

HAMLET, human α-lactalbumin made lethal to tumor cells; BAMLET, bovine α-lactalbumin made lethal to tumor cells; Cys, cysteine; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DEAE, diethylaminoethyl; FPLC, fast protein liquid chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; GP-HPLC, gel permeation high-performance liquid chromatography; DD, degree of denaturation; Tris, tris(hydroxymethyl)aminomethane; FBS, fetal bovine serum; RPMI, Roswell Park Memorial Institute; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

ACKNOWLEDGMENT

We thank Padraig McStay for his assistance in measuring protein denaturation curves.

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Received for review August 18, 2009. Revised manuscript received January 13, 2010. Accepted February 22, 2010. The project was supported by Irish Dairy Levy Seed Funding.