α-LACTALBUMIN SPECIES VARIATION, HAMLET FORMATION AND TUMOR CELL DEATH

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HAMLET (human α-lactalbumin made lethal to tumor cells) is a tumoricidal complex of apo α-lactalbumin and oleic acid, formed in casein after low pH treatment of human milk. This study examined if HAMLET-like complexes are present in casein from different species and if isolated α-lactalbumin from those species can form such complexes with oleic acid. Casein from human, bovine, equine and porcine milk were separated by ion exchange chromatography and active complexes were only found in human casein. This was not explained by α-lactalbumin sequence variation, as purified bovine, equine, porcine and caprine α-lactalbumins formed complexes with oleic acid with biological activity similar to HAMLET. We conclude that structural variation of α-lactalbumins does not preclude the formation of HAMLET-like complexes and that natural HAMLET formation in casein was unique to human milk, which also showed the highest oleic acid content.

Keywords: α-lactalbumin, oleic acid, HAMLET, tumor cell death.

[1]Abbrevations used are: kDa, kilo Dalton; HAMLET, human α-lactalbumin made lethal to tumor cells; BAMLET, bovine α-lactalbumin made lethal to tumor cells; K_a, association constant; CD, circular dichroism.

Introduction

HAMLET[1] (human α-lactalbumin made lethal to tumor cells) is a complex of apo α-lactalbumin and oleic acid (C18:1) [1] which kills tumor cells while healthy cells are spared. The lethal effect on tumor cells was discovered in a casein fraction, obtained after low pH precipitation of human milk [2]. To identify the active component, the casein fraction was fractionated by ion exchange chromatography. The active component showed higher affinity than the other casein proteins for the ion exchange matrix, and eluted only after 0.8M NaCl. Surprisingly, the major component of this fraction was identified as α-lactalbumin, the major whey protein, which had not been recognized as a component of the casein fraction. The fraction was named MAL (multimeric α-lactalbumin), due to the oligomeric nature on SDS-PAGE [2, 3]. Subsequently, MAL was shown to also contain oleic acid, and the conditions required for complex formation were defined by deliberate conversion of native α-lactalbumin to HAMLET in the presence of oleic acid [1]. HAMLET was formed after partial unfolding of α-lactalbumin by EDTA-treatment and ion exchange chromatography on an oleic acid conditioned matrix. Complex formation was specific for oleic acid suggesting that a new fatty acid binding domain becomes exposed in partially unfolded α-lactalbumin [4].

α-Lactalbumin is the major protein in human milk whey and is secreted by the mammary gland epithelium. The native protein is one of two components in the lactose synthase complex which catalyses the final step in lactose biosynthesis in the lactating mammary gland [5]. α-Lactalbumin has a molecular size of 14 kDa and consists of 122-

123 amino acids, divided into two domains [6]. The large α-helical domain contains three major α-helices (amino acids 5-11, 23-34, 86-98) and two short 3_{10}-helices (amino acids 18-20, 115-118). The small β-domain consists of a triple-stranded anti-parallel β-sheet (amino acids 40-50) and a short 3_{10}-helix (amino acids 76-82). The two domains are connected by a calcium-binding loop and the native conformation is stabilized by four disulfide bonds (amino acids 6-120, 61-77, 73-91, 28-111). The release of Ca^{2+}, at low pH or by chelators such as EDTA, forces the molecule to a partially unfolded state, which maintains a native-like secondary structure but lacks well-defined tertiary packing [7-9]. Due to its stability in these partially unfolded states, α-lactalbumin has been extensively used to study protein folding [10, 11]. A native-like molten globule state is present at high salt, whereas other molten globules probably exist as a population of structurally less defined states. At physiological solvent conditions and in the presence of Ca^{2+}, apo α-lactalbumin reverts to the native state.

α-Lactalbumins are structurally conserved among species suggesting that HAMLET-like complexes might be formed from other species variants of α-lactalbumin. In a previous study, bovine α-lactalbumin was shown to bind oleic acid and form BAMLET (Bovine alpha-lactalbumin made lethal to tumor cells), which has anti-tumor activity [12]. There is, however, considerable inter-species variation in the fatty acid content of milk, which might influence the formation of active complexes in casein. This study examined if HAMLET-like complexes are formed in casein from different species, and if purified α-lactalbumin species variants can form HAMLET-like complexes with oleic acid.

Materials and methods

Purification of casein by low pH precipitation. The protocol used for casein precipitation was developed for bovine milk by Melander [13]. The milk was defatted by centrifugation at 4,200 ×*g* for 15 min (Sorvall RC5C Plus, Du Pont Instruments, Wilmington, DE) and filtration through glass wool. Fat free milk was precipitated with 10% potassium oxalate (27 ml/l fat free milk) and incubated at 4°C over night. After centrifugation (4,200 ×*g*, 15 min), the supernatant was collected and diluted twice in water. The pH was set to 4.6 with hydrochloric acid and the sample was incubated at 30°C for 2 hours followed by overnight incubation at 4°C. The pellet (4,200 ×*g*, 15 min) was washed and dissolved in water prior to lyophilisation. The casein yield obtained for bovine and porcine milk was 13 and 28 g/l fat free milk, respectively. As a control, casein was precipitated from bovine milk at pH 4.3.

Separation of casein by SDS-PAGE electrophoresis. The casein proteins were separated by SDS-PAGE electrophoresis (NuPAGE Gel System, Invitrogen, Carlsbad, CA). Casein samples (10 mg/ml) were sonicated (2 min using Branson 2200 Ultrasonic cleaner, Branson Ultrasonics Corporation, Danbury, CT) and mixed with sample buffer before application to the gel (NuPAGE 4-12% Bis-Tris Gel). The gel was run at 200 V for 35 min in MES buffer and stained with Coomassie blue. α-Lactalbumin was used as standard (0.6, 0.4 and 0.2 mg/ml).

Fractionation and conversion of casein by ion exchange chromatography. Ten milligrams of casein were dissolved in 20 ml of 10 mM Tris/HCl pH 8.5 with or without 25 mM EDTA and sonicated for 2 min. A column (14 cm x 1.6 cm) packed with 10 ml of DEAE-Trisacryl M (BioSepra, Ville-neuf, France) was attached to a Bio-Logic chromatography system (Bio-Rad Laboratories, Hercules, CA). Sample was applied and eluted with NaCl: 40 ml linear gradient (100-85 % A, 0-15% B), 20 ml A/B (85/15%), 10 ml A/B (20/80%) and 20 ml A (100%), (buffer A: 10 mM Tris/HCl pH 8.5, 0.1 M NaCl; buffer B: 10 mM Tris/HCl pH 8.5, 1 M NaCl). Fractions eluted with high salt were desalted by dialysis against distilled water with at least four changes of water in 100-fold volume excess, (Spectra/Pore, Spectrum Medical Industries, Laguna Hills, CA, membrane cut off 3.5 kDa) and lyophilised.

Clean DEAE-Trisacryl M matrix was conditioned with oleic acid (C18:1:9cis) (Larodan, Malmö, Sweden). Ten microliters were dissolved in 100 µl of 99.5% ethanol and 10 ml of buffer A was added during mixing. The lipid solution was applied to then column and dispersed through out the matrix using a NaCl gradient as described above.

Ten mg casein was dissolved in 20 ml of buffer A, sonicated for 2 min and applied to

individual oleic acid conditioned columns. After elution with high salt, the fractions were desalted by dialysis against distilled water and lyophilised.

Apoptosis assay. A murine leukemia cell line (L1210 cells, ATCC, CCL 219) was cultured in suspension in RPMI1640 medium supplemented with 5% foetal calf serum, 2 mM glutamine, 1:100 of nonessential amino acids, 1 mM sodium pyruvate, 50 μg/ml of gentamicin and 50 μM 2-mercaptoethanol as described [2]. The cells were harvested by centrifugation (400 ×*g*, 10 min, Multifuge 1 L-R, Heraeus, Germany), washed in phosphate-buffered saline (PBS), re-suspended in cell culture medium without foetal calf serum, seeded into 24 well plates (Falcon, Becton Dickinson, New Jersey) at a density of $2x10^6$ cells/well and incubated at 37°C in 5% CO_2 atmosphere. The lyophilised material was dissolved in cell culture medium without foetal calf serum at 10 mg/ml and added to wells at a final concentration of 0.2 or 0.5 mg/ml. After 1 hour, 50 μl of foetal calf serum was added to each well at a final concentration of 5%. Cell viability was determined after 5 hours as Trypan blue exclusion by interference contrast microscopy (Ortolux II, Leitz Wetzlar, Germany).
Oligonucleosome length DNA fragments in the same samples were detected by agarose gel electrophoresis. The remainder of the cell suspension (990 μl, $2x10^6$ cells/ml) was lysed in 5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0 at 4°C over night and centrifuged at 16,000 ×*g* for 15 minutes. DNA was ethanol precipitated over night at -20°C, treated with proteinase K (25 μg/ml) and RNAse (50 μg/ml), loaded on 1.8% agarose gels and subjected to electrophoresis under constant voltage set at 130 V for 1 h. DNA fragments were visualized with ethidium bromide using a 305 nm UV-light source and recorded using Geldoc equipment (Bio-Rad Laboratories, Hercules, CA).

Purification of α-lactalbumin. Native α-lactalbumin was purified from frozen human, bovine, caprine, porcine and equine milk by ammonium sulphate precipitation and phenyl sepharose chromatography [14]. Ammonium sulphate was added at 264 g/l milk, the mixture was incubated over night at 4 °C and centrifuged at 4,200 ×*g* for 15 minutes. The supernatant was complemented with 50 mM Tris/HCl and 35 mM EDTA, pH 7.5. The sample was loaded onto a phenyl-sepharose column (Pharmacia Biotech, Uppsala, Sweden) packed in 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, washed with 50 mM Tris/HCl, pH 7.5, 1 mM EDTA and eluted with 50 mM Tris/HCl, pH 7.5, 1 mM $CaCl_2$. The eluate containing native α-lactalbumin was dialyzed against distilled water.

Formation of HAMLET-like complexes on oleic acid conditioned matrices. A column packed with DEAE-Trisacryl M was attached to a chromatography system and conditioned with oleic acid as described above. Apo α-lactalbumin was prepared by addition of EDTA (20 x mole eqvivalent) dissolved in 20 ml of 10 mM Tris/HCl pH 8.5 and applied to oleic acid conditioned columns and eluted as described above.

Near UV CD spectroscopy on α-lactalbumin variants. Lyophilised material was dissolved in 5 mM Tris, pH 7.0 at 1 mg/ml and concentrations were determined as the absorbance at 280 nm. CD spectra were obtained on a JASCO J-720 spectro-polarimeter with a JASCO PTC-343 Peltier type thermostated cell holder using quartz cuvettes with 1-cm path length. Spectra were recorded between 240 and 320 nm with a wavelength step of 1 nm, a response time at 8 sec and scan rate at 10 nm/min. An average of six scans is presented where the mean residue ellipticity, θ_m in $deg \cdot cm^2 \cdot dmol^{-1}$, was calculated using the following equation:

$$\theta_m = \theta / (c \cdot n \cdot l)$$

where θ is the recorded ellipticity in degrees; c, the protein concentration in $dmol/cm^3$; n, number of residues in the protein (123 in this case) and l, cuvette length in cm.

Results

Casein fractions and tumor cell apoptosis. Newly secreted human milk does not trigger apoptosis, as α-lactalbumin is in its native conformation and oleic acid is bound to triglycerides. Active complexes are formed in human milk at low pH, however, and may be isolated from human milk casein by ion exchange chromatography using a salt gradient. Low pH treatment of bovine, equine and

porcine milk was used to collect the casein fractions and to investigate if active complexes were formed in casein. The milk was exposed to pH 4.6, as the pI of α-lactalbumin from different species varies between 4.9-5.2, with human α-lactalbumin having the lowest pI of 4.88. Casein precipitates were formed in all milk samples, harvested and washed with water. The yield of casein from bovine and porcine milk was 13 and 28 g per litre of fat free milk, respectively. The α-lactalbumin content of the casein fractions was examined by SDS PAGE and quantified in relation to an α-lactalbumin standard (Fig. 1A). Equine casein contained about 0.6 mg/ml, human casein about 0.2 mg/ml and porcine and bovine caseins <0.2 mg/ml of α-lactalbumin.

The biological activity of the precipitated casein fractions was examined in the lymphoma cell apoptosis assay (Fig. 1B and C). All caseins were tested at 0.5 mg/ml, with cell death and DNA fragmentation as end points. Human casein decreased tumor cell viability to 31 % after 5 hours (n= 6, SD=19.6) but the remaining casein fractions were inactive (93%$\pm$1.3) resulting in a significant difference ($p<0.001$, one-way ANOVA). The human casein fraction also induced DNA fragmentation with oligo nucleosome bands starting at 200 bp (Fig. 1C). The other casein fractions did not trigger DNA fragmentation.

The difference in biological activity between the casein fractions was supported by a difference in yield of HAMLET-like complexes (Fig. 1D). Each casein fraction was subjected to ion exchange chromatography on a clean matrix, according to the protocol used to isolate HAMLET from human milk casein (Fig. 1E). Human casein served as a positive control. Most of the added protein eluted in the void, but 16% showed high affinity for the ion matrix and eluted after high salt (0.8M NaCl). The other casein fractions contained less material with high affinity for the matrix and $\leq$5% of added casein eluted after high salt. The yields were too low to test the biological activity of those complexes. The peaks eluting at lower salt concentrations were also tested in the apoptosis assay, but no apoptotic activity was found. The results suggested that the formation of a functionally active, HAMLET-like complex at low pH is a characteristic of human milk.

α-Lactalbumin sequence variants. Subsequent experiments examined if differences in α-lactalbumin structure might determine active complexes formation. The sequences of human, bovine, equine, caprine and porcine α-lactalbumins were compared (Fig. 2, Table 1) and sequence alignment showed about 71% sequence homology and 63% sequence identity between them. The high affinity Ca^{2+}-binding site is 100% conserved (amino acids 79, 82, 84, 87-88), probably reflecting the structural importance of this motif. The sequence homology is most extensive at the N-terminal, at positions 48-58, 75-88 and 91-97 and at the carboxy-terminal end at positions 103-121. Variable domains are located in the interface between the α-helical and the β-sheet domains (amino acids 39-47), in the loop of the β-domain (amino acids 66-68) and at the top of the C helix at position 98 and 99. Human α-lactalbumin shows 85% sequence homology with the bovine, equine, porcine and caprine proteins, respectively. The caprine and bovine proteins are the most closely related, with 95% sequence homology with amino acid differences in the α-helical region, at position 10, 11, 17, 30, 70 and 90. The porcine protein lacks one amino acid at position 68 and has seven unique amino acid residues throughout the sequence (amino acids 20, 33, 39, 44, 67, 89 and 123).

Conversion of the α-lactalbumin species variants to HAMLET-like complexes. The ability to form HAMLET-like complexes was compared between the α-lactalbumin species variants (Fig. 3). Human, bovine, equine, caprine and porcine α-lactalbumins were unfolded by EDTA-treatment and the apo proteins were subjected to ion exchange chromatography on oleic acid conditioned column matrices. Complexes were eluted with a salt gradient and their structure and biological activity was examined after dialysis and lyophilisation. The conversion of human α-lactalbumin to HAMLET was used as a control and about 60 % of applied protein eluted as a sharp peak after high salt. Similar elution profiles were obtained for α-lactalbumins from the other species but with lower conversion yields (between 46% and 24%, table in Figure 3). The results show that all the α-lactalbumin species variants can bind oleic acid and form

HAMLET-like complexes but convert less efficiently than human α-lactalbumin.

Near UV CD spectroscopy on converted complexes. The tertiary structure of the α-lactalbumin species variants and the HAMLET-like complexes was examined by near UV CD spectroscopy (Fig. 4A and B). The human α-lactalbumin and HAMLET spectra were used as controls and showed the expected maximum at 293 nm and minimum at 270 nm, reflecting tyrosine and tryptophan signals. HAMLET showed the expected loss of signal compared to native human α-lactalbumin, confirming the partially unfolded state of the protein in the complex (Fig. 4B). The other holo proteins showed a lower signal at 293 nm. At 270 nm, the bovine and caprine proteins showed the lowest minimum, while the equine and porcine spectra gave a weaker signal than human α-lactalbumin. The spectra of equine and porcine α-lactalbumin resembled the other species variants, as previously reported [8, 12, 15-19]. The HAMLET-like complexes showed a loss of signal compared to each holo protein. The spectrum of porcine complex resembled that of HAMLET most closely, while the converted equine complex had the least defined spectra indicating a poorly defined tertiary structure.

Apoptosis induction by HAMLET and HAMLET-like complexes from other species. The cell death response to the HAMLET-like species variants was examined (Fig. 4C) with each holo protein as a control (Fig. 4D). HAMLET-like complexes from all species caused a reduction in cell viability from about 90% (range: 82-94) in the control to about 35% (range: 7-62) at 0.2 mg/ml (Fig. 4C). The difference between control and the HAMLET variants was significant ($p<0.001$, one-way ANOVA) but no significant differences between HAMLET and the HAMLET-like complexes was obtained. In addition, all complexes caused detectable DNA fragmentation (data not shown). The native α-lactalbumins had no effect on cell viability or chromatin structure (Fig. 4D). The results show that purified α-lactalbumin species variants can form HAMLET-like complexes with anti-tumor activity after partial unfolding and coupling to oleic acid.

Fatty acids content of milk from different species. Triglycerides comprise about 98 per cent of the total milk lipid. Only traces of free fatty acids are found in fresh milk [20], but lipases are activated at low pH and may hydrolyse the triglycerides and release fatty acids. Human milk casein thus contains high amounts of free fatty acids, including oleic acid, which is important for HAMLET formation. The fatty acid content of milk was therefore examined. The reported fatty acid composition of milk from different species is summarized in Table 2. Human and porcine milk contain a high percentage of unsaturated cis fatty acids, including oleic acid but short and saturated fatty acids are less abundant. In contrast, bovine, equine and caprine milk contain mainly short saturated fatty acids, and the oleic acid content is lower than in human and porcine milk. The total lipid content in bovine and human milk is similar, around 3-5% [20]. This analysis suggested that the availability of oleic acid might influence the formation of HAMLET-like complexes in caseins from different species

Can HAMLET-like complexes be formed from casein if oleic acid is provided? To examine if oleic acid is the critical factor for complex formation, different casein fractions were subjected to ion exchange chromatography on oleic acid conditioned matrices (Fig. 5A). All of the casein variants formed complexes with oleic acid, which eluted as sharp peaks after high salt. The porcine and bovine caseins gave the highest yield (50 and 40 % of the applied casein, respectively). Human casein gave a low conversion yield (27 %) and the lowest yield was obtained with equine casein (25 %). The results show that α-lactalbumin in the different casein fractions was unfolded at low pH and free to bind oleic acid. This suggests α-lactalbumin is not occupied by other cofactors than oleic acid.

The cellular response to the converted casein complexes was compared in the L1210 apoptosis assay (Fig. 5B). All complexes showed high activity with virtually complete loss of cell viability at 0.5 mg/ml, significantly different from the control ($p<0.001$, one-way ANOVA). No significant difference in activity was seen between the complexes. In addition, all complexes induced DNA fragmentation (data not shown). The results show that

complex formation with oleic acid is the critical event for activity.

Discussion

α-Lactalbumin is a structurally conserved milk protein which functions as a co-enzyme in lactose synthesis [5] and as a Ca^{2+} carrier in milk [21]. In the absence of α-lactalbumin, the milk cannot be secreted, due to high viscosity, and the deletion of the *ala* gene in mice is not compatible with survival of the offspring [22]. In addition, α-lactalbumins form stable folding intermediates when Ca^{2+} is released [23]. We have shown that the partially unfolded form of the human protein attains a new function when bound to oleic acid [1]. HAMLET kills tumor cells *in vitro* and the anti-tumor activity is retained *in vivo,* in a brain tumor model and in patients with skin papillomas [24, 25]. This study examined if α-lactalbumins from different species can form HAMLET-like with similar biological activity as HAMLET. In addition, the natural complex formation in milk from the different species was investigated. The results suggest that HAMLET formation is unique to casein from human milk despite the sequence similarity between α-lactalbumin sequence variants.

The casein fractions were shown to differ considerably in α-lactalbumin content and in the ability to form active HAMLET-like complexes. The difference was examined as a function of the protein and the oleic acid content. The α-lactalbumin content was not directly correlated with the yield of active complex, however. For example, human casein contained intermediate amounts of α-lactalbumin compared to the other species, but had the highest yield. Complex formation was also not determined by the amount of oleic acid in the different caseins. For example, porcine milk contained high amounts of oleic acid, but did not form active complexes. All of the α-lactalbumins in the casein fraction could form a complex with oleic acid on a preconditioned matrix, however, showing that they were unfolded and available for binding. It thus remains unclear why the active complex is formed uniquely in human milk.

The difference in conversion yield and biological activity suggested that sequence variation might influence the unfolding and fatty acid binding of α-lactalbumins. The unfolding is influenced by the readiness of the protein to release Ca^{2+} but this step is not likely to vary as the sequence in the Ca^{2+}-binding site is conserved and the K_a of bovine, caprine and human α-lactalbumins are similar ($2.5 \cdot 10^8$, $2.8 \cdot 10^8$ and $3 \cdot 10^8$ M^{-1}) [26]. Furthermore, near UV CD spectroscopy suggested that the proteins undergo a similar conformational change after the release of Ca^{2+}. Therefore, the yield may reflect amino acid differences in the domains involved in fatty acid binding and resulting in changes in affinity of individual α-lactalbumins for the fatty acid cofactor. Native human α-lactalbumin contains two hydrophobic clusters. The one in the α-helical domain is formed by helices A, B and C (residues Phe3, Leu8, Leu12, Asp16, Gly17, Ile21, Ala22, Leu23, Leu26, Ile27 and Ile85). The cluster is capped by the basic residues Lys1 and Lys93, which theoretically might coordinate a polar fatty acid head group. The other cluster is located in the interface between the α-helical and the β-sheet domains. The crystal and NMR structures of bovine apo α-lactalbumin have revealed a significant change in the cleft between the two domains, compared to the native conformation [11, 27]. The α-domain, in contrast, remains structured in both the native and the apo state, with near native side chain packing [8, 28]. We have proposed that oleic acid binds in the interface between the α- and β-domains as this hydrophobic pocket only becomes exposed after partially unfolding of the protein [4].

The sequence variation in this region of α-lactalbumin was examined, as the fit of oleic acid to this domain might vary extensively between species. Bovine α-lactalbumin carries three amino acid substitutions in the proposed fatty acid binding site compared to the human sequence. R70S, I98K and K99V are located adjacent to this area and the charge difference that they create might influence fatty acid binding, either through the conformation or through a repulsion of the fatty acid head group. Equine α-lactalbumin lacks one hydrophobic and one basic residue is this region (I98S and K99E), which might repel the negatively charged lipid head group. Porcine α-lactalbumin lacks one amino acid at position 68, which might influence the relative packing of the side chains. Like the equine protein, porcine α-lactalbumin had a polar amino acid at position 98 and an acidic residue at position 99 while the caprine protein had a non-polar

residue at position 90. The opposite is seen the porcine sequence at position 89. This sequence variation was exclusive for the caprine and porcine proteins compared to the human sequence. The fatty acid binding efficiency may be determined either by the conformation of this domain or by amino acid residues that interact directly with the fatty acid.

Oleic acid supports the formation of HAMLET in human milk. Saturated fatty acids and unsaturated fatty acids in trans conformation could not form complex with α-lactalbumin. Cis fatty acids other than oleic acid could, however, form HAMLET-like complexes with oleic acid but with lower biological activity [4]. As the fatty acid composition in milk differs between species, the availability might be a limiting factor in bovine, equine and caprine milk. For example, human milk contains mainly long unsaturated fatty acids while bovine, equine and caprine milk contain less oleic acid, [20, 29, 30] than porcine and human milk [20, 30-33]. The spontaneous formation of HAMLET-like complexes at low pH did not directly reflect the oleic acid content, however. Despite the high percentage of oleic acid in porcine milk, low amount of complex were formed at low pH but conversion of casein on the oleic acid matrix gave a complex with high activity. This may indicate that the fatty acids are bound to triglycerides and not available for complex formation. Furthermore, the amino acid sequence and fold of porcine α-lactalbumin may not be optimal, due to the amino acid deletion at position 68 and the lack of basic amino acids at positions 98 and 99. This is consistent with the low yield after conversion of purified porcine α-lactalbumin. The results of the porcine milk casein and α-lactalbumin illustrate that HAMLET formation depends both on the protein structure and oleic acid accessibility. Human milk contained the highest amounts of oleic acid among the species tested and human α-lactalbumin bound oleic acid more efficiently than the species variants.

Fatty acids are released from milk triglycerides by gastric lipase in the stomach or lipases in milk [34, 35]. This includes a variety of fatty acids but oleic acid is the most abundant one. Conceivably, milk might contain species-specific cofactors that replace oleic acid and fit the α-lactalbumin structure of each species. To identify such alternative milk cofactors, casein was precipitated from horse, cow and pig and the casein fractions were applied to tumor cells. No activity was detected except in human casein. To further exclude that the proteins might be occupied by naturally occurring cofactors, which prevent them from binding oleic acid, the casein fractions were subjected to ion exchange chromatography on oleic acid conditioned matrices. In this case, conversion was achieved, showing that the proteins can form complexes if exposed to the proper fatty acid. The results suggested that the other tested species lack the ability to form natural biologically active complexes due mainly to the lack of cofactors in milk.

HAMLET is not present in newly secreted human milk, as α-lactalbumin is in the native conformation and oleic acid is bound in milk triglycerides. The prerequisites for HAMLET-formation *in vivo* are present in the stomach of the breastfed child, however, as Ca^{2+} is released at low pH and the protein unfolds. Furthermore, lipases hydrolyse triglycerides, and oleic acid becomes available to form HAMLET. HAMLET is more resistant to proteolytic degradation compared to the unfolded protein without the fatty acid [36], suggesting that the complex may be stable in the gastro-intestinal tract. We have proposed that this may be an essential mechanism to control rapidly growing cells in the intestinal mucosa and to purge tumor cell precursors, but direct evidence in support of this theory is lacking, so far. The lack of apoptosis-inducing activity in the bovine, equine or porcine caseins is surprising, considering the potential importance of this tumor surveillance mechanism. On the other hand, population biology has identified cancer as a fairly neutral cause of death for the survival of the species**.** With increased longevity, cancer is becoming increasingly prevalent in humans but cancer is not a common cause of death in the reproductive age groups. The evolutionary pressure to evolve lipid cofactors for the formation of HAMLET-like complexes may not be as strong in species with a shorter life span or a lower cancer-related mortality.

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Figure legends

Figure 1. Apoptosis assay and ion exchange chromatography on casein. (A) Quantification of α-lactalbumin in caseins from different species. Equine, human, bovine and porcine casein fractions (10 mg/ml) were separated by SDS-PAGE electrophoresis and stained with Coomassie blue. α-Lactalbumin (0.6, 0.4 and 0.2 mg/ml) was used as standard and ladder is shown in kDa. The L1210 mouse lymphoma cell line was exposed to the different casein fractions (0.5 mg/ml) for 5h. Cell viability (B) and DNA fragmentation (C) were used as end points. Apoptosis activity was only seen in human casein (*** = $p<0.001$, one-way ANOVA). Cell viability (%) is given as average with standard deviation as error bars and number of experiments is shown. Cell viability is shown below gel and ladder is given in base pairs. (D, E) Casein from the different species was applied on unconditioned ion exchange matrices. Conversion yield was determined as the area under the curve for fraction 1 (0-110 min) and 2 (110-140 min). An arrow marks elution of active complex.

Figure 2. Sequence variation in α-lactalbumins from different species. Letters indicate individual amino acids with basic amino acids shown in red, acidic in green, polar in pink and aromatic and non-polar amino acids in dark and light blue. The white box at position 68 indicates the deletion in the porcine sequence. Protein sequence alignments were carried out using ClustalW (http://www.ebi.ac.uk/clustalw/). Pubmed accession numbers are NP_002280 (human), NP_999525 (porcine), LAHO (equine), P00712 (caprine) and NP_776803 (bovine).

Figure 3. Conversion of α-lactalbumin species variants on oleic acid conditioned matrices. Equine, bovine, caprine and porcine α-lactalbumin were treated with EDTA, subjected to ion exchange chromatography on an oleic acid conditioned column and complexes eluted with a NaCl gradient. Human α-lactalbumin was used as control, and HAMLET eluted as a sharp peak at a conductivity of about 60 mS/cm. Table shows conversion yield, determined as the area under the curve from 0-110 min (fraction 1) and 110-140 min (fraction 2). In addition, α-lactalbumin which had not bound to the column was harvested from the void volume and reapplied to the same oleic acid conditioned column. In each case, the protein readily formed a complex with a similar yield as in the first run (data not shown), suggesting that the low yield was not due to structural α-lactalbumin heterogeneity. Arrows mark elution of HAMLET and HAMLET-like complexes.

Figure 4. Near UV CD spectra and apoptosis assay on α-lactalbumins, HAMLET and HAMLET-like complexes. Near UV CD spectra were recorded in 5 mM Tris, pH 7.0 on (A) human, equine, bovine, caprine and porcine α-lactalbumin and (B) HAMLET and HAMLET-like complexes. The spectra of human, bovine and caprine α-lactalbumins have previously been described [8, 12, 15-19] but spectra of equine and porcine α-lactalbumin have not previously been reported. The L1210 mouse lymphoma cell line was exposed to (C) HAMLET and HAMLET-like complexes (0.2 mg/ml) or to (D) the corresponding native α-lactalbumin (0.5 mg/ml) for 5h. The cells died rapidly when exposed to HAMLET and HAMLET-like complexes from bovine, equine, porcine and caprine α-lactalbumin showed similar effect ($p<0.001$, one-way ANOVA). The native proteins did not affect cell viability. Cell viability (%) is given as average with standard deviation as error bars and number of experiments is shown.

Figure 5. Ion exchange chromatography of casein fractions and apoptosis assay on converted casein complexes. (A) Casein from the different species was applied on oleic acid

conditioned ion exchange matrices. The conversion yield was determined as the area under the curve from 0-110 min (fraction 1) and 110-140 min (fraction 2). Arrows mark elution of HAMLET-like complexes. (B) The L1210 mouse lymphoma cell line was exposed to the converted complexes (0.5 mg/ml) for 5h. All of the converted casein complexes were active ($p<0.001$, one-way ANOVA). Cell viability (%) is given as average with standard deviation as error bars and number of experiments is shown.

Figure 1

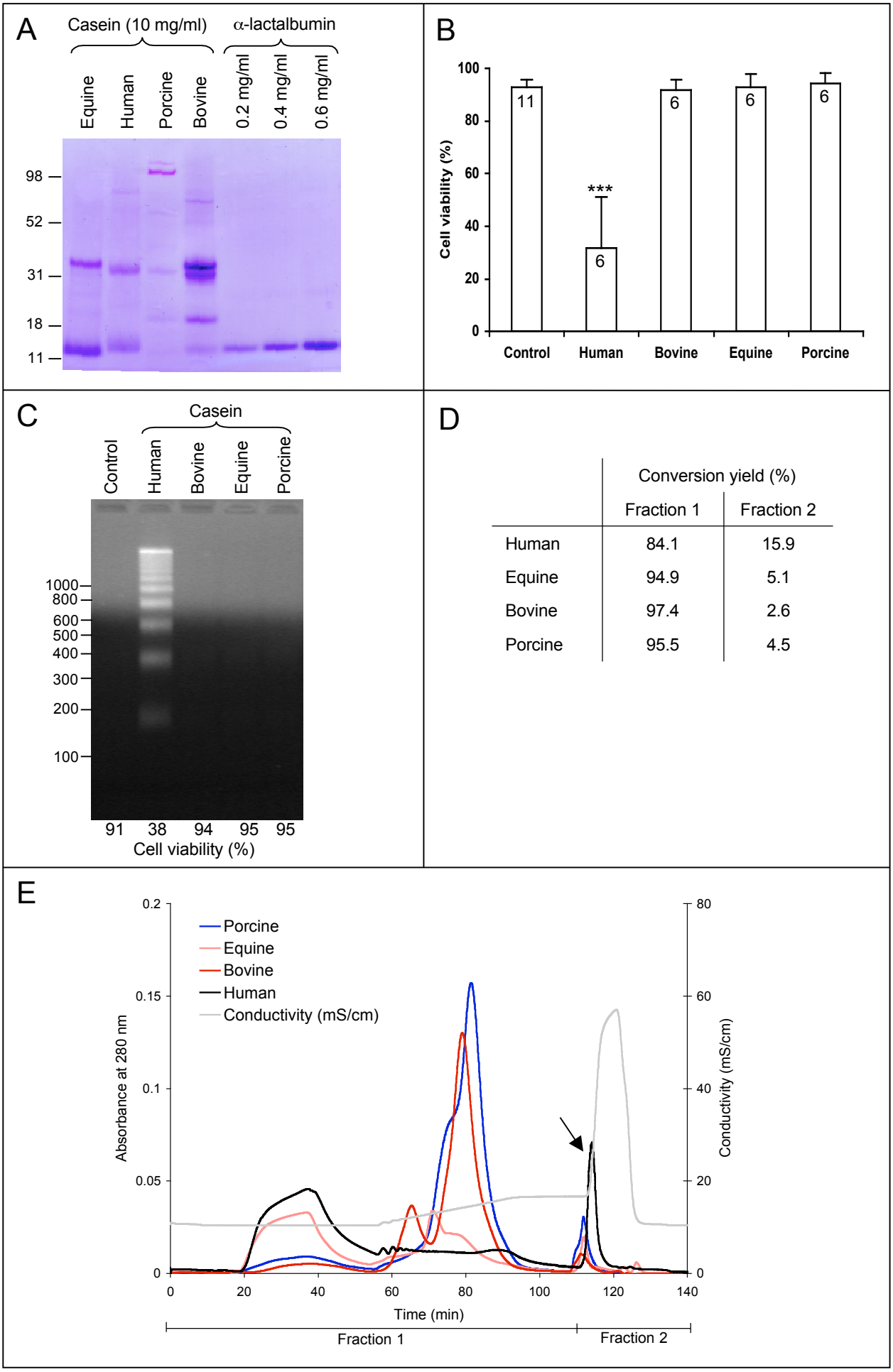

A
Casein (10 mg/ml)
α-lactalbumin
Equine
Human
Porcine
Bovine
0.2 mg/ml
0.4 mg/ml
0.6 mg/ml
98
52
31
18
11
B
Cell viability (%)
100
80
60
40
20
0
11
***
6
6
6
6
Control
Human
Bovine
Equine
Porcine
C
Casein
Control
Human
Bovine
Equine
Porcine
1000
800
600
500
400
300
200
100
91
38
94
95
95
Cell viability (%)
D
Conversion yield (%)
Fraction 1
Fraction 2
Human 84.1 15.9
Equine 94.9 5.1
Bovine 97.4 2.6
Porcine 95.5 4.5
E
Porcine
Equine
Bovine
Human
Conductivity (mS/cm)
Absorbance at 280 nm
0.2
0.15
0.1
0.05
0
80
60
40
20
0
Conductivity (mS/cm)
0
20
40
60
80
100
120
140
Time (min)
Fraction 1
Fraction 2

Figure 2

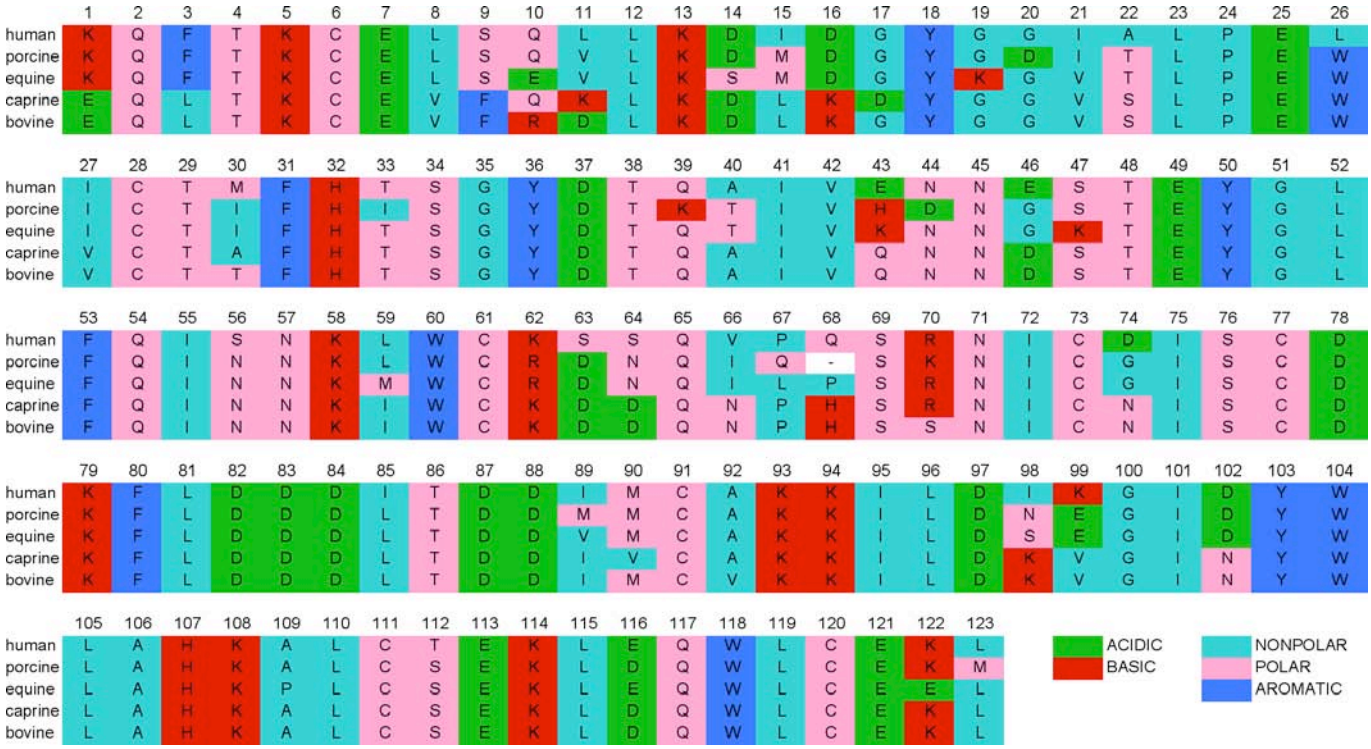
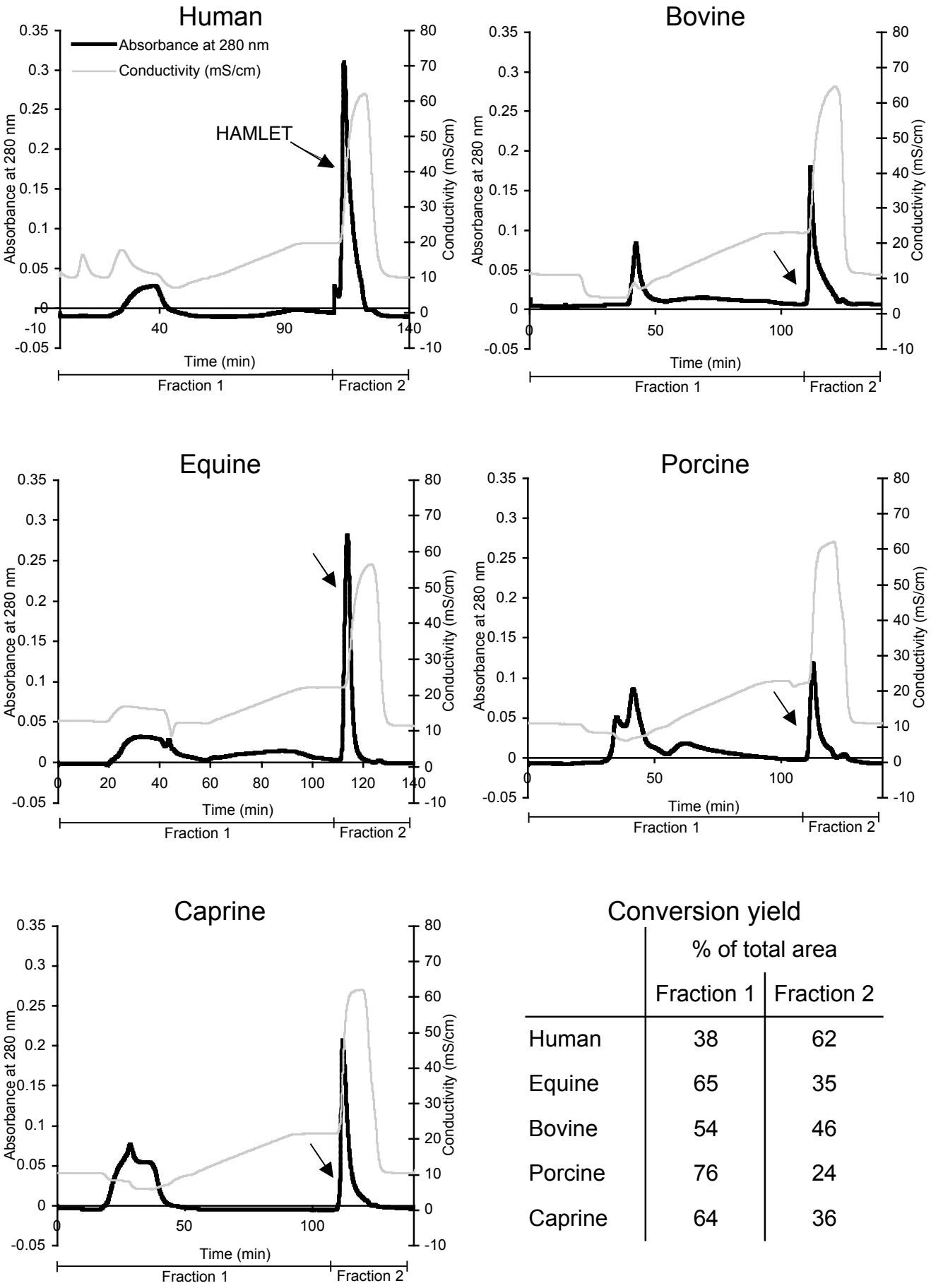

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
human K Q F T K C E L S Q L L K D I D G Y G G I A L P E L
porcine K Q F T K C E L S Q V L K D M D G Y G D I T L P E W
equine K Q F T K C E L S E V L K S M D G Y K G V T L P E W
caprine E Q L T K C E V F Q K L K D L K D Y G G V S L P E W
bovine E Q L T K C E V F R D L K D L K G Y G G V S L P E W
27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52
human I C T M F H T S G Y D T Q A I V E N N E S T E Y G L
porcine I C T I F H I S G Y D T K T I V H D N G S T E Y G L
equine I C T I F H T S G Y D T Q T I V K N N G K T E Y G L
caprine V C T A F H T S G Y D T Q A I V Q N N D S T E Y G L
bovine V C T T F H T S G Y D T Q A I V Q N N D S T E Y G L
53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78
human F Q I S N K L W C K S S Q V P Q S R N I C D I S C D
porcine F Q I N N K L W C R D N Q I Q - S K N I C G I S C D
equine F Q I N N K M W C R D N Q I L P S R N I C G I S C D
caprine F Q I N N K I W C K D D Q N P H S R N I C N I S C D
bovine F Q I N N K I W C K D D Q N P H S S N I C N I S C D
79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104
human K F L D D D I T D D I M C A K K I L D I K G I D Y W
porcine K F L D D D L T D D M M C A K K I L D N E G I D Y W
equine K F L D D D L T D D V M C A K K I L D S E G I D Y W
caprine K F L D D D L T D D I V C A K K I L D K V G I N Y W
bovine K F L D D D L T D D I M C V K K I L D K V G I N Y W
105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123
human L A H K A L C T E K L E Q W L C E K L
porcine L A H K A L C S E K L D Q W L C E K M
equine L A H K P L C S E K L E Q W L C E E L
caprine L A H K A L C S E K L D Q W L C E K L
bovine L A H K A L C S E K L D Q W L C E K L
ACIDIC
BASIC
NONPOLAR
POLAR
AROMATIC

Figure 3



Conversion yield

	% of total area	
	Fraction 1	Fraction 2
Human	38	62
Equine	65	35
Bovine	54	46
Porcine	76	24
Caprine	64	36

Figure 4

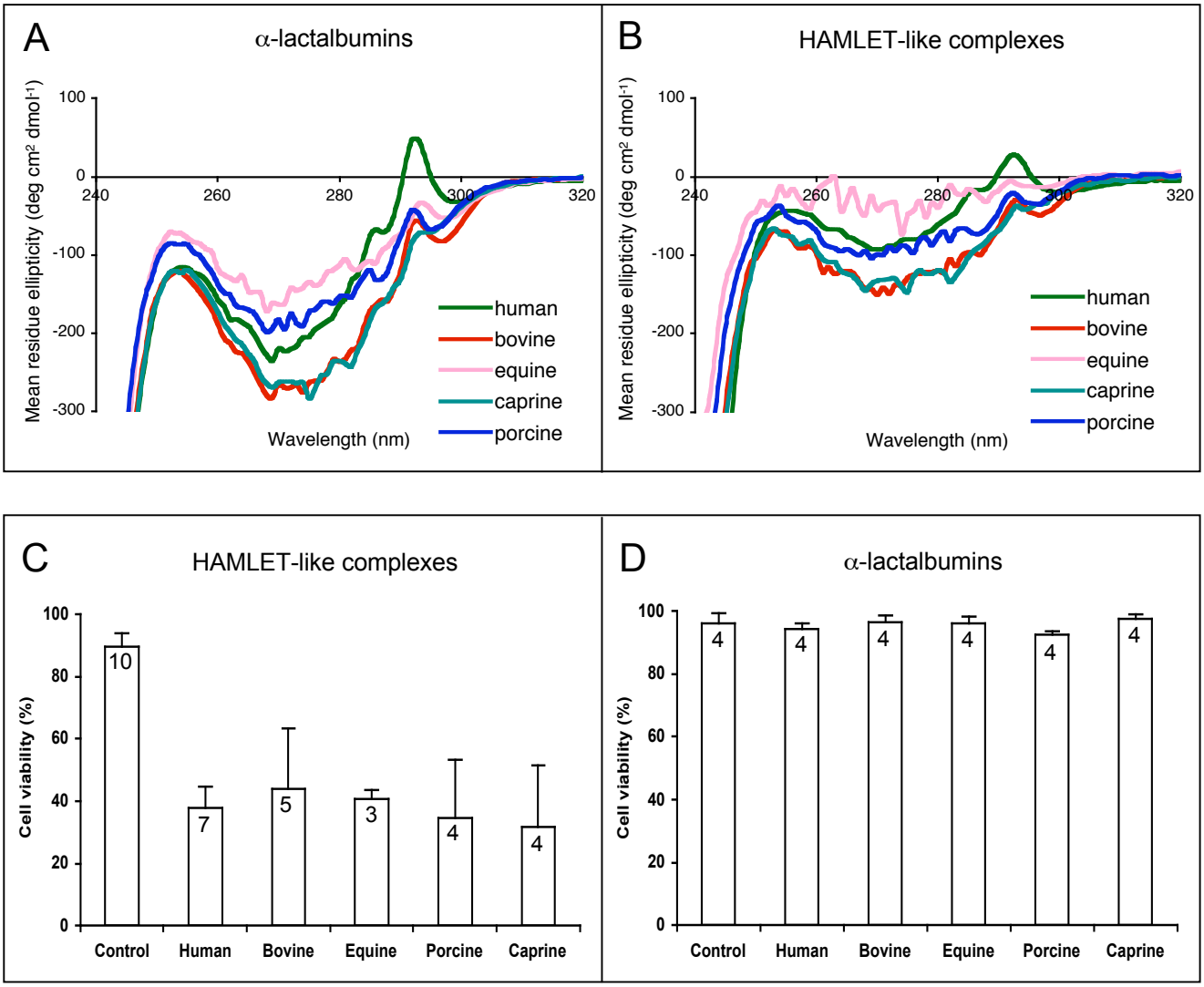
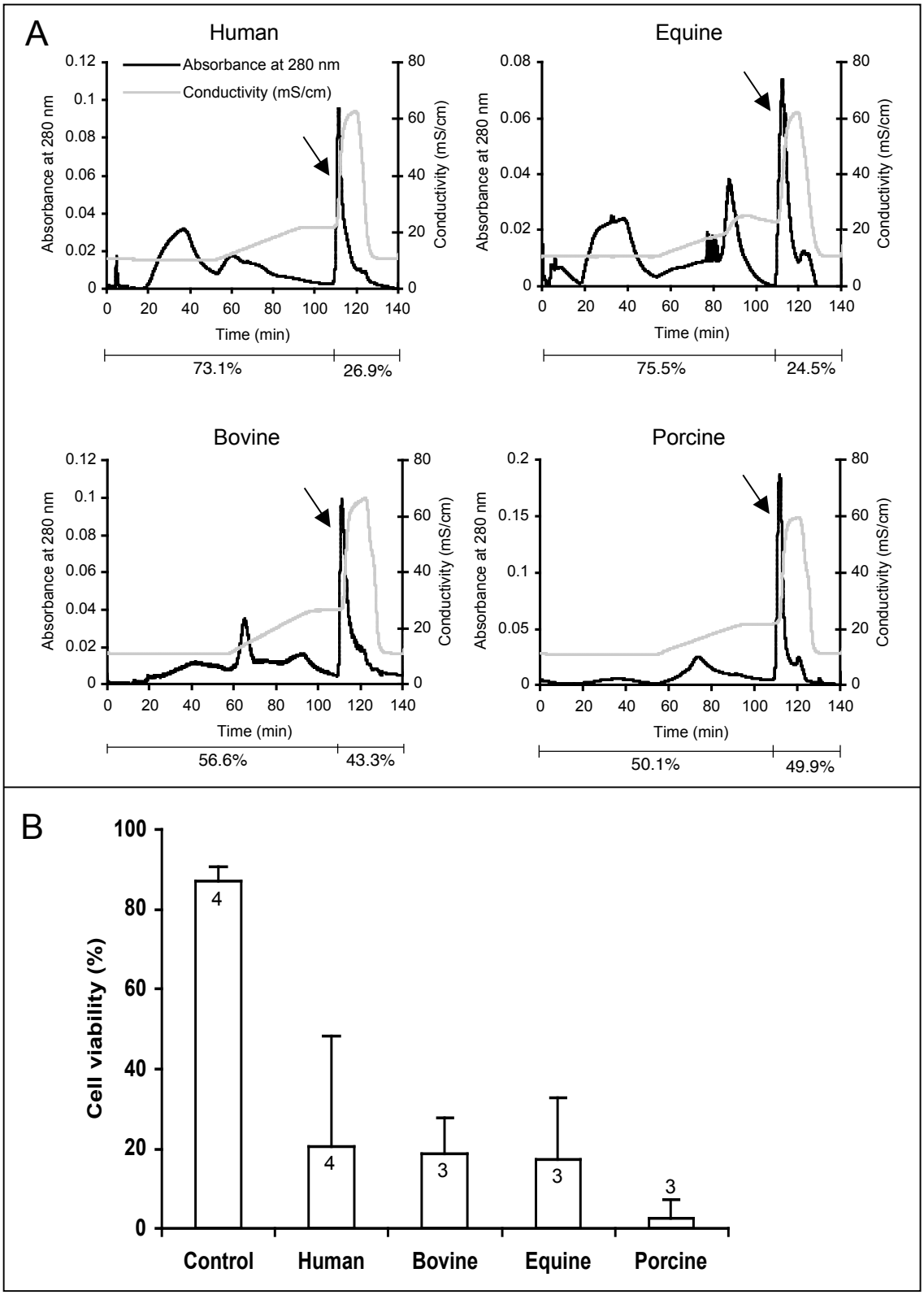

A
α-lactalbumins
Mean residue ellipticity (deg cm² dmol⁻¹)
100
0
-100
-200
-300
240
260
280
300
320
Wavelength (nm)
human
bovine
equine
caprine
porcine
B
HAMLET-like complexes
Mean residue ellipticity (deg cm² dmol⁻¹)
Wavelength (nm)
human
bovine
equine
caprine
porcine
C
HAMLET-like complexes
Cell viability (%)
100
80
60
40
20
0
10
7
5
3
4
4
Control
Human
Bovine
Equine
Porcine
Caprine
D
α-lactalbumins
Cell viability (%)
4
4
4
4
4
4
Control
Human
Bovine
Equine
Porcine
Caprine

Figure 5


A
Human
Absorbance at 280 nm
Conductivity (mS/cm)
Absorbance at 280 nm
Conductivity (mS/cm)
Time (min)
73.1%
26.9%
Equine
Absorbance at 280 nm
Conductivity (mS/cm)
Time (min)
75.5%
24.5%
Bovine
Absorbance at 280 nm
Conductivity (mS/cm)
Time (min)
56.6%
43.3%
Porcine
Absorbance at 280 nm
Conductivity (mS/cm)
Time (min)
50.1%
49.9%
B
Cell viability (%)
4
4
3
3
3
Control
Human
Bovine
Equine
Porcine