Title: PREPARATION OF COMPLEXED LACTALBUMIN

Abstract: The present invention relates to a method for the preparation of a biologically active alpha-lactalbumin complex comprising alpha-lactalbumin and a fatty acid or a lipid said method comprising the steps of: obtaining an alpha-lactalbumin composition; conversion of said alpha-lactalbumin to alpha-lactalbumin complex by mixing alpha-lactalbumin or a functional homologue thereof and a fatty acid or a lipid in the absence of an ion exchange medium, and subsequently exposing the mixture to an ion exchange medium. The active alpha-lactalbumin complex comprising alpha-lactalbumin and a fatty acid or a lipid are suitable for use in the manufacture of medicaments for use in therapy. Medicaments, comprising monomeric LAC are for use in the treatment of bladder cancer, papilloma and actinic keratosis.
Preparation of complexed lactalbumin

All patent and non-patent references cited in the application, or in the present application, are also hereby incorporated by reference in their entirety.

Field of invention

The present invention relates to a method for the preparation of a biologically active complex of alpha-lactalbumin and a fatty acid or a lipid. Particularly it relates to a method for the preparation of LAC, which is an active complex of alpha-lactalbumin and a fatty acid or lipid that is capable of inducing apoptosis in tumour cells and/or immature cells.

Background of invention

Alpha-lactalbumin (LA) is the major protein in human milk whey. Mature monomeric alpha-lactalbumin consists of 123 amino acid residues (14.2 kDa) in many mammalian species. Human, bovine, equine, caprine, and camelide alpha-lactalbumin all consist of 123 amino acid residues, whereas porcine alpha-lactalbumin consists of 122 amino acids. Human, bovine, caprine and porcine alpha-lactalbumin also comprise a 19 amino acid leader sequence. This 14 KDa protein has been extensively characterised and the crystal structure has been resolved.

The crystal structure of alpha-lactalbumin has revealed that the protein consists of four \(\alpha\)-helices and one triple stranded \(\beta\)-sheet, which is found at the C-terminal end of the protein. The major alpha-helical domain contains amino acid 5-11, 23-34, 86-98, and the short alpha-helical segments; amino acid 18-20, 115-118. The beta-domain contains the triple-stranded anti-parallel sheet that consists of amino acids 40-50 and the short 76-82 helix.

Alpha-lactalbumin is a metalloprotein and comprises a high affinity Ca\(^{2+}\) binding site as well as several zinc binding sites. The high affinity Ca\(^{2+}\) binding site spans amino acid residues 77-89. In particular, residues 79, 82, 84, 87 and 88 appear to be involved in Ca\(^{2+}\) binding (Permyakov et al., \(\alpha\)-Lactalbumin: structure and function. FEBS Letters 473 (2000) 269-274.). Alpha-lactalbumin binds other physiologically significant cations
such as Mg$^{2+}$, Mn$^{2+}$, Na$^+$ and K$^+$, which can compete with Ca$^{2+}$ for the high affinity binding site.

The native monomer is the regulatory subunit of the lactose synthase complex, and alters the acceptor specificity of β-galactosyltransferase from N-acetylglucosamine to glucose, with subsequent synthesis of lactose.

It has been shown that complexes of alpha-lactalbumin and a fatty acid or lipid may have cell killing abilities. A fraction from human milk containing an oligomeric complex was described as multimeric alpha-lactalbumin or MAL or HAMLET (human a-lactalbumin made lethal to tumour cells) and was shown to induce in vitro apoptosis selectively in tumour cells, but not in healthy differentiated cells. Danish patent application number PA 2006 01512 describes complexes of alpha-lactalbumin and fatty acid or lipid, which also have cell killing abilities.

Alpha-lactalbumin may undergo conformational switching and may adopt the so called apo state when exposed to low pH, or in the presence of chelators, that release the strongly bound Ca$^{2+}$ ion. The apo state or molten globule state has native secondary structure, but less well defined tertiary structure than the native state. Similar states of alpha-lactalbumin can also form at neutral pH, upon removal of the tightly bound Ca$^{2+}$ ion, reduction of disulphide bonds or at elevated temperatures (the apo-state).

The apoptotic activity of this folding variant was discovered by serendipity. During a study regarding the effect of human milk on bacterial adherence, it was surprisingly discovered that human milk induced apoptosis in transformed and nontransformed immature cell lines. The apoptotic activity in human milk was isolated and found to be partially unfolded alpha-lactalbumin in an apo-like conformation with native-like secondary structure, but lacking specific tertiary packing of the side chains.

A complex of alphalactalbumin and fatty acid or lipid (LAC) has been shown to bind to the surface of tumour cells, translocate into the cytoplasm and accumulate in cell nuclei, where it causes DNA fragmentation.

LAC is reported as having therapeutic applications both in the field of antibiotics and cancer therapy. In particular, LAC may induce apoptotic cell death in cancer cells and
immature cells, but not (or only to a low extent) in mature, healthy cells. These observations suggested that the protein acquires novel biological properties when forming an active complex with a fatty acid or lipid. Thus, reagents such as fatty acids or lipids, such as oleic acid, may be useful in the conversion of LA to LAC.

Binding of Ca\(^{2+}\) to a single very high affinity Ca\(^{2+}\) binding site is important for the protein to maintain a native conformation. The high affinity Ca\(^{2+}\) binding site is 100% conserved across many mammalian species including human, bovine, equine, porcine, caprine and camelide alpha-lactalbumin. It appears that five of the seven oxygens that ligate the Ca\(^{2+}\) are contributed by side chain carboxylates of Asp residues at positions 82, 87 and 88 and by carbonyl oxygens of Lys 79 and Asp 84, and two water molecules supply the remaining ligands. The bound Ca\(^{2+}\) brings the \(\alpha\)-helical region and the \(\beta\)-sheet in close proximity, and two disulfide bonds flanking the Ca\(^{2+}\) binding site, make this part of the molecule fairly inflexible. Binding of other cations, such as Mg\(^{2+}\), Mn\(^{2+}\), Na\(^{+}\) and K\(^{+}\) also cause conformational changes in alpha-lactalbumin although these are smaller than for the binding of Ca\(^{2+}\).

A LAC complex has previously been produced by first exposing alpha-lactalbumin in the apo state to a DEAE Trisacyl resin that had been pre-conditioned with oleic acid causing the formation of active complex of alpha-lactalbumin and oleic acid (e.g. Svensson, et al., (2000) Proc Natl Acad Sci USA, 97,4221-6, WO 03/098223, WO 2005/082406, Danish patent application The previously used DEAE Trisacyl resin has proven difficult to use due to the softness of the resin, with which a working flow rate of just 25-80 cm/h is the maximum recommended for this type of resin. This process is furthermore time-consuming and the yield of LAC is low.

**Summary of invention**

The present invention describes new and effective methods for preparation of an active complex of lactalbumin and a fatty acid or lipid.

Surprisingly, the present invention discloses that selecting another type of ion exchange resin improved the load and the yield possible in the conversion of lactalbumin to an active complex of lactalbumin and a fatty acid or lipid; the conversion of LA to LAC. By changing the anion exchange resin from DEAE Trisacyl to another
resin, for example a resin with a carbohydrate based matrix both the load and the yield may be increased.

Thus, in one aspect the invention relates to a method for preparing LAC using an ion exchange medium comprising a matrix comprising carbohydrate.

Other features of the selected ion exchange medium may also be of relevance. For example, the present invention also discloses a method for preparing LAC comprising use of an anion exchange resin, wherein the particles of the resin have a mean size of at least 80 µm, such as in the range of 80-120 µm. Alternatively in certain embodiments an anion exchange resin, wherein the particles of the resin have a mean size of 120 µm, such as in the range of 100 to 130 µm may be used.

In addition the maximum recommended working flow rate for DEAE Trisacryl is 25-80 cm/h. In another aspect the invention discloses a method for preparing LAC comprising use of an anion exchange resin having a recommended maximum flow rate which is higher than 80 cm/h, for example having a maximum flow rate which is at least 300 cm/h.

Furthermore, DEAE Trisacryl is a weak anion exchange resin. It is an additional aspect of the invention to provide a method for preparing LAC comprising use of an anion exchange resin which is a strong anion exchange resin, for example a resin comprising a quaternary ammonium group (Q).

It is also comprised within the invention that said "another resin" may have at least two, such as at least 3, for example all four of the aforementioned properties.

In particular the invention describes a method for the preparation of an active complex of lactalbumin and a fatty acid or lipid by the exposure of lactalbumin to an ion exchange medium comprising a resin, wherein the mean size of the particles of the resin is larger than 80 µm, for example in the range of 80 µm to 120 µm, comprising the steps of:
obtaining an alpha-lactalbumin composition comprising alpha-
lactalbumin of SEQ ID NO: 1 or SEQ ID NO: 2 or a functional homologue thereof comprising a sequence at least 70% identical thereto,

- conversion of said alpha-lactalbumin or a functional homologue thereof to lactalbumin complex

1) by release of calcium from said alpha-lactalbumin or a functional homologue thereof and

2) binding of a fatty acid or a lipid to said alpha-lactalbumin or a functional homologue thereof

3) exposing the alpha-lactalbumin or a functional homologue thereof to an ion exchange medium comprising a carbohydrate based matrix

wherein steps 2. and 3. may be performed sequentially or simultaneously.

The methods for preparing an active complex of alphalactalbumin and a fatty acid described in the prior art customary involves binding a fatty acid or lipid to the anion exchange resin firstly and then subsequently applying LA to the resin with bound fatty acid or lipid thereby converting LA to LAC. Even more surprisingly it is disclosed by the present invention that by mixing LA and a fatty acid or lipid prior to the exposure to the anion exchange resin it is possible to increase both the load and the yield remarkably. This new method not only increases the load and the yield, but also decreases the time for the conversion as the resin does not have to be pre-treated with the fatty acid or lipid as previously, a process that generally takes several hours.

Another aspect of the present invention relates to a new and effective method for preparation of an active complex of alpha-lactalbumin and a fatty acid or lipid. In particular the invention describes a method for the preparation of an active complex of alpha-lactalbumin and a fatty acid or lipid where alpha-lactalbumin and a fatty acid or a lipid is mixed in the absence of an ion exchange medium, comprising the steps of:
- obtaining an alpha-lactalbumin composition comprising lactalbumin of SEQ ID NO: 1 or SEQ ID NO: 2 or a functional homologue thereof comprising a sequence of at least 70% identical therewith,
- conversion of said lactalbumin or a functional homologue thereof to alpha-lactalbumin complex
  - by mixing alpha-lactalbumin or a functional homologue thereof and a fatty acid or a lipid in the absence of an ion exchange medium, and subsequently
  - exposing the mixture to an ion exchange medium.

When used herein:

The term "Alpha-lactalbumin" as used herein has the meaning of the alpha-lactalbumin polypeptide independent of the tertiary structure of the polypeptide. The sequences of bovine and human alpha-lactalbumin are defined by SEQ ID NO 2 and SEQ ID NO 1 respectively. Figure 1 B shows a sequence alignment of human and bovine alpha-lactalbumin. Thus human alpha-lactalbumin is any polypeptide of the sequence SEQ ID NO 1 with any tertiary structure. Similarly, bovine alpha-lactalbumin is any polypeptide of the sequence SEQ ID NO 2 with any tertiary structure.

The term "LA" as used herein has the meaning of the alpha-lactalbumin polypeptide preferably in the native tertiary structure and preferably with calcium bound to the high affinity calcium-binding site. LA is not in complex with any fatty acids or lipids and does preferably not have cell killing abilities. The terms "hLA" and "bLA" as used herein have the meaning of human LA and bovine LA, respectively.

A-state of alpha-lactalbumin has the meaning of partially folded state of alpha-lactalbumin adopted for example when dissolved at low pH, whereas the apo-state is the partially folded state alpha-lactalbumin adopted for example upon removal of the protein bound calcium at neutral pH and low salt concentration].

The term "LAC" as used herein has the meaning of an active complex of alpha-lactalbumin and a fatty acid or a lipid. By "active" is meant that the complex has capacity of apoptosis induction (see more details herein below in the section "Alpha-
Lactalbumin”). The terms hLAC and bLAC as used herein has the meaning of human LAC and bovine LAC, respectively. Preferably, LAC has cell killing activity, more preferably, the LD50 of said LAC is at the most 100 pg/cell, preferably at the most 75 pg/cell, more preferably at the most 50 pg/cell, such as at the most 42 pg/cell, when determined as described in Example 7 herein below. It is furthermore preferred that LAC has histone binding ability, preferably LAC can bind histones, so that the absorption at 450 nm after performing a histone binding assay is at least 3 times the absorption of the negative control, such as at least 0.1 , preferably at least 1, more preferably at least 1.5, wherein the histone binding assay is performed as described in Example 6 below.

Description of Drawings

Figure 1: A. Sequence alignments of equine, porcine, camelide, human, bovine and caprine alpha-lactalbumin. B: Sequence alignments of human and bovine alpha-lactalbumin.

Figure 2: Chromatograms for the conversion with DEAE Trisacryl Plus M.

Figure 3: Chromatograms for the conversion with alternative resins; A: Capto Q, B: UnoSphere Q, C: Q Sepharose XL.

Figure 4: Conversion with Q Sepharose XL with a linear gradient from 0 to 100% B-buffer (left) and SE-HPLC identification of peaks 1, 2 and 3 (right).

Figure 5: Conversion with Q Sepharose XL with steps gradient (45, 70 and 100% B-buffer) (left) and SE-HPLC identification of peaks land 2 (right).

Figure 6: Conversion with Q Sepharose XL with step gradient (45, 70 and 100% B-buffer) and higher bLA load.

Figure 7: Yield of conversion versus load.
Figure 8: Chromatograms for the conversion with Q sepharose XL. Four different bl_A loads were tested. Run LAC-031 was performed with a lower amount of oleic acid. Cutting criteria at 280 nm are indicated. bl_A is eluted at 45% B-buffer and bLAC is eluted at 70% B-buffer.

Figure 9: Characterization of peaks eluted at different salt concentration during conversion of bl_A to bLAC. Retention time of bLA/bLAC dimer = 23 min; Retention time of bLA/bLAC monomer = 25 min.

Figure 10: Histone assay of bLAC converted at different bLA loads. Histone assays N274-28A for bLAC ID. No. N277-07E and N277-09G; N274-36A for bLAC ID. No. N277-18A and N262-35B (described in Example 7).

Figure 11: Cell killing assay (N274-26A) of bLAC. N262-35B (described in Example 8), pre-conditioned ion exchange resin -old conversion method, N277-09G (described in Example 8), un-conditioned ion exchange resin -new conversion method).

Figure 12: Chromatograms of the conversion runs with bLA start material N277-64A (described in Example 9), un-conditioned ion exchange resin.

Figure 13: Chromatograms of the conversion runs with bLA start material N289-56A (described in Example 9), un-conditioned ion exchange resin.

Figure 14: Summary of conversion, histone binding and cell killing of bLA in complex with the respective fatty acids (un-conditioned ion exchange resin).

Figure 15: Chromatograms of the conversion runs. Standard step gradient was applied with Q sepharose XL. Linear gradient was applied with the other resins (un-conditioned ion exchange resin).

Detailed description of the invention

The present invention regards a method for the production of an active complex of alpha-lactalbumin with a fatty acid or a lipid. The wild-type human alpha-lactalbumin i.e.
the naturally occurring non-mutated version of the protein is identified as SEQ ID NO: 1 and the wild-type bovine alpha-lactalbumin i.e. the naturally occurring non-mutated version of the protein is identified as SEQ ID NO: 2. The present invention also covers functional homologues of alpha-lactalbumin comprising a sequence identity of at least 70% to SEQ ID NO: 1 or comprising a sequence identity of at least 70% to SEQ ID NO:2 as well as an active complex of functional homologues of alpha-lactalbumin with a fatty acid or a lipid. The wild-type human alpha-lactalbumin including the leader sequence i.e. the naturally occurring non-mutated version of the protein including the 19 amino acid leader sequence is identified as SEQ ID NO: 3 and the wild-type bovine alpha-lactalbumin including the leader sequence i.e. the naturally occurring non-mutated version of the protein including the 19 amino acid leader sequence is identified as SEQ ID NO: 4.

A functional homologue can be defined as alpha-lactalbumin that differs in sequence from the wild-type alpha-lactalbumin, such as wild-type human alpha-lactalbumin or wild-type bovine lactalbumin, but is still functionally competent. A functional homologue may be a mutated version or an alternative splice variant of the wild-type alpha-lactalbumin. In another aspect functional homologues of alpha-lactalbumin are defined as described herein below. A functional homologue may be, but is not limited to, a recombinant version of alpha-lactalbumin with one or more mutations and/or one or more sequence deletions and/or additions introduced ex vivo.

In preferred embodiments of the invention the alpha-lactalbumin may be human or bovine alpha-lactalbumin, wherein the alpha-lactalbumin is either naturally occurring milk alpha-lactalbumin or the alpha-lactalbumin has been recombinantly produced.

**Alpha-lactalbumin**

Alpha-lactalbumin is as described in the background section highly abundant in milk. The sequence of alpha-lactalbumin from different mammal species is well conserved. Sequences from rodents (mouse, rat, rabbit, guinea pig), primates, cats and dogs show a high degree of identity. The amino acid sequence from equine, caprine, bovine, porcine and humans show approximately 75-95 % identity (Pettersson, Jenny, BBRC 345 (2006) 260-270 and Figure 1 A).
Alpha-lactalbumin from any species, preferably any mammalian species, may
according to the invention be used for the preparation of a biologically active complex
of alpha-lactalbumin and a fatty acid or a lipid. For the present invention alpha-
lactalbumin from any species different from bovine or human species are considered
functional equivalents (see below) of bovine or human alpha-lactalbumin. Alpha-
lactalbumin is evolutionary related to and share around 35 to 40% of sequence
homology as well as the positions of the four disulfide bonds with lysozyme C.

In an embodiment of the invention the functional equivalent of bovine or human alpha-
lactalbumin is selected from the group consisting of alpha-lactalbumin from equine,
caprine, bovine, camelide and porcine. In most and equally preferred embodiments the
alpha-lactalbumin is bovine or human. Figure 1B shows an alignment of the protein
sequences of bovine and human alpha-lactalbumin.

Human wild-type alpha-lactalbumin is identified as SEQ ID NO: 1 and bovine wild-type
alpha-lactalbumin is identified as SEQ ID NO: 2. In one preferred embodiment of the
invention alpha-lactalbumin is human alpha-lactalbumin and in another preferred
embodiment of the invention alpha-lactalbumin is bovine alpha-lactalbumin. In a more
preferred embodiment alpha-lactalbumin is human wild-type alpha-lactalbumin as
identified by SEQ ID NO: 1 and in an equally preferred embodiment of the invention
alpha-lactalbumin is bovine alpha-lactalbumin.

In another preferred embodiment alpha-lactalbumin is recombinant wild type human
alpha-lactalbumin and in an equally preferred embodiment of the invention alpha-
lactalbumin is recombinant wild type bovine alpha-lactalbumin. Alpha-lactalbumin
variants include any form of alpha-lactalbumin known to a person skilled in the art and
any functional homologue thereof. For example, alpha-lactalbumin variants include
splice variants and allelic variants and single nucleotide polymorphisms.

A functional homologue of alpha-lactalbumin may be any protein that exhibits at least
some sequence identity with SEQ ID NO: 1 or SEQ ID NO: 2, and when complexed
with a fatty acid or a lipid shares one or more functions with alpha-lactalbumin, such as
the capacity of apoptosis induction (see more details herein below).

The capacity of alpha-lactalbumin of induction of apoptosis can for example be
measured as described in Danish patent application PA 2006 015 12 in Example 7: Cell
killing assay. Danish patent application PA 2006 0 1512 is incorporated herein by reference in its entirety. The capacity of alpha-lactalbumin of DNA fragmentation can be visualised as described in (Pettersson, Jenny, BBRC 345 (2006) 260-270) for example with ethidium bromide using a 305 nm UV-light source. Histone binding activity of alpha-lactalbumin, which is a function of wild type LAC can be measured as described in Example 6: Histone assay.

Alpha-lactalbumin to be used with the present invention may be derived from any suitable source, for example alpha-lactalbumin may be naturally occurring alpha-lactalbumin or alpha-lactalbumin may be recombinantly produced alpha-lactalbumin as described in detail herein below. In a preferred embodiment alpha-lactalbumin is human alpha-lactalbumin purified from human milk and in another equally preferred embodiment alpha-lactalbumin is bovine alpha-lactalbumin purified from bovine milk. In a preferred embodiment alpha-lactalbumin is recombinant human alpha-lactalbumin and in another equally preferred embodiment alpha-lactalbumin is recombinant bovine alpha-lactalbumin. In a more preferred embodiment alpha-lactalbumin is recombinant human wild-type alpha-lactalbumin and in another preferred embodiment alpha-lactalbumin is recombinant bovine wild-type alpha-lactalbumin.

Functional homoloques of alpha-lactalbumin

In one preferred embodiment of the invention alpha-lactalbumin is human alpha-lactalbumin, in a more preferred embodiment alpha-lactalbumin is human wild-type alpha-lactalbumin as identified by SEQ ID NO: 1. In a very preferred embodiment alpha-lactalbumin is recombinant wild type human alpha-lactalbumin. In another preferred embodiment of the invention alpha-lactalbumin is bovine alpha-lactalbumin, in a more preferred embodiment alpha-lactalbumin is bovine wild-type alpha-lactalbumin as identified by SEQ ID NO: 2. In a very preferred embodiment alpha-lactalbumin is recombinant wild type bovine alpha-lactalbumin.

A functional homologue of alpha-lactalbumin may be any protein that exhibits at least some sequence identity with SEQ ID NO. 1 or SEQ ID NO.2 and shares one or more functions with alpha-lactalbumin, such as:

• Acting as a co-factor in the synthesis of lactose
• Exhibiting cell killing activity when converted to LAC

• Capability to induce apoptosis when converted to LAC

• Histone binding activity when converted to LAC

Several methods may be used to determine whether LAC has cell killing activity. Preferably, LAC is said to have cell killing activity, when said LAC has a LD50 of at the most 100 pg/cell, preferably at the most 75 pg/cell, more preferably at the most 50 pg/cell, such as at the most 42 pg/cell, when determined as described in Example 7 herein below.

Several methods may be used to determined whether LAC has histone binding activity. Preferably, LAC is said to have histone binding activity, when said LAC results in an absorption at 450 nm after performing the histone binding assay as described in Example 6, which is at least 3 times the absorption of the negative control, such as at least 0.1, preferably at least 1, more preferably at least 1.5.

Preferably the functional homologue exhibits at least some sequence identity with SEQ ID NO. 1 or SEQ ID NO.2 and has cell killing abilities.

Preferably, evolutionary conservation between alpha-lactalbumin of different closely related species, e.g. assessed by sequence alignment, can be used to pinpoint the degree of evolutionary pressure on individual residues. Preferably, alpha-lactalbumin sequences are compared between species where alpha-lactalbumin function is conserved, for example but not limited to mammals including rodents, monkeys and apes. Residues under high selective pressure are more likely to represent essential amino acids that cannot easily be substituted than residues that change between species. For example, such an alignment may be performed using ClustalW from EBML-EBI comparing porcine alpha-lactalbumin and human alpha-lactalbumin (Figure 1 A). It is evident from the above that a reasonable number of modifications or alterations of the bovine or human alpha-lactalbumin sequence does not interfere with the activity of the alpha-lactalbumin molecule according to the invention. Such alpha-
lactalbumin molecules are herein referred to as functional equivalents of bovine or human alpha-lactalbumin, and may be such as variants and fragments of native bovine or human alpha-lactalbumin as described here below.

Functional assays can for example be used in order to determine if alpha-lactalbumin function is conserved. Functional assays known to a skilled person can be used to verify the functional conservation of uncomplexed alpha-lactalbumin. Such functional assays determine the ability of alpha-lactalbumin to act as a regulatory subunit of the lactose synthase complex in the production of lactose.

Functional assays known to a skilled person can be used to verify the functional conservation of alpha-lactalbumin in complex with a fatty acid or a lipid. Functional assays for evaluating alpha-lactalbumin function known to persons skilled in the art include, but are not limited to, assays described herein above and in examples 6 and 7, such as the cell killing assay or the histone assay.

As used herein the expression "variant" refers to polypeptides or proteins which are homologous to the basic protein, which is suitably bovine or human alpha-lactalbumin, but which differs from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Figure 1A shows an alignment of the protein sequences of bovine, human, equine, caprine, bovine, camelide and porcine alpha-lactalbumin wherein identical residues (".") and residues with conservative (.:) and semi-conservative (:.*) substitutions are marked.

Accordingly, in one embodiment of the invention it is preferred that functional homologues of alpha-lactalbumin comprises a sequence with high sequence identity to SEQ ID NO: 1 or SEQ ID NO:2, wherein none of the conserved residues marked with ":" in figure 1A are substituted. It is furthermore preferred within this embodiment that the residues marked with ":." in figure 1A are either not substituted or only substituted
by conservative substitution, more preferably by substitution with an amino acid with a high level of similarity as defined herein below.

Thus in one embodiment it is preferred that functional homologues of human alpha-lactalbumin have a sequence with high sequence identity to SEQ ID NO: 1, wherein residues K1, F2, E9, L8, 115, 121, A22, I27, Q39, A40, 141, N44, L59, K62, Q65, I85, M90, D102, E116, K122 are either not substituted or substituted only by conservative substitution, more preferably substituted only an amino acid with a high level of similarity as defined herein below.

In a more preferred embodiment functional homologues of human alpha-lactalbumin have a sequence with high sequence identity to SEQ ID NO: 1, wherein residues K1, F3, E7, L8, 115, 121, A22, I27, Q39, A40, 141, N44, L59, K62, Q65, I85, M90, D102, E116, K122 are either not substituted or substituted only by conservative substitution, more preferably substituted only an amino acid with a high level of similarity as defined herein below.

It is even further preferred within the present invention that functional homologues of alpha-lactalbumin have a sequence with high sequence identity to SEQ ID NO: 1 or SEQ ID NO: 2, wherein residues marked with "." in figure 1A are either not substituted or are only substituted by conservative substitutions, such as with amino acids with lower levels or high level of similarity as defined herein below. Accordingly, it is preferred that functional homologues of human alpha-lactalbumin have a sequence with high sequence identity to SEQ ID NO: 1, wherein residues D14, D16, G17, G20, P24, S47, S56, S63, S64, D74, A92, and A109 are either not substituted or only substituted by conservative substitutions, such as with amino acids with lower level or high level of similarity as defined herein below.

It is also comprised within the present invention that functional homologues of alpha-lactalbumin may have a sequence with high sequence identity to SEQ ID NO: 1 or SEQ ID NO: 2, wherein the unmarkes residues in Figure 1A may be substituted with any other amino acid. Thus, functional homologues of human alpha-lactalbumin may have a sequence with high sequence identity to SEQ ID NO: 1, wherein residues S9, Q10, L11, G19, L25, T29, M30, T33, E43, E46, T48, V66, P67, Q68, R70, I89, I98, K99, L118, and L123 are either not substituted or substituted with any other amino acid.
A person skilled in the art will know how to make and assess 'conservative' amino acid substitutions, by which one amino acid is substituted for another with one or more shared chemical and/or physical characteristics. Conservative amino acid substitutions are less likely to affect the functionality of the protein. Amino acids may be grouped according to shared characteristics. A conservative amino acid substitution is a substitution of one amino acid within a predetermined group of amino acids for another amino acid within the same group, wherein the amino acids within a predetermined groups exhibit similar or substantially similar characteristics.

Within the meaning of the term "conservative amino acid substitution" as applied herein, one amino acid may be substituted for another within the groups of amino acids indicated herein below:

**Lower levels of similarity:**

**Polarity:**

i) Amino acids having polar side chains (Asp, Glu, Lys, Arg, His, Asn, Gln, Ser, Thr, Tyr, and Cys)

ii) Amino acids having non-polar side chains (Gly, Ala, Val, Leu, lie, Phe, Trp, Pro, and Met)

**Hydrophilic or hydrophobic:**

iii) Hydrophobic amino acids (Ala, Cys, Gly, lie, Leu, Met, Phe, Pro, Trp, Tyr, Val)

iv) Hydrophilic amino acids (Arg, Ser, Thr, Asn, Asp, Gln, Glu, His, Lys)

**Charges:**

v) Neutral amino acids (Ala, Asn, Cys, Gln, Gly, lie, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val)

vi) Basic amino acids (Arg, His, Lys)

vii) Acidic amino acids ((asp, Glu)
High level of similarity:

viii) Acidic amino acids and their amides (Gln, Asn, Glu, Asp)
ix) Amino acids having aliphatic side chains (Gly, Ala Val, Leu, Ile)
x) Amino acids having aromatic side chains (Phe, Tyr, Trp)

xi) Amino acids having basic side chains (Lys, Arg, His)

xii) Amino acids having hydroxy side chains (Ser, Thr)

xiii) Amino acids having sulphor-containing side chains (Cys, Met),

Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

It is clear from the above outline that the same functional homologue or fragment thereof may comprise more than one conservative amino acid substitution from more than one group of conservative amino acids as defined herein above.

Aside from the twenty standard amino acids and two special amino acids, selenocysteine and pyrrolysine, there are a vast number of "nonstandard amino acids" which are not incorporated into protein in vivo. Examples of nonstandard amino acids include the sulfur-containing taurine and the neurotransmitters GABA and dopamine. Other examples are lanthionine, 2-Aminoiso butyric acid, and dehydroalanine. Further non standard amino are ornithine and citrulline.

Non-standard amino acids are usually formed through modifications to standard amino acids. For example, taurine can be formed by the decarboxylation of cysteine, while dopamine is synthesized from tyrosine and hydroxyproline is made by a posttranslational modification of proline (common in collagen). Examples of non-natural
amino acids are those listed e.g. in 37 C.F.R. section 1.822(b)(4), all of which are incorporated herein by reference.

Both standard and non standard amino acid residues described herein can be in the "D" or or "L" isomeric form.

It is contemplated that a functional equivalent according to the invention may comprise any amino acid including non-standard amino acids. In preferred embodiments a functional equivalent comprises only standard amino acids.

The standard and/or non-standard amino acids may be linked by peptide bonds or by non-peptide bonds, preferably however by peptide bonds. The term peptide also embraces post-translational modifications introduced by chemical or enzyme-catalyzed reactions, as are known in the art. Such post-translational modifications can be introduced prior to partitioning, if desired. Amino acids as specified herein will preferentially be in the L-stereoisomeric form. Amino acid analogs can be employed instead of the 20 naturally-occurring amino acids. Several such analogs are known, including fluorophenylalanine, norleucine, azetidine-2-carboxylic acid, S-aminoethyl cysteine, 4-methyl tryptophan and the like.

Sequence identity can be calculated using a number of well-known algorithms and applying a number of different gap penalties. The sequence identity is calculated relative to full-length SEQ ID NO: 1 or SEQ ID NO: 2. In the alternative, it is calculated relative to SEQ ID NO: 1 or SEQ ID NO: 2, wherein the sequence encoding the signal peptide is not included. Without being bound by theory, the signal peptide is predicted to comprise amino acids 1 to 24 of SEQ ID NO: 1 and SEQ ID NO: 2. Any sequence alignment tool, such as but not limited to FASTA, BLAST, or LALIGN may be used for searching homologues and calculating sequence identity. Moreover, when appropriate any commonly known substitution matrix, such as but not limited to PAM, BLOSSUM or PSSM matrices may be applied with the search algorithm. For example, a PSSM (position specific scoring matrix) may be applied via the PSI-BLAST program. Moreover, sequence alignments may be performed using a range of penalties for gap opening and extension. For example, the BLAST algorithm may be used with a gap opening penalty in the range 5-12, and a gap extension penalty in the range 1-2.
A functional homologue within the scope of the present invention is a polypeptide that exhibits some sequence identity with human alpha-lactalbumin or with bovine alpha-lactalbumin as identified by SEQ ID NO: 1 or SEQ ID NO: 2, preferably they have a high sequence identity to SEQ ID NO: 1 or SEQ ID NO: 2, for example functional homologues may have a sequence sharing at least 70% sequence identity preferably functional homologues have at least 75% sequence identity, for example at least 80% sequence identity, such as at least 85% sequence identity, for example at least 90% sequence identity, such as at least 91% sequence identity, for example at least 91% sequence identity, such as at least 92% sequence identity, for example at least 93% sequence identity, such as at least 94% sequence identity, for example at least 95% sequence identity, such as at least 96% sequence identity, for example at least 97% sequence identity, such as at least 98% sequence identity, for example 99% sequence identity with SEQ ID NO: 1 or SEQ ID NO: 2.

Functional homologues may in one embodiment further comprise chemical modifications such as ubiquitination, labeling (e.g., with radionuclides, various enzymes, etc.), pegylation (derivatization with polyethylene glycol), or by insertion (or substitution by chemical synthesis) of amino acids (amino acids) such as ornithine, which do not normally occur in human proteins.

In addition to the peptidyl compounds described herein, sterically similar compounds may be formulated to mimic the key portions of the peptide structure and that such compounds may also be used in the same manner as the peptides of the invention. This may be achieved by techniques of modelling and chemical designing known to those of skill in the art. For example, esterification and other alkylations may be employed to modify the amino terminus of, e.g., a di-arginine peptide backbone, to mimic a tetra peptide structure. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

Peptides with N-terminal alkylations and C-terminal esterifications are also encompassed within the present invention. Functional equivalents also comprise glycosylated and covalent or aggregative conjugates formed with the same molecules, including dimers or unrelated chemical moieties. Such functional equivalents are prepared by linkage of functionalities to groups which are found in fragment including at any one or both of the N- and C-termini, by means known in the art.
Suitable fragments may be deletion or addition mutants. The addition of at least one amino acid may be an addition of from preferably 2 to 250 amino acids, such as from 10 to 20 amino acids, for example from 20 to 30 amino acids, such as from 40 to 50 amino acids.

A functional homologue may be a deletion mutant of alpha-lactalbumin as identified by SEQ ID NO: 1 or SEQ ID NO: 2, sharing at least 70% and accordingly, a functional homologue preferably have at least 75% sequence identity, for example at least 80% sequence identity, such as at least 85 % sequence identity, for example at least 90 % sequence identity, such as at least 91 % sequence identity, for example at least 91% sequence identity, such as at least 92 % sequence identity, for example at least 93 % sequence identity, such as at least 94 % sequence identity, for example at least 95 % sequence identity, such as at least 96 % sequence identity, for example at least 97% sequence identity, such as at least 98 % sequence identity, for example 99% sequence identity.

Deletion mutants suitably comprise at least 20 or 40 consecutive amino acid and more preferably at least 80 or 100 consecutive amino acids in length. Accordingly such a fragment may be a shorter sequence of the sequence as identified by SEQ ID NO: 1 or SEQ ID NO: 2 comprising at least 20 consecutive amino acids, for example at least 30 consecutive amino acids, such as at least 40 consecutive amino acids, for example at least 50 consecutive amino acids, such as at least 60 consecutive amino acids, for example at least 70 consecutive amino acids, such as at least 80 consecutive amino acids, for example at least 90 consecutive amino acids, such as at least 95 consecutive amino acids, such as at least 100 consecutive amino acids, such as at least 105 amino acids, for example at least 110 consecutive amino acids, such as at least 115 consecutive amino acids, for example at least 120 consecutive amino acids, wherein said deletion mutants preferably share at least 75% sequence identity, for example at least 80% sequence identity, such as at least 85 % sequence identity, for example at least 90 % sequence identity, such as at least 91 % sequence identity, for example at least 91% sequence identity, such as at least 92 % sequence identity, for example at least 93 % sequence identity, such as at least 94 % sequence identity, for example at least 95 % sequence identity, such as at least 96 % sequence identity, for example at
least 97% sequence identity, such as at least 98% sequence identity, for example 99% sequence identity with SEQ ID NO: 1 or SEQ ID NO:2.

It is preferred that functional homologues of alpha-lactalbumin comprises at the most 500, more preferably at the most 400, even more preferably at the most 300, yet more preferably at the most 200, such as at the most 175, for example at the most 160, such as at the most 150 amino acids, for example at the most 142 amino acids.

The term "fragment thereof" may refer to any portion of the given amino acid sequence. Fragments may comprise more than one portion from within the full-length protein, joined together. Portions will suitably comprise at least 5 and preferably at least 10 consecutive amino acids from the basic sequence. They may include small regions from the protein or combinations of these.

In an embodiment of the invention the alpha-lactalbumin fragment comprise one or more amino acid segments. The segments may be selected from the major alpha-helical domain containing amino acid 5-1 1, 23-34, 86-98, and the short alpha-helical segments; amino acid 18-20, 115-1 18, or from the beta-domain containing the triple-stranded anti-parallel sheet: amino acids 40-50 and the short 76-82 helix or the calcium binding domain 76-89 or any segments between these domains: amino acid 1-4, 12-17, 21-22, 35-39,51-76, 82-84, 99-1 14 or 119-1 23. Preferably an alpha-lactalbumin fragment comprises at least two of the above mentioned segments, more preferably at least three of the indicated segments, more preferably four or most preferably five or all sixth mentioned segments.

The region which forms the interface between the alpha and beta domains is, in human alpha-lactalbumin, defined by amino acids 35-39 and 83-87 in the structure. Thus by parallel thereto suitable fragments of human or bovine alpha-lactalbumin will include these regions, such as the entire region from amino acid 35-87 of the native protein, for example from amino acid 20-1 00 of the native protein, for example from amino acid 10-110 of the native protein, for example from amino acid 5-1 15 of the native protein, for example from amino acid 1-123. This region of the molecule differs between the bovine and the human proteins, in that one of the three basic amino acids (R70) is changed to S70 in bovine \(\alpha\)-lactalbumin, thus eliminating one potential coordinating side chain.
The deletion and/or the addition may - independently of one another - be a deletion and/or an addition within a sequence and/or at the end of a sequence.

5 The high affinity Ca$^{2+}$ binding site is 100% conserved in alpha-lactalbumin from different species (Acharya K. R., et al., (1991) J Mol Biol, 221,571-581), illustrating the importance of this function for the protein. It is co-ordinated by five different amino acids (K79, D82, D84, D87 and D88) and two water molecules as described in the background section.

10 In a particular embodiment, the functional homologue according to the invention is one in which the calcium binding site has been modified so that the affinity for calcium is reduced, or it is no longer functional. The calcium binding site in alpha-lactalbumin is coordinated by the residues K79, D82, D84, D87 and D88. Thus modification of these residues, by for example removing one of more of the acidic residues, can reduce the affinity of the site for calcium, or eliminate the function completely and mutants of this type are an embodiment of the invention. In a specific embodiment, the aspartic acid residue at amino acid position 87 within the protein sequence is mutated to a non-acidic residue, and in particular a non-polar or uncharged polar side chain. In order to minimize the structural distortion in the mutant protein, D87 may also be replaced by an asparagine (N). Thus variants for use in the complexes of the invention may be D87A and D87N variants of a-lactalbumin, or fragments which include this mutation.

Alpha-lactalbumin complexes appear to be active with and without calcium present.

25 Two explanations for this are plausible. In the first and most likely scenario, the alpha-lactalbumin complex is formed by unfolding and binding of fatty acid (se below) with little disturbance of the $\alpha$-helical domain. The Ca$^{2+}$-binding site may then retain a similar conformation as in the absence of fatty acid and Ca$^{2+}$ may be bound there to. A second possibility is that the Ca$^{2+}$ site is disrupted and that the observed Ca$^{2+}$ binding is explained by the generation of a new Ca$^{2+}$ site in the alpha-lactalbumin complex. The head group of the fatty acid might potentially coordinate calcium together with amino acid residues.

It appears therefore that the Ca$^{2+}$-binding site is not involved in the conversion of alpha-lactalbumin to an apoptosis-associated conformation, and that the structural
changes associated with Ca\textsuperscript{2+} binding to alpha-lactalbumin complex do not hinder the biological function. Thus in an alternative embodiment the Ca\textsuperscript{2+} binding site is preserved by the inclusion of amino acid segment 76-89 as described above.

5

**Purification of alpha-lactalbumin**

Natural sources of LA are milk from different mammalian species, preferably selected from the group of: equine, caprine, human, bovine and porcine, most preferably bovine or human even more preferably bovine. Alternatively LA may be produced recombinantly (see more details herein below in the section "Recombinant Production") or obtained as a commercial product from several companies.

Purification of proteins in general involves one or more steps of removal of or separation from contaminating nucleic acids, phages and/or viruses, other proteins and/or other biological macromolecules. The obtaining of LA from a composition comprising LA, such as milk or a culture medium or an extract of host cells (see herein below in the section "Recombinant production") may comprise one or more protein isolation steps. Any suitable protein isolation step may be used with the present invention. The skilled person will in general readily be able to identify useful protein isolation steps for LA if such are required.

The protein isolation steps useful with the present invention may be commonly used methods for protein purification including for example chromatographic methods such as for example gas chromatography, liquid chromatography, ion exchange chromatography and/or affinity chromatography; filtration methods such as for example gel filtration and ultrafiltration; precipitation, such as ammonium sulphate precipitation and/or gradient separation such as sucrose gradient separation. Purification of LA may comprise one or more of the aforementioned methods in any combination.

The aforementioned methods are well known to the skilled person and may for example be performed as described in the "Protein Separation Handbook Collection" including the titles "Antibody Purification", "The Recombinant Protein Handbook", "Protein Purification", "Ion Exchange Chromatography", "Affinity Chromatography", "Hydrophobic Interaction Chromatography", "Gel Filtration", "Reversed Phase..."
Chromatography”, “Expanded Bed Adsorption” and “Chromatofocusing” prepared by Amersham Biosciences and available from GE.

In particular, purification of LA may for example comprise one or more centrifugation steps. Said centrifugation may be employed for example for defattening purposes and/or to remove cells/cellular debris or the like and/or to separate supernatant from precipitate.

In particular, purification of LA may for example comprise one or more precipitation steps, for example precipitation using ammonium sulphate, for example at a concentration in 10 to 75%, preferably in the range of 30 to 60%, such as in the range of 40-45%. When precipitation is performed using an ammonium sulphate concentration of in the range of 40-145%, LA will generally be present in the supernatant.

Purification of LA may comprise one or more steps of filtration, for example filtration through a filter paper and/or filtration using another filter with a pore size of the range pf 0.1 μm to 100 μm, for example in the range of 0.5 to 50 μm, such as in the range of 0.5 to 20 μm, such as in the range of 0.5-1 μm.

Purification of LA may comprise one or more chromatographic steps, for example any of the chromatographic methods mentioned above. In one preferred embodiment the method comprises a hydrophobic interaction chromatography.

An example of a preferred embodiment of purification of LA from milk is described in Example 1 of Danish Patent application PA 2006 01512, which is incorporated herein as a reference. In the example LA is prepared from bovine milk, first by defatting of the milk followed by ammonium sulphate precipitation steps prior to centrifugation. LA was purified using hydrophobic interaction chromatography.

LA thus purified may then be employed in the preparation of LAC for example as described herein below.

Recombinant production
Functional equivalents of LA are preferably produced recombinantly. Wild type LA may be recombinantly produced. Useful recombinant production methods include conventional methods known in the art, such as by expression of heterologous LA of functional homologues thereof in suitable host cells such as *E. coli*, *S. cerevisiae* or *S. pombe* or insect or mammalian cells suitable for production of recombinant proteins (see below). The skilled person will in general readily be able to identify useful recombinant techniques for the production of recombinant proteins in general and LA specifically.

In one embodiment LA is produced in a transgene plant or animal. By a transgenic plant or animal in this context is meant a plant or animal which has been genetically modified to contain and express a nucleic acid encoding human or bovine LA or functional homologues thereof.

In a preferred embodiment of the invention, LA or a functional homologue thereof is produced recombinantly by host cells.

Thus, in one aspect of the present invention, LA is produced by host cells comprising a first nucleic acid sequence encoding alpha-lactalbumin or a functional homologue thereof operably associated with a second nucleic acid capable of directing expression in said host cells. The second nucleic acid sequence may thus comprise or even consist of a promoter that will direct the expression of protein of interest in said cells. A skilled person will be readily capable of identifying useful second nucleic acid sequence for use in a given host cell.

The process of producing recombinant LA or a functional homologue thereof in general comprises the steps of:

- providing a host cell

- preparing a gene expression construct comprising a first nucleic acid encoding LA or a functional homologue thereof operably linked to a second nucleic acid capable of directing expression of said protein of interest in the host cell
-transforming the host cell with the construct,

cultivating the host cell, thereby obtaining expression of LA or the functional homologue thereof.

The composition comprising LA may thus be an extract of said host cells or a composition purified from an extract of said host cells and/or from the culture medium.

The recombinant LA thus produced may be isolated by any conventional method for example by any of the protein purification methods described herein above. The skilled person will be able to identify a suitable protein isolation steps for purifying any protein of interest.

In one embodiment of the invention, the recombinantly produced LA or the functional homologue thereof is excreted by the host cells.

When the LA or the functional homologue thereof is excreted the process of producing a recombinant protein of interest may comprise the following steps

- providing a host cell

-preparing a gene expression construct comprising a first nucleic acid encoding LA or a functional homologue thereof operably linked to a second nucleic acid capable of directing expression of said LA or functional homologue thereof in said host cell

-transforming said host cell with the construct,

cultivating the host cell in a culture medium, thereby obtaining expression of LA or the functional homologue thereof and secretion of the protein into the culture medium,

-thereby obtaining culture medium comprising LA or a functional homologue thereof.
The composition comprising LA or a functional homologue thereof may thus in this embodiment of the invention be the culture medium or a composition prepared from the culture medium.

In another embodiment of the invention said composition is an extract prepared from animals, parts thereof or cells or an isolated fraction of such an extract.

In a preferred embodiment of the invention, LA is recombinantly produced in vitro in host cells and is isolated from cell lysate, cell extract or from tissue culture supernatant. In a more preferred embodiment LA is produced by host cells that are modified in such a way that they express the protein of interest. In an even more preferred embodiment of the invention said host cells are transformed to produce and excrete LA.

Thus in a preferred embodiment, the LA preparation is preferably a recombinant preparation, wherein the LA preparation is obtained by:

- preparing a gene expression construct comprising a first nucleic acid encoding human or bovine alpha-lactalbumin peptide or a functional homologue thereof, operably linked to a second nucleic acid capable of directing expression in a host cell,

- transforming a host cell culture with the construct,

- cultivating the host cell culture in a culture medium, thereby obtaining expression and secretion of the polypeptide into the culture medium

- obtaining a composition comprising a variety of alpha-lactalbumin molecules and nucleic acids

In one embodiment the LA preparation is preferably a recombinant preparation, wherein the LA preparation is obtained by:

- preparing a gene expression construct comprising a first nucleic acid encoding human or bovine alpha-lactalbumin peptide or a functional homologue thereof,
operably linked to a second nucleic acid capable of directing expression in a host cell,

- transforming a host cell culture with the construct,

- cultivating the host cell culture either in vitro or in the form of a transgenic plant or animal thereby obtaining expression of LA

- obtaining a composition comprising a plurality of LA molecules and nucleic acids

According to the invention, the nucleic acid encoding alpha-lactalbumin may be derived from the human or bovine alpha-lactalbumin gene or from alpha-lactalbumin genes of other animal species as defined herein above.

In a preferred embodiment the gene expression construct is suitable for expression in mammalian cell lines or transgenic plants or animals. In one embodiment the host cell culture is cultured in a transgene animal. By a transgenic plant or animal in this context is meant a plant or animal which has been genetically modified to contain and express a nucleic acid encoding human or bovine alpha-lactalbumin or a functional homologue thereof as defined herein above.

In a preferred embodiment the gene expression construct of the present invention comprises a viral based vector, such as a DNA viral based vector, a RNA viral based vector, or a chimeric viral based vector. Examples of DNA viruses are cytomegalovirus, Herpex Simplex, Epstein-Barr virus, Simian virus 40, Bovine papillomavirus, Adeno-associated virus, Adenovirus, Vaccinia virus, and Baculo virus. However, the gene expression construct may for example only comprise a plasmid based vector.

In one aspect the invention provides an expression construct encoding human or bovine alpha-lactalbumin or functional homologues thereof, featured by comprising one or more intron sequences from the human or bovine human or bovine alpha-lactalbumin gene including functional derivatives hereof. Additionally, it may contain a promoter region derived from a viral gene or an eukaryotic gene, including mammalian and insect genes.
The promoter region is preferably selected to be different from the native human or bovine human or bovine alpha-lactalbumin promoter, and preferably in order to optimize the yield of human or bovine alpha-lactalbumin, the promoter region is selected to function most optimally with the vector and host cells in question.

In a preferred embodiment the promoter region is selected from a group comprising Rous sarcoma virus long terminal repeat promoter, and cytomegalovirus immediate-early promoter, and elongation factor-1 alpha promoter.

In another embodiment the promoter region is derived from a gene of a microorganism, such as other viruses, yeasts and bacteria.

In order to obtain a greater yield of recombinant LA or functional homologue thereof, the promoter region may comprise enhancer elements, such as the QBI SP163 element of the 5’ end untranslated region of the mouse vascular endothelial growth factor gene.

One process for producing recombinant LA according to the invention is characterised in that the host cell culture is may be eukaryotic, and for example a mammalian cell culture or a yeast cell culture.

Useful mammalian cells may for example be human embryonal kidney cells (HEK cells), such as the cell lines deposited at the American Type Culture Collection with the numbers CRL-1 573 and CRL-1 0852, chick embryo fibroblast, hamster ovary cells, baby hamster kidney cells, human cervical carcinoma cells, human melanoma cells, human kidney cells, human umbilical vascular endothelium cells, human brain endothelium cells, human oral cavity tumor cells, monkey kidney cells, mouse fibroblast, mouse kidney cells, mouse connective tissue cells, mouse oligodendritic cells, mouse macrophage, mouse fibroblast, mouse neuroblastoma cells, mouse pre-B cell, mouse B lymphoma cells, mouse plasmacytoma cells, mouse teratocacinoma cells, rat astrocytoma cells, rat mammary epithelium cells, COS, CHO, BHK, 293, VERO, HeLa, MDCK, WI38, and NIH 3T3 cells.
It is however preferred that the host cells are either prokaryotic cells or yeast cells. Prokaryotic cells may for example be *E. coli*. Yeast cells may for example be *Saccharomyces, Pichia or Hansenula.*

When recombinantly produced alpha-lactalbumin is used with the present invention it is preferred that said recombinantly produced alpha-lactalbumin has a size distribution profile that is similar to naturally occurring alpha-lactalbumin.

The aforementioned methods are well known to the skilled person and may for example be performed as described in the Current Protocols in Molecular Biology, 2001, by John Wiley and Sons, Inc. edited by Frederick M. Ausubel et al.

Recombinantly produced LA may for example be purified as described herein above in the section "Purification of Alpha-lactalbumin" and recombinantly produced LA may be used for preparing LAC, for example as described herein below.

**Method of producing alpha-lactalbumin complex**

LAC according to the present invention is an active complex pf alpha-lactalbumin and a fatty acid or lipid. Functional assays known to a skilled person can be used to verify the functional activity of alpha-lactalbumin in complex with a fatty acid or a lipid. Functional assays for evaluating alpha-lactalbumin function known to persons skilled in the art include, but are not limited to, assays described herein above and in examples 6 and 7, such as the cell killing assay or the histone assay.

The present invention in one embodiment regards the preparation of a biologically active LAC comprising:

a. alpha-lactalbumin of SEQ ID NO: 1 or SEQ ID NO: 2 or a functional homologue thereof comprising a sequence of at least 70% identical therewith (for example any of the functional homologues described herein above), and

b. a fatty acid or a lipid (as defined herein below),
said method comprising the steps of:

i. obtaining an alpha-lactalbumin composition comprising alpha-lactalbumin of SEQ ID NO: 1 or SEQ ID NO: 2 or a functional homologue thereof comprising a sequence of at least 70% identical thereof (as defined herein above),

ii. conversion of said alpha-lactalbumin or a functional homologue thereof to alpha-lactalbumin complex

1. by release of calcium from said alpha-lactalbumin or a functional homologue thereof and

2. binding of a fatty acid or a lipid to said alpha-lactalbumin or a functional homologue thereof

3. exposing the alpha-lactalbumin or a functional homologue thereof to an ion exchange medium

comprising a matrix comprising carbohydrate.

The steps 1, 2. and 3. may be performed sequentially in any order, or simultaneously, such as pairwise simultaneously or all three steps may be performed simultaneously.

In one preferred embodiment, the steps are performed sequentially in the order 1., 2. and 3. In another preferred embodiment the steps 1. and 2. are performed simultaneously and step 3 is performed subsequently. In yet another embodiment step 1 is performed first and steps 2. and 3. are performed simultaneously afterwards. In an even further embodiment steps 1. 2. and 3 are all performed simultaneously.

In one embodiment of the invention the conversion of LA or a functional homologue thereof to LAC, comprises as a first step release of calcium from LA or a functional homologue thereof, followed by binding of a fatty acid or a lipid, followed by exposure of LAC to an ion exchange medium, such as any of the ion exchange mediums described herein below. Release of calcium may be performed by any of the methods described herein below in the section "Release of Calcium", for example a calcium chelating agent may be added to LA or a functional homologue thereof thereby mediating the release of Ca$^{2+}$ from LA.
In another embodiment of the invention the conversion method comprises as a first step release of calcium from LA, followed by the simultaneous binding of a lipid or a fatty acid to LA and exposure of LAC to an ion exchange medium comprising a resin where the mean size of the particles of the resin is at least 80 μm, for example in the range of 80 μm to 120 μm. In this embodiment the ion exchange medium is frequently pre-conditioned with said fatty acid or lipid (see more details regarding preconditioning of a ion exchange medium herein below). In a preferred embodiment of the invention LA may be exposed to a calcium chelating agent and a fatty acid or a lipid simultaneously, followed by exposure of LAC to an ion exchange medium comprising a resin as described in more detail herein below.

In a particular embodiment of the invention the conversion the conversion of alpha-lactalbumin of SEQ ID NO: 1 or SEQ ID NO.2 or a functional homologue thereof comprising a sequence at least 70% identical therewith, to a biologically active lactalbumin complex with a fatty acid or a lipid comprise the steps of:

a. releasing calcium from said alpha-lactalbumin or a functional homologue thereof, comprising contacting said alpha-lactalbumin or a functional homologue thereof with a calcium chelating agent, thereby inducing alpha-lactalbumin to form a molten globule-like state

b. pre-conditioning an ion exchange column with a fatty acid or a lipid

c. binding said fatty acid or lipid to said molten globule-like alpha-lactalbumin by loading said molten globule-like lactalbumin on to an ion exchange medium comprising a matrix comprising carbohydrate.

Another embodiment of the invention relates to a method for the preparation of a biologically active lactalbumin complex comprising:

a. alpha-lactalbumin of SEQ ID NO: 1 or SEQ ID NO.2 or a functional homologue thereof comprising a sequence of at least 70% identical therewith (defined herein above) and

b. a fatty acid or a lipid (defined herein below)
i. obtaining an alpha-lactalbumin composition comprising
lactalbumin of SEQ ID NO: 1 or SEQ ID NO.2 or a functional
homologue thereof comprising a sequence of at least 70%
identical therewith,

ii. conversion of said alpha-lactalbumin or a functional homologue
thereof to alpha-lactalbumin complex

1. by mixing alpha-lactalbumin or a functional homologue
thereof and a fatty acid or a lipid in the absence of an ion
exchange medium, and subsequently

2. exposing the mixture to an ion exchange medium.

In a preferred version of this embodiment of the invention the method also comprise a
step of release of calcium from LA, which preferably is performed either before or
simultaneously with step 1. Thus, for example a calcium chelating agent may be added
to the alpha-lactalbumin or a functional homologue thereof before the addition of a fatty
acid or a lipid. In other equally preferred embodiments the calcium chelating agent is
added to the mixture of alpha-lactalbumin or a functional homologue thereof and a fatty
acid or a lipid after or simultaneous with the addition of a fatty acid or a lipid.

In this embodiment it is furthermore preferred that the ion exchange medium is not
preconditioned with oleic acid prior to exposing the ion exchange medium to the
mixture of alpha-lactalbumin and fatty acid and/or lipid. It is even more preferred that
the ion exchange medium is not preconditioned with fatty acid selected from the group
consisting of vaccenic acid and oleic acid prior to exposing the ion exchange medium
to the mixture of alpha-lactalbumin and fatty acid and/or lipid. It is even more preferred
that the ion exchange medium is not preconditioned with any fatty acid and/or lipid prior
to exposing the ion exchange medium to the mixture of alpha-lactalbumin and fatty acid
and/or lipid.

In an embodiment of the present invention the mixing of alpha-lactalbumin or a
functional homologue thereof, and a fatty acid or a lipid and optionally a calcium
chelating agent may be carried out for any suitable time period for example in the
range of 1 minute to 24 hours, such as in the range of 5 minutes to 12 hours, for
example in the range of 10 minutes to 6 hours, such as in the range of 15 minutes to 3
hours, for example in the range of 20 minutes to 1 hour, for example approximately 30 minutes.

In an embodiment of the present invention the mixing of alpha-lactalbumin or a functional homologue thereof, and a fatty acid or a lipid and optionally a calcium chelating agent may be carried at any suitable temperature for example in the range of 1°C to 40°C, such as in the range of 5°C to 35°C, for example in the range of 10°C to 30°C, such as in the range of 15°C to 28°C, for example in the range of 20°C to 25°C, such as around room temperature.

Frequently, the mixing of alpha-lactalbumin or a functional homologue thereof and a fatty acid or a lipid and optionally a calcium chelating agent is carried out in the presence of a buffer, for example at a pH in the range of 5 to 10, such as in the range of 6 to 9, for example in the range of 7 to 8, such as around 7.5. The buffer may be any buffer suitable for buffering to aforementioned pH, for example a Tris buffer.

**Release of calcium**

Release of calcium may be obtained by any suitable method known to the skilled person.

In one embodiment release of calcium may be achieved by contacting LA with a calcium chelating agent. The calcium chelating agent may be selected from the group of calcium chelators comprising, but not limited to, 1,2-Bis(2-aminophenoxy)ethane-$\Lambda_\Lambda_\Lambda_\Lambda$-$\lambda_\lambda_\lambda_\lambda$-tetraacetic acid (BAPTA) or Ethylene glycol-bis(amineylether)-$\Lambda_\Lambda_\Lambda_\Lambda$-$\lambda_\lambda_\lambda_\lambda$-tetraacetic (EGTA) or Ethylene diamine tetraacetic acid (EDTA). In a very preferred embodiment of the invention the calcium chelator is Ethylene diamine tetraacetic acid (EDTA).

In an embodiment a calcium chelating agent is added in molar excess over alpha-lactalbumin or a functional homologue thereof. Molar excess means that there are more moles of a calcium chelating agent than there is of alpha-lactalbumin. The molar excess of a calcium chelating agent over alpha-lactalbumin or a functional homologue thereof may be that for one mole alpha-lactalbumin at least two moles of a calcium
chelating agent are added, for example for one mole alpha-lactalbumin at least three moles of a calcium chelating agent are added, such as for one mole alpha-lactalbumin at least five moles of a calcium chelating agent are added, for example for one mole alpha-lactalbumin at least seven moles of a calcium chelating agent are added, such as for one mole alpha-lactalbumin at least ten moles of a calcium chelating agent are added, for example for one mole alpha-lactalbumin at least 15 to 20 moles of a calcium chelating agent are added, such as in the range of 2 to 30 moles, for example in the range of 5 to 30 moles, such as in the range of 10 to 30 moles, for example in the range of 15 to 20 moles of calcium chelating agent are added.

In one preferred embodiment the calcium chelating agent is ethylene diamine tetraacetic acid and said EDTA is added in molar excess over alpha-lactalbumin or a functional homologue thereof. Molar excess means that there are more moles of ethylene diamine tetraacetic acid than there is of alpha-lactalbumin. The molar excess of ethylene diamine tetraacetic acid over alpha-lactalbumin or a functional homologue thereof may be that for one mole alpha-lactalbumin at least two moles of ethylene diamine tetraacetic acid are added, for example, for one mole alpha-lactalbumin at least three moles of ethylene diamine tetraacetic acid are added, such as for one mole alpha-lactalbumin at least five moles of ethylene diamine tetraacetic acid are added, for example for one mole alpha-lactalbumin at least seven moles of ethylene diamine tetraacetic acid are added, for example for one mole alpha-lactalbumin at least ten moles of ethylene diamine tetraacetic acid are added, for example for one mole alpha-lactalbumin at least 15 to 20 moles of ethylene diamine tetraacetic acid are added.

In a very preferred embodiment the molar excess of ethylene diamine tetraacetic acid over alpha-lactalbumin corresponds to a 15 to 20 fold molar excess. Thus, in a preferred embodiment of the invention the concentration of ethylene diamine tetraacetic acid is in the range of 0.01 mM to 500 mM, for example in the range of 0.05 mM to 50 mM, such as in the range of 0.1 mM to 40 mM, for example in the range of 0.2 mM to 25 mM, such as in the range of 0.5 mM to 10 mM, preferably in the range of 0.75 mM to 5 mM, such as around 1 mM.

In a special embodiment of the invention release of calcium is obtained by using a functional homologue of alpha-lactalbumin, wherein the calcium binding site has been modified in a manner that reduces the ability of said functional homologue of alpha
lactalbumin to bind calcium. In particular, the amino acids of the calcium-binding site (K79, D82, D84, D87 and D88) may be modified so that the affinity for calcium is reduced, or it is no longer functional. In this special embodiment the step involving the release of calcium from LA is obsolete and the conversion of LA to LAC comprises of the binding of a fatty acid or a lipid to LA with either simultaneous or subsequent exposure to an anion exchange medium.

**Preconditioning of ion exchange medium**

In some embodiments of the present invention the ion exchange medium is preconditioned with a fatty acid or lipid, for example any of the fatty acids or lipids described herein below in the section "Fatty Acid".

Preconditioning may be performed by any conventional method, for example as described in Svensson, et al., (2000) Proc Natl Acad Sci USA, 97,4221-6, WO 03/098223 or WO 2005/082406.

In general, pre-conditioning is performed by adding one or more fatty acids or lipids to the ion exchange medium, for example the fatty acids or lipids may be added in an amount corresponding to in the range of 1 to 30, such as in the range of 2 to 20, for example in the range of 4 to 12, such as in the range of 6 to 10, for example in the range of 7 to 9, such as approximately 8 mg/cm² ion exchange resin. Thus, for example in the range of 1 to 30, such as in the range of 2 to 20, such as in the range of 3 to 10, for example in the range 5 to 8, such as in the range of 6 to 7, for example approximately 6.4 ml fatty acid or lipid 8 (for example oleic acid or vaccenic acid) may be added per 8 ml column material.

After addition of fatty acid or lipid to the ion exchange material then the column may be washed, using any suitable wash solution, preferably at least one column volume (CV) wash solution, for example in the range of 2 to 5 CV. The wash solution may comprise suitable buffer and optionally salt, such as in the range pf 0.001 to 0.2M salt, such as around 0.1 M salt (for example NaCl) for example the wash buffer may be the equilibration buffer described in Example 2 herein below.
The method may also comprise an isocratic step, for example a step of washing with elution buffer. Preferably, at least one column volume (CV) is used for washing, such as in the range of 2 to 5 CV. The elution buffer may be any suitable elution buffer, which in general comprises a buffer and salt, preferably at least 0.1 M, such as at least 0.5M for example around 1M salt (for example NaCl), such as solvent B described herein below in Example 2.

Following an isocratic step an additional wash with a washing solution may be performed as described above.

In one embodiment the preconditioning is performed essentially as described in Example 2 herein below.

**Ion exchange chromatography**

The present invention regards a method for the preparation of active alpha-lactalbumin complex i.e. alpha-lactalbumin in complex with a fatty acid or a lipid, wherein one step is exposing alpha-lactalbumin or a mixture of alpha-lactalbumin and a fatty acid or a lipid to an ion exchange medium.

Ion exchange chromatography is one of the most frequently used techniques for purification of proteins, peptides, nucleic acids and other charged biomolecules. The technique is capable of separating molecular species that have only minor differences in their charge properties.

Ion exchange chromatography separates molecules on the basis of differences in their net surface charge. Molecules vary considerably in their charge properties and will exhibit different degrees of interaction with charged chromatography media according to differences in their overall charge, charge density and surface charge distribution.

Since all molecules with ionizable groups can be titrated, their net surface charge is highly pH dependent. In the case of proteins, which are built up of many different amino acids containing weak acidic and basic groups, their net surface charge will change gradually as the pH of the environment changes i.e. proteins are amphoteric.
Ion exchange chromatography takes advantage of the fact that the relationship between net surface charge and pH is unique for a specific protein. In ion exchange chromatography separation, reversible interactions between charged molecules and oppositely charged ion exchange chromatography media are controlled in order to favour binding or elution of specific molecules and achieve separation. A protein that has no net charge at a pH equivalent to its isoelectric point (pI) will not interact with a charged medium. However, at a pH above its isoelectric point, a protein will bind to a positively charged medium or anion exchanger and, at a pH below its pi, a protein will behind to a negatively charged medium or cation exchanger.

An ion exchange medium comprises a matrix of spherical particles substituted with ionic groups that are negatively (anionic) or positively (cationic) charged. The matrix is usually porous to give a high internal surface area. The medium is packed into a column to form a packed bed. The bed is then equilibrated with buffer which fills the pores of the matrix and the space in between the particles.

The pH and ionic strength of the equilibration buffer are selected to ensure that, when sample is loaded, proteins of interest bind to the medium and as many impurities as possible do not bind. The proteins which bind are effectively concentrated onto the column while proteins that do not have the correct surface charge pass through the column at the same speed as the flow of buffer, eluting during or just after sample application, depending on the total volume of sample being loaded.

When the entire sample has been loaded and the column washed so that all non-binding proteins have passed through the column (i.e. the UV signal has returned to baseline), conditions are altered in order to elute the bound proteins. Most frequently, proteins are eluted by increasing the ionic strength (salt concentration) of the buffer or, occasionally, by changing the pH. As ionic strength increases, the salt ions (typically Na+ or Cl-) compete with the bound components for charges on the surface of the medium and one or more of the bound species begin to elute and move down the column. The proteins with the lowest net charge at the selected pH will be the first ones eluted from the column as ionic strength increases.

Similarly, the proteins with the highest charge at a certain pH will be most strongly retained and will be eluted last. The higher the net charge of the protein, the higher the
ionic strength that is needed for elution. By controlling changes in ionic strength using different forms of gradient, proteins are eluted differentially in a purified, concentrated form. A wash step in very high ionic strength buffer removes most tightly bound proteins at the end of an elution. The column is then re-equilibrated in start buffer before applying more sample in the next run. Alternatively, conditions can be chosen to maximize the binding of contaminants and allow the target protein(s) to pass through the column thus removing contaminants.

Matrix

The term "matrix" as used herein in relation to ion exchange chromatography relates to the material of the resin without ion exchange ligand. Thus, the matrix is the base material to which different ion exchange ligands may be bound. By way of example, the matrix of a Q Sepharose Fast Flow is Sepharose Fast Flow.

Similarly, the term "ligand" as used herein in relation to ion exchange chromatography relates to an ion exchange group coupled to a given matrix. Thus, the ligand is main responsible for the ion exchange properties of a given resin. By way of example, the ligand of a Q Sepharose Fast Flow is the quaternary ammonium ion Q (see below).

A high porosity of a matrix offers a large surface area covered by charged groups and so ensures a high binding capacity. High porosity is may also an advantage when separating large biomolecules.

In a preferred aspect the invention relates to a method for preparing LAC using an ion exchange medium comprising a matrix which is carbohydrate based. Preferably, said matrix comprises carbohydrate.

The matrix is preferably a polymer of residues, wherein at least some residues are monosaccharide residues, for example at least 25%, such as at least 50%, for example at least 75%, such as at least 95%, for example essentially all, preferably all residues are monosaccharide residues.

The monosaccharide residues may for example be aldose or ketose residues or derivatives thereof, such as derivatives obtained by oxidation, deoxygenation,
dehydration, introduction of other substituents, alkylation or acylation of hydroxygroups. Preferably, the monosaccharide residues are aldose or ketose residues.

In a preferred embodiment at least some of the monosaccharide residues are galactose or glucose residues or galactose derived residues, such as galactopyranose or anhydrogalactopyranose. Thus, for example at least 25%, such as at least 50%, for example at least 75%, such as at least 95%, for example essentially all, for example all residues are galactose or glucose residues. Thus, for example the matrix may be selected from the group consisting of cellulose (such as sephacel), dextran (such as sephadex) and agarose based matrices such as sepharose and mixtures of the aforementioned such as Capto, which is based on dextran and agarose. Preferably, the matrix is selected from the group consisting of agarose based matrices such as sepharose and matrices based on mixtures of agarose and dextran, such as Capto.

In another preferred embodiment for example at least 25%, such as at least 50%, for example at least 75%, such as at least 95%, for example essentially all, for example all residues are galactose residues or galactose derived residues, preferably galactopyranose and/or anhydrogalactopyranose. Thus, for example the matrix may be agarose or derived from agarose, for example the matrix may be sepharose.

Sepharose media are based on chains of agarose, arranged in bundles and with different degrees of intra-chain cross-linking.

The term "essentially all" as used above is meant to mean "all detectable".

In one embodiment it is preferred that the matrix is not a Trisacryl matrix. It is also preferred that the matrix is not polystyrene. It is also preferred that the matrix is not polystyrene divinyl benzene. It is also preferred that the matrix is not an acryl amide. It is also preferred that the matrix is not ceramic and/or coated with a ceramic material.

Examples of anion exchangers functional group
Quaternary ammonium (Q) strong -O-CH$_2$N+(CH$_3$)$_3$
Diethylaminoethyl (DEAE)* weak -O-CH$_2$CH$_2$N+H(CH$_2$CH$_3$)$_2$
Diethylaminopropyl (ANX)* weak -O-CH$_2$OHCH$_2$N+H(CH$_2$CH$_3$)$_2$
In preferred embodiments of the present invention the ion exchange medium is an anion exchange resin. In a more preferred embodiment the ion exchange medium is a strong anion exchanger. In an even more preferred the ion exchange medium is a strong Quaternary ammonium (Q) based resin.

Particle size is a significant factor in resolution and, in general, the smallest particles will produce the narrowest peaks under the correct elution conditions and in a well-packed column. Although resolution in terms of efficiency can be improved by decreasing the particle size of the matrix, using a smaller particle size often creates an increase in back pressure so that flow rates need to be decreased, lengthening the run time. Thus, the optimal particle size must in general be determined for each individual purification scheme depending on the compound to be purified.

Several different mean particle sizes may be useful for purification of a given compound. Thus, it is possible that for example very small and very large particles may both be useful in the purification of a given compound, whereas particles of an intermediary size may be less useful. It is also possible that only particles of a particular size range are useful or particles with a particular minimum and/or maximum are useful.

<table>
<thead>
<tr>
<th>Particle sizes of various anion exchange columns</th>
<th>Mean particle size</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE Trisacryl M (Pall BioSepra)</td>
<td>40-80 µm</td>
<td></td>
</tr>
<tr>
<td>DEAE Trisacryl Plus M (Pall BioSepra)</td>
<td>40-80 µm</td>
<td></td>
</tr>
<tr>
<td>DEAE Ceramic HyperD F (Pall BioSepra)</td>
<td>50 µm</td>
<td></td>
</tr>
<tr>
<td>Capto Q (GE Healthcare)</td>
<td>90 µm</td>
<td>45-165</td>
</tr>
<tr>
<td>DEAE Sepharose Fast Flow (GE Healthcare)</td>
<td>90 µm</td>
<td>45-165</td>
</tr>
<tr>
<td>Q Sepharose XL (GE Healthcare)</td>
<td>90 µm</td>
<td></td>
</tr>
<tr>
<td>Source 30Q</td>
<td>30 µm</td>
<td></td>
</tr>
<tr>
<td>Unosphere Q (BioRad)</td>
<td>120 µm</td>
<td></td>
</tr>
</tbody>
</table>

In the prior art (e.g. Svensson, et al. (2000) Proc Natl Acad Sci USA, 97,4221-6, WO 03/098223, WO 2005/082406,) the weak anion exchanger DEAE Trisacryl where the particles of the resin are in the range of 40-80 µm and with which a maximum
recommended working flow rate of 25-80 cm/h has been the resin of choice for preparation of LAC.

However, surprisingly the present invention discloses that in one embodiment it may be preferable to use ion exchange resins with a larger mean particle size for the preparation of LAC.

In an embodiment of the present invention, the ion exchange medium comprises a resin, comprising particles with a mean size of at least 80 μm, such as a resin wherein the mean size of the particles are at least 85 μm, for example at least 90 μm, for example in the range of 80 to 300 μm, such as in the range of 80 to 200 μm, for example in the range of 80 to 150 μm, such as in the range of 80 μm to 120 μm, for example in the range of 90 to 120 μm, such as in the range of 90 μm to 100 μm, such as in the range of 85 μm to 120 μm, for example in the range of 87 μm to 95 μM, such as around 90 μm. In a very preferred embodiment of the invention the mean size of the particles of the ion exchange resin is 90 μm.

In another embodiment of the present invention, the ion exchange medium comprises a resin, comprising particles with a mean size of at least 80 μm, such as a resin wherein the mean size of the particles are at least 85 μm, for example at least 90 μm, for example in the range of 80 to 300 μm, such as in the range of 80 to 200 μm, for example in the range of 80 to 150 μm, such as in the range of 90 μm to 140 μm, for example in the range of 100 to 130 μm, for example 110 μm to 128 μm, such as in the range of 115 μm to 125 μm, for example in the range of 118 μm to 123 μM, such as around 120 μm. In a very preferred embodiment of the invention the mean size of the particles of the ion exchange resin is 120 μm.

In another embodiment of the invention it is disclosed that it may be preferably to use ion exchange resins with a smaller mean particle size for preparation of LAC. In particular, a resin with a mean particle size smaller than 40 μm, for example in the range of 1 to 29 μm or in the range of 31 to 40 μm may be useful.

In a very preferred embodiment of the invention, the ion exchange medium comprises Capto Q sepharose. In an equally preferred embodiment of the invention, the ion exchange medium comprises Q Sepharose XL resin.
In one embodiment of the invention the ion exchange medium has been pre-conditioned with a fatty acid or a lipid as described herein below. In an equally preferred embodiment the ion exchange medium has not been pre-conditioned with a fatty acid or a lipid.

In one embodiment where the ion exchange medium has not been pre-conditioned with a fatty acid or a lipid; Unosphere Q is another preferred ion exchange medium.

Flow rates
High physical stability and uniformity of particle size facilitate high flow rates, particularly during cleaning or re-equilibration steps, to improve throughput and productivity. High chemical stability ensures that the matrix can be cleaned using stringent cleaning solutions if required.

The maximum flow rate applied during a separation can vary according to the stage of the separation. For example, during sample application and elution, lower flow rates allow time for sample components to diffuse in and out of the pores as they to bind to or dissociate from the functional groups.

Flow rate can be measured in simple volume terms, e.g. ml/min, but when comparing results between columns of different sizes or when scaling-up, it is useful to use linear flow: cm/hour.

The present invention discloses that it may be advantageous to use an ion exchange medium with a high recommended working flow rate for preparation of LAC.

Thus, in a preferred embodiment of the invention the ion exchange medium comprises a resin with a recommended maximum working flow rate of at least 80 cm/h, such as a recommended maximum working flow rate of at least 90 cm/h, for example a recommended maximum working flow rate of at least 100 cm/h, for example a recommended maximum working flow rate of at least 150 cm/h, such as a recommended maximum working flow rate of at least 200 cm/h such as a recommended maximum working flow rate of at least 250 cm/h, such as a recommended maximum working flow rate of at least 300 cm/h, such as a
recommended maximum working flow rate of at least 350 cm/h, for example a
recommended maximum working flow rate of at least 400 cm/h, such as a
recommended maximum working flow rate of at least 450 cm/h, for example a
recommended maximum working flow rate of at least 500 cm/h.

Regardless of the recommended maximum working flow rate of the resin used, it is
frequently preferred within the present invention to use an actual flow rate in the range
of 5 to 1000 cm/h, preferably in the range 5 to 500 cm/h, such as in the range of 5 to
250 cm/h, for example in the range of 10 to 100 cm/h, such as in the range of 10 to 60
cm/h, for example in the range og 15 to 40 cm/h for preparation of LAC.

Elution
Gradient elution is often used when starting with an unknown sample (as many
components as possible are bound to the column and eluted differentially to see a total
protein profile) and for high resolution separation or analysis. In some embodiments of
the present invention gradient elution may be used to elute LAC from an ion exchange
medium.

Step elution may be used in several ways. When an ion exchange separation has been
optimized using gradient elution, changing to a step elution speeds up separation times
and reduces buffer consumption while retaining the required purity level. Step elution
can also be used for group separation in order to concentrate the proteins of interest
and rapidly remove them from unwanted substances.

In one embodiment of the present invention a step gradient elution is used to elute
lactalbumin complex and lactalbumin or a functional homologue thereof separately,
from the ion exchange medium. In an embodiment step one of the step gradient may
be in the range of 30 to 60% of buffer, for example 35 to 55% of buffer, such as 40 to
50% of buffer, for example around 45% of buffer. In an embodiment step two of the
step gradient may be in the range of 61 to 80% of buffer, for example 65 to 75% of
buffer, such as around 70% of buffer. In an embodiment step three of the step gradient
may be at least 81% of buffer, for example at least 85% of buffer, such as at least 90%
of buffer, for example at least 95% of buffer, such as at least 99% of buffer, for
example around 100% of buffer.
In a very preferred embodiment of the invention the step gradient comprises steps of 45, 70 and 100% of buffer.

Said afore-mentioned buffer may be any buffer suitable for eluting LAC from an ion exchange medium. For example, said buffer may comprise:

a) in the range of 100 mM to 10 M salt, such as in the range of 100 mM to 5M salt, for example in the range of 500 mM to 2 M salt, such as approximately 1 M salt, wherein said salt may be any suitable salt, such as KCl, NaCl, MgCl₂, CaCl₂, preferably NaCl; and

b) a buffer capable of buffering to a pH in the range of 5 to 10, preferably in the range of 6 to 9, such as in the range of 8 to 9, for example around 8.5, wherein said buffer for example may be a TRIS buffer, for example at a concentration of 1 mM to 1M, for example around 10 mM.

In one embodiment where the ion exchange medium has not been pre-conditioned with a fatty acid or a lipid and Unosphere Q is used as the ion exchange medium it is preferred to use gradient elution.

Load/yield

One of advantages of the present invention is that using the methods of the present invention is that it is possible to increase both the load of alpha-lactalbumin and the yield of active alpha-lactalbumin complex, compared to the methods described in the art. The relationship between load and yield must be well balanced, if the load is too high the yield will generally decrease, in particular this is the case for the methods described in the prior art.

In the following the yield is generally indicated as % yield, wherein the % yield indicates the % of LAC obtained compared to the input of alpha-lactalbumin. Thus, loading a column with for example 90 mg/cm² alpha-lactalbumin and obtaining 76 mg/cm² LAC indicates a yield of 84%.

In a preferred embodiment of the present invention the column is loaded with more than 1,5 mg alpha-lactalbumin/cm² ion exchange medium, such as at least 5 mg/cm², for example more than 10 mg/cm², such as at least 15 mg/cm², for example more than
20 mg/cm², such as with more than 22 mg/cm², for example at least 25 mg/cm², for example in the range of 23 to 27 mg alpha-lactalbumin/cm² ion exchange medium. In an embodiment of the present invention the yield of alpha-lactalbumin complex using aforementioned load is at least 50%, such as at least 55%, for example more than 60%, such as at least 65%, for example more than 70%, such as at least 75%, for example more than 80%. Thus, in one preferred embodiment of the invention the yield is preferably at least 50%, preferably at least 60%, more preferably at least 70%, even more preferably at least 75%, yet more preferably at least 80%, when the load is 25 mg alpha-lactalbumin/cm² ion exchange medium. It is also preferred that the yield is at least 75%, such as at least 80%, when the load is 20 mg alpha-lactalbumin/cm² ion exchange medium. Thus, a preferred ion exchange medium according to the invention is a material wherein aforementioned yields may be achieved.

Above-mentioned paragraph is in particular relevant in embodiments of the invention wherein the methods comprise preconditioning an ion exchange material prior to addition of alpha-lactalbumin to said ion exchange medium.

In another, equally preferred embodiment of the present invention, the column may be loaded with more than 20 mg alpha-lactalbumin/cm² ion exchange medium, such as at least 30 mg/cm², for example more than 40 mg/cm², such as at least 50 mg/cm², for example more than 60 mg/cm², such as at least 70 mg/cm², for example more than 80 mg/cm², such as at least 90 mg alpha-lactalbumin/cm² ion exchange medium. In an embodiment of the present invention the yield of lactalbumin complex with aforementioned load is at least 50%, such as at least 55%, for example more than 60%, such as at least 65%, for example more than 70%, such as at least 75%, for example more than 80%. Thus, in one preferred embodiment the yield is at least 60%, preferably at least 70%, for example at least 75, such as at least 80%, when the load is 30 mg alpha-lactalbumin/cm² ion exchange medium. It is also preferred that the yield is at least 60%, preferably at least 70%, for example at least 75, such as at least 80%, for example at least 90%, when the load is 42 mg alpha-lactalbumin/cm² ion exchange medium. In another embodiment it is preferred that the yield is at least 20%, preferably at least 30%, such as at least 60%, preferably at least 70%, for example at least 75, such as at least 80%, when the load is 90 mg alpha-lactalbumin/cm² ion exchange medium.
In another preferred embodiment of the present invention the column is loaded with more than 1.5 mg alpha-lactalbumin/cm² ion exchange medium, such as at least 5 mg/cm², for example more than 10 mg/cm², such as at least 15 mg/cm², for example more than 20 mg/cm², such as with more than 22 mg/cm², for example at least 25 mg/cm², for example in the range of 25 to 150 mg alpha-lactalbumin/cm² ion exchange medium, such as in the range of 30 to 100 mg alpha-lactalbumin/cm² ion exchange medium, preferably 34 to 54 mg alpha-lactalbumin/cm² ion exchange medium.

In an embodiment of the present invention the yield of alpha-lactalbumin complex using aforementioned load is in the range in the range of 35 to 100%, such as at least 40%, for example at least 50%, such as at least 55%, for example more than 60%, such as at least 65%, for example more than 70%, such as at least 75%, for example more than 80%, such as more than 90% depending on the fatty acid or lipid used. Thus, a preferred ion exchange medium according to the invention is a material wherein aforementioned yields may be achieved.

In some embodiments where for example Oleic acid, Vaccenic acid, Linoleic acid or alfa Linolenic acid it is preferred that the yield is at least 60%, preferably at least 70%, for example at least 75%, when the load is 54 mg alpha-lactalbumin/cm² ion exchange medium.

In some embodiments where for example Palmitoleic acid, Eicosapentaenoic acid, Heptadecenoic acid, gamma Linolenic acid, Gondoic acid, Stearidonic acid or Oleic acid have been used in the conversion, it is preferred that the yield is at least 45%, preferably at least 50%, for example at least 55%, such as at least 60%, for example at least 65% preferably at least 70%, for example at least 75%, such as at least 80%, for example at least 90%, preferably 95% when the load is 44 mg alpha-lactalbumin/cm² ion exchange medium.

Above-mentioned paragraph is in particular relevant in embodiments of the invention wherein the methods comprise mixing fatty acid or lipid with alpha-lactalbumin prior to exposing the mixture to an ion exchange medium.

Regeneration
In one embodiment of the invention the method may include a regeneration procedure. The regeneration procedure may follow the exposure of the mixture of lactalbumin, and fatty acid or lipid and optionally calcium chelating agent to an ion exchange medium. Alternatively, the regeneration procedure may be performed prior to the conversion of LA to LAC, thus allowing reuse of a used column. In a preferred embodiment, the regeneration procedure comprises sequential CIP (cleaning in place) with acetic acid, sodium hydroxide and ethanol 70%. In an even more preferred embodiment, the regeneration procedure comprise applying increasing and decreasing ethanol concentrations in steps to avoid air bubble formation.

It is preferred that the regeneration does comprise cleaning with more than one of the aforementioned. In particular, it is preferred that the regeneration procedure does not only consist of regeneration with acetic acid.

**Fatty acids**

The present invention regards new methods for the production of biologically active LAC; an alpha-lactalbumin complex of bovine or human alpha-lactalbumin of SEQ ID NO.1 or SEQ ID NO:2 or a functional equivalent there of with a lipid or a fatty acid.

In preferred embodiments of the present invention alpha-lactalbumin is complexed with a fatty acid.

Fatty acids are carboxylic acids, which often have a long unbranched aliphatic chain. As the biosynthesis of fatty acids involves acetyl-CoA, in which the acetic unit contains two C-atoms, most natural fatty acids have an even number of C atoms ranging from 4 to 80 C atoms. The aliphatic chain of a fatty acid can be either saturated or unsaturated. Saturated fatty acids are saturated with hydrogen and thus have no double bonds. Unsaturated fatty acids can be either mono-unsaturated (or MUFAs), having one double bond or poly-unsaturated (PUFAs), having 2 or more double bonds. The fatty acids of the present invention may be a saturated fatty acid or an unsaturated fatty acid.
In preferred embodiments of the invention the fatty acid is selected from the group of C4 to C30, for example from C6 to C28, such as from C8 to C26, for example from C10 to C24, such as from C12 to C22, for example from C14 to C20, such as from C16 to C20, for example from the group of C16, C17, C18 and C20., such as from the group of C16, C18 and C20. Even more preferred fatty acid is selected from the group of C16, C17, C18 and C20.

Fatty acids are often described using the number of C-atoms of the chain and the number, location and conformation of double bonds. Steric acid, for example, has a chain of 18 C-atoms and no double bonds and can be described as C18:0, oleic acid has a chain of 18 C-atoms and one double bond and can be described as C18:1, linoleic acid has a chain of 18 C-atoms and two double bonds and can be described as C18:2 and so forth.

The double bond is located on the xth carbon-carbon bond, counting from the carboxyl terminus. The Latin prefixes cis (on this side) and trans (across) describe the conformation of the double bonds by describing the orientation of the hydrogen atoms with respect to said double bond. Double bonds in the cis conformation are preferred. The position of the double bond is frequently indicated as the last number, following the integer indicating the number of double bonds. Thus, for example oleic acid having a 18 carbon chain with one double bond between carbon 9 and 10 may be described as C18:1 :9cis and α-linolenic acid having a 18 carbon chain with three double bonds between carbon 9 and 10, 12 and 13 and 15 and 16, respectively, may be described as C18:3:9,12,15. Cis or trans may be indicated after the position of the double bond. If there is more than one double bond and they all are of the same conformation, then the term cis or trans may be indicated after indication of the position of all double bonds and thus relates to the conformation of all double bonds. Thus, for example Linoleic acid having a 18 carbon chain with 2 double bonds, which are both cis double bonds between carbons 9 and 10 and 12 and 13, respectively may be described as C18:2:9,12cis

In preferred embodiments of the present invention the fatty acid has in the range of 0 to 6 double bonds, for example in the range of 1 to 5 double bonds, such as the number of double bonds is selected from the group of 1, 2, 3 or 4 double bonds. In more
preferred embodiments of the invention the fatty acid has 1 or 3 double bonds. In a most preferred embodiment of the invention the fatty acid has one double bond.

Examples of saturated fatty acids are:

Butyric (butanoic acid): CH3(CH2)2COOH or C4:0
Caproic (hexanoic acid): CH3(CH2)4COOH or C6:0
Caprylic (octanoic acid): CH3(CH2)6COOH or C8:0

Capric (decanoic acid): CH3(CH2)8COOH or C10:0
Laurie (dodecanoic acid): CH3(CH2)10 COOH or C12:0
Myristic (tetradecanoic acid): CH3(CH2)12 COOH or C14:0
Palmitic (hexadecanoic acid): CH3(CH2)14COOH or C16:0
Stearic (octadecanoic acid): CH3(CH2)16COOH or C18:0

Arachidic (eicosanoic acid): CH3(CH2)18 COOH or C20:0
Behenic (docosanoic acid): CH3(CH2)20COOH or C22:0

Unsaturated fatty acids are preferred for the present invention.

Examples of unsaturated fatty acids that may be used with the invention includes for example:

Oleic acid: CH3(CH2)7CH=CH(CH2)7COOH or C18:1 :9cis
Elaidic acid: CH3(CH2)7CH==CH(CH2)7COOH or C18:1 :9trans

Linoleic acid: CH3(CH2)4CH=CH2CH=CH(CH2)7COOH or C 18:2:9,1 2cis
Alpha-linolenic acid:
CH3CH2CH=CHCH2CH=CHCH2CH=CH(CH2)7COOH or C18:3:6,9, 12, 15 cis

Gamma linolenic acid: C 18:3:6,9, 12cis
Arachidonic acid:
CH3(CH2)4CH=CHCH2CH=CHCH2CH=CH(CH2)3COOH or C20:4:5, 8,1 1 ,14cis

Eicosapentaenoic acid:
CH3CH2CH=CHCH2CH=CHCH2CH=CHCH2CH=CH(CH2)3COOH or C20:5: 5,8,1 1,14,17Cis
Docosahexaenoic acid:
CH₃CH₂CH=CHCH₂CH=CHCH₂CH=CHCH₂CH=CHCH₂CH=CH(CH₂)₂COO
H or C22:6 4,7,10,13,16,19 9Cis
Erucic acid: CH₃(CH₂)₇CH=CH(CH₂)₉COOH or C22:1
5 Vaccenic acid: CH₃(CH₂)₅CH=CH(CH₂)₉COOH or C18:1:1cis
Palmitoleic acid: CH₃(CH₂)₉CH=CH(CH₂)₇COOH or 6:1:9cis
Petroselinic acid: CH₃(CH₂)₁₀0.CH=CH(CH₂)₉COOH or C18:1:6cis
Stearidonic acid: C18:4:6, 9, 12, 15cis
Heptadecenoic Acid: C17:1:1cis
10 Eicosenoic Acid: · · · N , · · ·, · · ·, · · ·, V , · · ·, · · ·, or 20:1:1cis
Gondoic acid or C11-EICOSENOIC ACID: CH₃(CH₂)₁₀0.CH=CH(CH₂)₉COOH or C29:1:9cis

In an embodiment a mono-saturated fatty acid is complexed with alpha-lactalbumin.

More preferred are mono-saturated fatty acids selected from the group of: C17:1:1cis or trans, C16:1:6cis or trans, C16:1:9cis or trans, C16:1:1cis or trans, C18:1:6cis or trans, C18:1:9cis or trans, C18:1:1cis or trans, C18:1:13cis or trans, C20:1:9cis or trans, C20:1:1cis and trans, C20:1:13cis or trans.

In a preferred embodiment the mono-saturated fatty acid complexed with alpha-lactalbumin is in the cis conformation such a fatty acid selected from the group of:

In another preferred embodiment the fatty acid complexed with alpha-lactalbumin is an unsaturated fatty acid in the cis conformation, preferably selected from the group consisting of C17:1:1cis, C18:1:9cis, C18:1:1cis, C18:1:6cis, C16:1:9cis, C18:3:6,9,12cis, C18:3:9,12,15cis, C18:2:9,12cis.

In another preferred embodiment the fatty acid complexed with alpha-lactalbumin is selected from the group consisting of C16 to C20 fatty acids comprising in the range of 1 to 5 cis double bonds. Thus, the fatty acid may for example be selected from the group consisting of Vaccenic Acid C18:1:1cis, Linoleic Acid C18:2:9,12cis, Alpha Linolenic Acid C18:3:9,12cis, Palmitoleic Acid C16:1:9cis, Heptadecenoic Acid C17:1:1cis, Gamma Linolenic Acid C18:3:6,9,12cis, Stearidonic acid C18:4:6, 9, 12, 15cis.
C18:4:6,9,12,15cis, Eicosenoic Acid C20:1:11cis and Eicosapentaenoic Acid C20:5:5,8,11,14,17cis, such as from the group consisting of Vaccenic Acid C18:1:11cis, Linoleic Acid C18:2:9,12cis, Alpha Linolenic Acid C18:3:9,12,15cis, and C18:3:9,12,15cis, such as from the group consisting of Linoleic Acid C18:2:9,12cis, Alpha Linolenic Acid C18:3:9,12,15cis, and C20:5:5,8,11,14,17cis, such as from the group consisting of Eicosapentaenoic Acid C20:5:5,8,11,14,17cis.

In a very preferred embodiment of the invention the fatty acid complexed with alpha-lactalbumin is an unsaturated C16 or C18 fatty acid, preferably a C18 fatty acid, wherein all double bonds are cis double bonds. In this embodiment the fatty acid may preferably comprise 1, for example 2, such as 3, for example 4 double bonds, wherein all double bonds are cis double bonds. Thus, the fatty acid may for example be selected from the group consisting of C18:1:9cis, C18:1:11cis, C18:1:6cis, C16:1:9cis, C18:3:6,9,12cis, C18:3:9,12,15cis, C18:2:9,12cis and C18:4:6,9,12,15cis, preferably selected from the group consisting of C18:1:9cis, C18:1:11cis, C18:1:6cis, C18:3:6,9,12cis, C18:3:9,12,15cis, C18:2:9,12cis and C18:4:6,9,12,15cis, for example selected from the group consisting of C18:1:9cis, C18:1:11cis, C18:3:6,9,12,15cis and C18:2:9,12cis.

In another very preferred embodiment of the invention the fatty acid complexed with alpha-lactalbumin is an unsaturated C16, C17 or C18 fatty acid with no more than three unsaturated bonds.

In another preferred embodiment of the invention the fatty acid complexed with alpha-lactalbumin is an unsaturated C17 fatty acid, preferably C17:1:10cis.

Most preferred fatty acids are according to the invention C17:1:10cis, C18:1:9cis and C18:1:11cis. C18:1:9cis is highly preferred for the complex of the invention.

In an alternative embodiment a polyunsaturated fatty acid is complexed with alpha-lactalbumin. Preferably a polyunsaturated acid selected from the group of C18:2:9,12cis, C18:3:9,12,15cis, C18:3:6,9,12cis, and C20:4:5,8,11,15cis.

In one embodiment the fatty acid is an artificial fatty acid.

The fatty acid or lipid binding site in alpha-lactalbumin may be located in the groove between the α-helical and β-sheet domains, which becomes exposed in the apo-protein. The applicant without being bound by theory believes that the fatty acid or lipid
such as oleic acid binds in the interface between the alpha and the beta domains, and that the bound fatty acid or lipid locks this region of the molecule, while allowing the \( \alpha \)-domain to maintain a native-like conformation.

The active complex is preferably produced in local environments that favour the altered protein fold, and where fatty acid or lipid cofactors are available.

In an embodiment a fatty acid or a lipid is in molar excess over alpha-lactalbumin or a functional homologue thereof. Molar excess means that there are more moles of a fatty acid or a lipid than there is of alpha-lactalbumin. The molar excess of a fatty acid or a lipid is over alpha-lactalbumin or a functional homologue thereof may for one mole alpha-lactalbumin be in the range of 1.5 moles and 50 moles of a fatty acid or a lipid are added, such as for one mole alpha-lactalbumin in the range of 1 moles and 45 moles of a fatty acid or a lipid are added, for example in the range of 1 moles and 40 moles of a fatty acid or a lipid are added. Thus, in a preferred embodiment the concentration of a fatty acid or lipid, preferably oleic acid is in the range of 0.01 mM to 200 mM, such as in the range of 0.05 mM to 100 mM, for example in the range of 0.1 mM to 50 mM, such as in the range of 0.2 mM to 25 mM, for example in the range of 0.5 mM to 10 mM, such as in the range of 1 mM to 5 mM, such as in the range of 2 mM to 3 mM.

In some embodiments the molar ratio of fatty acid to emulsifier is at least 1:2, for example at least 3:1, such as at least 4:1, for example at least 5:1, such as at least 6:1, for example at least 10:1, such as at least 11:1, for example at least 12:1, such as at least 13:1, for example at least 14:1, such as at least 15:1, for example at least 16:1, such as at least 17:1, for example at least 18:1, such as at least 19:1, for example at least 20:1.

In some embodiments the molar ratio of fatty acid to emulsifier is approximately 1:2, for example approximately 3:1, such as approximately 4:1, for example approximately 5:1, such as approximately 6:1, for example approximately 10:1, such as approximately 11:1, for example approximately 12:1, such as approximately 13:1, for example approximately 14:1, such as approximately 15:1, for example approximately 16:1, such as approximately 17:1, for example approximately 18:1, such as approximately 19:1, for example approximately 20:1.
In an embodiment oleic acid is in molar excess over alpha-lactalbumin or a functional homologue thereof. Molar excess means that there are more moles of oleic acid than there is of alpha-lactalbumin. The molar excess of oleic acid over alpha-lactalbumin or a functional homologue thereof may for one mole alpha-lactalbumin be in the range of 1.5 moles and 50 moles of oleic acid is added, such as for one mole alpha-lactalbumin in the range of 1 moles and 45 moles of oleic acid is are added, for example in the range of 1 moles and 40 moles of oleic acid is are added. Thus, in a preferred embodiment the concentration of oleic acid is in the range of 0.01 mM to 200 mM, such as in the range of 0.05 mM to 100 mM, for example in the range of 0.1 mM to 50 mM, such as in the range of 0.2 mM to 25 mM, for example in the range of 0.5 mM to 10 mM, such as in the range of 1 mM to 5 mM, such as around in the range of 2 mM to 3 mM.

When used herein "approximately" means a given value +/- 10%.

Cytotoxicity

As described in the background section an active complex of alpha-lactalbumin and a fatty acid or a lipid; LAC has been demonstrated to posses selective cytotoxic activities towards cancer cells and immature cells besides its effect on bacterial and viral infections. LAC selectively kills tumour cells in vitro while sparing healthy cells and activity that has been retained in vivo.

The dose of alpha-lactalbumin capable of killing 50% of a given cell population is calculated based on the measured luminescence data. The potency of the alpha-lactalbumin composition is reflected by the LD50 dose, where a low LD50 dose is characteristic for a composition with high potency, i.e. a composition highly effective in killing cancer cells. In this situation a cancer cell line L1210 is used, although it is clear that several different cell lines are suitable for the purpose. Results from such an analysis are depicted in figure 11 and in a table format in table 5.

Composition
An embodiment of the present invention regards a composition of alpha-lactalbumin that has been prepared by a method comprising the steps of:

i. obtaining an lactalbumin composition comprising alpha-lactalbumin of SEQ ID NO: 1 or SEQ ID NO:2 or a functional homologue thereof comprising a sequence of at least 70% identical thereof,

ii. conversion of said alpha-lactalbumin or a functional homologue thereof to lactalbumin complex
   1. by release of calcium from said alpha-lactalbumin or a functional homologue thereof and
   2. binding of a fatty acid or a lipid to said alpha-lactalbumin or a functional homologue thereof
   3. exposing the alpha-lactalbumin or a functional homologue thereof to an ion exchange medium comprising a resin, wherein the mean size of the particles of the resin is in the range of 80 \( \mu \)m to 120 \( \mu \)m,

wherein steps 2. and 3. may be performed sequentially or simultaneously.

An equally preferred embodiment of the invention regards a composition of alpha-lactalbumin that has been prepared by a method comprising the steps of:

i. obtaining an lactalbumin composition comprising lactalbumin of SEQ ID NO: 1 or SEQ ID NO:2 or a functional homologue thereof comprising a sequence of at least 70% identical therewith,

ii. conversion of said lactalbumin or a functional homologue thereof to lactalbumin complex
   1. by mixing lactalbumin or a functional homologue thereof and a fatty acid or a lipid in the absence of an ion exchange medium, and subsequently
   2. exposing the mixture to an ion exchange medium.

The composition in general comprises alpha-lactalbumin in complex with a fatty acid or lipid, such as any of the fatty acids or lipids mentioned herein above in the section "Fatty Acid".

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It is preferred that the composition comprises monomeric LAC, such as at least 50%, such as at least 60%, for example at least 70%, such as at least 80%, for example at least 90% monomeric LAC. In certain embodiments of the invention essentially all LAC is in the form of monomeric LAC. The amount of monomeric versus oligomeric LAC, such for example dimeric LAC may be determined by SE-HPLC as described in the Examples below.

It is furthermore preferred that the composition comprises only very little-if any-contaminating biomacromolecules such as proteins. Thus it is preferred that at least 50%, such as at least 60%, for example at least 70%, such as at least 80%, for example at least 90% by weight of the proteins of the composition is LAC.

It is preferred that the composition has cell killing activity, preferably the composition comprises LAC, wherein the LD50 of said LAC is at the most 100 pg/cell, preferably at the most 75 pg/cell, more preferably at the most 50 pg/cell, such as at the most 42 pg/cell, when determined as described in Example 7 herein below.

It is furthermore preferred that the composition has histone binding ability, preferably the composition comprises LAC, which can bind histones, so that the absorption at 450 nm after performing a histone binding assay is at least 3 times the absorption of the negative control, such as at least 0.1, preferably at least 1, more preferably at least 1.5, wherein the histone binding assay is performed as described in Example 6 below.

Treatment

The composition according to the invention may be used for the manufacture of a medicament for a clinical disorder wherein selective cytotoxicity is desirable. The clinical disorder may be selected from the group consisting of respiratory tract infections, cancer and warts or for the inhibition of angiogenesis. In a preferred embodiment the cancer is bladder cancer. In another preferred embodiment the warts are papiloma infection.

Infection of the respiratory tract
In one embodiment of the invention the composition of LAC may be used in the treatment of Infections of the respiratory tract, e.g., meningitis, otitis and sinusitis, which are caused by bacteria which enter via the nasopharynx.

Viral infections of the respiratory tract may be caused by such as adenovirus, influenzavirus, respiratory syncytial virus (RSV), parainfluenza, Phinoviruses and coronaviruses.

In an embodiment the composition according to the invention is for the treatment of infections of the respiratory tract. The medicament according to the invention may be inhaled in the form of a mist into the upper respiratory airways.

Treatment of tumors
In one embodiment of the invention tumors of both the benign or malignant type may further be treated using the LAC composition according to the invention, based on the selective cytotoxic activity of alpha-lactalbumin complexes.

Wart
A wart is generally a small, rough tumour, typically on hands and feet, that resembles a cauliflower. Warts are common, and are caused by a viral infection, specifically by the human papillomavirus (HPV). They typically disappear after a few months but can last for years and can recur.

A range of different types of wart have been identified, which differ in shape and site affected, including:

Common wart (verruca vulgaris): a raised wart with roughened surface, most common on hands and knees.

Flat wart (verruca plana): a small, smooth flattened wart, tan or flesh coloured, which can occur in large numbers; most common on the face, neck, hands, wrists and knees.

Filiform or digitate wart: a thread- or finger-like wart, most common on the face, especially near the eyelids and lips.

Plantar wart (verruca, verruca pedis): a hard sometimes painful lump, often with multiple black specks in the centre; usually only found on pressure points on the soles of the feet.
Mosaic wart: a group of tightly clustered plantar-type warts, commonly on the hands or soles of the feet.

Genital wart (venereal wart, condyloma acuminatum, verruca acuminata): wart affecting the genital areas.

In a preferred embodiment the composition according to the invention is for the treatment of warts, which is preferably treated by topical application of a medicament according to the invention.

Papillomas

Papilloma refers to a benign epithelial tumor, which may or may not be caused by Human papillomavirus. Alternative causes are such as Choroid plexus papilloma (CPP).

Two types of papilloma often associated with HPV are squamous cell papilloma and transitional cell papilloma (also known as "bladder papilloma"). Subtypes of Papillomas include but are not limited to Skin papillomas, 

In a preferred embodiment the LAC composition according to the invention is for the treatment of papillomas, which is preferably treated by topical application of a medicament according to the invention.

Cancer

Cancerous diseases are scientifically designated neoplasia or neoplasms and may be benign or malignant. Cancers are classified by the type of cell that resembles the tumor and, therefore, the tissue presumed to be the origin of the tumor. The following general categories are applied:

Carcinoma: malignant tumors derived from epithelial cells. This group includes the most common cancers, comprising the common forms of breast, prostate, lung and colon cancer.

Lymphoma and Leukemia: malignant tumors derived from blood and bone marrow cells.

Sarcoma: malignant tumors derived from connective tissue, or mesenchymal cells
Mesothelioma: tumors derived from the mesothelial cells lining the peritoneum and the pleura.

Glioma: tumors derived from glia, the most common type of brain cell

Germinoma: tumors derived from germ cells, normally found in the testicle and ovary.

Choriocarcinoma: malignant tumors derived from the placenta.

In a preferred embodiment the LAC composition according to the invention is for the treatment of cancer.

Medicaments, as defined herein below, for treatment of cancer are according to the invention preferably applied directly to the tumour.

Mucosal tumors
The conditions found at mucosal surfaces can be quite unique in terms of properties such as p. H. and the like. Mucosal surfaces are found inter alia in the nasal passages, in the mouth, throat, oesophagus, lung, stomach, colon, vagina and bladder.

Particular mucosal surfaces that may be treated with in accordance with the invention include throat, lung, colon and bladder surfaces which tumours.

Bladder cancer
Bladder cancer refers to any of several types of malignant growths of the urinary bladder. The most common type of bladder cancer begins in cells lining the inside of the bladder and is called urothelial cell or transitional cell carcinoma (UCC or TCC).

In a more preferred embodiment the LAC composition according to the invention is for the treatment of bladder cancer.

Glioblastome
A glioma is a type of primary central nervous system (CNS) tumor that arises from glial cells. The most common site of involvement of a glioma is the brain, but they can also affect the spinal cord, or any other part of the CNS, such as the optic nerves.

In a more preferred embodiment the LAC composition according to the invention is for the treatment of glioma/glioblastome.
Angiogenesis.

Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. The process of angiogenesis is initiated when tumor cells release molecules signalling to the normal host tissue, activating genes and proteins to encourage growth of new blood vessels. A series of natural inhibitors of angiogenesis have been identified, and are believed to prevent and/or inhibit the growth and spread of cancer cells.

The finding that alpha-lactalbumin complexes may inhibit angiogenesis further spread the applicability of alpha-lactalbumin in treatment and/or inhibition of cancer.

In an embodiment the composition according to the invention is for inhibition of angiogenesis.

Actinic keratosis

Actinic keratosis (AK) is a UV light-induced lesion of the skin that may progress to invasive squamous cell carcinoma. Clinically, actinic keratoses range from barely perceptible rough spots of skin to elevated, hyperkeratotic plaques several centimeters in diameter. Most often, they appear as multiple discrete, flat or elevated, keratotic lesions. Lesions typically have an erythematous base covered by scale (hyperkeratosis). They are usually 3-10 mm in diameter and gradually enlarge into broader, more elevated lesions. With time, actinic keratoses may develop into invasive squamous cell carcinoma.

In a more preferred embodiment the LAC composition according to the invention is for the treatment of actinic keratosis.

Pharmaceutical composition

The present invention provides pharmaceutical compositions comprising LAC. In one aspect the present invention relates to a pharmaceutical composition. The pharmaceutical composition may be formulated in a number of different manners, depending on the purpose for the particular pharmaceutical composition.
For example the pharmaceutical composition may be formulated in a manner so it is useful for a particular administration form. Preferred administration forms are described herein below.

In one embodiment the pharmaceutical composition is formulated so it is a liquid. For example the composition may be a protein solution or the composition may be a protein suspension. Said liquid may be suitable for parenteral administration, for example for injection or infusion.

The liquid may be any useful liquid, however it is frequently preferred that the liquid is an aqueous liquid. For many purposes, in particular when the liquid should be used for parenteral administration, it is furthermore preferred that the liquid is sterile. Sterility may be conferred by any conventional method, for example filtration, irradiation or heating. Furthermore, it is preferred that the liquid has been subjected to a virus reduction step, in particular if the liquid is formulated for parenteral administration.

Virus reduction may for example be performed by nanofiltration or virus filtering over a suitable filter, such as a Planova filter consisting of several layers. The Planova filter may be any suitable size for example 75N, 35N, 20N or 15N or filters of different size may be used, for example Planova 2ON. Virus reduction may also comprise a step of prefiltering with another filter, for example using a filter with a pore size of the the range of 0.01 to 1 µm, such as in the range of 0.05 to 0.5 µm, for example around 0.1 µm. Virus reductions may also include an acidic treatment step.

The pharmaceutical composition may be packaged in single dosage units, which may be more convenient for the user. Hence, pharmaceutical compositions for bolus injections may be packages in dosage units of for example at the most 10 ml, preferably at the most 8 ml, more preferably at the most 6 ml, such as at the most 5 ml, for example at the most 4 ml, such as at the most 3 ml, for example around 2.2 ml.

The pharmaceutical composition may be packaged in any suitable container. In one example a single dosage of the pharmaceutical composition may be packaged in injection syringes or in a container useful for infusion.
In another embodiment of the present invention the pharmaceutical composition is a dry composition. The dry composition may be used as such, but for most purposes the composition is a dry composition for storage only. Prior to use the dry composition may be dissolved or suspended in a suitable liquid composition, for example sterile water.

The pharmaceutical composition according to the present invention may also comprise a first nucleic acid sequence encoding LAC, such as any of the LAC mentioned herein above. Said first nucleic acid sequence is preferably operably associated with a second nucleic acid sequence directing expression of the first nucleic acid in the individual to be treated with the pharmaceutical composition, more preferably in the cells of said individual, which are diseased. Thus it is preferred that the second nucleic acid sequence is capable of directing expression of the first nucleic acid sequence in a human being. In embodiments of the invention wherein the clinical condition is cancer, it is preferred that the second nucleic acid sequence is capable of directing expression of the first nucleic acid sequence in cancer cells, such as malignant cells. It is furthermore preferred that the first and the second nucleic acid sequences are included in a suitable vector.

It is also comprised within the invention that the pharmaceutical composition may be applied topically to the site of the site, for example in the form of a lotion, a crème, an ointment, a spray, such as an aerosol spray or a nasal spray, rectal or vaginal suppositories, drops, such as eye drops or nasal drops, a patch, an occlusive dressing or the like.

**Pharmaceutically acceptable additives**

The pharmaceutical compositions containing LAC may be prepared by any conventional technique, e.g. as described in Remington: The Science and Practice of Pharmacy 1995, edited by E. W. Martin, Mack Publishing Company, 19th edition, Easton, Pa.

The terms "medicament" and "pharmaceutical compostions" are used interchangeably herein.
The pharmaceutical acceptable additives may be any conventionally used pharmaceutical acceptable additive, which should be selected according to the specific formulation, intended administration route etc. For example the pharmaceutical acceptable additives may be any of the additives mentioned in Nema et al, 1997. Furthermore, the pharmaceutical acceptable additive may be any accepted additive from FDA’s “inactive ingredients list”, which for example is available on the internet address http://www.fda.gov/cder/drug/iig/default.htm.

In some embodiments of the present invention it is desirable that the pharmaceutical composition comprises an isotonic agent. In particular when the pharmaceutical composition is prepared for administration by injection or infusion it is often desirable that an isotonic agent is added.

Accordingly, the composition may comprise at least one pharmaceutically acceptable additive which is an isotonic agent.

The pharmaceutical composition may be isotonic, hypotonic or hypertonic. However it is often preferred that a pharmaceutical composition for infusion or injection is essentially isotonic, when it is administrated. Hence, for storage the pharmaceutical composition may preferably be isotonic or hypertonic. If the pharmaceutical composition is hypertonic for storage, it may be diluted to become an isotonic solution prior to administration.

The isotonic agent may be an ionic isotonic agent such as a salt or a non-ionic isotonic agent such as a carbohydrate.

Examples of ionic isotonic agents include but are not limited to NaCl, CaCl₂, KCl and MgCl₂. Examples of non-ionic isotonic agents include but are not limited to mannitol and glycerol.

It is also contained within the present invention that at least one pharmaceutically acceptable additive is a buffer. For some purposes, for example, when the pharmaceutical composition is meant for infusion or injection, it is often desirable that
the composition comprises a buffer, which is capable of buffering a solution to a pH in the range of 4 to 10, such as 5 to 9, for example 6 to 8.

However, in other embodiments of the invention the pharmaceutical composition may comprise no buffer at all or only micromolar amounts of buffer.

The buffer may for example be selected from the group consisting of TRIS, acetate, glutamate, lactate, maleate, tartrate, phosphate, citrate, carbonate, glycinate, histidine, glycine, succinate and triethanolamine buffer.

In a preferred embodiment the buffer is TRIS. TRIS buffer is known under various other names for example tromethamine including tromethamine USP, THAM, Trizma, Trisamine, Tris amino and trometamol. The designation TRIS covers all the aforementioned designations.

The buffer may furthermore for example be selected from USP compatible buffers for parenteral use, in particular, when the pharmaceutical formulation is for parenteral use. For example the buffer may be selected from the group consisting of monobasic acids such as acetic, benzoic, gluconic, glyceric and lactic, dibasic acids such as aconitic, adipic, ascorbic, carbonic, glutamic, malic, succinic and tartaric, polybasic acids such as citric and phosphoric and bases such as ammonia, diethanolamine, glycine, triethanolamine, and TRIS.

The pharmaceutical compositions may comprise at least one pharmaceutically acceptable additive which is a stabiliser. The stabiliser may for example be a detergent, an amino acid, a fatty acid, a polymer, a polyhydric alcohol, a metal ion, a reducing agent, a chelating agent, a sugar, a protein or an antioxidant, however any other suitable stabiliser may also be used with the present invention.

For example the stabiliser may be selected from the group consisting of poloxamers, Tween-20, Tween-40, Tween-60, Tween-80, Brij, metal ions, amino acids, polyethylene glycol, Triton, EDTA, ascorbic acid, Triton X-100, NP40 or CHAPS.

Furthermore, the stabiliser may be selected from the group consisting of amino acids such as glycine, alanine, arginine, leucine, glutamic acid and aspartic acid, surfactants
such as polysorbate 20, polysorbate 80 and poloxamer 407, fatty acids such as phosphotidyl choline, ethanolamine and acetyltryptophanate, polymers such as polyethylene glycol and polyvinylpyrrolidone, polyhydric alcohol such as sorbitol, mannitol, glycerin, sucrose, glucose, propylene glycol, ethylene glycol, lactose and trehalose, antioxidants such as ascorbic acid, cysteine HCl, thioglycerol, thioglycolic acid, thiosorbitol and glutathione, reducing agents such as several thiols, chelating agents such as EDTA salts, glutamic acid and aspartic acid and metal ions such as Ca++, Ni++, Mg++ and Mn++.

Other examples of antioxidants and reducing agents useful with the present invention includes acetone sodium bisulfite, ascorbate, bisulfite sodium, butylated hydroxy anisole, butylated hydroxy toluene, cystein/cysteinate HCl, dithionite sodium, gentisic acid, gentisic acid ethanolamine, glutamate monosodium, formaldehyde sulfoxylate sodium, metabisulfite potassium, metabisulfite sodium, monothioglycerol, propyl gallate, sulfite sodium and thioglycolate sodium.

The pharmaceutical composition according to the invention may also comprise one or more cryoprotectant agents. In particular, when the composition comprises freeze-dried protein or the composition should be stored frozen it may be desirable to add a cryoprotecting agent to the pharmaceutical composition.

The cryoprotectant agent may be any useful cryoprotectant agent, for example the cryoprotectant agent may be selected from the group consisting of dextran, glycerin, polyethylenglycol, sucrose, trehalose and mannitol.

Accordingly, the pharmaceutically acceptable additives may comprise one or more selected from the group consisting of isotonic salt, hypertonic salt, buffer and stabilisers. Furthermore, the pharmaceutically acceptable additives may comprise one or more selected from the group consisting of isotonic agents, buffer, stabilisers and cryoprotectant agents. For example, the pharmaceutically acceptable additives comprise glucosemonohydrate, glycine, NaCl and polyethylenglycol 3350.

Administration
The pharmaceutical composition may be prepared so it is suitable for one or more particular administration methods. Furthermore, the method of treatment described herein may involve different administration methods.

In general any administration method, wherein LAC may be administered to an individual in a manner so that active LAC may reach the site of disease may be employed with the present invention.

For example, the pharmaceutical compositions of the invention may be administered parenterally, that is by intravenous, intramuscular, subcutaneous intranasal, intrarectal, intravaginal or intraperitoneal administration.

In general, for injection and infusion the pharmaceutical composition should be a sterile liquid, which preferably also has been subjected to a virus reduction step.

Injection may be injection to any preferred site, for example injection may be selected from the group consisting of intravenous, subcutaneous, intra-arterial, intra-muscular and intra-peritonal injection. Infusion is generally intra-venous infusion. Injection may also be directly to site of the disease. This may in particular be applicable when treating a cancer which is a solid tumour.

Furthermore, the route of administration may be topical administration to for example a mucosal membrane or to the skin. The mucosal membrane to which the pharmaceutical preparation of the invention is administered may be any mucosal membrane of the mammal to which the biologically active substance is to be given, e.g. in the nose, vagina, eye, mouth, genital tract, lungs, gastrointestinal tract, or rectum.

Topical administration to the skin may for example be in the form of a lotion, cream, ointment, drops, transdermal patch or the like.

The pharmaceutical compositions according to the present invention may be administered once or more than once, for example they may be administered in the range of 2 to 5 times, such as 5 to 10 times, for example 10 to 20 times, such as 20 to 50 times, for example 50 to 100 times, such as more than 100 times.
Dosage

The dosage of LAC to be administered depends on the individual to be treated as well as on the clinical condition and the mode of administration. In general, in the range of 0.5 µg to 50 mg, such as in the range of 1 µg to 20 mg, for example in the range of 1 µg to 2 mg, such as in the range of 1 µg to 100 µg isolated LAC may be administered per administration to a given individual, such as a human being.

Detailed description of the drawings

Detailed description of figures

Figure 1A. Sequence alignments of equine, porcine, camelide, human, bovine and caprine alpha-lactalbumin. 1B: Sequence alignments of human and bovine alpha-lactalbumin.

Figure 2 Chromatograms of alpha-lactalbumin composition after conversion to the alpha-lactalbumin complex using DEAE Trisacryl Plus M anion exchange resin. (A): bLAC produced using DEAE Trisacryl Plus M (load 20 mg/cm²), (B) bLAC produced using DEAE Trisacryl Plus M (load 25 mg/cm²). bLa elutes during the isocratic step gradient at 15% elution solution (Tris 10 mM, NaCl 1M. pH 8.5). bLAC elutes when 100% Tris 10 mM, NaCl 1M. pH 8.5 is applied.

Figure 3 Chromatograms of alpha-lactalbumin composition after conversion to the alpha-lactalbumin complex using (A): Capto Q resin, (B): UnoSphere Q resin, (C): Q Sepharose XL resin. The linear gradient from 0 to 15% Tris 10 mM, NaCl 1M. pH 8.5 has been suppressed in comparison to Figure 2. With the Unosphere Q, the main peak eluted during the first isocratic step gradient at 15% (B), for the two other resins, capto Q (A) and Q Sepharose XL (C), a main peak eluted when the step gradient of 100% B-buffer was applied.

Figure 4 Chromatograms of alpha-lactalbumin composition after conversion to the alpha-lactalbumin complex using Q Sepharose XL with a linear gradient from 0 to 100% Tris 10 mM, NaCl 1M. pH 8.5 (left) and Size Exclusion- High Performance Liquid
Chromatography (SE-HPLC) identification of peaks 1, 2 and 3 (right). From the elution of the conversion run with a linear gradient, it can be seen that peak 1 that it is expected to be unconverted bl_A contains monomer and bl_A dimer. Peak 3, that it is expected to contain bLAC, is mainly seen as a monomer of bLAC.

Figure 5: Conversion with Q Sepharose XL with steps gradient (45, 70 and 100% Tris 10 mM, NaCl 1M, pH 8.5) (left) and SE-HPLC identification of peaks land 2 (right). The step gradients elution allowed, a separation between a peak containing equivalent amount of monomer and bLA dimer (peak 1) and a peak containing mainly monomer of bLA (peak 2).

Figure 6: Conversion with Q Sepharose XL with steps gradient (45, 70 and 100% Tris 10 mM, NaCl 1M, pH 8.5) and higher bLA load. The step gradients elution allowed, a separation between a peak containing equivalent amount of monomer and bLA dimer (peak 1) and a peak containing mainly monomer of bLA (peak 2).

Figure 7: Yield of conversion versus load. There is a linear relation between the bLAC recovered and the bLA load when looking at the three conversion runs.

Figure 8: Chromatograms for the conversion with Q sepharose XL. Four different bLA loads were tested. Run LAC-031 was performed with a lower amount of oleic acid. Cutting criteria at 280 nm are indicated. bLA is eluted at 45% Tris 10 mM, NaCl 1M, pH 8.5 and bLAC is eluted at 70% Tris 10 mM, NaCl 1M, pH 8.5. (A) LAC-031 : 1mM EDTA/0.2 mM Oleic acid, load 30 mg bLA/cm², recovery 5 mg bLAC/ cm², Yield 15%. (B) LAC-041 : 1mM EDTA/2 mM Oleic acid, load 30 mg bLA/cm², recovery 28 mg bLAC/ cm², Yield 93%. (C): LAC-042: 1mM EDTA/2 mM Oleic acid, load 42 mg bLA/cm², recovery 38 mg bLAC/ cm², Yield 90%. (D): LAC-047: 1mM EDTA/2 mM Oleic acid, load 90 mg bLA/cm², recovery 76 mg bLAC/ cm², Yield 84%.

Figure 9: Characterization of peaks eluted at different salt concentration during conversion of bLA to bLAC. Retention time of bLA/bLAC dimer = 23 min; Retention time of bLA/bLAC monomer = 25 min.

Figure 10: Histone assay of bLAC converted at different bLA loads. Histone assays N274-28A for bLAC ID. No. LAC-041 - N277-07E and LAC-042 - N277-09G; N274-
36A for blAC ID. No. LAC-047 - N277-18A and LAC-016 - N262-35B. When bl_A has complexed with oleic acid, binding will occur resulting in an increase in absorbance.

Figure 11: Cell killing assay (N274-26A) of blAC (LAC-016 - N262-35B, old conversion method i.e. pre-conditioned ion exchange resin, LAC-042 - N277-09G, new conversion method i.e. mixing of blA and oleic acid prior ion exchange chromatography, described in Example 8). The cell killing abilities of the blAC samples are expressed by the relative amount of ATP determined using the ViaLight PLUS kit (luminescence) giving an indication of the percentage of viable cells. The cell killing abilities of the two blAC samples expressed as a decrease in luminescence were found to be similar.

Figure 12: Chromatograms of the conversion runs with blA start material N277-64A (un-conditioned ion exchange resin, described in Example 9) with step gradient (45, 70 and 100% Tris 10 mM, NaCl 1M. pH 8.5). (A): Oleic acid, (B): Vaccenic acid, (C): Linoleic acid, (D): alfa-linoleic acid. The step gradients elution allowed, a separation between a peak containing blA (peak 1) and a peak containing blAC (peak 2).

Figure 13: Chromatograms of the conversion runs with blA start material N289-56A (un-conditioned ion exchange resin, described in Example 9) with step gradient (45, 70 and 100% Tris 10 mM, NaCl 1M. pH 8.5). (A): Oleic acid, (B): Palmitoleic acid, (C): Eicosapentaenoic acid, (D): stearidonic acid, (E) heptadecenoic acid, (F) gamma-linoleic acid, (G) Gondoic acid. The step gradients elution allowed, a separation between a peak containing blA (peak 1) and a peak containing blAC (peak 2).

Figure 14: Summary of conversion, histone binding and cell killing of blA in complex with the respective fatty acids (un-conditioned ion exchange resin).

Figure 15: Chromatograms of the conversion runs. Standard step gradient was applied with Q sepharose XL. Linear gradient was applied with the other resins un-conditioned ion exchange resin). (A) Q sepharose XL, (B) Unosphere Q, (C) DEAE Trisacryl Plus M, (D) DEAE Sepharose FF. Converted blAC is recovered in peak 2 from Q sepharose XL and Unosphere Q and in peaks 2 and 3 from DEAE Trisacryl Plus M and DEAE Sepharose FF.
Examples

Example 1: Conversion with DEAE Trisacryl Plus M

The bl_A was converted at laboratory scale as for example essentially described in Svensson et al., Lipids as cofactors in protein folding: Stereo-specific lipid-protein interactions are required to form HAMLET (human α-lactalbumin made lethal to tumor cells). Protein Science (2003), 12:2805-2814.

<table>
<thead>
<tr>
<th>Column ID</th>
<th>N 263</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Tricorn 10/10 packed with 8 mL:</td>
</tr>
<tr>
<td></td>
<td>Resin 1: DEAE Trisacryl Plus M (Pall # 26709-14) - Lac-01</td>
</tr>
<tr>
<td></td>
<td>Resin 5: DEAE Trisacryl Plus M (Sigma # D2540) - Lac-024</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Equilibration Solution (Solvent A)</th>
<th>Tris (10mM) NaCl (0.1 M) pH=8.5 (220°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elution Solution (Solvent B)</td>
<td>Tris (10mM) NaCl (1 M) pH=8.5 (220°C)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>bLA loads</th>
<th>16 mg (from bLA batch N262-14C) corresponding to 20 mg/cm² - LAC-01</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>20 mg (from bLA batch N262-14C) corresponding to 25 mg/cm² - LAC-024</td>
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</table>

<table>
<thead>
<tr>
<th>bLAC Elution profile</th>
<th>Wash: Equilibration solution (2CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient step 1:</td>
<td>linear 0-15% solvent B (in Solvent A) (1 CV)</td>
</tr>
<tr>
<td>Gradient step 2:</td>
<td>linear 15% solvent B (in Solvent A) (2 CV)</td>
</tr>
<tr>
<td>Gradient step 3:</td>
<td>linear 100% solvent B (in Solvent A) (4 CV)</td>
</tr>
</tbody>
</table>
Example 2: Conversion with alternative resins

The bl_A was converted at laboratory scale essentially as described in Svensson, 2003. However, the conversion was done with a variety of different resins and the linear gradient from 0 to 15% B buffer was suppressed, i.e. it was changed from 0 to 15% without delay.

Column | Tricorn 10/10 packed with 8 ml:
Resin 2: Capto Q (GE Healthcare # 17-531-6-99) −
Lac-020
Resin 3: UNPsphere Q (BioRad # 156-1010) −
Lac-021
Resin 4: Q Sepharose XL (GE Healthcare # 17-5072-99) −
Lac-023

Column ID | N263

Equilibration Solution | Tris (10mM)
(Solvent A) NaCl (0.1 M)
pH=8.5 (22°C)

Elution Solution | Tris (10mM)
(Solvent B) NaCl (1 M)
pH=8.5 (22°C)

bl_A load | 20 mg (from bl_A batch N262-14C) corresponding to 25 mg/cm²

bLAC Elution profile | Wash: Equilibration solution (2CV)
Gradient step 2: linear 15% solvent B (in Solvent A) (2CV)
Gradient step 3: linear 100% solvent B (in Solvent A) (4CV)

RESULTS: Comparisons of the conversion runs

The yields of the conversion runs were determined by size exclusion HPLC (SE-HPLC) according to standard methods.
The potency of the converted bl_A was determined by the cell killing assay according to Example 7. The LD50 is given in pg bLAC per cell. Before testing the bLAC solutions were desalted with NaCl (0.9%) using NAP-25 desalting column (GE Healthcare).

The chromatograms for the different conversion runs can be seen in Figures 2 and 3. The conversion with the DEAE Trisacryl Plus M (obtained from either Pall or Sigma) are seen in Figure 2. The conversion runs with the alternative ion exchange resins are seen in Figure 3.

The profiles of the two conversions performed with the DEAE Trisacryl resins are similar. Nevertheless, as shown in Table 1, the yield of conversion was 32% with the DEAE Triscaryl Plus M with a load of 25 mg/cm². A peak corresponding to unconverted bl_A can be seen during the step gradient of 15% B-buffer. It seems that the column was overloaded. Therefore the load of bl_A on DEAE Triscaryl Plus M should not exceed 20 mg/cm². The LD50 for the bLAC recovered from the two resins were 19 and 39 pg/cell, respectively.

Table 1: Summary of the conversion runs

<table>
<thead>
<tr>
<th>Prod ID</th>
<th>Item no</th>
<th>Resin</th>
<th>Load (mg/cm²)</th>
<th>Yield (mg/cm²)</th>
<th>Yield (%)</th>
<th>Killing Assay LD50 (pg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC-018</td>
<td>N263-49G</td>
<td>DEAE Trisacryl +M</td>
<td>20</td>
<td>14</td>
<td>70%</td>
<td>19</td>
</tr>
<tr>
<td>LAC-024</td>
<td>N263-65G</td>
<td>DEAE Trisacryl +M</td>
<td>25</td>
<td>8</td>
<td>32%</td>
<td>39</td>
</tr>
<tr>
<td>LAC-020</td>
<td>N263-53G</td>
<td>Capto Q</td>
<td>25</td>
<td>18</td>
<td>70%</td>
<td>23</td>
</tr>
<tr>
<td>LAC-021</td>
<td>N263-58G</td>
<td>Unosphere Q</td>
<td>25</td>
<td>21</td>
<td>82%,(2)</td>
<td>&gt;200</td>
</tr>
<tr>
<td>LAC-023</td>
<td>N263-61 G</td>
<td>Q Sepharose XL</td>
<td>25</td>
<td>23</td>
<td>92%</td>
<td>33</td>
</tr>
</tbody>
</table>


The profiles of the three conversions performed with alternative resins rather than the DEAE Trisacryl Plus M are shown in Figure 3. The step gradient was identical as for the DEAE Trisacryl Plus M conversions, but the linear gradient from 0 to 15% was suppressed. The three resins were pre-conditioned with the same oleic acid solution as for the DEAE Trisacryl Plus M resin, but the subsequent wash and elution were different.

The conversion profiles with the three resins are rather different. With the Unosphere Q, the main peak eluted during the first isocratic step gradient at 15%. This peak
contains mainly unconverted bl_A, as assessed by SE-HPLC and cell killing assay, where no activity was detected.

The conversion profiles for the two other resins, Capto Q and Q Sepharose XL, showed a main peak that eluted when the step gradient of 100% B-buffer was applied. For the Capto Q, some bLA, probably unconverted started to elute during the step gradient at 15% B-buffer. The recovered bLAC, showed a LD50 similar to the one obtained with the DEAE Trisacryl. The yield of the conversion was also similar, however, the load was higher. For the Q Sepharose XL, a higher yield was obtained in the main peak than for the other resin. Nevertheless, a shoulder in the main peak can be seen.

The purity of the conversion recovery was determined by SE-HPLC. Main impurities consist of a bLA dimer.

<table>
<thead>
<tr>
<th>Prod ID</th>
<th>Sample ID</th>
<th>Sample Description</th>
<th>FP1 %</th>
<th>FP2 %</th>
<th>Main %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC-018</td>
<td>N263-49G</td>
<td>DEAE Trisacryl Plus M</td>
<td>0</td>
<td>17</td>
<td>83</td>
</tr>
<tr>
<td>LAC-024</td>
<td>N263-65G</td>
<td>DEAE Trisacryl Plus M</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>LAC-020</td>
<td>N263-53G</td>
<td>Capto Q</td>
<td>0</td>
<td>9</td>
<td>91</td>
</tr>
<tr>
<td>LAC-021</td>
<td>N263-58G</td>
<td>Unosphere Q</td>
<td>0</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>LAC-023</td>
<td>N263-61G</td>
<td>Q Sepharose XL</td>
<td>0</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

1 Corresponding to unconverted bLA
2 FP2 = Front Peak corresponding of bLA/bLAC dimers

It is apparent from Table 2 that purification with either Capto Q, Unosphere Q or Q Sepharose XL obtain a more pure fraction than purification with DEAE Trisacryl Plus M.

Example 3: Alternative gradient with Q Sepharose XL

The column packed with Q Sepharose XL was pre-conditioned with the oleic acid solution as described in the previous section. bLA and bLAC were separated by a linear gradient from 0 to 100% elution solution. From the linear gradient new isocratic steps were re-defined for the elution. Then two different bLA loads were tested (LAC-027 and LAC-029).
Column                  Tricorn 10/1 0 packed with 8 ml:
                        Resin 4 : Q Sepharose XL (GE Healthcare # 17-5072-99)

Column ID

Equilibration Solution Tris (10mM)
(Solvent A)    NaCl (0.1 M)
               pH=8.5 (22°C)

Elution Solution     Tris (10mM)
(Solvent B)   NaCl (1 M)
               pH=8.5 (22°C)

bL_A load 20 mg (from bLA N262-14C) corresponding to 25 mg/ cm² -
            LAC-025

15.5 mg (from bLA N262-26D corresponding to 16 mg/ cm² -
            LAC-027

29 mg (from bLA N262-26D) corresponding to 37 mg/ cm² -
            LAC-029

bLAC Elution Wash: Equilibration solution  (2CV)
profile    Gradient step 2: Linear from 0 - 100% solvent B (in Solvent A
Linear gradient ~ (5CV)
Lac-025       Gradient step 3: Step 100% solvent B (4CV)

bLAC Elution Wash: Equilibration solution  (2CV)
profile    Gradient step 1: Step 45% solvent B (in Solvent A) (5CV)
Step gradient - Gradient step 2: Step 70% solvent B (in Solvent A) (2CV)
Lac-027/029 Gradient step 3: Step 100% solvent B (2CV)

RESULTS: Optimization of the conversion of bLA with Q Sepharose XL

From the different ion exchange resins tested, the Q Sepharose XL was chosen, as it showed some possibility to increase the yield of the conversion of bLA to bLAC in comparison to the current DEAE Triscaryl Plus M resin.
In the first test with the Q Sepharose XL, a shoulder, maybe containing unconverted bl_A, was eluted with bLAC. In order to find new steps gradient that could separate bl_A from bLAC, a linear gradient was applied. The result is shown in Figure 4.

From the elution of the conversion run with the linear gradient, it can be seen that peak 1 that is expected to be unconverted blA contains monomer and blA dimer. Peak 3, that is expected to contain bLAC, is mainly seen as a monomer of bLAC. No cell killing assay activity was recovered on the three peaks of this run. After desalting on NAP-25, the three peaks were concentrated on microcon YM-3 (3,000 Da Cut off) from Milipore.

Oleic acid might have been removed from the concentrated bLAC either because the traces of glycerol used for the YM-3 membrane storage or by the YM-3 membrane itself consisting of regenerated cellulose.

Based on the results of the linear gradient elution, steps gradient of 45, 70 and 100% B-buffer (in Solvent A) were defined and the conversion was repeated. By using a step gradient elution, blA and bLAC could be eluted in a smaller volume and then be used directly in cell killing assay without the concentration step that might inactivate bLAC.

As seen in Figure 5, the step gradients elution allowed, a separation between a peak containing equivalent amount of blA (peak 1) and a peak containing mainly monomer (peak 2). The last was tested for cell killing assay potency and was found to be active as seen in Table 3. As peak 1 was too diluted for the assay, a new conversion run was performed with higher load of blA. The results are seen in Figure 6.

The first peak eluted during the step gradient in Figure 6 did not show after desalting (N263-83A) any cell killing potency (LD50>158 pg/cell). The cell killing activity was recovered in the peak eluted in the second step gradient (LD50 = 28 pg/cell) as expected.

Comparing the conversion performed on Figures 4, 5 and 6, it can be seen that higher loads of blA from 16 to 37 mg/cm² resulted in lower recoveries of bLAC. Then the peak corresponding to unconverted blA increased (cf. Figure 6).

As seen in Figure 7, there is a linear relation between the blAC recovered and the blA load when looking at the three conversion runs (cf. Table 3). From Figure 7, it can be
concluded that to maintain a yield for conversion over 80% on Q Sepharose XL, the load of bl_A should not exceed 20 mg/cm^2, and to maintain a yield of 70% even 25 mg/cm^2 may be loaded.

Table 3: Summary of optimization runs with Q Sepharose XL

<table>
<thead>
<tr>
<th>Prod ID</th>
<th>Item no</th>
<th>Gradient</th>
<th>Load (mg/cm^2)</th>
<th>Yield (mg/cm^2)</th>
<th>Yield (%)</th>
<th>Killing Assay LD50 (pg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC-025</td>
<td>N263-72C</td>
<td>Linear 0-100% B-buffer</td>
<td>25</td>
<td>14</td>
<td>70%</td>
<td>&gt;338</td>
</tr>
<tr>
<td>LAC-027</td>
<td>N263-74I</td>
<td>Steps 45-70-100% B-buffer</td>
<td>16</td>
<td>14</td>
<td>88%</td>
<td>25</td>
</tr>
<tr>
<td>LAC-029</td>
<td>N263-82I</td>
<td>Steps 45-70-100% B-buffer</td>
<td>37</td>
<td>18</td>
<td>48%</td>
<td>28</td>
</tr>
</tbody>
</table>


In this study three ion exchange resins were tested for the conversion of bLA to bLAC, as an alternative for the current DEAE Trisacryl Plus M resin. The resins were chosen from a first screening experiment in microtiter plate.

From the three resins tested, Unosphere Q from BioRad was less suitable for conversion, because the obtained complex had a very high LD50. With Capto Q and Q Sepharose XL from GE Healthcare, bLAC was recovered with similar LD50 in cell killing assays, as for bLAC formed with the current resin, however with a better recovery, in particular with a higher loading. Q Sepharose XL was chosen for further optimization, as it gave the better recovery.

Optimization work with Q Sepharose XL disclosed to new step gradients for the separation of bLA and bLAC, which may be preferable to use. The unconverted bLA recovered was seen both in monomeric and dimeric forms whereas bLAC recovered mainly as a monomer.

Example 4: bLA sample conditioning

An oleic acid solution of 3.5 mM was freshly prepared prior each sample conditioning. Oleic acid (20 µL) was mixed with Ethanol 96% (0.25 mL) and Tris-HCl (10 mM) pH 8.5, NaCl (0.1 M) (20 mL).
bl_A purified by hydrophobic interaction chromatography (HIC) was conditioned with EDTA, Tris (0.1 M pH 8.5) and oleic acid solution. A description of the different samples conditioning are shown in Table 4.

Table 4: bl_A sample conditioning

<table>
<thead>
<tr>
<th>Run ID</th>
<th>Sample ID</th>
<th>bLA</th>
<th>Tris pH 8.5 (1 M)</th>
<th>EDTA (0.25 M)</th>
<th>Oleic acid (3.5 mM)</th>
<th>MIIIQ H2O</th>
<th>Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC-031</td>
<td>N262-26D</td>
<td>8.6 mL</td>
<td>3.1 mL</td>
<td>0.124 mL</td>
<td>(1 mM)</td>
<td>0.2 mL</td>
<td>1.71 mL</td>
</tr>
<tr>
<td>LAC-034</td>
<td>N262-26D</td>
<td>9 mL</td>
<td>3.6 mL</td>
<td>0.144 mL</td>
<td>(1 mM)</td>
<td>0.2 mL</td>
<td>2.7 mL</td>
</tr>
<tr>
<td>LAC-039</td>
<td>N262-54D</td>
<td>6.2 mL</td>
<td>2.48 mL</td>
<td>0.099 mL</td>
<td>(1 mM)</td>
<td>0.2 mL</td>
<td>1.86 mL</td>
</tr>
<tr>
<td>LAC-040</td>
<td>N262-54D</td>
<td>9 mL</td>
<td>3.6 mL</td>
<td>0.144 mL</td>
<td>(1 mM)</td>
<td>0.2 mL</td>
<td>2.7 mL</td>
</tr>
<tr>
<td>LAC-042</td>
<td>N262-54D</td>
<td>9 mL</td>
<td>3.6 mL</td>
<td>0.144 mL</td>
<td>(1 mM)</td>
<td>0.2 mL</td>
<td>2.7 mL</td>
</tr>
</tbody>
</table>

The optimum EDTA concentration to deplete bl_A of Ca++ prior conversion to bLAC was found to be 1-10 mM. No real difference between 1 and 10 mM EDTA could be seen. Up to 25 mM EDTA was tested, but then the conversion recovery was lower. In this study a concentration of 1 mM EDTA was chosen for sample conditioning. This corresponds to a 15/20-fold molar excess EDTA/bLA in the examples in Table 4.

The oleic acid concentration to add to bLA prior conversion to bLAC was tested in the range 0.2-2 mM. 2 mM oleic acid for the conversion gave the best results. In this study concentrations of 0.2-2 mM oleic acid were tested for sample conditioning. This corresponds to a 3/40-fold molar excess oleic acid/bLA in the examples in Table 4.

Prior to application on the AIEC column the conditioned samples were mixed for approximately 30 min. at room temperature.
Example 5: AIEC step

A column was newly packed with Q Sepharose XL resin (GE healthcare). The different bl_A samples shown in Table 4 were applied on this column. The chromatographic parameters for the AIEC runs were the following. The Equilibration solution (solvent A) and solvent B were as described in Example 3.

Column
Tricorn 10/100 with 8 mL packed with Q Sepharose XL (GE HealthCare #17-5072-99)

Column ID
N263-87A

bl_A loads
LAC-031: 29 mL conditioned sample (30 mg/cm^2)
LAC-034: 34 mL conditioned sample (31 mg/cm^2)
LAC-035/037/041: 23 mL conditioned sample (30 mg/cm^2)
LAC-042: 33 mL conditioned sample (42 mg/cm^2)
LAC-038/047: 70 mL conditioned sample (90 mg/cm^2)

bLAC Elution profile
Wash: Equilibration solution (2 CV)

Step gradient
Gradient step 1: Step 45% solvent B (in Solvent A) (2CV)
Gradient step 2: Step 70% solvent B (in Solvent A) (2CV)
Gradient step 3: Step 100% solvent B (2CV)

Several regenerations strategies were tested:

Regeneration
Acetic acid (1 M) (2CV)

After LAC03 1/034
Wash with washing solution (2CV)
NaOH (0.5M) (2CV)
Wash with washing solution (2CV)
Ethanol (20%) (2CV)

Regeneration
Acetic acid (1 M) (2CV)

After LAC035/037
Wash with washing solution (2CV)
<table>
<thead>
<tr>
<th>Regeneration</th>
<th>Acetic acid (1 M)</th>
<th>(2CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After Wash</td>
<td>Wash with washing solution</td>
<td>(2CV)</td>
</tr>
<tr>
<td>LAC038/04 1/042/047</td>
<td>NaOH (0.5 M)</td>
<td>(2CV)</td>
</tr>
<tr>
<td>Wash with washing solution</td>
<td>(2-10CV)</td>
<td></td>
</tr>
<tr>
<td>Ethanol (20%)</td>
<td>(2CV)</td>
<td></td>
</tr>
<tr>
<td>Ethanol (70%)</td>
<td>(2CV)</td>
<td></td>
</tr>
<tr>
<td>Ethanol (20%)</td>
<td>(2CV)</td>
<td></td>
</tr>
</tbody>
</table>

The regeneration procedure with 70% ethanol is applied in order to remove hydrophobically bound substances like oleic acid or bLAC. Increasing and decreasing ethanol concentrations were done in steps to avoid air bubbles formation.

**Example 6: Yield and potency**

The yields of the conversion runs were determined by size exclusion HPLC (SE-HPLC) run according to standard procedures. The potency of the converted bLA was determined by cell killing and histone binding abilities. Cell killing and histone assays were performed as described herein below in Examples 6 and 7. The potency of bLAC determined by cell killing assay is given in pg bLAC per cell (LD50). Before testing in cell killing assay, the bLAC solutions were desalted against NaCl (0.9%) using NAP-25 desalting column (GE HealthCare).

**RESULTS: Comparison of the conversion runs**

The chromatograms of several conversion runs on Q sepharose XL are seen in Figure 9. In all the runs, the final concentration of EDTA was 1 mM.

The first run performed (LAC-031) was with a concentration of oleic acid of 0.2 mM (3 fold molar excess/bLA). The UV profile of LAC-031 showed a main peak eluted in the first step gradient (45% B-buffer) and a smaller peak eluted at 70% B-buffer. Both peaks were tested for cell killing ability. The LD50 in the peak at 70% B-buffer (N263-89B) was 39 pg/cell, whereas no cell killing ability was detected in the first main peak (N263-89A). From this result, it could be concluded that the preconditioning of the Q sepharose XL resin with oleic acid is not necessary to convert bLA to bLAC. Mixing of bLA, EDTA and oleic acid during the sample conditioning seems sufficient.
The yield of the conversion of LAC-031 was 17%. In order to increase the yield, the amount of oleic acid was increased during sample conditioning. An optimum concentration of 2 mM oleic acid (approximately 30 fold molar excess/bLA) was found in a previous experiment performed in microwell format. This amount of oleic acid was used during the sample conditioning in all the runs performed after LAC-031. The results of all the conversion runs are resumed in Table 5. The yield calculations and the cell killing abilities were performed on the peak eluted at 70% solvent B (in Solvent A).

Table 5: Summary of the conversion runs

<table>
<thead>
<tr>
<th>Prod ID</th>
<th>Item no</th>
<th>Load (mg/cm²)</th>
<th>Yield (mg/cm²)</th>
<th>Yield (%)</th>
<th>SE-HPLC (mg/mL)</th>
<th>Purity SE-HPLC (%)</th>
<th>Monomer</th>
<th>Killing Assay LD50 (pg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC-031*</td>
<td>N263-88F (89B)</td>
<td>30</td>
<td>5</td>
<td>17</td>
<td>1.0</td>
<td>100</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>LAC-034</td>
<td>N263-95G (96B)</td>
<td>31</td>
<td>26</td>
<td>84</td>
<td>3.3</td>
<td>93</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>LAC-035*</td>
<td>N263-99E (99G)</td>
<td>30</td>
<td>25</td>
<td>83</td>
<td>3.2</td>
<td>100</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>LAC-041*</td>
<td>N277-07C (07E)</td>
<td>30</td>
<td>28</td>
<td>93</td>
<td>3.2</td>
<td>100</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>LAC-042</td>
<td>N277-09F (99G)</td>
<td>42</td>
<td>38</td>
<td>90</td>
<td>4.0</td>
<td>100</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>LAC-047</td>
<td>N277-17G (18A)</td>
<td>90</td>
<td>76</td>
<td>84</td>
<td>8.4</td>
<td>100</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

*In bracket the sample ID after desalting used for cell killing assay

From the results showed in Table 5, it can be seen that the yield of the conversion is over 80% in the conditions tested, except for LAC-031, due to a lower amount of oleic acid used. Before LAC-041 the column was regenerated with a sequence of acetic acid (1 M), NaOH (0.5 M), ethanol (20% v/v), ethanol (70% v/v) and again ethanol (20% v/v) before re-equilibration. With this regeneration procedure a yield for the conversion over 80% was restored. Regeneration with only acetic acid (1 M) did not restore the yield for conversion, but gave a yield as low as 33-70%. Thus, regeneration with only acetic acid is not preferable.

Three different bLA loads 30, 42 and 90 mg/cm² corresponding to 2.9, 4.1 and 8.8 mg/mL Q sepharose XL were tested in LAC-041, -042 and -047. The yield of the conversion was found still over 80% at the highest load (LAC-047). This confirms the results of the previous microwell format experiment where it could be deducted a load of bLA of approximately 10 mg/mL resin.
RESULTS: Characterization of bLA and bLAC

SE-HPLC
The peaks eluted from the different conversion runs of bLA to bLAC were analyzed by SE-HPLC. These analyses were used for the quantification of bLA/bLAC before and after conversion in order to determine the yield of the runs (cf. Table 5). This analysis gives also an indication of the purity of bLAC after conversion, i.e. monomer, dimer, or aggregates forms. This is particularly important when the conversion run were performed at different bLA loads.

When looking at the SE-HPLC chromatogram (Figure 9), it is seen that the bLAC recovered at 70% B-buffer was mainly in monomer form. There was a tendency that at higher load, only bLAC monomer was recovered. The bLA dimer was then recovered in the first step gradient at 45% B-buffer, with bLA monomer in lower amount. No cell killing activity was recovered in the 45% B-buffer elution as mentioned previously.

Example 7: Histone assay

The histone binding abilities of the different bLAC recovered after different loads was determined by a histone assay, which was performed essentially as described below

The assay is designed to determine if bovine alpha-lactalbumin (bLA) has been successfully converted to bovine alpha-lactalbumin oleic acid complex (bLAC). bLAC is bound to histone H3 coated onto the 96-well ELISA plate and subsequently detected using a HRP-labelled polyclonal goat anti-bovine alpha-lactalbumin.

bLA that has not been converted to bLAC will not bind histone H3 and therefore not detected by the HRP-labelled anti-bovine alpha-lactalbumin antibody.

Capture antibody Coating Procedure
1. Coat FluoroNunc microplates with 100 µL/well of a solution containing 50 µg/mL of histone H3 dissolved in 50 mM NaCO₃ buffer pH 9.6. Incubate the plates 1 hour at room temperature without agitation.
2. Wash the plates three times in TBST-buffer in Plate Washer.
3. Block the plates by adding 200 µL/well of blocking solution.
   Cover the plates with a self adhesive plastic lid in order to avoid evaporation. Leave the plates for 30 minutes at room temperature.
4. Wash the plates three times in TBST-buffer in Plate Washer.
5. Use the plates immediately. If stored for short period, always cover plates with a self adhesive plastic lid.

bl_A/bLAC Sample Application
6. Apply the bLA/bLAC samples in duplicates as visualized in the plate layout in Table 6.
7. All prediluted bLA/bLAC samples should be further diluted in TBS buffer. Blanks are added in duplicate by applying TBS buffer.
8. Pre-dilution of bLA reference: Initially, dilute the bLA reference to a concentration of 70 µg/mL.
9. Reference and sample dilution series: Dilute the bLAC reference and test samples as recommended in Table 6 using TBS buffer.
10. Add 100 µL of each standard curve sample per well. Apply samples on the histone H3 coated plates immediately after preparation.
11. Add 100 µL of each bLA/bLAC sample per well. Apply samples on the histone H3-coated plate immediately after preparation.
12. Cover the plate with a self adhesive lid in order to avoid evaporation. Incubate the plates on plate shaker (gently shaking) for 60 minutes at room temperature.

Table 6: Histone assay plate setup with application of the standards, blanks and unknown samples. S1- S11 are dilutions of the bLA reference, see above. X to Z are samples to be assayed.

<table>
<thead>
<tr>
<th></th>
<th>1+2</th>
<th>3+4</th>
<th>5+6</th>
<th>7+8</th>
<th>9+10</th>
<th>11+12</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>S1</td>
<td>S2</td>
<td>S3</td>
<td>S4</td>
<td>S5</td>
<td>S6</td>
</tr>
<tr>
<td>B</td>
<td>S7</td>
<td>S8</td>
<td>S9</td>
<td>S10</td>
<td>S11</td>
<td>S12</td>
</tr>
</tbody>
</table>
Initially, pre-dilute the bl_A reference to obtain 70 µg/mL (for a 9.0 mg/mL reference this would be a 40 µL + 360 µL, followed by a 80 µL + 935 µL (totally a 127x dilution).

<table>
<thead>
<tr>
<th>C</th>
<th>X1</th>
<th>X2</th>
<th>X3</th>
<th>X4</th>
<th>X5</th>
<th>X6</th>
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</thead>
<tbody>
<tr>
<td>D</td>
<td>X7</td>
<td>X8</td>
<td>X9</td>
<td>X10</td>
<td>X11</td>
<td>X12</td>
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<tr>
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<td>Y1</td>
<td>Y2</td>
<td>Y3</td>
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<td>Y5</td>
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<td>Y10</td>
<td>Y11</td>
<td>Y12</td>
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<td>Z3</td>
<td>Z4</td>
<td>Z5</td>
<td>Z6</td>
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<tr>
<td>H</td>
<td>Z7</td>
<td>Z8</td>
<td>Z9</td>
<td>Z10</td>
<td>Z11</td>
<td>Z12</td>
</tr>
</tbody>
</table>

**Table 7: bLA/bLAC Standard curve and sample dilutions**

<table>
<thead>
<tr>
<th>S1 (X1-Z1) (=7 µg/well)</th>
<th>S2 (X2-Z2) (=5.0 µg/well)</th>
<th>S3 (X3-Z3) (=3.6 µg/well)</th>
<th>S4 (X4-Z4) (=2.55 µg/well)</th>
<th>S5 (X5-Z5) (=1.82 µg/well)</th>
<th>S6 (X6-Z6) (=1.30 µg/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prediluted bl_A reference</td>
<td>600 µL S1 + 240 µL buffer</td>
<td>600 µL S2 + 240 µL buffer</td>
<td>600 µL S3 + 240 µL buffer</td>
<td>600 µL S4 + 240 µL buffer</td>
<td>600 µL S5 + 240 µL buffer</td>
</tr>
<tr>
<td>Start Conc.: 70 µg/mL</td>
<td>Dilution factor: 1.4</td>
<td>Dilution factor: 1.96</td>
<td>Dilution factor: 2.74</td>
<td>Dilution factor: 3.84</td>
<td>Dilution factor: 5.38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S7 (X7-Z7) (~0.93 µg/well)</th>
<th>S8 (X8-Z8) (~0.66 µg/well)</th>
<th>S9 (X9-Z9) (~0.47 µg/well)</th>
<th>S10 (X10-Z10) (~0.34 µg/well)</th>
<th>S11 (x11-Z11) (~0.24 µg/well)</th>
<th>S12 (X12-Z12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>600 µL S6 + 240 µL buffer</td>
<td>600 µL S7 + 240 µL buffer</td>
<td>600 µL S8 + 240 µL buffer</td>
<td>600 µL S9 + 240 µL buffer</td>
<td>600 µL S10 + 240 µL buffer</td>
<td>Buffer</td>
</tr>
<tr>
<td>Dilution factor: 7.53</td>
<td>Dilution factor: 10.5</td>
<td>Dilution factor: 14.8</td>
<td>Dilution factor: 20.7</td>
<td>Dilution factor: 28.9</td>
<td>Conc.: 0 µg/mL</td>
</tr>
</tbody>
</table>
bLA/bLAC Detection

13. Wash the plates three times in TBST-buffer in a plate washer.

14. Add 100 µl per well of HRP-labelled anti-bLA antibodies (A10-1 28P) diluted 1+60,000 in TBST-buffer and incubate on plate shaker (gently shaking) 60 min at room temperature.

15. Wash the plates three times in TBST-buffer in Plate Washer.

16. Add 100 µl per well of TMB substrate and leave the plate in the dark for 10 min.

17. Stop the reaction with 100 µL/well of 1 M H₂SO₄.

18. Read plate at 450 nm using the Biotek EL808IU plate reader.

Evaluation

19. For all measurements, list the dilution of bLA/bLAC (X-values) versus the absorbance (Y-values).

   Example: Dilution = 0.01 when dilution factor is 100

20. Plot the dilutions versus the absorbance in SigmaPlot ver. 10.0 for Windows (see figure 1)

21. If bLA has complexed with oleic acid, binding will occur resulting in an increase in absorbance. Absorbance values above 3 times the absorbance of the background (blank) is defined as positive and oleic acid has complexed with bLA.

No difference in histone binding ability (cf. Figure 10) could be seen between the bLAC produced at different bLA loads. bLAC N262-35B produced with the former process and the former AIEC resin (DEAE-Trisacryl) was used as control. The histone binding ability was then the same.

Example 8: Cell killing assay

The cell killing abilities of the different bLAC produced in this study was determined by a cell killing assay. The following assay is designed to measure LACs potency to kill cancer cells and, was performed essentially as described herein below:
The lymphocytic leukaemia cell line from mouse called L1210 (ATCC cat.no CCL-21 9) is used as a model cell. A certain number of cells are mixed with different doses of LAC in RPMI medium without HEPES buffer and serum (both can affect the activity of LAC).

After 1 hour incubation serum is added to inactivate all extracellular LAC. Another 1 hour incubation allow cells to undergo apoptosis, and the ATP concentration is then determined, since there is a direct correlation between the relative level of ATP and the percentage of viable cells. The relative amount of ATP is determined using the ViaLight PLUS kit from Cambrex and a luminometer.

Solvents

0.9% NaCl
9 g NaCl is dissolved in MilliQ H₂O to a final volume of 1000 mL
Filter the solution through a 0.2 µm VacuCap filter.
Store at room temperature. Estimated storage time: long

RPMI medium without FBS
To 490 mL RPMI 1640 add 5 mL 100x NEAA and 5 mL 200 mM sodium pyruvate.
Store at 2-8°C. Estimated storage time 3 months.

RPMI medium with 33.4% FBS
To 20 mL RPMI 1640 without FBS (but with NEAA and sodium pyruvate) add 10 mL fetal bovine serum.
Store at 2-8°C. Estimated storage time 1 month.
AMR PLUS (ATP Monitoring 1. Add Assay buffer into the vial containing the lyophilized AMR PLUS until the vial is approximately 75% full.

2. Replace the yellow screw cap and mix gently.

3. Pour the reconstituted reagent into the remaining Assay buffer.

4. Repeat the above process to ensure all the lyophilized reagent has been transferred into the Assay buffer.

5. Allow the reagent to equilibrate for 15 minutes at room temperature to ensure complete rehydration.

Can be stored at room temperature for up to 8 hours or for 24 hours at 2 - 8°C. Unused reagent can be stored at -20°C for up to 2 months. Once thawed, reagent must not be refrozen, and reagents should be allowed to reach room temperature without the aid of artificial heat before use.

Allow all solvents to reach room temperature before use unless stated otherwise.

Procedure:

1. Count the culture that supplies cells for the assay on day 2 after last sub-culture (set up at \(1 \cdot 10^5\) AnL), and check viability with trypan blue exclusion - viability must be >90% in order to perform the assay!

2. Predilution: Initially, all LAC samples (i.e. control and test samples) should be diluted to 1.0 mg/mL. This initial dilution should be done in 0.9% NaCl solution. If the LAC concentration is unknown, assume 10 mg/mL.

   Example: 10 mg/mL LAC samples and bLAC control are diluted 40\(\mu\)L+360\(\mu\)L in 0.9% NaCl solution.
3. Prepare bLAC Control dilution series as described in Table 8. 0.9% NaCl solution is used as diluent.

Comment: The dilution ratio of 2^1 is applied to cover the measurement interval from 20 µg/well to 0.35 µg/well in 11 dilutions.

4. Prepare LAC test samples as described in Table 8. 0.9% NaCl solution is used as diluent.

Comment: One sample can be applied on one plate. The dilution ratio of 2^1 is applied to cover the measurement interval from 20 µg/well to 0.35 µg/well in 11 dilutions.

5. Gently spin down the cells at room temperature for 10 minutes at 200 g (take 0.10-1.06 cells for each well in a 96-well plate).

6. Carefully wash the cells once with an equal volume of PBS and spin at 200 g for 10 minutes.

7. Carefully remove the supernatant from the pelleted cells and discard it.

8. Resuspend the cells to a concentration of approximately 2.5-10^6 cells/mL (or as required) in RPMI medium without serum. Take a sample and count the cells.

9. Adjust the cell density to 2.0-10^6 cells/mL with RPMI medium without serum, and take a new sample for determination of actual cell density (count later).

10. While preparing the cells apply 20 µl of the different samples in triplicate to the 96-well plate according to the scheme in Table 8. Include a negative control sample (0.9% NaCl).

11. Dispense 50 µl cell suspension to each used well of the plate (100,000 cells per well).

12. Incubate the plate for 60 minutes at 37°C and 5% CO₂.

13. After the 1 hour incubation add 30 µl RPMI medium with 33.4% fetal bovine serum to each well.
14. Incubate for another 60 minutes (or as required) at 37°C and 5% CO₂, and then determine cell viability.

**Table 8**: bLAC Control curve dilutions (2+1 dilution series in 0.9% NaCl solution).

<table>
<thead>
<tr>
<th></th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(≥20 µg/well)</td>
<td>(≥13.3 µg/well)</td>
<td>(≥8.9 µg/well)</td>
<td>(≥5.9 µg/well)</td>
<td>(≥4.0 µg/well)</td>
<td>(≥2.6 µg/well)</td>
</tr>
<tr>
<td>Prediluted LAC reference</td>
<td>200 µL S1 + 100 µL 0.9% NaCl</td>
<td>200 µL S2 + 100 µL 0.9% NaCl</td>
<td>200 µL S3 + 100 µL 0.9% NaCl</td>
<td>200 µL S4 + 100 µL 0.9% NaCl</td>
<td>200 µL S5 + 100 µL 0.9% NaCl</td>
<td></td>
</tr>
<tr>
<td>Start Conc.: 500 ng/mL</td>
<td>Dilution factor: 1.50</td>
<td>Dilution factor: 2.25</td>
<td>Dilution factor: 3.38</td>
<td>Dilution factor: 5.06</td>
<td>Dilution factor: 7.59</td>
<td></td>
</tr>
</tbody>
</table>

Initially, pre-dilute the bLAC Control with 0.9% NaCl solution.

**Table 9**: LAC test sample dilutions (2+1 dilution series in 0.9% NaCl solution).

<table>
<thead>
<tr>
<th></th>
<th>S7</th>
<th>S8</th>
<th>S9</th>
<th>S10</th>
<th>S11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(≥1.8 µg/well)</td>
<td>(≥1.2 µg/well)</td>
<td>(≥0.78 µg/well)</td>
<td>(≥0.52 µg/well)</td>
<td>(≥0.35 µg/well)</td>
</tr>
<tr>
<td>200 µL S6 + 100 µL 0.9% NaCl</td>
<td>200 µL S7 + 100 µL 0.9% NaCl</td>
<td>200 µL S8 + 100 µL 0.9% NaCl</td>
<td>200 µL S9 + 100 µL 0.9% NaCl</td>
<td>200 µL S10 + 100 µL 0.9% NaCl</td>
<td></td>
</tr>
<tr>
<td>Dilution factor: 11.39</td>
<td>Dilution factor: 17.09</td>
<td>Dilution factor: 25.63</td>
<td>Dilution factor: 38.44</td>
<td>Dilution factor: 57.67</td>
<td></td>
</tr>
</tbody>
</table>

Initially, pre-dilute the LAC test sample with 0.9% NaCl solution.
Table 10: LAC cell killing assay plate setup with application of the bLAC Control, blank and samples. S1-S11 are dilutions of the bLAC Control, see above. X1 to X11 are dilutions of the sample to be assayed. B is 0.9% NaCl.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
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</table>

Determining viability

Allow all solvents to reach room temperature before use unless stated otherwise.

*Procedure:*

Remove the culture plate from the incubator and allow cooling to room temperature for 10 minutes.

Add 50 µL of cell lysis reagent to each well and incubate for 10 minutes at room temperature.

Add 100 µL AMR PLUS solution to each well and incubate the plate for 5 minutes at room temperature.

Read the plate in the luminometer with an integrated reading time of 1
second and evaluate the data.

Data evaluation

1. For both sample and reference measurements, list the dose of LAC in µL per well (20 µL/dilution factor) (X-values) versus the luminescence (Y-values).

2. Evaluate the fit for both control and sample curves using a 4 parameter logistic fit in SigmaPlot Ver.10 for Windows or similar:
   \[ Y = Y_0 + A \left( \frac{X}{X_0^B} + 1 \right) \]
   where the following 4 parameters are estimated:
   - \( A \) = Difference between maximum luminescence at highest cell viability and minimum luminescence at background,
   - \( Y_0 \) = luminescence at background when 0% viability.
   - \( X_0 \) = LAC dose in well (in µL) when luminescence is \( Y_0 + A/2 \).
   - \( B \) = the tangential slope of the curve in \([X, Y] = [X_0, Y_0 + A/2] \).

3. Determine LD_{50} expressed as pL/cell at the L1210 cell concentration applied (cells/mL) after 1 hour incubation for both the reference and the sample by LD_{50} = X_0 \cdot 10^{6} C_{ceil}
   where \( C_{ceil} \) is the actual number of viable cells added per well in the assay.

4. LD_{50} expressed as pg/cell at the L1210 cell concentration applied (cells/mL) after 1 hour incubation can be calculated by multiplying the concentration of LAC in the undiluted sample or reference (LAC concentration based on SEC assay) to the LD_{50} expressed as pL/cell.

5. An assay should be discarded if LD_{50} of the bLAC control is below 9 pg/cell or above 50 pg/cell.

The cell killing abilities of the different bLAC produced in this study are reported in Table 5. In Figure 11, the cell killing ability of bLAC N277-09G from LAC-042 was compared to a previous bLAC sample (N262-35B) converted with an other resin (DEAE...
Trisacryl) than the one used in this study and with the previous conversion method requiring a preconditioning of the resin with oleic acid.

The cell killing abilities of the bLAC samples are expressed by the relative amount of ATP determined using the ViaLight PLUS kit (luminescence) giving an indication of the percentage of viable cells. The cell killing abilities of the two bLAC samples expressed as a decrease in luminescence were found to be similar.

CONCLUSION

In this study a new method for the conversion of bLA to bLAC was found. The AIEC resin (Q sepharose XL) was no longer preconditioned with oleic acid, but bLA, EDTA and oleic acid were mixed prior the chromatographic step. The optimum conditions for the conversion were a 15 fold molar excess of EDTA/bLA and a 29 fold molar excess of oleic acid/bLA, giving final concentrations of EDTA and oleic acid of 1 and 2 mM respectively.

With this method, it was then possible to achieve a maximum load of 90 mg bLA per cm² on the AIEC column, or 9 mg bLA/mL resin which is an increase of 4.5 times of the bLA load. Then the column was pre-conditioned with oleic acid.

Cell killing and histone binding abilities of the bLAC obtained in this way were verified and found to be similar as bLAC converted previously with other method and other AIEC resin.

The bLAC recovered from the anion exchange step was found to be mainly in a monomeric form.

Eight conversion runs were performed on the same column packed with Q sepharose XL resin. A regeneration procedure consisting of sequential CIP with acetic acid, sodium hydroxide and ethanol 70% was necessary to maintain high conversion yield.

Example 9: Conversion of bLA to bLAC with different fatty acids
The method for conversion of bl_A in complex with fatty acids was tested in this study with mono or polyunsaturated cis fatty acids other than C18:1 (n-9) cis (oleic acid), and with fatty acids of shorter or longer carbon chains (C16 to C20) using the conversion method described herein above where the AIEC resin (Q sepharose XL) was no longer preconditioned with oleic acid.

The method for conversion of bl_A with fatty acids was tested with mono or polyunsaturated cis fatty acids other than C18:1 (n-9) cis (oleic acid), and with fatty acids of shorter or longer carbon chains (C16, C17 and C20).

Cell killing and histone binding abilities of the complex obtained between bl_A and the different fatty acids were determined.

*Conversion of bLA to complex with fatty acids*

Two batches of bl_A were used for the conversion.

**Table 11**: Batches of bl_A used for conversion

<table>
<thead>
<tr>
<th>bl_A batch</th>
<th>Amount</th>
<th>Storage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N277-64A</td>
<td>7x1.7 mL aliquot</td>
<td>-20°C prepared from bl_A batch N278-50C produced at Natimmune.</td>
<td>bLA concentration: 4.01 mg/mL (SEC-HPLC) with bLA reference N262-06A</td>
</tr>
<tr>
<td>N289-56A (=N289-78B)</td>
<td>6x1.8 mL aliquot</td>
<td>-20°C prepared from bl_A batch N177-76B produced at Biovian.</td>
<td>bLA concentration: 2.5 mg/mL (SEC-HPLC) with bLA reference N286-28A</td>
</tr>
</tbody>
</table>

*From the same bLA batch (N1 77-76B), but from different bags of this batch.*

The following fatty acids were purchased from Larodan (Sweden) for the conversion of bLA to complex.

**Table 12**: Fatty acids used for conversion
<table>
<thead>
<tr>
<th>Name</th>
<th>No. of C:Double bond</th>
<th>Configuration</th>
<th>Larodan no.</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitoleic Acid</td>
<td>16:1 (n-7)</td>
<td>Cis</td>
<td>10-1601-30</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Heptadecenoic Acid</td>
<td>17:1 (n-7)</td>
<td>Cis</td>
<td>10-1701-30</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>18:1 (n-9)</td>
<td>Cis</td>
<td>10-1801-17</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Elaidic acid</td>
<td>18:1 (n-9)</td>
<td>Trans</td>
<td>10-1810-13</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Vaccenic acid</td>
<td>18:1 (n-7)</td>
<td>Cis</td>
<td>10-1812-30</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>18:2(n-6)</td>
<td>Cis</td>
<td>10-1802-13</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Alfa Linolenic Acid</td>
<td>18:3(n-3)</td>
<td>Cis</td>
<td>10-1803-30</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Gamma Linolenic Acid</td>
<td>18:3(n-6)</td>
<td>Cis</td>
<td>10-1830-30</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Stearidonic acid</td>
<td>18:4(n-3)</td>
<td>Cis</td>
<td>10-1840-4</td>
<td>&gt;97%</td>
</tr>
<tr>
<td>Gondoic acid</td>
<td>20:1 (n-9)</td>
<td>Cis</td>
<td>10-2001-30</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
<td>20:5(n-3)</td>
<td>Cis</td>
<td>10-2005-30</td>
<td>&gt;99%</td>
</tr>
</tbody>
</table>

The conversion of bl_A to a complex with the different fatty acid was performed according to Examples 4 and 5 described herein above.

On the day of conversion, a solution was prepared with the respective fatty acid according to the following Table:

**Table 13: Preparation of fatty acid solution**

<table>
<thead>
<tr>
<th></th>
<th>Melting point</th>
<th>Volume Ethanol</th>
<th>Ethanol (mg)</th>
<th>Equilibration buffer (mL)</th>
<th>Total volume (mL)</th>
<th>FA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid</td>
<td>13</td>
<td>20</td>
<td>0.25</td>
<td>20</td>
<td>20.27</td>
<td>3.5</td>
</tr>
<tr>
<td>Vaccenic acid</td>
<td>39</td>
<td>20</td>
<td>0.25</td>
<td>20</td>
<td>20.27</td>
<td>3.5</td>
</tr>
<tr>
<td>Elaidic acid</td>
<td>44</td>
<td>20</td>
<td>0.25</td>
<td>20</td>
<td>20.27</td>
<td>3.5</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>-5</td>
<td>20</td>
<td>0.25</td>
<td>20</td>
<td>20.27</td>
<td>3.5</td>
</tr>
</tbody>
</table>
bl_A, EDTA and fatty acid were mixed with the following ratio:

<table>
<thead>
<tr>
<th>Mix ID</th>
<th>Sample ID</th>
<th>Fatty acid</th>
<th>Molar ratio EDTA/bLA</th>
<th>Molar ratio Oleic acid/bLA</th>
<th>AIEC load mL</th>
<th>AIEC load mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC-078</td>
<td>N277-64C</td>
<td>Oleic acid</td>
<td>15</td>
<td>30</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>LAC-080</td>
<td>N277-74C</td>
<td>Vaccenic acid</td>
<td>15</td>
<td>30</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>LAC-082</td>
<td>N277-77D</td>
<td>Linoleic acid</td>
<td>15</td>
<td>30</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>LAC-083</td>
<td>N277-80B</td>
<td>Alfa Linolenic acid</td>
<td>15</td>
<td>30</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>LAC-125</td>
<td>N289-56C</td>
<td>Palmitoleic acid</td>
<td>19</td>
<td>37</td>
<td>54</td>
<td>34**</td>
</tr>
<tr>
<td>LAC-126</td>
<td>N289-59B</td>
<td>Eicosapentaenoic acid</td>
<td>19</td>
<td>37</td>
<td>54</td>
<td>34**</td>
</tr>
<tr>
<td>LAC-127</td>
<td>N289-62B</td>
<td>Heptadecenoic acid</td>
<td>19</td>
<td>37</td>
<td>54</td>
<td>34**</td>
</tr>
<tr>
<td>LAC-128</td>
<td>N289-64B</td>
<td>Gamma Linolenic acid</td>
<td>19</td>
<td>37</td>
<td>54</td>
<td>34**</td>
</tr>
<tr>
<td>LAC-132</td>
<td>N289-67B</td>
<td>Gondoic acid</td>
<td>19</td>
<td>37</td>
<td>54</td>
<td>34**</td>
</tr>
<tr>
<td>LAC-134</td>
<td>N289-70B</td>
<td>Stearidonic acid</td>
<td>19</td>
<td>37</td>
<td>54</td>
<td>34**</td>
</tr>
<tr>
<td>LAC-140</td>
<td>N289-78D</td>
<td>Oleic acid</td>
<td>19</td>
<td>37</td>
<td>54</td>
<td>34**</td>
</tr>
</tbody>
</table>

* Sample conditioning with bl_A N277-64A

** Sample conditioning with bl_A N289-56A/N289-78B

Conversion with Elaidic acid was not performed, as the conditioned sample made from this fatty acid was not soluble. Elaidic acid is the fatty acid with the highest melting point among those tested and the double bond is a trans double bond.

Conversion with oleic acid was done twice with the two different bl_A start materials. The molar ratio between EDTA and bl_A and fatty acid and bl_A should have been 15 and 30 respectively. Other molar ratios were applied from LAC-125 (cf. Table 14). The bl_A reference for the SE-HPLC analysis was changed. With the new reference the bl_A
concentration is approximately 15% lower. The sample conditioning calculations were done on basis of the concentration measured.

Each conditioned sample was applied on a 0.78 cm² column (Tricorn 10/100) newly packed and CIP'ed with Q sepharose XL (10 cm bed height). The load of bl_A during the purifications was approximately 54 mg/cm² (LAC-078 to LAC-083) and 44 mg/cm² (LAC-125 to LAC-140).

bLAC recovery

In every purification the whole bLAC recovery was stored frozen, except for one small sample (1 mL) that was desalted against milliQ-H2O using a NAP-10 column (GE Healthcare). The desalted sample was used for cell killing assay and GC analysis.

bLAC recovery characterization:

Yield

The yields of the conversion runs were determined by size exclusion HPLC (SE-HPLC) run according to standard methods for example thos described in the GE healthcare book: "Gel Filtration: Principles and Methods".

Potency

The potency of the converted bLA was determined by cell killing abilities. Cell killing was run according to Example 8. The potency of bLAC determined by cell killing assay is given in pg bLAC per cell (LD50). Before testing in cell killing assay, the bLAC solutions were desalted against milli-Q H2O using NAP-10 desalting column (GE HealthCare).

Identity

The presence of complex bLA and fatty acid was determined by histone binding assay. The histone binding assays was run according to Example 7. Only complex between bLA and fatty acid have histone biding abilities.
GC-Analysis

The amount of lipid and the lipid composition of the bLAC samples were determined by Net-Food lab (Finland) after conventional methods: After esterification by the Boron trifluoride-methanol method, the fatty acids methyl esters (FAME) in the bLAC samples were analyzed by Gas Chromatography. Lipid content was calculated in relation to the concentration of the internal standard. The method is based on the European Pharmacopoeia (5.6) protocol 2.4.22 (Composition of Fatty Acids by Gas Chromatography, Method C).

RESULTS

Conversion runs

The chromatograms of the conversion of bLAC with the different fatty acids can be seen in Figure 12 and 13.

When bLA N289-56A was used as start material the first eluted peak corresponding to non converted bLA was lower. This can be due to the higher molar ratio between bLA and EDTA and bLA and oleic acid, and also the lower amount of bLA loaded on the column (cf. Table 14). With these considerations, oleic acid and gondoic acid showed the same elution profile, while with all the other fatty acids the first peak is increased. When stearidonic acid was used for conversion, no peak corresponding to a complex between fatty acid and bLA was obtained. Here all protein eluted in the 1st step gradient.

The yield of the conversions runs based on the SE-HPLC analysis can be seen in next Table:

<table>
<thead>
<tr>
<th>Pro Fatty acid</th>
<th>Recovery</th>
<th>bLAC</th>
<th>Volume</th>
<th>bLA load</th>
<th>Yield</th>
<th>Yield</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>d. ID</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(mg/mL)</td>
<td>(mL)</td>
<td>(mg/cm²)</td>
<td>(mg/cm²)</td>
<td>(%)</td>
<td>Oleic</td>
<td></td>
</tr>
<tr>
<td>Acid Type</td>
<td>Start Material</td>
<td>New Start Material</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
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<td>--------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>N267-64A</td>
<td>N289-56A/78B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccenic acid</td>
<td>N277-64H</td>
<td>N277-74H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>N289-57B</td>
<td>N289-59G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>N289-63A</td>
<td>N289-64G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**LA C-078**

- Oleic acid
  - N277-64A: 4.1, 6.5, 54, 34, 63%, 1 (def)
  - N277-65A: 2.9

**LA C-080**

- Vaccenic acid
  - N277-74A: 4.9, 6.5, 54, 41, 76%, 1.20

**LA C-082**

- Linoleic acid
  - N277-78A: 4.4, 7, 54, 39, 73%, 1.16

**LA C-083**

- Linolenic acid
  - N277-80A: 4.1, 7.5, 54, 39, 73%, 1.16

**LA C-125**

- Palmitoleic acid
  - N289-57B: 3.6, 6.5, 44, 30, 68%, 0.83

**LA C-126**

- Eicosapentaenoic acid
  - N289-59B: 2.6, 6.5, 44, 22, 49%, 0.60

**LA C-127**

- Heptadecenoic acid
  - N289-63B: 4.0, 6.5, 44, 33, 76%, 0.93

**LA C-128**

- Gamma Linolenic acid
  - N289-64B: 2.8, 6.5, 44, 23, 53%, 0.65
The yield for the two conversion runs with oleic acid was rather different. Conversion was then performed with different sample conditioning and different bl_A start material as discussed earlier. The lower load and different sample conditioning for LAC-140 might explain the better yield obtained.

In Table 16, the yield of conversion with oleic acid was compared to the yield obtained with the other fatty acids for similar sample conditioning, sample load and bl_A start material.

**Table 16: Yield ranking compared to oleic acid (18:1 (n-9))**

<table>
<thead>
<tr>
<th>Fatty acid (From + to -)</th>
<th>Rank (from Table 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Vaccenic acid</td>
<td>18:1 (n-7)</td>
</tr>
<tr>
<td>2. Gondoic acid</td>
<td>20:1 (n-9)</td>
</tr>
<tr>
<td>3. Linoleic acid</td>
<td>18:2(n-6)</td>
</tr>
<tr>
<td>4. Alfa-Linolenic acid</td>
<td>18:3(n-3)</td>
</tr>
<tr>
<td>5. OLEIC ACID</td>
<td>18:1 (n-9)</td>
</tr>
<tr>
<td>6. Heptadecenoic acid</td>
<td>17:1 (n-7)</td>
</tr>
</tbody>
</table>

* Item no. after desalting
** No converted peak recovered
Lipid composition by GC analysis

The lipid composition and lipid content of the bl_A in complex with the different fatty acids obtained was determined by GC analysis. As shown in Table 17, the fatty acid used for the conversion was detected in all the corresponding samples analysed by Gas chromatography, except for the sample converted with gamma Linolenic acid. For this sample the amount of fatty acid was below the detection limit of the analysis. As the conversion performed normally for this fatty acid, the sample was sent for retest by GC analysis. Stearidonic acid was not detected in the sample recovered from the conversion run with this fatty acid. In this case no converted bl_A peak was obtained, and the sample corresponded to non-converted bl_A was sent to GC analysis.

Table 17: Lipid composition by GC analysis

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>m-1</th>
<th>pal</th>
<th>hpa</th>
<th>heptadecanoic</th>
<th>vaccenic</th>
<th>CL</th>
<th>18:1c9t11</th>
<th>18:1c11</th>
<th>18:2c6t11</th>
<th>18:3c6t11</th>
<th>18:4c6t11</th>
<th>18:5c6t11</th>
<th>18:6c6t11</th>
<th>Docosahexaenoic</th>
<th>Eicosapentaenoic</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sa Fatty acid</td>
<td>14 0</td>
<td>16 16</td>
<td>17 18</td>
<td>18 18</td>
<td>18 18</td>
<td>18 20</td>
<td>20 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mpl used for conversi on</td>
<td>(n-7)</td>
<td>(n-7)</td>
<td>(n-7)</td>
<td>(n-9)</td>
<td>(n-6)</td>
<td>(n-6)</td>
<td>(n-9)</td>
<td>(n-6)</td>
<td>(n-9)</td>
<td>(n-3)</td>
<td>(n-5)</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>N2 Oleic acid 77-65A</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>98</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
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<td></td>
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</tr>
<tr>
<td>N2 Vaccenic acid 77-741</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>95</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td></td>
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</tr>
<tr>
<td>N2 Linoleic acid 77-78F</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2 Alfa-Linolenic acid 77-80H</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
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<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>N2 Palmitoleic acid 89-57C</td>
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<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td>N2 Eicosapentaenoic acid 89-59H</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2 Heptadecenoic acid 89-63B</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2 Gamma-Linolenic acid 89-64H</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2 Gondoic acid 89-68C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2 Steazdonic acid 89-7OF</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>92</td>
<td>0</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2 Oleic acid 89-79D</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Below detection limit

Only non-converted product tested; below detection limit

Not done

The molar Npid/bLA ratios in the samples were calculated from the results of the GC analysis. The calculations are shown in Table 18.

**Table 18: Molar ratio Npid/bLA**
The molar ratio between bLA and lipid was found highest when oleic acid was used to conversion. Similar molar ratios to oleic acid were obtained for fatty acids giving the highest yield of conversion, i.e. gondoic acid, vaccenic and linoleic acid. Then the molar ratio Npid/bLA decrease for the two fatty acids were the yields of the conversion was the lowest, i.e. Palmitoleic acid and Eicosapentaenoic acid.

The Npid/bLA ratio in the sample prepared using gamma-Linolenic acid could not be measured.

*Identity by Histone assay*

The histone binding abilities of the bLAC samples converted with the different fatty acids were compared by histone binding assay. For each plate, a bLAC reference converted with oleic was run. From the binding curve EC50 was determined for each sample based on the SE-HPLC concentration. The EC50 were normalized to the EC50 of the bLAC reference applied in the plate. The results are shown in Table 19.

**Table 19**: EC50 by histone binding assay

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Plate ID:</th>
<th>Fatty acid used</th>
<th>EC50 (µg/mL)</th>
<th>Binding (%) to ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N277-65A</td>
<td>N281 -20A</td>
<td>Oleic acid</td>
<td>9</td>
<td>107%</td>
</tr>
<tr>
<td>N277-30A</td>
<td>N281 -20A</td>
<td>Oleic acid (reference)</td>
<td>9.6</td>
<td>100%(def)</td>
</tr>
<tr>
<td>N277-74I</td>
<td>N281 -22A</td>
<td>Vaccenic acid</td>
<td>7.4</td>
<td>142%</td>
</tr>
<tr>
<td>N277-78F</td>
<td>N281 -22A</td>
<td>Linoleic acid</td>
<td>19.1</td>
<td>55%</td>
</tr>
<tr>
<td>Sample</td>
<td>Fatty Acid</td>
<td>EC50 (%)</td>
<td>Histone Binding Capacity</td>
<td>Molar Ratio Npid/bLA</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------------</td>
<td>----------</td>
<td>--------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>N277-80H N281-22A</td>
<td>Alfa-Linolenic acid</td>
<td>71.6</td>
<td>15%</td>
<td></td>
</tr>
<tr>
<td>N277-30A N281-22A</td>
<td>Oleic acid (reference)</td>
<td>10.5</td>
<td>100% (def)</td>
<td></td>
</tr>
<tr>
<td>N289-57C N291-80B</td>
<td>Palmitoleic acid</td>
<td>17.5</td>
<td>35%</td>
<td></td>
</tr>
<tr>
<td>N289-59H N291-80B</td>
<td>Eicosapentaenoic acid</td>
<td>41.3</td>
<td>15%</td>
<td></td>
</tr>
<tr>
<td>N289-63B N291-80B</td>
<td>Heptadecenoic acid</td>
<td>10.1</td>
<td>61%</td>
<td></td>
</tr>
<tr>
<td>N277-30A N291-80B</td>
<td>Oleic acid (reference)</td>
<td>6.2</td>
<td>100% (def)</td>
<td></td>
</tr>
<tr>
<td>N289-64H N291-80C</td>
<td>Gamma-Linolenic acid</td>
<td>41.5</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td>N289-68C N291-80C</td>
<td>Gondoic acid</td>
<td>3.1</td>
<td>174%</td>
<td></td>
</tr>
<tr>
<td>N277-30A N291-80C</td>
<td>Oleic acid (reference)</td>
<td>5.4</td>
<td>100% (def)</td>
<td></td>
</tr>
<tr>
<td>N289-70H N296-04B</td>
<td>Stearidonic acid</td>
<td>No binding</td>
<td>No binding</td>
<td></td>
</tr>
<tr>
<td>N277-30A N296-04B</td>
<td>Oleic acid (reference)</td>
<td>5.6</td>
<td>100% (def)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 20:** EC50 ranking by histone binding assay

<table>
<thead>
<tr>
<th>Fatty acid histone binding ranking</th>
<th>Histone binding capacity</th>
<th>Molar ratio Npid/bLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Gondoic acid 20:1 (n-9)</td>
<td>174%</td>
<td>16</td>
</tr>
<tr>
<td>2. Vaccenic acid 18:1 (n-7)</td>
<td>142%</td>
<td>14</td>
</tr>
<tr>
<td>3. Oleic acid 18:1 (n-9)</td>
<td>107%</td>
<td>18</td>
</tr>
<tr>
<td>4. Heptadecenoic acid 17:1 (n-7)</td>
<td>61%</td>
<td>11</td>
</tr>
<tr>
<td>5. Linoleic acid 18:2(n-6)</td>
<td>55%</td>
<td>13</td>
</tr>
<tr>
<td>6. Palmitoleic acid 16:1 (n-7)</td>
<td>35%</td>
<td>6</td>
</tr>
<tr>
<td>7. alfa-Linolenic acid 18:3(n-3)</td>
<td>15%</td>
<td>12</td>
</tr>
<tr>
<td>7. Eicosapentaenoic acid 20:5(n-3)</td>
<td>15%</td>
<td>3</td>
</tr>
<tr>
<td>9. gamma-Linolenic acid 18:3(n-6)</td>
<td>13%</td>
<td>no data</td>
</tr>
<tr>
<td>10. Stearidonic acid 18:4(n-3)</td>
<td>No binding</td>
<td>no data</td>
</tr>
</tbody>
</table>
As shown in Table 20, it seems that there is a good correlation between the molar ratio Npid/bLA and histone binding of the bLA-lipid complex. The higher the molar ratio, the higher binding is obtained, with the exception of the product obtained when the conversion was performed with gamma Linolenic acid (which again behaves differently to the other fatty acids tested).

**Potency by cell killing assay**

The cell killing ability of the bl_A in complex with the different fatty acids was verified. From the cell killing analysis, LD50 was determined for each bLA-fatty acid complex. The results are shown in Table 21:

<table>
<thead>
<tr>
<th>SampleID</th>
<th>Plate D:</th>
<th>Fatty acid used</th>
<th>S&lt;sub&gt;E-HFPLCC&lt;/sub&gt; for conversion</th>
<th>LD50 (mg/mL)</th>
<th>LD50 (pL/cell)</th>
<th>LD50 (pg/cell)</th>
<th>Binding % to ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>N277-65A</td>
<td>N281 -24B</td>
<td>Oleic acid</td>
<td></td>
<td>2.94</td>
<td>6.33</td>
<td>19</td>
<td>100%</td>
</tr>
<tr>
<td>N277-74I</td>
<td>N281 -24B</td>
<td>Vcacinic acid</td>
<td></td>
<td>3.26</td>
<td>6.55</td>
<td>21</td>
<td>90%</td>
</tr>
<tr>
<td>N277-78F</td>
<td>N281 -24C</td>
<td>Linoleic acid</td>
<td></td>
<td>2.76</td>
<td>6.34</td>
<td>17</td>
<td>112%</td>
</tr>
<tr>
<td>N277-80H</td>
<td>N281 -24C</td>
<td>Alpha Linolenic acid</td>
<td></td>
<td>2.68</td>
<td>7.92</td>
<td>21</td>
<td>90%</td>
</tr>
<tr>
<td>N289-57C</td>
<td>N291 -76A</td>
<td>Palmitoleic acid</td>
<td></td>
<td>2.32</td>
<td>13.28</td>
<td>31</td>
<td>126%</td>
</tr>
<tr>
<td>N277-30A</td>
<td>N291 -76A</td>
<td>Oleic acid</td>
<td></td>
<td>7.55</td>
<td>5.1 4</td>
<td>39</td>
<td>100%</td>
</tr>
<tr>
<td>N289-59H</td>
<td>N291 -84A</td>
<td>Eicosapentaenoic acid</td>
<td></td>
<td>1.84</td>
<td>37.44</td>
<td>69</td>
<td>48%</td>
</tr>
<tr>
<td>N277-30A</td>
<td>N291 -84A</td>
<td>Oleic acid</td>
<td></td>
<td>7.55</td>
<td>4.33</td>
<td>33</td>
<td>100%</td>
</tr>
<tr>
<td>N289-63B</td>
<td>N291 -84B</td>
<td>Heptadecenoic acid</td>
<td></td>
<td>2.56</td>
<td>8.5</td>
<td>22</td>
<td>145%</td>
</tr>
<tr>
<td>N277-30A</td>
<td>N291 -84B</td>
<td>Oleic acid</td>
<td></td>
<td>7.55</td>
<td>4.2</td>
<td>32</td>
<td>100%</td>
</tr>
<tr>
<td>N289-64H</td>
<td>N291 -90A</td>
<td>Gamma Linolenic acid</td>
<td></td>
<td>1.56</td>
<td>26.75</td>
<td>42</td>
<td>90%</td>
</tr>
<tr>
<td>N277-30A</td>
<td>N291 -90A</td>
<td>Oleic acid</td>
<td></td>
<td>7.55</td>
<td>5.01</td>
<td>38</td>
<td>100%</td>
</tr>
<tr>
<td>N289-68C</td>
<td>N291 -90B</td>
<td>Gondoic acid</td>
<td></td>
<td>3.83</td>
<td>34.3</td>
<td>13.1</td>
<td>25%</td>
</tr>
<tr>
<td>N277-30A</td>
<td>N291 -90B</td>
<td>Oleic acid</td>
<td></td>
<td>7.55</td>
<td>4.41</td>
<td>33</td>
<td>100%</td>
</tr>
<tr>
<td>N289-70H</td>
<td></td>
<td>Stearidonic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Not tested</td>
</tr>
</tbody>
</table>
Also used as reference in the cell killing assay

From the results presented in Table 21, it can be seen that most of the bl_A converted with the fatty acid tested have similar LD50 to the bl_A converted with oleic acid with the exception of the complex obtained with eicosapentaenoic acid and gondoic acid, which are the fatty acids with the longest carbon chains.

**Table 22:** LD50 ranking by cell killing assay

<table>
<thead>
<tr>
<th>bl_A and Fatty acid histone binding ranking</th>
<th>Molar ratio NpId/blA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Heptadecenoic acid 17:1(n-7)</td>
<td>145%</td>
</tr>
<tr>
<td>2. Palmitoleic acid 16:1(n-7)</td>
<td>126%</td>
</tr>
<tr>
<td>3. Linoleic acid 18:2(n-6)</td>
<td>112%</td>
</tr>
<tr>
<td>4. Oleic acid 18:1(n-9)</td>
<td>100% (def)</td>
</tr>
<tr>
<td>5. Alfa Linolenic acid 18:3(n-3)</td>
<td>90%</td>
</tr>
<tr>
<td>5. Vaccenic acid 18:1(n-7)</td>
<td>90%</td>
</tr>
<tr>
<td>5. Gamma Linolenic acid 18:3(n-6)</td>
<td>90%</td>
</tr>
<tr>
<td>6. Eicosapentaenoic acid 20:5(n-3)</td>
<td>48%</td>
</tr>
<tr>
<td>7. Gondoic acid 20:1(n-9)</td>
<td>25%</td>
</tr>
<tr>
<td>8. Stearidonic acid 18:4(n-3)</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

*Not tested because no binding to histone and no lipid detected in the sample.

**Example 10:** Test of AIEC Resins with New Conversion Method of bl_A to blAC
In this study, the conversion method, where the ion exchange resin was not pre-conditioned with a fatty acid or a lipid, of bLA to bLAC was tested with several other anion exchange resins than Q sepharose XL. Of the three anion exchange resins tested, only Unosphere Q showed similar results to Q sepharose XL, despite a slightly lower conversion yield. The bLAC recovered had similar molar ratio Npid/bLA and histone binding abilities.

The two other resins, DEAE Trisacryl and DEAE sepharose Fast Flow gave very low conversion yields. The molar ratio Npid/bLA was about five times lower than the one seen with Unosphere Q. Nevertheless the converted bLAC with these two resins showed some histone binding abilities.

The conversion of bLA to bLAC (bovine lactalbumin in complex with oleic acid) is currently done by mixing bLA, EDTA and oleic acid in solution prior anion exchange chromatography, where bLAC is separated from unconverted bLA.

The Q sepharose XL resin was chosen in Examples 1 and 2. In those experiments the conversion was done with the column pre-conditioned with oleic acid. The aim of this study was to test the new conversion method (Examples 3, 4 and 5) of bLA to bLAC with other anion exchange resins than Q sepharose XL.

METHODS

Conversion of bLA to complex with fatty acids

The same bLA batch aliquoted in different bags was used for the conversion experiment.

Table 23: Batch of bLA used for conversion

| bLA batch N289-29A | 7x15 mL aliquot stored at -20°C prepared from bLA batch bags N177-76B-04 and N177-76B-05 produced at Biovian. bLA concentration: 2.5 mg/mL (SEC-HPLC) with bLA reference N286-28A |
bl_A batch N289-78B 6x1 8 ml aliquot stored at -20°C prepared from bl_A batch N177-76B-06 and N177-76B-07 produced at Biovian.

bl_A concentration: 2.5 mg/mL (SEC-HPLC) with bl_A reference N286-28A

Table 24: Resins used for conversion (only the ones not in cursive were used for conversion)

<table>
<thead>
<tr>
<th>Resins</th>
<th>Manufacturer</th>
<th>Part No.</th>
<th>Particle Size (µm)</th>
<th>Matrix structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE Trisacryl Plus M</td>
<td>Pall BioSepra</td>
<td>26709-01 4</td>
<td>40-80</td>
<td>semi-rigid microbeads of hydrophilic acrylic copolymers</td>
</tr>
<tr>
<td>Capto Q</td>
<td>GE Healthcare</td>
<td>17-5316</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>DEAE Sepharose Fast Flow</td>
<td>GE Healthcare</td>
<td>170709</td>
<td>45-1 65</td>
<td>Agarose based</td>
</tr>
<tr>
<td>Q Sepharose Fast Flow</td>
<td>GE Healthcare</td>
<td>170510</td>
<td>45-165</td>
<td></td>
</tr>
<tr>
<td>Q Sepharose XL</td>
<td>GE Healthcare</td>
<td>17-5072</td>
<td>45-1 65</td>
<td>Agarose based</td>
</tr>
<tr>
<td>Unosphere Q</td>
<td>BioRad</td>
<td>156-01 01</td>
<td>120</td>
<td>Acrylamide based hydrophilic spherical polymeric bead</td>
</tr>
</tbody>
</table>

The conversion of bl_A to a complex with oleic acid (bLAC) was performed according to Examples 3, 4 and 5.

On the day of conversion, a solution was prepared with oleic acid (Purity > 99% from Larodan) according to the following Table:

Table 25: Preparation of fatty acid solution
The molar ratio between EDTA and bLA and fatty acid and bLA should have been 15 and 30 respectively, as described in Examples 3, 4 and 5. Other molar ratios were applied due to the fact that the standard reference for SE-HPLC analysis was changed. With the new reference the bLA concentration is approximately 15% lower (cf. Table 1). The sample conditioning calculations were done on basis of the concentration measured with the previous reference.

Each conditioned sample was applied on 0.78 cm² column (Tricorn 10/100) newly packed and CIP’ed (10 cm bed height) with the respective resins. The load of bLA during the purifications was approximately 54 mg/cm².

**bLAC recovery**

For each purification, eluted peaks were pool according UV at 280 nm, and stored at -20°C prior analysis.
**bLAC recovery characterization**

**Yield**
The yields of the conversion runs were determined by size exclusion HPLC (SE-HPLC) run according to standard procedures for example as described in GE healthcare book "Gel Filtration: Principles and Methods".

**Potency**
The potency of the converted bLA is generally determined by cell killing abilities. Cell killing is run according to Example 7. The potency of bLAC determined by cell killing assay is given in pg bLAC per cell (LD50). Cell killing was not performed on the bLAC samples recovered in this study.

**Identity**
The presence of complex bLA and fatty acid was determined by histone binding assay. The histone binding assays was run according to Example 6. Only complex between bLA and oleic acid have histone binding abilities.

**GC-Analysis**
The amount of lipid and the lipid composition of the bLAC samples were determined at Net-Food lab (Finland) after conventional methods: After esterification by the Boron trifluoride-methanol method, the fatty acids methyl esters (FAME) in the bLAC samples were analyzed by Gas Chromatography. Lipid content was calculated in relation to the concentration of the internal standard.

**RESULTS**

**Conversion runs**
The chromatograms of the conversion of bLAC with the different resins can be seen in Figure 15.

When conversion is performed with Q sepharose XL, step gradients are applied to elute the bLAC complex which is recovered during the 2nd step gradient (cf LAC-140). With the other resins tested, a linear gradient was applied as the elution profile of bLAC was then not known.
A similar profile to Q sepharose XL was obtained with Unosphere Q. Profiles were rather different with the two other resins tested (Figure 15).

Identity by Histone assay

In order to identify the complex between bl_A and oleic acid in the runs shown in Figure 1, histone binding abilities were tested on the recovered samples. Only converted blAC can bind to histone. The results are shown in the next Table:

Table 27: EC50 by histone binding assay

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Plate ID:</th>
<th>Resin used</th>
<th>EC50 (µg SEC/mL)</th>
<th>Binding (%) to ref.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N289-79D</td>
<td>N291 -93A</td>
<td>Q sepharose XL, peak 2</td>
<td>-</td>
<td>Not tested</td>
</tr>
<tr>
<td>N289-85G</td>
<td>N291 -93A</td>
<td>Unosphere Q, peak 1</td>
<td>-</td>
<td>Not tested</td>
</tr>
<tr>
<td>N289-85H</td>
<td>N291 -93A</td>
<td>Unosphere Q, peak 2</td>
<td>5.5</td>
<td>100%</td>
</tr>
<tr>
<td>N289-87G</td>
<td>N291 -93A</td>
<td>DEAE Trisacryl Plus M, peak 1</td>
<td>-</td>
<td>No binding</td>
</tr>
<tr>
<td>N289-87H</td>
<td>N291 -93A</td>
<td>DEAE Trisacryl Plus M, peak 2</td>
<td>9.6</td>
<td>57%</td>
</tr>
<tr>
<td>N289-87I</td>
<td>N291 -93B</td>
<td>DEAE Trisacryl Plus M, peak 3</td>
<td>7.0</td>
<td>74%</td>
</tr>
<tr>
<td>N289-90G</td>
<td>N291 -93B</td>
<td>DEAE sepharose FF, peak 1</td>
<td>-</td>
<td>No binding</td>
</tr>
<tr>
<td>N289-90H</td>
<td>N291 -93B</td>
<td>DEAE sepharose FF, peak 2</td>
<td>14.3</td>
<td>36%</td>
</tr>
<tr>
<td>N289-90I</td>
<td>N291 -93C</td>
<td>DEAE sepharose FF, peak 3</td>
<td>4.4</td>
<td>118%</td>
</tr>
</tbody>
</table>

*blAC N277-30A was used as reference in the assay. EC50 was 5.5 µg/mL (plate N291 -93A) and 5.2 µg/mL (plates N291 -93A/B).

Based on the histone binding assay, converted blAC is recovered in peak 2 from Unosphere Q, peaks 2 and 3 from DEAE Trisacryl Plus M and DEAE sepharose FF.

Yield of conversion

The yield of the conversion runs was calculated from the peaks where histone binding abilities were detected. Yield calculations are based on SE-HPLC analysis:
Table 28: Yield of the conversion runs

<table>
<thead>
<tr>
<th>Prod. ID</th>
<th>Resins used</th>
<th>Recovery</th>
<th>bLAC (mg/mL)</th>
<th>Volume (ml)</th>
<th>bLA load (mg/cm²)</th>
<th>Yield (mg/cm²)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC-140</td>
<td>Q sepharose XL</td>
<td>N289-79D</td>
<td>5.1</td>
<td>5.5</td>
<td>44</td>
<td>36</td>
<td>82%</td>
</tr>
<tr>
<td>LAC-141</td>
<td>Unosphere Q</td>
<td>N289-85H</td>
<td>3.1</td>
<td>8.0</td>
<td>44</td>
<td>32</td>
<td>73%</td>
</tr>
<tr>
<td>LAC-142</td>
<td>DEAE Trisacyl Plus M</td>
<td>N289-87H</td>
<td>1.7</td>
<td>7.5</td>
<td>44</td>
<td>21</td>
<td>48%</td>
</tr>
<tr>
<td>LAC-143</td>
<td>DEAE sepharose FF</td>
<td>N289-90H</td>
<td>0.5</td>
<td>7.0</td>
<td>44</td>
<td>10</td>
<td>23%</td>
</tr>
</tbody>
</table>

The highest yield was obtained with Q sepharose XL. Very low yields are obtained for the two DEAE resins tested, independently of their different matrix structure (agarose vs. acrylamide).

In a previous experiment, it was not possible to obtained converted bLAC with Unosphere Q, when this matrix was pre-conditioned with oleic acid (Examples 1 and 2).

Lipid composition by GC analysis

The lipid composition (Table 29) and lipid content (Table 30) of the bLAC obtained with the different resins was determined by GC analysis.

Table 29: Lipid composition by GC analysis

<table>
<thead>
<tr>
<th>Sampled D</th>
<th>Sampled D*</th>
<th>Resins used</th>
<th>Assay D</th>
<th>palmitic acid</th>
<th>stearic acid</th>
<th>oleic acid</th>
<th>linoleo acid</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C18:1</th>
<th>C18:2</th>
<th>Lipid content (n-9)</th>
<th>Lipid content (n-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ desalting</td>
<td>+ desalting</td>
<td>Q sepharose XL</td>
<td>Not performed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N289-79D</td>
<td>-</td>
<td>Q sepharose XL</td>
<td>Not performed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N289-85H</td>
<td>N289-85I</td>
<td>Unosphere Q</td>
<td>N068-12G</td>
<td>0%</td>
<td>0%</td>
<td>93%</td>
<td>0%</td>
<td>7%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N289-88A</td>
<td>N289-88A</td>
<td>DEAE trisacyl Plus M</td>
<td>N068-</td>
<td>Below detection limit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
GC analysis was performed on desalted samples.

The molar ratio Npid/bLA in the samples was calculated from the results of the GC analysis. The calculations are shown in Table 30.

**Table 30:** Molar ratio Npid/bLA

<table>
<thead>
<tr>
<th>SampleID</th>
<th>SampleID</th>
<th>Fatty acid</th>
<th>Lipid (mg/mL)</th>
<th>Lipid (µmol/mL)</th>
<th>bLA (µmol/mL)</th>
<th>Molar ratio Lipid/bLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>N289-85H</td>
<td>N289-85I</td>
<td>Unosphere Q</td>
<td>0.54</td>
<td>1.92</td>
<td>0.15</td>
<td>13.2</td>
</tr>
<tr>
<td>N289-87G</td>
<td>N289-88A</td>
<td>DEAE trisacyl Plus M</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N289-87H</td>
<td>N289-88B</td>
<td>DEAE trisacyl Plus M</td>
<td>0.12</td>
<td>0.44</td>
<td>0.12</td>
<td>3.7</td>
</tr>
<tr>
<td>N289-87I</td>
<td>N289-88C</td>
<td>DEAE trisacyl Plus M</td>
<td>0.07</td>
<td>0.27</td>
<td>0.06</td>
<td>4.3</td>
</tr>
<tr>
<td>N289-90H</td>
<td>N297-1 0A</td>
<td>DEAE sepharose FF</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N289-90I</td>
<td>N297-1 0B</td>
<td>DEAE sepharose FF</td>
<td>0.03</td>
<td>0.11</td>
<td>0.03</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Molar ratio between lipid and bLA is typically 15-20 when Q sepharose XL is used for the conversion (see above). Similar molar ratio Npid/bLA was obtained when the conversion was performed with Unosphere Q.

The molar ratio was Npid/bLA was approximately 4, for the two other resins tested, DEAE trisacyl Plus M and DEAE sepharose FF.

The results of the GC analysis confirmed the results of the histone binding assay, i.e. histone binding abilities are found in the peak containing lipid (oleic acid).
**Potency by cell killing assay**

None of the samples described in this report have been tested in the cell killing assay.

The yield of bLAC was slightly lower with Unosphere Q than with Q sepharose XL, but the bLAC recovered showed similar molar ratio Npid/bLA and similar histone binding abilities. In a previous study, when the conversion was done on resins pre-conditioned with oleic acid, it was shown that Unosphere Q resin could not be used (Examples 1 and 2).

The two other resins, DEAE Trisacryl and DEAE sepharose Fast Flow gave very low yield of bLAC conversion. Furthermore molar ratio Npid/bLA was about five time lower than the one seen with Unosphere Q. Nevertheless the converted bLAC with these two resins showed some histone binding abilities.
Claims

1. A method for the preparation of a biologically active alpha-lactalbumin complex comprising
   a. alpha-lactalbumin of SEQ ID NO: 1 or SEQ ID NO:2 or a functional homologue thereof comprising a sequence of at least 70% identical therewith; and
   b. a fatty acid or a lipid

   said method comprising the steps of:
   iii. obtaining an alpha-lactalbumin composition comprising alpha-lactalbumin of SEQ ID NO: 1 or SEQ ID NO:2 or a functional homologue thereof comprising a sequence of at least 70% identical therewith,
   iv. conversion of said alpha-lactalbumin or a functional homologue thereof to alpha-lactalbumin complex
      1. by mixing alpha-lactalbumin or a functional homologue thereof and a fatty acid or a lipid in the absence of an ion exchange medium, and subsequently
      2. exposing the mixture to an ion exchange medium.

2. The method according to claim 1, wherein a calcium chelating agent is added to the alpha-lactalbumin or a functional homologue thereof before the addition of a fatty acid or a lipid.

3. The method according to claim 1, wherein a calcium chelating agent is added to the mixture of alpha-lactalbumin or a functional homologue thereof and a fatty acid or a lipid after or simultaneous with the addition of a fatty acid or a lipid.

4. The method according to any of claims 1 to 3 wherein the mixing of alpha-lactalbumin or a functional homologue thereof, and a fatty acid or a lipid and optionally a calcium chelating agent is carried out for in the range of 5 minutes to 24 hours, preferably in the range of 15 minutes to 12 hours, for example approximately 30 minutes.
5. The method according to any of claims 1 to 4, wherein the mixing of alpha-lactalbumin or a functional homologue thereof, and a fatty acid or a lipid and optionally a calcium chelating agent is carried out at in the range of 1°C to 40°C, preferably in the range of 5°C to 35°C, more preferably in the range of 10°C to 30°C, such as around room temperature.

6. The method according to any of the preceding claims 1 to 5, wherein the ion exchange medium comprises a matrix comprising carbohydrate.

7. The method according to any of the preceding claims 1 to 6, wherein the ion exchange medium comprises a matrix comprising a polymer comprising galactose derived residues.

8. The method according to any of the preceding claims 1 to 7, wherein the ion exchange medium is an anion exchange resin.

9. The method according to claims 1 to 8, wherein the ion exchange medium comprises a strong anion exchanger.

10. The method according to claim 9, wherein the strong anion exchanger is quaternary ammonium Q.

11. The method according to any of the preceding claims 1 -10, wherein the ion exchange medium comprises a resin wherein the mean size of the particles are in the range of 80 µm to 120 µm, for example 85 µm to 110 µm, such as around 90 µm.

12. The method according to any of claims 1 to 10, wherein the ion exchange medium comprises a resin wherein the mean size of the particles are in the range of 1 to 29 µm or in the range of 31 to 40 µm.

13. The method according to claims 1 to 12, wherein the ion exchange medium is Q Sepharose XL resin.
14. The method according to claims 1 to 12, wherein the ion exchange medium is Capto Q sepharose.

15. The method according to any of the preceding claims 1 to 14, wherein the ion exchange medium comprises a resin with a recommended maximum working flow rate of at least 80 cm/h, such as a working flow rate of at least 90 cm/h, for example a working flow rate of at least 100 cm/h, for example a working flow rate of at least 150 cm/h, such as a working flow rate of at least 200 cm/h such as a working flow rate of at least 250 cm/h, such as a working flow rate of at least 300 cm/h, such as a working flow rate of at least 350 cm/h, for example a working flow rate of at least 400 cm/h, such as a working flow rate of at least 450 cm/h, for example a working flow rate of at least 500 cm/h.

16. The method according to claims 2 to 15, wherein a calcium chelating agent is in molar excess over alpha-lactalbumin or a functional homologue thereof.

17. The method according to claim 2 to 16, wherein the molar excess of a calcium chelating agent/alpha-lactalbumin corresponds to a 15 to 20 fold molar excess.

18. The method according to any of the preceding claims 2 to 17 wherein the calcium chelating agent is ethylene diamine tetraacetic acid.

19. The method according to claims 18, wherein ethylene diamine tetraacetic acid is in molar excess over alpha-lactalbumin or a functional homologue thereof.

20. The method according to claims 18 to 19, wherein the molar excess of ethylene diamine tetraacetic acid / alpha-lactalbumin corresponds to a 15 to 20 fold molar excess.

21. The method according to any of the preceding claims 18 to 20, wherein the concentration of ethylene diamine tetraacetic acid is in the range of 0.1 mM to 50 mM, preferably in the range of 0.5 mM to 25 mM, more preferably in the range of 1 mM to 10 mM, such as around 1 mM.
22. The method according to any of claims 1 to 21, wherein the fatty acid or lipid is selected from the group of C14 to C20.

23. The method according to any of claims 1 to 22, wherein the fatty acid or lipid has in the range of 1 to 4 double bonds.

24. The method according to any of claims 1 to 23, wherein the fatty acid or lipid is a C16, C17 or C18 fatty acid comprising in the range of 1 to 4 cis double bonds.

25. The method according to claim 24, wherein the fatty acid is selected from the group consisting of C17:1:1Ocis, C18:1:9cis, C18:1:11cis, C18:1:6cis, C16:1:9cis, C18:3:6,9,12cis, C18:3:9,1:2,15cis, C18:2:9,12cis and C18:4:6,9,12,15cis.

26. The method according to claims 1 to 25, wherein the fatty acid or lipid is in molar excess over alpha-lactalbumin or a functional homologue thereof.

27. The method according to claims 1 to 26, wherein the molar excess of fatty acid or lipid /alpha-lactalbumin corresponds to a 3 to 40 fold molar excess.

28. The method according to any of the preceding claims 1 to 27, wherein the fatty acid is oleic acid.

29. The method according to any of the preceding claim 28, wherein the concentration of oleic acid is in the range of 0.01 mM to 20 mM, preferably in the range of 0.02 mM to 10 mM, more preferably in the range of 0.1 mM to 5 mM, even more preferably in the range of 0.2 mM to 2 mM, such as around 2 mM.

30. The method according to any of the preceding claims 1 to 29, wherein the alpha-lactalbumin is human alpha-lactalbumin of SEQ ID NO: 1 or a functional homologue thereof comprising a sequence at least 70% identical therewith.
31. The method according to any of the claims 1 to 29, wherein the alpha-lactalbumin is bovine alpha-lactalbumin of SEQ ID NO: 2 or a functional homologue thereof comprising a sequence at least 70% identical therewith.

32. The method according to any of the claims 1 to 29, wherein the functional homologue of alpha-lactalbumin is selected from the group of; equine, caprine, camelide and porcine alpha-lactalbumin.

33. The method according to any of the preceding claims 1 to 32, wherein the alpha-lactalbumin is monomeric alpha-lactalbumin.

34. The method of any of the preceding claims 1 to 33, wherein alpha-lactalbumin or a functional homologue thereof has been recombinantly produced.

35. The method according to any of the claims 1 to 33, wherein alpha-lactalbumin or a functional homologue thereof is exposed of the mixture of alpha-lactalbumin or a functional homologue thereof and fatty acid or lipid and optionally a calcium chelating agent to an ion exchange medium is achieved by loading lactalbumin or a functional homologue thereof onto the ion exchange medium.

36. The method according to any of the preceding claims 1 to 35, wherein the load of alpha-lactalbumin or a functional homologue thereof is at least 20 mg/cm², such as at least 30 mg/cm², for example at least 40 mg/cm², for example at least 50 mg/cm², such as at least 60 mg/cm², for example at least 70 mg/cm², such as at least 80 mg/cm², for example at least 90 mg/cm² ion exchange resin.

37. The method according to any of the preceding claims 1 to 36 wherein the load of alpha-lactalbumin complex is at least 50%, such as at least 60%, for example at least 70%, such as at least 80%.
39. The method according to any of the preceding claims 1 to 38, wherein a regeneration procedure follows the exposure of the mixture of alpha-lactalbumin, and fatty acid or lipid and optionally calcium chelating agent to an ion exchange medium.

40. The method according to claim 39, wherein the regeneration procedure comprises sequential CIP (cleaning in place) with acetic acid, sodium hydroxide and ethanol 70%.

41. A composition comprising a biologically active alpha-lactalbumin complex produced by the method according to any of the preceding claims 1 to 40.

42. Use of a composition according to claim 41 for the preparation of a medicament for treatment of clinical disorders selected from the group consisting of respiratory tract infections, cancer and warts or for the inhibition of angiogenesis.

43. The use according to claim 42, wherein the clinical condition is bladder cancer.

44. The use according to claim 42, wherein the clinical condition is papiloma.

45. The use according to claim 42, wherein the clinical condition is actinic keratosis.

46. A method for the preparation of a biologically active lactalbumin complex comprising

a. lactalbumin of SEQ ID NO: 1 or SEQ ID NO:2 or a functional homologue thereof comprising a sequence of at least 70% identical therewith, and

b. a fatty acid or a lipid,

said method comprising the steps of:

v. obtaining an lactalbumin composition comprising alpha-lactalbumin of SEQ ID NO: 1 or SEQ ID NO: 2 or a functional homologue thereof comprising a sequence of at least 70% identical thereof,

vi. conversion of said alpha-lactalbumin or a functional homologue thereof to alpha-lactalbumin complex
1. by release of calcium from said alpha-lactalbumin or a functional homologue thereof and
2. binding of a fatty acid or a lipid to said alpha-lactalbumin or a functional homologue thereof
   a. exposing the alpha-lactalbumin or a functional homologue thereof to an ion exchange medium
      comprising a matrix comprising carbohydrate,

wherein steps 2. and 3. may be performed sequentially or simultaneously.

47. The method according to claim 46, wherein the ion exchange medium is an anion exchange resin.

48. The method according to any of claims 46 to 47, wherein the ion exchange medium comprises a matrix essentially consisting of a polymer of residues, wherein the residues are selected from the group of monosaccharides.

49. The method according to claim 48, wherein at least some residue are galactose derived residues.

50. The method according to any of the preceding claims 46 to 49, wherein the ion exchange medium comprises a resin wherein the mean size of the particles are at least 80 µm, such as in the range of 80 µm to 120 µm, for example 85 µm to 110 µm, such as around 90 µm.

51. The method according to any of the preceding claims 46 to 49, wherein the ion exchange medium comprises a resin wherein the mean size of the particles are at least 90 µm, for example in the range of 80 to 150 µm, such as 100 to 130 µm, for example around 120 µm

52. The method according to any of claims 46 to 49, wherein the ion exchange medium comprises a resin wherein the mean size of the particles are at the most 40 µm, such as in the range of 1 to 29 µm or in the range of 31 to 40 µm.
53. The method according to any of the preceding claims 46 to 52, wherein the ion exchange medium comprises a strong anion exchanger.

54. The method according to claim 53, wherein the strong anion exchanger is quaternary ammonium Q.

55. The method according to any of the preceding claims 46 to 54, wherein the ion exchange medium comprises Capto Q sepharose.

56. The method according to any of the claims 46 to 54 wherein the ion exchange medium comprises Q Sepharose XL resin.

57. The method according to any of the claims 46 to 54, wherein the ion exchange medium comprises Unosphere Q resin.

58. The method according to any of the preceding claims 46 to 57, wherein the ion exchange medium comprises a resin with a recommended maximum working flow rate of at least 80 cm/h, such as a working flow rate of at least 90 cm/h, for example a working flow rate of at least 100 cm/h, for example a working flow rate of at least 150 cm/h, such as a working flow rate of at least 200 cm/h such as a working flow rate of at least 250 cm/h, such as a working flow rate of at least 300 cm/h, such as a working flow rate of at least 350 cm/h, for example a working flow rate of at least 400 cm/h, such as a working flow rate of at least 450 cm/h, for example a working flow rate of at least 500 cm/h.

59. The method according to any of the preceding claims 46 to 58, wherein the ion exchange medium has been pre-conditioned with a fatty acid or lipid.

60. The method according to any of the preceding claims 46 to 59, wherein the fatty acid or lipid is selected from the group of C4 to C30.

61. The method according to any of the preceding claims 46 to 60, wherein the fatty acid or lipid is selected from the group of C14 to C20.
62. The method according to any of the preceding claims 46 to 61, wherein the fatty acid or lipid is selected from the group of C16, C17, C18 and C20.

63. The method according to any of the preceding claims 46 to 62, wherein the fatty acid or lipid has in the range of 1 to 4 double bonds.


65. The method according to any of claims 46 to 59, wherein the fatty acid or lipid is a C16 or C18 fatty acid comprising in the range of 1 to 4 cis double bonds.

66. The method according to claim 66, wherein the fatty acid is selected from the group consisting of C17:1:0cis, C18:1:9cis, C18:1:11cis, C18:1:6cis, C16:1:9cis, C18:3:6,9,1 2cis, C18:3:9,1 2cis, C18:2:9,1 2cis and C18:4:6, 9, 12, 15cis.

67. The method according to any of the preceding claims 46 to 59, wherein the fatty acid or lipid is oleic acid or vaccenic acid.

68. The method according to any of the preceding claims 46 to 67, wherein the conversion of alpha-lactalbumin of SEQ ID NO: 1 or SEQ ID NO: 2 or a functional homologue thereof comprising a sequence at least 70% identical therewith, to a biologically active alpha-lactalbumin complex with a fatty acid or a lipid comprise the steps of:

a. releasing calcium from said alpha-lactalbumin or a functional homologue thereof, comprising contacting said alpha-lactalbumin or a functional homologue thereof with a calcium chelating agent, thereby inducing alpha-lactalbumin to form a molten globule-like state
b. pre-conditioning an ion exchange column with a fatty acid or a lipid
c. binding said fatty acid or lipid to said molten globule-like alpha-lactalbumin by loading said molten globule-like alpha-lactalbumin on to an ion exchange medium comprising a matrix comprising carbohydrate.
69. The method according to any of the preceding claims 46 to 68, wherein the load of alpha-lactalbumin or a functional homologue thereof is at least 1.5 mg/cm², such as at least 5 mg/cm², for example at least 10 mg/cm², such as at least 15 mg/cm², for example at least 20 mg/cm² ion exchange resin.

70. The method according to any of the preceding claims 46 to 69, wherein the yield of alpha-lactalbumin complex is at least 50%, such as at least 60%, for example at least 70%, such as at least 80%.

71. The method according to any of the preceding claims 46 to 70, wherein the alpha-lactalbumin is human alpha-lactalbumin of SEQ ID NO: 1 or a functional homologue thereof comprising a sequence at least 70% identical therewith.

72. The method according to any of the claims 46 to 70, wherein the alpha-lactalbumin is bovine alpha-lactalbumin of SEQ ID NO: 2 or a functional homologue thereof comprising a sequence at least 70% identical therewith.

73. The method according to any of the claims 46 to 70, wherein the functional homologue of alpha-lactalbumin is selected from the group of; equine, caprine, camelide and porcine alpha-lactalbumin.

74. The method according to any of the preceding claims 46 to 73, wherein the alpha-lactalbumin is monomeric alpha-lactalbumin.

75. The method according to any of the preceding claims 46 to 74, wherein alpha-lactalbumin is purified naturally occurring milk alpha-lactalbumin.

76. The method according to any of the preceding claims 46 to 75 wherein alpha-lactalbumin or a functional homologue thereof has been recombinantly produced.

77. The method according to any of the preceding claims 46 to 76, wherein the release of calcium from said alpha-lactalbumin or a functional homologue
thereof comprises contacting the alpha-lactalbumin or a functional homologue thereof with a calcium chelating agent.

78. The method according to claim 77, wherein the calcium chelating agent is ethylene diamine tetraacetic acid.

79. The method according to any of the preceding claims 46 to 78, wherein a step gradient elution is used to elute alpha-lactalbumin complex and lactalbumin or a functional homologue thereof separately from the ion exchange medium.

80. The method according to claim 79, wherein the step gradient is 40, 70 and 100% of Tris 10 mM, NaCl 1M, pH 8.5.

81. The method according to any of the preceding claims 46 to 78, wherein a linear gradient elution is used to elute alpha-lactalbumin complex and lactalbumin or a functional homologue thereof separately from the ion exchange medium.

82. A composition comprising a biologically active alpha-lactalbumin complex prepared by the method according to any of the preceding claims 46 to 81.

83. Use of the composition according to claim 82 for the preparation of a medicament for treatment of a clinical disorder selected from the group consisting of respiratory tract infections, cancer and warts or for the inhibition of angiogenesis.

84. The use according to claim 83, wherein the clinical disorder is bladder cancer.

85. The use according to claim 83, wherein the clinical disorder is papiloma.

86. The use according to claim 83, wherein the clinical disorder is actinic keratosis.
Figure 2

A

LAC-024
DEAE Trisacryl Plus M
20 mg/cm²

B

LAC-018
DEAE Trisacryl Plus M
25 mg/cm²
Figure 7

\[ y = -0.019x + 1.1809 \]

\[ R^2 = 0.9994 \]
Figure 9

Peak eluted at 45% B-buffer (Low load – LAC-034)

Peak eluted at 70% B-buffer (Low load – LAC-034)

Peak eluted at 70% B-buffer (High load – LAC-047)
Figure 10

Absorbance

bLAC (µg/mL)

- LAC-041 - N277-07E
- LAC-042 - N277-09G
- LAC-047 - N277-18A
- LAC-016 - N262-35B
Figure 12

A  LAC-078: Oleic acid

B  LAC-080: V vaccenic acid

C  LAC-082: Linoleic acid
Figure 13

A. LAC-140: Oleic acid

B. LAC-125: Palmitoleic acid

C. LAC-126: Eicosapentaenoic acid

D. LAC-134: Stearidonic acid

E. LAC-127: Heptadecenoic acid

F. LAC-128: Υ-Linolenic acid

G. LAC-132: Gondoic acid
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/76 " A61K38/38
ADD. A61K38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>WO 99/26979 A (SVANBORG CATHARINA [SE]; SVENSSON MALIN WILHELMINA [SE]; HAAKANSSON PE) 3 June 1999 (1999-06-03)</td>
<td>1,4-8, 22-25, 28,30, 33,34, 41-48, 59-68, 71, 74-77, 81-86</td>
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X Further documents are listed in the continuation of Box C. X See patent family annex.

Special categories of cited documents:

'A' document defining the general state of the art which is not considered to be of particular relevance
'E' earlier document but published on or after the international filing date
'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
'O' document referring to an oral disclosure, use, exhibition or other means
'P' document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search: 1 October 2008

Date of mailing of the international search report: 10/10/2008

Authorized officer: Sirim, Pinar

Form PCT/ISA/210 (second sheet) (Amp-2008I)
### INTERNATIONAL SEARCH REPORT

**Documents Considered to be Relevant**

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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
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<td>X</td>
<td>wo 2005/082406 A (HAMLET PHARMA AB [SE]; SVANBORG CATHARINA [SE]); 9 September 2005 (2005-09-09); cited in the application</td>
<td>1.4-8, 22-25, 28-30, 33-34, 41-48, 59-68, 71, 74-77, 81-86</td>
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<td></td>
<td>SVENSSON M ET AL: &quot;CONVERSION OF ALPHA-LACTALBUMIN TO A PROTEIN INDUCING APOPTOSIS&quot; PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC; US; vol. 97, no. 8; 11 April 2000 (2000-04-11), pages 4221-4226, XP002250705</td>
<td>71, 74-77, 81-86</td>
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<td></td>
<td>SVENSSON MALIN ET AL: &quot;Molecular characterization of alpha-lactalbumin folding variants that induce apoptosis in tumor cells&quot; JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOCHEMICAL BIOLOGISTS, BIRMINGHAM, UK; US; vol. 274, no. 10; 5 March 1999 (1999-03-05), pages 6388-6396, XP002455417</td>
<td>71, 74-77, 81-86</td>
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<td>SVENSSON MALIN ET AL: &quot;Lipids as co-factors in protein folding: Stereo-specific lipid-protein interactions are required to form HAMLET (human alpha-lactalbumin made lethal to tumor cells)&quot; PROTEIN SCIENCE, CAMBRIDGE UNIVERSITY PRESS, CAMBRIDGE, GB; vol. 12, no. 12; 1 December 2003 (2003-12-01), pages 2805-2814, XP002455419</td>
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