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ORIGINAL PAPER

Pilot-scale purification of α-lactalbumin from enriched whey protein concentrate by anion-exchange chromatography and ultrafiltration

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Abstract A method based on anion exchange chromatography (AIEX) in combination with ultrafiltration (UF) was developed to obtain kilogram-scale amounts of bovine α-lactalbumin (α-La) of high purity from α-La-enriched whey protein concentrate (αWPC). Initially, α-La was successfully purified, at laboratory scale, from a 10% solution of αWPC. Removal of casein and denatured whey protein by acid precipitation resulted in a α-La purity of 78%. A further purification by AIEX eluting with sodium acetate (NaAc) buffer led to a final purity and recovery of 100 and 85%, respectively. Based on this, the process was simplified and optimized for pilot-scale purification by selective binding of β-lactoglobulin (β-Lg). Batchwise expanded bed AIEX using a column with 5 L of Q Sepharose matrix was performed. Pure α-La was obtained in the run through buffer. In the end, 1100 g of *holo* form α-La was obtained with a purity of 97.4% in protein and a recovery of 80%, calculated based on the protein detectable in reversed phase high-performance liquid chromatography (RP-HPLC). Thus, by combining AIEX and UF, heating was avoided allowing production of α-La in kilo-scale with high purity and recovery from αWPC with a minimum volume of buffer solutions.

Keywords Bovine α-lactalbumin · Purification · Anion exchange chromatography · Ultrafiltration

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1 Introduction

In bovine milk, α-lactalbumin (α-La) is one of the principal whey proteins and constitutes ~20% (*w*/*w*) of the total whey protein or 3.5% of total milk protein (Fox and McSweeney 1998). It is a small (14.2 kg.mol^{-1}), acid (pI 4.2), and compact globular, metal binding (one Ca^{2+} per mole) protein, consisting of 123 amino acids in a single chain, with four di-sulphide bonds and no free thiol groups. Its structure and functionality have been extensively studied (Chatterton et al. 2006; Madureira et al. 2007; Barbana et al. 2011). α-La functions as a coenzyme for synthesis of lactose in the lactating mammary gland. When isolated, it has also been shown to possess a wide range of biofunctionalities, such as improved cognitive functioning (Booij et al. 2006; Markus et al. 2005; Schmitt et al. 2005) and anticancer properties when forming complexes with oleic acid (Svensson et al. 2003). Furthermore, it represents the best characterized model of the intermediate folding structure, the molten globule state, formed upon removal of Ca^{2+} by lowering the pH to below 4, or by using chelating agent such as EDTA at neutral pH (Kuwajima 1996). In addition, α-La has been shown to form nanotubular structures upon limited proteolysis (Ipsen et al. 2001a), which in turn can result in formation of strong transparent gels and provide the possibility for α-La to be used as a thickening agent or gelling agent in food systems or for encapsulation of bioactive compounds (Ipsen and Otte 2007). However, this nanostructure only forms when α-La occurs in high purity without the presence of β-Lg (Ipsen et al. 2001b). Hence, in order to study the conditions for formation of α-La nanostructures and their potential application, pure, native α-La should be available in the kilogram scale.

The methods applied to obtain enriched fractions or purified α-La have recently been reviewed by Kamau et al. (2010) and El-Sayed and Chase (2011). Extraction of α-La with high purity is not an easy task, especially at large- or pilot-scale purification, primarily due to the presence of the other major whey protein β-lactoglobulin (β-Lg). The amount of β-Lg in milk is almost 2.5 times higher than that of α-La, and the two proteins have molecular weights in a similar range (~14–18 kg.mol^{-1}). However, the isoelectric point, net charge, heat and enzyme sensitivity are different enough for separation to be feasible. A summary of studies aiming to purify α-La in a large scale is given in Table 1. Isolation of α-La from milk or whey protein can be achieved based on three principles. Selective precipitation of α-La or β-Lg was developed during the 1980s (Pearce 1983), and it is a popular method both in lab and pilot scale. By lowering the pH to around the isoelectric point of α-La, with mild heating (around 50 °C), α-La precipitates together with other minor whey proteins. Further washing and re solubilisation of the precipitate can be used to isolate α-La. Such a process is relatively simple and easy to apply, although the purity of α-La is normally low, around 20–89% (Lucena et al. 2006; Alomirah and Alli 2004). Although Tolkach et al. (2005) have successfully achieved an α-La preparation with a purity of 98% by heating whey protein concentrate (WPC) to induce aggregation of β-Lg, the quantity of pure α-La was only a few grams. A recent study by Toro-Sierra et al. (2013) used thermal aggregation, membrane separation, and a resolubilization technique to purify α-La at a large scale, but the purity was somewhat lower (91.3%). Another strategy is based on the different sensitivity of the whey proteins to enzymatic digestion. Schmidt and Poll

Table 1 Summary of studies on pilot- or large-scale purification of bovine α-lactalbumin (α-La)

Method	Starting material	Purity (%)	Recovery (%)	Amount of α-La (g)	Reference
Precipitation	WPI	91	60–80	~500	Toro-Sierra et al. (2013)
Precipitation	WPC	74	86	~20	Lucena et al. (2006)
Precipitation and chromatography	Liquid whey	38–54	Low	~2000	Outinen et al. (1996)
Precipitation	Liquid whey	52–83	6	–	Gesan-Guiziou et al. (1999)
Enzymatic digestion	WPC	90–95	15	~100	Konrad and Kleinschmidt (2008)
Anion exchange	Raw milk	100	–	~400	Kristiansen et al. (1998)
Gel filtration	WPC	Low	Low	~20	Forsum et al. (1973)

WPC whey protein concentrate, *WPI* whey protein isolate

(1991) reported that native α-La was highly resistant to trypsin while β-Lg was not. At lab scale, tryptic hydrolysis of β-Lg and discarding the fractions by membrane filtration seem suitable for achieving 100% purity of α-La (Lisak et al. 2013; Cheison et al. 2011); however, when upscaling, the purity drops to 90–95% and the recovery is quite low (Konrad and Kleinschmidt 2008). In order to achieve high purity, both at laboratory and pilot scale, chromatographic methods seem to be the best choice (Gurgel et al. 2000; Ye et al. 2000; Kristiansen et al. 1998), and most often ion exchange chromatography is used. At lab scale, the bound proteins are usually eluted by increasing concentrations of salt or by altering the pH of the eluting buffer. The method is limited by the binding capacity of ion-exchange resins, high costs, and requirement of high amounts (thousands of liters) of elution buffer for producing 1 kg pure α-La (Kristiansen et al. 1998). Although ion exchange purification of whey proteins can also be performed using charged ultrafiltration membranes (Arunkumar and Etzel 2014), this process has not been upscaled, and the purity of the α-La obtained (around 87%) was inferior to the level reached by traditional chromatographic methods.

The aim of this study was to produce pure α-La in amounts above 1 kg using a simple and gentle process. For this purpose, a process was developed and optimized for batchwise expanded bed anion exchange chromatography (AIEX) for selective binding of β-Lg and stepwise elution to recover α-La with a minimum consumption of eluting buffer. The temperature was kept below 25 °C and the pH above the isoelectronic point 4.2 in order to preserve the properties of α-La.

2 Materials and methods

2.1 Materials

Commercial α-La-enriched whey protein concentrate (αWPC; Lacprodan® Alpha-20) from Arla Foods Ingredients, Denmark, was used as raw material for purification. According to the manufacturer's specification, this αWPC contains a minimum of 60% α-La in total protein and maximum 3% lactose and 2% fat. Q Sepharose® Fast Flow

resin (Pharmacia, Uppsala, Sweden) was used for the AIEX processes. All other chemicals used in this study were of analytical grade.

2.2 Laboratory-scale purification of α-La

For laboratory-scale purification, 16 mL of resin was packed in a HiLoad ™ Q Sepharose® Fast Flow, XK 26/10 column (Pharmacia, Uppsala, Sweden), and fitted in a Pharmacia fast-performance liquid chromatography (FPLC) system (Pharmacia LKB, Uppsala, Sweden). The αWPC powder was dissolved at 10% (*w/w*) in 100 mL of demineralized water and stored at 4 °C overnight. The pH was adjusted to 4.6 by 1 mol.L^{-1} HCl, and it was kept at 4 °C overnight to give precipitation of casein and denatured whey proteins. The solution was then centrifuged at 12,470×*g* for 3 min (Sigma 1-14, Germany) and filtered through filter paper (Grade 42, Whatman®, UK) followed by a syringe filter (Frisenette, Denmark) with cellulose acetate membrane and pore size of 0.45 μm. Before loading on the FPLC system, the pH was increased to 7 with 1 mol.L^{-1} sodium hydroxide (NaOH), and 5 mL of this pre-purified αWPC solution was injected onto the AIEX column with a bed volume of 16 mL. Elution of the major whey proteins was achieved by stepwise changes in ionic strength and pH according to Table 2. The different pH values of the 0.2 mol.L^{-1} sodium acetate (NaAc) buffers were obtained by adding 0.2 mol.L^{-1} acetic acid with pH 3. The flow rate was 10 mL.min^{-1} and the UV absorbance was monitored at 280 nm. The α-La fraction was collected, weighed, and analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) as described in Sect. 2.5. The fractionation was repeated three times to make sure that it was reproducible.

2.3 Lab-scale process optimization for pilot-scale purification

In order to have a clear pre-purified αWPC solution for pilot-scale purification, a test was made to find the highest concentration of αWPC that could be used. Five solutions containing various concentrations of αWPC (1, 2, 4, 7, and 10% *w/w*) were made by adding αWPC to 100 mL of demineralized water. After acidification to pH 4.6 with 1 mol.L^{-1} HCl and storage overnight at 4 °C, without filtration, the particle size of these

Table 2 The process steps for lab-scale anion exchange chromatographic purification of α-La from enriched whey protein concentrate (αWPC)

Process step	No. of bed volumes	Time (min)
10% pre-purified αWPC, pH 7.0	0.3	0.5
0.1 mol.L^{-1} NaAc, pH 7.0	2.7	4.7
0.2 mol.L^{-1} NaAc, pH 7.0	6.9	10.3
0.2 mol.L^{-1} NaAc, pH 5.3	4.2	7.2
0.2 mol.L^{-1} NaAc, pH 4.8	4.2	7.2
0.2 mol.L^{-1} Acetate, pH 3.0	1.8	3.2
1.0 mol.L^{-1} NaCl	1.8	3.2
0.1 mol.L^{-1} NaAc, pH 7.0	1.8	3.2

solutions was measured using a MasterSizer (Malvern Instruments, UK). Each solution was mixed well prior to the measurement.

In order to reduce the elution buffer volume for the pilot-scale purification, the lab-scale AIEX process was adapted to selectively bind β-Lg so that α-La could be collected in the run-through from the column. This process was initially developed in lab scale using an ion exchange resin with a bed volume of 100 mL packed in a funnel fitted with filter paper (Grade 42, Whatman®, UK). The resin was regenerated by adding 60 mL of 1 mol.L^{-1} NaCl and draining by gravity. This was repeated five times. The resin was equilibrated by washing with 60 mL NaAc buffer (0.2 mol.L^{-1}, pH 7) five times. Before loading, NaAc was added to 60 mL of pre-purified αWPC to obtain concentrations between 0.2 and 0.4 mol.L^{-1}, and the pH was adjusted to 7 with 2 mol.L^{-1} NaOH. The NaAc-αWPC solution was loaded on the resin in the funnel, drained by gravity, and eluted twice with 0.2 mol.L^{-1} NaAc buffer at pH 7. Three eluates were collected (i.e., the run-through of NaAc-αWPC solution, and the two eluates with 0.2 mol.L^{-1} NaAc buffer) and analyzed by RP-HPLC to determine the purity of α-La. In total, five levels of NaAc (0.2, 0.23, 0.26, 0.3, and 0.4 mol.L^{-1}) in the NaAc-αWPC sample were tested in triplicate.

2.4 Pilot-scale purification of α-La

2.4.1 Preparation of NaAc-αWPC solution

Large-scale preparation of the NaAc-αWPC solution to be used for AIEX was performed as outlined in Fig. 1. The αWPC powder (20 kg) was dissolved in 1000 L of deionized water in a tank (Finncont Oy, Finland) with agitation for 8 h and stored overnight at room temperature. On the second day, the pH was adjusted to 4.6 with 1 mol.L^{-1} HCl, and again, the solution was stored overnight at room temperature. The third day, the 1000 L αWPC solution were subjected to ultrafiltration (UF) using a plant unit (DSS Silkeborg A/S, Denmark). Initially, concentration to 480 L was achieved using a spiral wound polyethersulfone UF membrane with 10 kg.mol^{-1} molecular cutoff and a membrane area of 17 m^2 (TriSep Corporation, USA). The concentrated αWPC solution was then separated using a 250 kg.mol^{-1} molecular weight cutoff polyvinylidene membrane (Synder Filtration, USA). About 440 L of permeate was collected and reconcentrated using the 10 kg.mol^{-1} molecular cutoff membrane to a final volume of 120 L. In order to remove salts, continuous dia-filtration was performed by pumping deionized water into the feeding tank while the UF process was running. The protein concentration of the permeate was monitored using a MilkoScan™ FT2 (FOSS, Denmark) during the whole process. From the final 120 L of pre-purified αWPC, batches of 3 L were added the desired amount of NaAc to achieve 0.3 mol.L^{-1} of NaAc and the pH was adjusted to 7.0 using 2 mol.L^{-1} NaOH. In total, 12 batches (36 L) of NaAc-αWPC were prepared for AIEX, and the rest (84 L) of the pre-purified αWPC solution was stored at 4 °C.

2.4.2 Batchwise expanded bed AIEX

For pilot-scale batchwise expanded bed ion exchange chromatography, 5 L of resin was packed in a column with 25-cm inside diameter (Pharmacia, Uppsala, Sweden), fitted

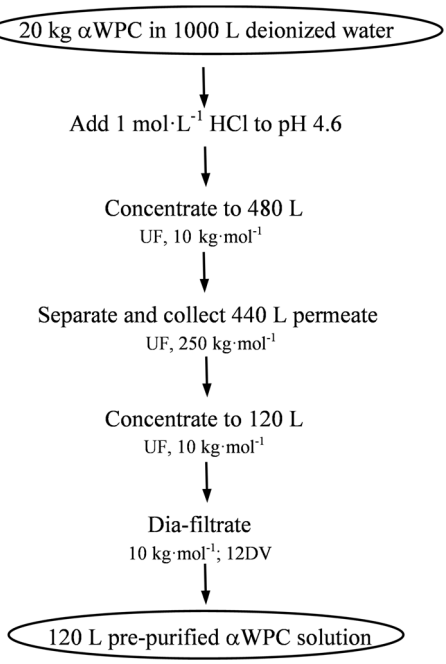

Fig. 1 Pilot-scale preparation of pre-purified α-lactalbumin-enriched whey protein concentrates (αWPCs) to be used for pilot-scale anion exchange chromatography

with an agitator 2 cm above the bottom membrane. The AIEX procedure used is illustrated in Fig. 2. Before loading, the resin was washed with 5 L of 1 mol.L^{-1} NaCl, gently stirred for 3 min, and drained by gravity. The procedure was repeated four times and the protein concentration in the eluted NaCl buffer was <0.5% as measured by the MilkoScan™ FT2 (FOSS, Denmark). Equilibration of the resin was done using the same procedure (five repetitions), but with 5 L of 0.2 mol.L^{-1} NaAc buffer at pH 7. A 3-L batch of NaAc-αWPC solution was then loaded on the column; stirring was done for 15 min ensuring sufficient absorption of other whey proteins onto the resin and that α-La was present in the collected eluate. The column was then washed two times with 3 L of 0.2 mol.L^{-1} NaAc buffer, pH 7, and the eluate collected. Samples from the first NaAc-αWPC run-through and the two subsequent NaAc buffer eluates were analyzed by RP-HPLC for purity and recovery (Sect. 2.5). The whole procedure was performed 12 times, and all the eluates were pooled resulting in 108 L of purified α-La solution.

2.4.3 Post-treatment of the α-La solution

The purified α-La solution was subsequently subjected to continuous dia-filtration as described above with 10 dia-filtration volumes (DVs) until the conductivity reached 0.2 mS.cm^{-1}. Final concentration was carried out in a plate-and-frame filtration system (The Danish Sugar Corporation Ltd., Denmark) with membranes having a pore size of 0.4 μm. The inlet pressure was 3 bar, and a flow rate of 4 m^3.h^{-1} was used. The final

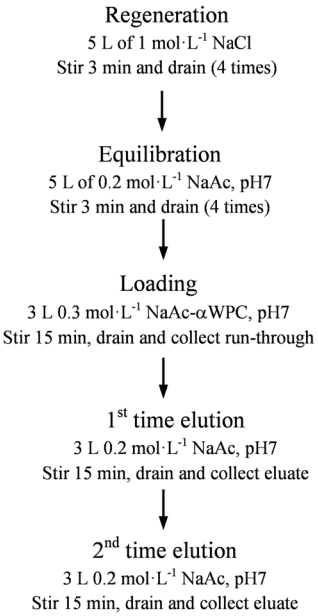

Fig. 2 Schematic outline of the steps performed during pilot-scale batchwise expanded bed anion exchange chromatography for isolation of α-La from pre-purified αWPC with 0.3 mol.L^{-1} NaAc (NaAc-αWPC). The whole process was performed 12 times, resulting in 108 L of eluate mixture to be further treated

purified α-La concentrate (11 L) was stored at 4 °C until freeze drying. For freeze drying, 1 L of concentrated α-La solution was evenly distributed into six petri dishes (20-cm diameter) and frozen at −45 °C overnight. The height of the frozen samples was ~10 mm. The frozen samples were immediately transferred to the freeze dryer (Edwards, Bush & Holm A/S, Denmark) and dried at −50 °C, for 72 h.

2.5 Determination of purity and recovery

The purity and recovery of all the fractions as well as the protein content of the initial whey solution was determined by RP-HPLC fitted with a PLRP-S 300 Å 8 μm column (Latek, Eppelheim, Germany). Samples (5 μL) were injected and separated at 40 °C with a flow rate of 1 mL.min^{-1} by gradient elution. Solvent A was 0.1% (*v/v*) trifluoroacetic acid (TFA) in water, and solvent B consisted of 20% (*v/v*) water, 80% (*v/v*) acetonitrile, and 0.05% (*v/v*) TFA. The gradient started with 45% of solvent B and increased every 5 min to 49, 51, 53, and 55%, respectively. The column was re-equilibrated with 100% solvent B for 3 min before returning to the initial conditions. Peaks detection was performed at 210 and 280 nm, and the resulting chromatograms were integrated by Agilent ChemStation software B.02.01 (Agilent technologies, US). The recovery was calculated as the ratio of the peak area of fractionated α-La to the peak area of α-La in the initial protein solution

taking into account the volumes of each. The purity of α-La was determined as the ratio of the peak area for α-La to the total peak area of all peaks in the chromatogram using the 280-nm molar absorption coefficients for α-La and β-Lg of 20.9 and 9.6, respectively (Pace et al. 1995).

2.6 Characterization of the final α-La powder

2.6.1 Gross composition

The composition of the final α-La powder was determined by standard methods. The moisture content was determined by drying at 100±2 °C for 4 h in an oven (Heraeus, Germany) and repeated drying for additional 1-h periods until a stable weight was obtained (FIL-IDF 26:1964). The protein content was obtained by the Kjeldahl method (FIL-IDF 20-1/2:2001) using a conversion factor of 6.38. For ash determination (FIL-IDF 27:1964), the powder was incinerated at 525±25 °C in a muffle furnace for 15 h and subjected to repeated heating for 1 h at a time until constant weight was attained. The calcium content was determined according to the IDF standard method (154:1992) by dissolving the remaining ash from the ash analysis in 4 N HNO_3 in a 20-mL volumetric flask and taking 2 mL of supernatant into a 50-mL volumetric flask, and adding adequate amounts La^{2+} and Milli-Q water. Atomic absorption spectrometry (PerkinElmer model 3300, USA) was used to detect the absorption of samples, and the calculation was based on a calcium standard curve.

2.6.2 Molecular weight of α-La

The molecular weight of the purified α-La was measured by liquid chromatography–mass spectrometry (LC-MS) essentially as described by Otte et al. (2007) using an Agilent 1100 LC-MSD Trap (Agilent Technologies A/S, Naerum, Denmark) mounted with a Zorbax 300SB C18 column (2.1×150 mm, 5 μm, Agilent Technologies A/S). The α-La powder was reconstituted to 1% with water and left overnight at 4 °C. Samples (5 μL) were injected into the column and eluted at 40 °C using a gradient consisting of 15–55% B in 47 min at a flow rate of 0.25 mL.min^{-1}. Buffer A was 0.1% (*v*/*v*) TFA in water and buffer B was 0.1% (*v*/*v*) TFA in 90% (*v*/*v*) acetonitrile. The online MS analyses were performed using the standard range from 200 to 2200 *m*/*z* and the target mass set to 1522 *m*/*z*. The molecular weight of the purified α-La was determined by deconvolution of an average mass spectrum taken over the peak for α-La.

2.6.3 Maximum denaturation temperature (T_{max})

The maximum denaturation temperature (T_{max}) of the purified α-La was determined by differential scanning calorimetry (DSC) using a Micro DSC III apparatus (SETARAM, Caluire, France). The purified α-La powder was dissolved in water at 50 mg.mL^{-1} and the pH was adjusted to 6.5 with 1 mol.L^{-1} HCl. The sample (700 μL) was placed in the DSC sample chamber and heated at 1 °C per minute from 25 to 90 °C. The measurements were performed triplicate.

3 Results and discussion

3.1 Laboratory-scale AIEX purification of α-La

As shown in Fig. 3, the lab-scale process initially applied was successful in giving a retention time for α-La around 18 min, nicely separated from β-Lg B (~30 min) and β-Lg A (~35 min). Thus, using a 0.2 mol.L^{-1} NaAc buffer at pH 7 resulted in elution of only α-La. By keeping the NaAc buffer at the same concentration but decreasing pH to 5.3 and 4.8 (Table 2), β-Lg B and β-Lg A, respectively, were eluted from the column. The α-La fraction was collected by pooling the fractions from 11 to 25 min. The purity of the pooled α-La fraction was analyzed by RP-HPLC as shown in Fig. 4 together with the profile of the sample applied. After pre-purification at pH 4.6, mainly α-La, β-Lg B, and β-Lg A form were present (Fig. 4a) as any remaining casein and other whey proteins had been denatured during heat treatment in the production of αWPC and were removed by sedimentation upon adjusting the pH to 4.6. Mayer et al. (2010) and Tolkach et al. (2005) applying a similar method to treat the initial whey protein solution also achieved removal of bovine serum albumin (BSA) and immunoglobulin. The minor whey proteins, especially BSA and immunoglobulin, like β-Lg, both contain free thiol groups, and irreversible denaturation of these already starts at 60 °C (Vermeer and Norde 2000; Boye et al. 1996). When the pH is adjusted to 4.6, most or all of the denatured minor whey proteins will be precipitated together with a small amount of denatured major whey proteins (Puyol et al. 1999; Hollar and Parris 1995). Figure 4b shows a typical 280-nm profile of α-La. A very similar profile was obtained at 210 nm (data not shown). The minor peaks found on both sides of the main peak were also seen by Toro-Sierra et al. (2013) and Cheison et al. (2012) who purified α-La from whey protein isolates. Heat treatment during milk and whey processing promote protein lactosylation causing the molecule to be more hydrophilic and elute earlier in RP-HPLC (Thomsen et al. 2012; Lund et al. 2005). Accordingly, the small peaks before the main peak for α-La are expected to contain mono-lactosylated α-La. The small peak appearing after the main α-La peak is believed to be another slightly modified form of

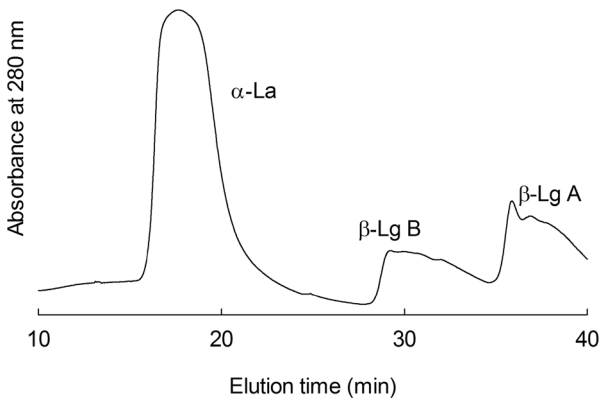

Fig. 3 Elution profile from the lab-scale anion exchange chromatography (AIEX) of 10% pre-purified αWPC. Elution steps are shown in Table 2

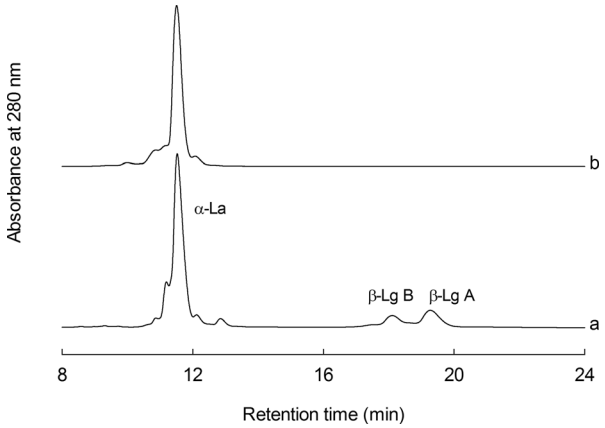

Fig. 4 RP-HPLC chromatograms of the initial 10% pre-purified αWPC solution loaded (**a**) and the α-La fraction collected (**b**) from the lab-scale AIEX shown in Fig. 3

α-La. By integration of the resulting peaks, the purity of α-La reached 100±0%, and the recovery was 85.1±6.4%.

3.2 Optimization of the particle size prior to UF

During membrane filtration, the particle size influences the permeability of membranes due to the fouling and hence affects the purification result. When upscaling the process, the 10% (*w*/*w*) pre-purified αWPC in a volume of 3 L appeared turbid, indicating that after centrifugation and filtration (0.45-μm pore size), there were still some small particles present in the solution, probably aggregated, denatured whey proteins. Large particles (≥1 μm) can easily be removed by filtration, but the smaller ones (≤1 μm) should be avoided in the solution. Turcaud et al. (1990) suggested that particles with diameters near 0.2 μm have maximum potential for fouling of membranes as they are too large for both permeation through the UF membrane and transportation back to the solution by Brownian diffusion. On the other hand, they will be too small for the lift force to remove them from the membrane surface (McLaugulin 1993). In order to minimize membrane fouling, the maximal αWPC concentration that could be present in the solution without forming small aggregates was determined. The particle size distribution at five concentrations of αWPC was measured, and the volume fractions of particles smaller than 1 μm in each sample are given in Table 3. It can be seen that solutions containing 1 and 2% pre-purified αWPC did not contain such small particles (0.1–1 μm). In solutions with concentrations above 2%, intensive particle formation was observed causing a high number of small particles, presumably sterically stabilized, which could not be removed by centrifugation or microfiltration. Therefore, a 2% αWPC solution was chosen as the starting point for the pilot-scale purification.

Table 3 The volume fraction of particles with size between 0.1 and 1 μm in solutions with various concentrations of α-La-enriched whey protein concentrate (αWPC)

αWPC (%, *w/w*)	Volume (%)
1	0
2	0
4	1.5±0.2
7	2.9±0.4
10	4.9±0.1

Values are expressed as means±SD from three independent replicates

3.3 Optimization of batchwise expended bed AIEX at lab scale

The lab-scale purification method described in Sect. 2.2 provided information about the appropriate conditions for separation of α-La, β-Lg B, and β-Lg A, such as the pH value, salt concentration, resin capacity, and buffer volume. Keeping the same flux, hence the same time scale for elution, and the same loading capacity, increasing the bed volume from 16 mL to 5 L for each batch (3 L, 10% protein load) would require 120 L of buffer for elution. From an economical point of view, this would not be optimal. Therefore, it was decided to add NaAc directly into the pre-purified αWPC causing the initial three process steps shown in Table 2 to be transformed into one. The effect of the various NaAc concentrations, at pH 7, on the purity of the α-La fraction collected as the run-through from the column tested at lab scale is shown in Table 4. The best result was obtained when NaAc was added to the pre-purified αWPC at around 0.3 mol.L^{-1}, which resulted in a purity of α-La of 98.6%. At this NaAc concentration, β-Lg appeared to be the major component interacting with the ion exchange resin. At neutral pH, β-Lg has a higher negative net charge than α-La (−17.6 and −15.6 for β-Lg A and β-Lg B, vs −3.7 for α-La; Lucas et al. 1998). At lower concentrations of NaAc (0.20,

Table 4 Purity of α-La, when using different NaAc concentrations in the α-La-enriched whey protein concentrate (αWPC) applied to the ion exchange column

NaAc (mol.L^{-1})	Purity of α-La (%)
0.20	96.6±0.45[a]
0.23	98.0±0.25[ab]
0.26	98.2±0.20[bc]
0.30	98.6±0.15 [c]
0.40	97.0±0.23[a]

Values are the mean of three independent replicates. Values with the same superscript letter are not significantly different ($p>0.05$)

0.23, and 0.26 mol.L^{-1}), the ionic environment did not provide optimum binding of β-Lg to the resin; consequently, a small amount of β-Lg eluted together with α-La. On the other hand, when further increasing the salt concentration to 0.4 mol.L^{-1}, excess Ac^- may compete with β-Lg for the binding sites, thus starting to displace β-Lg from the ion exchanger (Kisley et al. 2014). A concentration of 0.3 mol.L^{-1} NaAc was then chosen for the pilot-scale separation.

3.4 Pilot-scale purification of α-La

Upscaling the process was performed in two main steps: preparation of the NaAc-αWPC solution followed by batchwise expanded bed AIEX (of 3-L portions) performed 12 times. During concentration of the acidified αWPC (pH 4.6) from 1000 to 480 L, the protein content of the permeate was monitored and only 0.02% protein was present in the discarded permeate. The retentate was then UF-treated using a 250 kg.mol^{-1} cutoff membrane to remove precipitated casein and denatured whey protein. The retentate appeared milky and was discarded. After concentration of the collected permeate to 120 L and dia-filtration, the conductivity reached 40 μS.cm^{-1} and the pH value was increased from 4.6 to 5. At this pH value, which is near the isoelectric pH of β-Lg, the solution appeared turbid, indicative of protein aggregation. After adding 0.3 mol.L^{-1} NaAc, and adjusting the pH back to 7, the solution became clear and was ready for loading on the column.

Based on the optimization of the batchwise expended bed AIEX at lab scale, the whole process was scaled up to 50 times. The bed volume was increased from 100 mL to 5 L, and the loading capacity from 60 mL to 3 L. Loading 3 L of NaAc-αWPC caused the column bed to expand, i.e., increase the space between the adsorbent particles and thus the bed porosity. Figure 5a shows the protein composition of the NaAc-αWPC solution at pH 7 before loading to the column. There is a large peak for α-La and two smaller peaks representing β-Lg B and β-Lg A, respectively. This profile is almost identical to the one in Fig. 4a. The protein profiles of the eluates collected during the loading step and the two elution steps are shown in Fig. 5b–d, respectively. Based on the peak areas of α-La and volumes applied and collected, it was calculated that 70% of α-La ran through the column as the first fraction and only tiny peaks from β-Lg B and β-Lg A (enlarged part) were observed (Fig. 5b). By washing the column twice with 0.2 mol.L^{-1} NaAc at pH 7, another 7 and 3%, respectively, of α-La were obtained (Fig. 5c, d), giving a total recovery of 80± 1.2%. This recovery based on the AIEX method is satisfactory. It could have been increased by washing more times with 0.2 mol.L^{-1} NaAc at pH 7, but that would also increase the volume of buffer needed. In total, 1100 g of α-La with a purity of 97.4±0.4% in protein was obtained. Although this purity was slightly lower than that obtained at lab scale (98.6%), it is still the highest purity found when comparing with other pilot-scale purifications using WPC or WPI as raw material (Table 1). In addition, the total amount obtained (1100 g) is the highest amount obtained with reasonable purity (Table 1; Toro-Sierra et al. 2013; Konrad and Kleinschmidt 2008; Kristiansen et al. 1998). With the optimized method applied here, the total buffer volume used for recovery of α-La was 550 L (calculations not shown). If the process had been directly upscaled, the buffer volume necessary would have been 1440 L (120 L per AIEX), similar to what has been reported by Kristiansen et al. (1998).

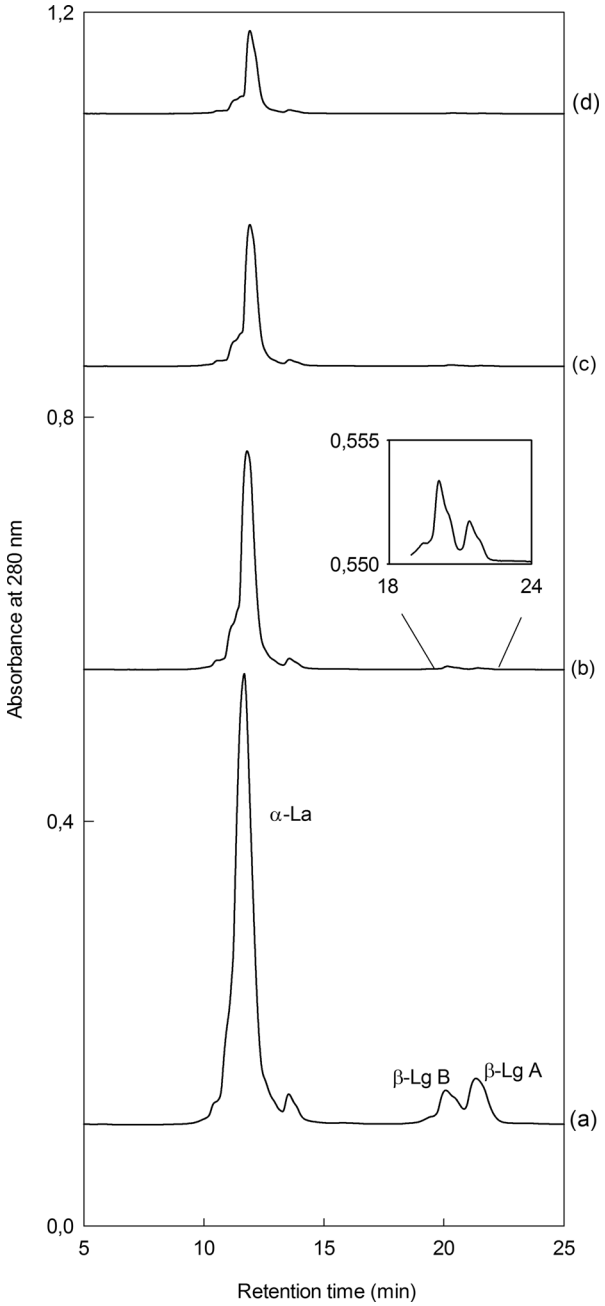

Fig. 5 RP-HPLC chromatograms from the pilot-scale purification of α-La showing the NaAc-αWPC solution before loading on the column (**a**), the run-through from loading of the NaAc-αWPC (**b**), and the eluates from the first (**c**) and second elution (**d**) with 0.2 mol.L^{-1} NaAc according to the process outlined in Fig. 2. The insert graph shows an expanded view of the region with β-Lg

Table 5 Composition (%) of produced α-La powder

Dry matter (DM)	98.3±0.4
Protein in DM	96.7±0.5
α-La in protein	97.4±0.4
Ash in DM	2.36±0.15
Calcium in DM	0.25±0.01

Values are expressed as means±SD from three independent replicates

3.5 Characteristics of the final α-La powder

A summary of the properties of the final α-La powder is shown in Table 5. After freeze drying, the dry matter of the powder reached 98.3%, containing 96.7% of protein in dry matter. The ash (2.36%) and calcium (0.25%) levels in the powder were similar to those of α-La purified by Kristiansen et al. (1998). The molecular weight of the purified α-La was 14,177 kg.mol^{-1}, which is exactly as expected for unmodified α-La, and the denaturation temperature T_m was 66.1 °C, which corresponds very well with the results obtained by others for the *holo* form of α-La (Konrad and Kleinschmidt 2008; Wang et al. 2006; Tolkach et al. 2005). Moreover, after converting calcium and α-La contents into moles, the molar ratio of Ca to α-La was 0.94, which is very close to 1. This agrees with the molar ratio for native α-La (Fox and McSweeney 1998). The average mass spectrum over all α-La peaks showed that the final powder contained 90% unmodified α-La and 10% monolactosylated α-La.

4 Conclusions

In this study, α-La with purity of 100% and recovery of 85% was obtained via a lab-scale AIEX process. When the method was upscaled and modified to selectively bind β-Lg and recover α-La in a small volume, a total amount of 1.1 kg of α-La (in its *holo* form) with a purity of 97.4% in protein was obtained with a recovery of 80% in the AIEX process. This is the highest purity and recovery among all studies on isolation of α-La from raw milk or whey protein powders at large or pilot scale. These results confirm that in order to obtain α-La in kilo-scale amounts with very high purity and recovery, ion exchange combined with membrane technique is the best solution. On the other hand, large-scale purification of α-La has not been extensively reported. One reason is probably due to the limited market for pure, native α-La. This work provided a way to obtain large amounts of pure α-La which can be used for further studies of biological and functional properties at industrial scale, e.g., for food, infant nutrition, and pharmaceutical applications.

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Conflict of interest The authors Xiao Lu Geng, Alexander Tolkach, Jeanette Otte, and Richard Ipsen declare that there are no competing interests.

Statement of human and animal rights This article does not contain any studies with human or animal subjects performed by any of the authors.

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