

Mannan interference and purification efficiency in downstream processing of precision-fermented milk proteins from *Komagataella phaffii*

Aryo D. Nugroho^a, Rensong Ji^{b,c}, Yi Ling Chin^a, Albert J.R. Heck^{b,c}, Karli R. Reiding^{b,c}, Remko M. Boom^{a,d}, Julia K. Keppler^{a,*}

^a Laboratory of Food Process Engineering, Wageningen University, Bornse Weilanden 9 6708 WG P.O. Box 17, 6700 AA Wageningen, The Netherlands

^b Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

^c Netherlands Proteomic Center, Padualaan 8, 3584 CH Utrecht, The Netherlands

^d Department of Food Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg, Denmark

ARTICLE INFO

Keywords:

Pichia pastoris
Mannoprotein
Exopolysaccharide
Heterologous proteins
Cellular agriculture
Recombinant β -lactoglobulin
Recombinant casein
Recombinant lactoferrin

ABSTRACT

Precision-fermented milk proteins from *Komagataella phaffii* are a well-established technology, but high downstream processing costs remain challenging. This study characterised extracellularly secreted recombinant β -lactoglobulin (rBLG), unphosphorylated α_{s1} -casein (rCSN), and lactoferrin (rLTF) based on protein and non-protein content, comparing them to their animal-derived counterparts. Three purification methods were evaluated. Two were charge-based, *i.e.*, isoelectric point (IEP) precipitation at a pH range of 2 to 7.5 for rBLG and rCSN and 5.5 to 11 for rLTF and anion-exchange (AEX) chromatography; one was size-based membrane separation. All target proteins matched ~95% of their animal-based counterparts in secondary structure. Irrespective of the protein, mannan (52–66% d.b., 2–242 kDa, 75–87% mannose) were the main impurity. Size-based membrane separation was ineffective due to the similar sizes of protein and mannan. Charge-based methods were more successful. AEX removed mannan effectively, increasing the protein purity from 20–41% to 64–81%, but recovered only 32–37% protein, limiting its use in the food industry. IEP precipitation worked only for rCSN, obtaining final protein purity up to 77% (in precipitated fraction) with only 7% of the casein remaining unprecipitated. Future work should focus on better mannan removal to meet purity demands for functional applications.

1. Introduction

The technology for producing recombinant proteins has developed rapidly over the last 40 years. Recombinant protein production was pioneered in 1977 with the synthesis of somatostatin (Itakura et al., 1977), followed by the production of the first commercial recombinant human insulin in 1982 (Johnson, 1983). This gradually broadened the range of recombinant proteins that can be produced (Gifre et al., 2017; Nielsen et al., 2024; Pham, 2018; Rosano and Ceccarelli, 2014), including milk proteins (Hettinga and Bijl, 2022; Keppler et al., 2021). Some of these recombinant milk proteins have already been produced at a similar structural level to the bovine reference proteins and should have similar technological functionalities (Hoppenreijns et al., 2024a; Keppler et al., 2021; Skoog et al., 2025).

Different microorganisms have been utilised to produce recombinant

proteins, such as the bacteria *Escherichia coli* and *Bacillus subtilis*, the yeasts *Saccharomyces cerevisiae* and *Komagataella phaffii* (formerly *Pichia pastoris*), and the filamentous fungi *Aspergillus oryzae* and *Trichoderma reesei* (Aro et al., 2023; Ichikawa et al., 2022; Mitsui and Yamada, 2021; Trabelsi et al., 2021; Valle and Bolívar, 2021; Yamada, 2021). Among these microbial hosts, *K. phaffii* is routinely used for recombinant protein production because of the relatively high yields that can be obtained (Besleaga et al., 2023; Eskandari et al., 2023; Karbalaei et al., 2020). This was, for example, demonstrated for the production of recombinant milk proteins like β -casein (Choi and Jiménez-Flores, 2001), lactoferrin (Skoog et al., 2025; Wang et al., 2002), β -lactoglobulin (Denton et al., 1998; Hoppenreijns et al., 2024a; Kalidas et al., 2001; Kim et al., 1997; Wilson et al., 2001), and α -lactalbumin (Saito et al., 2002).

The *K. phaffii* expression system is compatible for secreting recombinant proteins into the fermentation medium while keeping the

* Corresponding author.

E-mail address: julia.keppler@wur.nl (J.K. Keppler).

endogenous protein levels low (Cregg et al., 2000; Karbalaei et al., 2020). However, even in the extracellular medium, impurities accumulate. Soluble extracellular polysaccharides have been reported as the primary non-protein component of *K. phaffii* system (Denton et al., 1998). Recent research indicates that these polysaccharides primarily comprise mannan (Fischer et al., 2024; Hoppenreijns et al., 2024a).

Mannan originates from the outermost layer of the yeast cell wall (Baek et al., 2024; Bastos et al., 2022; Cuskin et al., 2015; Erwig and Gow, 2016; Gonzalez et al., 2010; Orlean, 2012). Orlean (2012) explained that the mannan was synthesised as mannoproteins (mannan bound with protein). The synthesis started in the endoplasmic reticulum with the assembly of precursor mannose and mannooligosaccharides, which were then attached as O- or N-glycosylation of a specific amino acid residue in the yeast cell wall protein (CWP), depending on the type of CWP. After elongation in the Golgi apparatus, the mannoproteins are secreted from the cell, where most are cross-linked with the cell wall β -glucan. Yeast mannans are typically highly branched and enriched with phosphomannose (mannose-6-phosphate) residues, which contribute to their exceptional solubility (Baek et al., 2024). Numerous studies have demonstrated that mannans are released from the cell wall during growth in various yeast species and strains (Domizio et al., 2014, 2017; Dupin et al., 2000; Fischer et al., 2024; Giovani et al., 2010; Wang et al., 2018). Knowing the effect of mannan on the protein separation when developing affordable downstream processes is important.

Despite the considerable potential of precision fermentation to produce animal-free food proteins, effectively purifying recombinant proteins at a low cost remains a significant challenge (Rudge and Ladisch, 2020; Saraswat et al., 2013). The current downstream processes have been adopted from the biopharmaceutical industry, which typically produces components with much higher value than bulk food components. The separation of the target protein from other components is generally performed through chromatography, especially ion-exchange chromatography, which aims to achieve high protein purity (Aro et al., 2023; Łojewska et al., 2016; Saraswat et al., 2013). Nonetheless, for food applications, high purity is not always necessary as long as functionality and safety are maintained. Transitioning to a functionality-driven downstream approach has shown that targeting functional fractions allows for more cost-effective and more sustainable production with plant proteins (Geerts et al., 2017; Lie-Piang et al., 2024, 2023; van der Goot et al., 2016). Developing alternative downstream processing methods is necessary to achieve this for precision fermentation-based proteins. However, currently, there is a dearth of literature to improve our understanding of the physicochemical characteristics and behaviour of recombinant milk proteins and the non-protein component to support the development of these alternative downstream processing methods.

Therefore, in this study, we aim to characterise three different excreted recombinant milk proteins, β -lactoglobulin, α_{s1} -casein, and lactoferrin, produced through precision fermentation with *K. phaffii*, as well as their non-protein components, to understand better how the separation can be conducted in a more sustainable and resource-efficient approach. We hypothesise that the recombinant milk proteins would closely resemble the structure and physicochemical properties of the animal-derived milk proteins. But variations in post-translational modifications (PTM) could alter their behaviour during downstream processing. Also, the extent to which yeast-derived components will affect the separation behaviour in the downstream processing remains unclear.

To solve these questions, we obtained crude (*i.e.*, unpurified but cell-free microfiltered and diafiltrated) recombinant β -lactoglobulin, α_{s1} -casein, and lactoferrin fractions from different producers, and characterised the overall composition and physicochemical properties of the protein and non-protein fractions. The protein structures were compared to their animal-derived counterparts. Furthermore, we investigated the behaviour of the crude recombinant milk protein fractions in response to downstream processing interventions, *i.e.*, isoelectric precipitation,

anion-exchange chromatography, and dead-end membrane filtration, to establish a basis for the development of future downstream processing. The overview of the experimental design is presented in Fig. 1.

2. Materials and methods

2.1. Materials

Crude recombinant milk protein fractions, *i.e.*, β -lactoglobulin variant A (rBLG), unphosphorylated α_{s1} -casein variant A (rCSN), and lactoferrin (rLTF), all extracellularly secreted by the yeast *K. phaffii*, were kindly provided by Formo (Berlin, Germany), Those Vegan Cowboys (Ghent, Belgium), and TurtleTree (Singapore), respectively. These recombinant milk proteins were non-commercialised test materials, and each was derived from a single production batch by the respective companies, and collected after centrifugation to remove the host cells. The materials were received as cell-free fermentation broths, either spray-dried (rBLG) or frozen (rCSN and rLTF). After resuspension or thawing, the broths were subjected to sterile filtration (0.2 mm) and diafiltration (10 kDa) to remove the leftover cell debris and small impurity molecules, respectively. The retentates after diafiltration were then freeze-dried and labelled as crude recombinant milk protein fractions. Bovine β -lactoglobulin (bBLG; mixed variant A and B, L3908, ≥ 90 % purity) and bovine α -casein (bCSN; mixed α_{s1} - and α_{s2} -casein variant B, C6780, ≥ 70 % purity) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), and purified bovine lactoferrin (bLTF; Vivinal, ≥ 95 % purity) was provided by FrieslandCampina (Amersfoort, The Netherlands). Yeast mannans from *S. cerevisiae* were purchased from Sigma-Aldrich (Merck; M7504, ≥ 99 % purity). Ultrapure water (MilliQ system; Merck) was used for all experiments. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Merck).

2.2. Characterisation methods

2.2.1. Composition analysis by ATR-FTIR spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was used to investigate material compositions, macromolecule profiles, and structural similarities. An Invenio-S system (Bruker Optik GmbH, Ettlingen, Germany) was used with OPUS software (Bruker). Samples were prepared at approximately 20 mg/mL dry matter content and loaded in a volume of 20 μ L on the surface of the thermostated attenuated total reflection (ATR) crystal (Bio-ATR; Bruker). Samples were left to equilibrate for 2 min before starting a measurement.

Measurements were conducted at 25 °C using ultrapure water as the background, with data averaged over 128 scans at a resolution of 4 cm^{-1} using a liquid nitrogen-cooled Photovoltaic-Mercury Cadmium Telluride (PV-MCT) detector. The fingerprint region of the FTIR spectrum, 1800–900 cm^{-1} (Table S. 1), indicates the macromolecules, including lipids, proteins, and carbohydrates. For the analysis of the protein conformation, the resulting spectra were vector normalised at the proteins (amide I) region (1700–1600 cm^{-1}). Then, the secondary derivatives were calculated using nine smoothing points. To analyse the similarity of mannan in the different samples, the spectra were cut at 1200–950 cm^{-1} and subjected to normalisation and offset correction to dismiss the difference in dry matter content and material purity.

2.2.2. Molecular weight distribution analysis by HPSEC-UV/RI

The molecular weight distribution profiles of proteins and polysaccharides were determined using high-performance size exclusion chromatography (HPSEC) on a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, Germering, Germany) equipped with an on-board ultraviolet (UV) detector and an additional refractive index (RI) detector (Shodex RI-501; Showa Denko K.K., Tokyo, Japan). All samples were prepared at 1–2 mg/mL dry matter content. To compensate for the possible concentration variability among materials, the chromatograms were normalised.

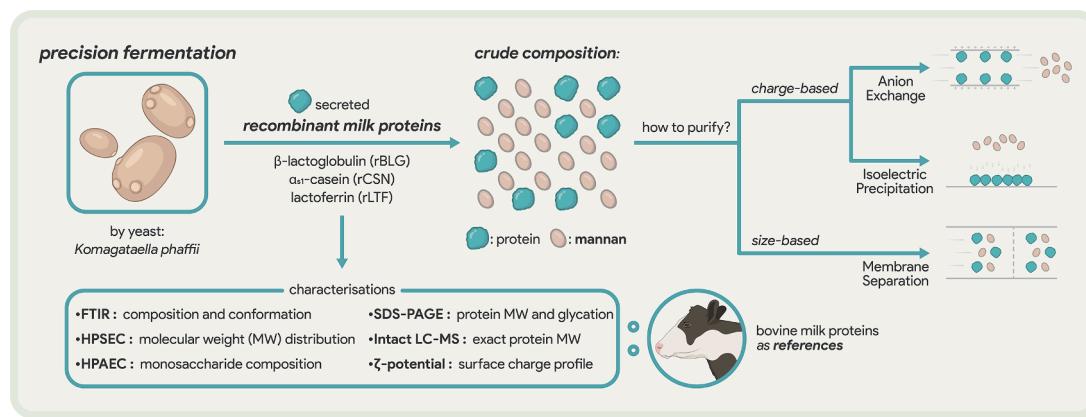


Fig. 1. Overview of the experimental design.

For protein analysis, the system was equipped with two TSKgel columns, G3000SWxl and G2000SWxl (each 7.8 mm ID \times 300 mm, 5 μ m), connected in series and preceded by a TSKgel SWxl guard column (6.0 mm ID \times 40 mm, 7 μ m), all from Tosoh Bioscience LLC (Tokyo, Japan). The column oven was maintained at 30 °C. An injection volume of 10 μ L sample was used, and the elution was carried out with a mobile phase of 30 % (v/v) acetonitrile (ACN) and 0.1 % (v/v) trifluoroacetic acid at a flow rate of 1.5 mL/min. The elution was detected using the UV detector at 214 nm. A series of globular proteins was used as molecular weight standards.

For polysaccharide analysis, we followed the method adapted from Pandeirada et al. (2021). A sequence of two TSKgel SuperAW4000 and SuperAW3000 (each 6 mm ID \times 150 mm, 6 μ m) was used, plus a TSKgel SuperAW-L guard column (4.6 mm ID \times 35 mm, 7 μ m), also from Tosoh Bioscience. The column oven was maintained at 55 °C. A 10 μ L sample volume was injected and eluted with a 0.2 M NaNO₃ at a 0.6 mL/min flow rate. The elution was detected using the RI detector. A series of dextran was used as molecular weight standards.

2.2.3. Sugar composition analysis by HPAEC-PAD

The sugar composition of the carbohydrates was determined after an acid hydrolysis, according to Jonathan et al. (2013). Pre-hydrolysis was performed with 72 % (12 M) sulphuric acid at 30 °C for 1 h, followed by the main hydrolysis in 1 M sulphuric acid for another 3 h at 100 °C.

The hydrolysates were cooled, diluted to approximately 0.1 mg/mL with ultrapure water, and analysed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS-6000 HPIC system (Thermo Fisher) equipped with a guard column (2 mm ID \times 50 mm, 10 μ m) and an analytical column (2 mm ID \times 250 mm, 10 μ m), both Dionex CarboPac™ PA1 IC from Thermo Fisher. The column oven was maintained at 20 °C. A 10 μ L sample volume was injected and eluted with a 0.3 mL/min flow rate.

The elution gradient was adapted from Pandeirada et al. (2021) with the following gradient of 0.1 M sodium hydroxide (NaOH; A) and 1.0 M NaOAc in 0.1 M NaOH (B): 0–5 min, 100 % A; 5–30 min, 0–15 % B; 30.1–35 min, 100 % B; 35.1–50 min 100 % A. Standards of mannose, glucose, and glucosamine (all from Sigma-Aldrich; Merck) were used for identification and quantification in the range of 25–200 μ g/mL. The collected data was analysed using Chromeleon 7.3 software (Thermo Fisher).

2.2.4. Protein content determination by BCA assay and Dumas

The soluble protein content was determined using the bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit; Thermo Fisher). The absorbance was measured using Thermo Scientific Multiskan FC (Thermo Fisher) at 560 nm. Besides, the total protein content was also determined using Dumas nitrogen combustion method (Rapid N exceed; Elementar Analysensysteme GmbH, Langenselbold, Germany) with

protein conversion factors of 6.29 for rBLG, 6.36 for rCSN, and 6.14 for rLTF, according to Walstra et al. (2005).

2.2.5. Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualise the molecular weight distribution and the possible glycosylation of the proteins in the materials. Mini-Protean Tetra System apparatus (Bio-Rad Laboratories Inc, Hercules, USA) was used with Mini-Protean TGXTM precast gels (4–20 %, 10-well; Bio-Rad). Samples were diluted twice using Laemmli sample buffer (Bio-Rad) with 2 M dithiothreitol (9:1 v/v) and heated at 95 °C for 5 min. The 10x Tris/Glycine/SDS (Bio-Rad) running buffer was diluted ten times with ultrapure water. At each well, 10 μ L of a sample (containing 10 μ g protein) or 5 μ L marker ladder (Precision Plus Protein™ Dual Xtra Standards; Bio-Rad) were loaded. Electrophoresis was run at 200 V for 35 min. The gel was then stained either with Bio-Safe Coomassie G-250 Stain (Bio-Rad) for protein staining or with Pierce™ Glycoprotein Staining Kit (Thermo Fisher) for glycan staining. Lastly, the gels were scanned using GS-900 Calibrated Densitometer (Bio-Rad). In addition to protein staining, glycan staining was used to detect the presence of glycoproteins such as mannoproteins and glycosylation on the milk proteins. Two identical gels were stained with either protein or glycan stain, resulting in a blue or a magenta gel, respectively. The positive responses are marked with bolder-coloured bands. The bands in both gels at a similar position indicated that the protein was glycosylated.

2.2.6. Protein molecular weight determination using intact LC-MS

The molecular weight of the purified recombinant milk protein fractions and their animal-derived references was determined using liquid chromatography-mass spectrometry (LC-MS). The samples were reconstituted in ultrapure water, and approximately 200 ng of proteins were injected for analysis. The method was modified from Gazi et al. (2022). The LC was performed using a Vanquish™ Flex UHPLC system (Thermo Fisher) equipped with an MAbPac™ Reversed Phase column (2.1 mm \times 50 mm; Thermo Fisher) heated to 40 °C. LC-MS/MS runtime was set to 29 min with a flow rate of 100 μ L/min. The elution gradient was performed using 0.1 % (v/v) fluoroacetic acid (FA) in ultrapure water (A) and 0.1 % FA in ACN (B): 0–5 min, 10 % B; 5–6 min, 10–31 % B; 6–20 min, 31–41 % B; 20–21 min, 41–95 % B; 21–24 min, 95 % B; 24–25 min, 95–10 % B; 25–29 min, 10 % B.

All intact protein MS experiments were performed on an Orbitrap Exploris™ 480 mass spectrometer (Thermo Fisher) set to intact protein mode with the low-pressure setting. The MS was acquired at resolutions (m/z 200) of 7500. The mass range was set to m/z 400–3000, with the automatic gain control (AGC) target set to 250 %. Maximum of injection was defined at 250 ms with 5 μ scans averaged for each scan. The raw files were analysed using BioPharma Finder (Thermo Fisher). The analysis method used was modified from the default ReSpect method.

Sliding windows were used for the deconvolution of each peak. The retention time range was set to 5 to 21 min. The output mass range was set to 10–90 kDa.

2.2.7. Surface charge measurement

The surface charge (ζ -potential) over a pH range of 2 to 7 or 5 to 11 in an interval of 0.5 was measured using the dynamic light scattering method (ZetaSizer Ultra; Malvern Panalytical Ltd, Malvern, UK) coupled with an MPT-2 Multi-Purpose Titrator (Malvern). Samples were prepared at 1 mg/mL protein content in a titration tube and connected to a folded capillary cell DTS1070 (Malvern). The cell temperature was controlled at 25 °C. Before each measurement, samples were equilibrated for 2 min. The isoelectric point (IEP) was determined as a pH value where the ζ -potential reached zero.

2.2.8. Ash content determination

The ash content of the material was determined using the dry ashing method. The samples were incinerated in a furnace (Carbolite AAF1100; Carbolite Gero Ltd, Hope Valley, UK) at 550 °C for 24 h. The ash content was calculated as a percentage of the initial sample weight.

2.3. Purification efforts

2.3.1. Isoelectric precipitation

The precipitation behaviour of the proteins was investigated across different pH values, as precipitation at the IEP may be an easy way to purify the target proteins. The protein solubility at a pH range of 2 to 7.5 or 5.5 to 11 in an interval of 0.5 was measured to determine whether the protein precipitates at its IEP. The pH of each material solution (containing 1 mg/mL protein) was adjusted using small amounts of diluted HCl or NaOH. A sample was taken and centrifuged at 20,000 g on every pH point for 10 min. The soluble protein fraction was quantified from the supernatant using BCA assay.

2.3.2. Preparative AEX-LC

Anion-exchange liquid chromatography (AEX-LC) was used as a reference purification method, since it is well-established for recombinant protein purification (Łojewska et al., 2016; Saraswat et al., 2013). The method also provided information on the net negative charge of the proteins upon elution. The method was adapted from Keppler et al. (2021). The ÄKTA pure™ 25 chromatography system (Cytiva Europe GmbH, Freiburg, Germany) equipped with a HiTrap™ Capto™ Q ImpRes column (Column Volume (CV): 5 mL; Cytiva) and coupled with Fraction Collector F9-R (Cytiva) was used. Two different running buffers, i.e., 20 mM sodium phosphate buffer pH 8.0 (for β -lactoglobulins and α -caseins) and 20 mM sodium carbonate-bicarbonate buffer pH 10.0 (for lactoferrins) with a 1 mL/min flow rate. After equilibration, the sample was injected, and several volumes of running buffer were applied to elute the unbound fractions. Then, a salt solution of 500 mM NaCl was eluted in a gradient from 0 to 100 % in 10 CV. The elution was detected with an onboard UV detector at 280 nm. The fractions (including the unbound ones) were collected every 3 mL. Fractions of the main peaks were then pooled, desalting, and analysed with FTIR.

Before the salt gradient was initiated, uncharged or positively charged molecules would elute as unbound fractions. Most of the carbohydrates were expected to elute in this period. Once the salt gradient began, the attached proteins would elute following their net negative charge. The more negatively charged the protein, the later it would be eluted.

2.3.3. Enzymatic hydrolysis of mannan

An enzymatic method was conducted to determine whether the mannan could be hydrolysed. Yeast mannan comprises the main α 1,6-mannose backbone with extensive branching of mainly α 1,2 residues and a lesser portion of α 1,3, which differs from plant mannan that consists of β -mannose residues (Baek et al., 2024; Cuskin et al., 2015;

Orlean, 2012). Therefore, it is expected that yeast mannan can be hydrolysed using endo- α -mannanase and exo- α -mannosidase. For this, a 1 mg/mL yeast mannan in phosphate buffer (50 mM, pH 6.7) was incubated with 20 % volume of α -Mannanase 76A and α -Mannosidase 92I (mixed 1:1; both from NZYtech, Lisbon, Portugal) at 37 °C up to 21 h.

Samples were taken at specific time points, and directly heated at 100 °C to inactivate the enzyme and cooled down to room temperature before the analysis. The enzymatic activity was measured as the amount of reducing ends released from the polysaccharide cleavage. In this case, the mannose content was monitored and analysed using the PAHBAH assay. Briefly, 25 μ L sample from each time point were mixed with 500 μ L of PAHBAH reagent (4-Hydroxybenzhydrazide) and were incubated at 70 °C for 35 min with shaking before the absorbance measurement at 405 nm.

2.4. Statistics

If not stated otherwise, all samples were prepared in technical triplicate. Statistical significance was determined by a one-way ANOVA and using the Tukey post hoc test. A significance level of 5 % was assumed. All tests were performed with IBM SPSS Statistics Version 28 (IBM, New York, USA).

3. Results

The recombinant β -lactoglobulin (rBLG), unphosphorylated α s1-casein (rCSN), and lactoferrin (rLTF) were analysed and compared to the bovine references (bBLG, bCSN, and bLTF). Section 3.1 describes the result of the overall composition and protein conformation. Molecular weight and sugar composition of the carbohydrate fractions are explained in Section 3.2. Subsequently, in Section 3.3, the protein fractions are analysed for their molecular weight and the possible glycosylation using HPSEC-UV, SDS-PAGE, and intact LC-MS. In Section 3.4, the results of surface charge analysis are presented. The purification efforts are described next to the characterisations. In Section 3.5, the precipitation behaviour of the proteins at their isoelectric point (IEP) is shown. The fractionation using anion-exchange is explained in Section 3.6. Lastly, a reflection on using size-based separation and enzymatic hydrolysis of mannan is presented in Section 3.7.

3.1. Overall composition and protein conformation

The overall compositions of the crude materials (listed in Table 1), including the fractions of rBLG, rCSN, and rLTF were estimated based on the dry matter content of protein, carbohydrate, and ash, and were then compared with the results from Fourier-transform infrared (FTIR) spectroscopy. Yeast mannan (YM) and isolated bovine milk proteins

Table 1

The overall composition of the crude recombinant milk protein fractions on the dry basis.

	Protein (% d.b.)		Carbohydrate (% d.b.)	Ash (% d.b.)	Other (% d.b.) ²
	BCA	DUMAS ¹			
rBLG	26.45 ± 1.04 ^b	31.68 ± 0.24 ^b	55.62 ± 5.69 ^b	3.59 ± 0.70 ^a	9.12
rCSN	39.79 ± 1.42 ^a	40.94 ± 0.29 ^a	52.41 ± 1.08 ^b	3.70 ± 0.65 ^a	2.95
rLTF	19.63 ± 0.70 ^c	26.09 ± 0.13 ^c	66.48 ± 4.35 ^a	0.93 ± 0.28 ^b	6.51

Letters in the same column indicate significant differences at $p < 0.05$.

¹ The protein conversion factors of 6.29, 6.36, and 6.14 were assumed for the quantification of rBLG, rCSN, and rLTF, respectively, based on Walstra et al. (2005).

² The “other” component content was calculated from the total dry matter (100 %) subtracted by protein (DUMAS), carbohydrate, and ash content.

(bBLG, bCSN, and bLTF) were used as references for the FTIR.

The averaged FTIR spectra of crude recombinant milk protein fractions, animal-derived milk protein references, and yeast mannan are shown in Fig. 2A. All milk protein references showed characteristic peaks in the amide I and II regions, confirming their high protein purities, which is consistent with their specifications. The yeast mannan reference had characteristic peaks in the carbohydrate region only, particularly in the 1200–950 cm⁻¹ range, typical for the C–O stretching of polysaccharides (Socrates, 2004). All three crude recombinant milk proteins possessed comparable spectral profiles with a lower absorption in the protein (amide I and II) region and more prominent peaks in the carbohydrate region, indicating that they contained both protein and carbohydrates. The spectral profile of the carbohydrate part of these crude recombinant milk proteins was comparable to yeast mannan with four matched peaks at ~1135, ~1075, ~1030, and ~975 cm⁻¹. The similarity of the carbohydrate region in those materials was >95 %, calculated using the Pearson Correlation Coefficient and Cosine Similarity method (Table S. 2). This finding is in accordance with previous research on yeast-based recombinant β -lactoglobulin, which also

contained mannan (Hoppenreijis et al., 2024a).

To further compare the recombinant proteins with their animal-derived references, the amide I secondary derivative profile of recombinant milk proteins was used, shown in Fig. 2B Table S. 3 shows that all recombinant milk proteins were similar (~95 %) to their bovine counterparts. To identify the main deviations, the relative percentages of α -helices and β -sheets on the purified recombinant milk protein fractions were calculated (Fig. 2C). The rBLG and bBLG were characterised by their main β -sheets peak (52–54 %), and a small portion (2–3 %) of α -helices, which is in line with previous findings (Hoppenreijis et al., 2024a; Keppler et al., 2021). The rBLG had 1 % fewer α -helices and 2 % fewer β -sheets than the bBLG. In contrast to β -lactoglobulin, caseins are less ordered, which is evident in their high proportion of “other” structural elements (McMahon and Oommen, 2008). Compared with its animal-derived reference, rCSN had 8 % fewer α -helices. It needs to be taken into account that the two caseins are different in the types and genetic variant, i.e., rCSN is an α_{s1} -casein variant A while bCSN is a mix of α_{s1} - and α_{s2} -casein variant B, which have different mass spectra profiles (Fig. 4C in Section 3.3). Lactoferrin (PDB: 1BLF) is a protein with

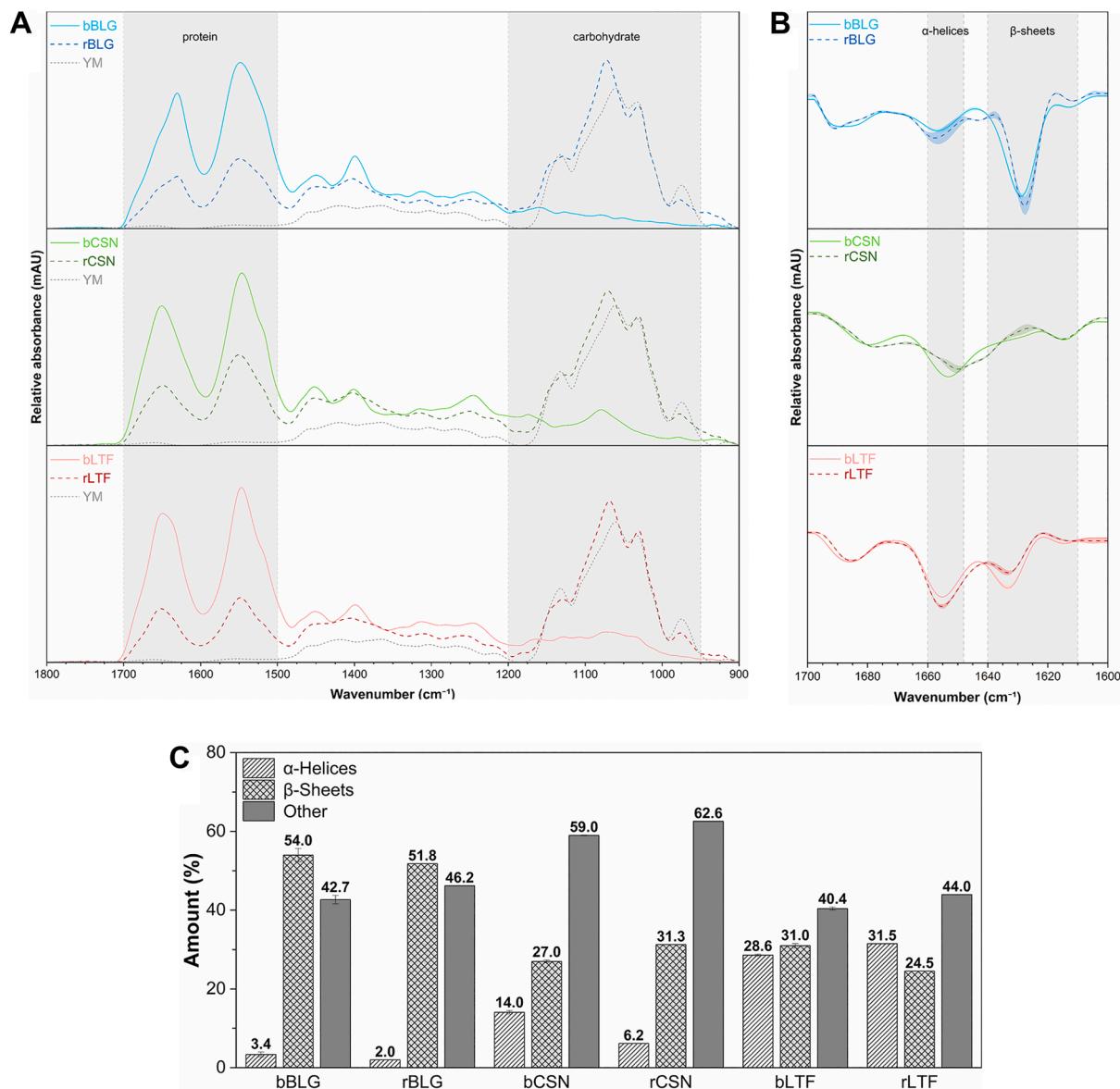


Fig. 2. FTIR spectra of crude recombinant milk protein fractions and the references: A) fingerprint region (1800–900 cm⁻¹) and B) amide I secondary derivative (1700–1600 cm⁻¹). C) Relative amounts of α -helices, β -sheets, and other structures calculated from the FTIR spectra of purified recombinant milk protein fractions and the references.

approximately 30 % α -helices (Moore et al., 1997), in line with the presented findings in Fig. 2C (31.52 % for rLTF and 28.61 % for bLTF), with rLTF having 6 % fewer β -sheets than bLTF.

3.2. Carbohydrates: molecular weight and sugar composition

The carbohydrate molecular weight distributions from all crude recombinant milk protein fractions were characterised by size exclusion chromatography (SEC) to assess their potential to be separated from the proteins by molecular size (Fig. 3A). The carbohydrates from the crude rLTF fraction had a broad molecular weight distribution ranging from 8 to 242 kDa with two main peaks at approximately 31.1 and 122.9 kDa. The carbohydrates in the crude rBLG fraction had the smallest molecular weight, ranging from 2 to 20 kDa with a peak at 7.4 kDa and shoulders at 3.9 and 10.0 kDa. Meanwhile, the carbohydrates from the crude rCSN fraction had a single peak at 43.5 kDa with a molecular weight range comparable to the yeast mannan reference (YM). However, the SEC

result should be interpreted thoroughly because it measures the size based on the hydrodynamic volume, and flexible elongated molecules such as mannans might vary in elution time based on their exact molecular structure.

The individual sugar building blocks were identified as mannose, glucosamine, and glucose in different proportions, as shown in Fig. 3B, using HPAEC. The carbohydrates of all crude recombinant milk proteins consisted of varying amounts of mannose (75–88 %) and glucosamine residues (10–23 %) with small proportions of glucose (1.5–2.3 %). These results are slightly different from previous reports (Hoppenreijns et al., 2024a), yet confirm that the carbohydrates of all of the recombinant milk proteins are mannans.

3.3. Proteins molecular weight and glycoprotein detection

To better understand whether the molecular weights of the recombinant proteins and mannans would differ sufficiently for size-based

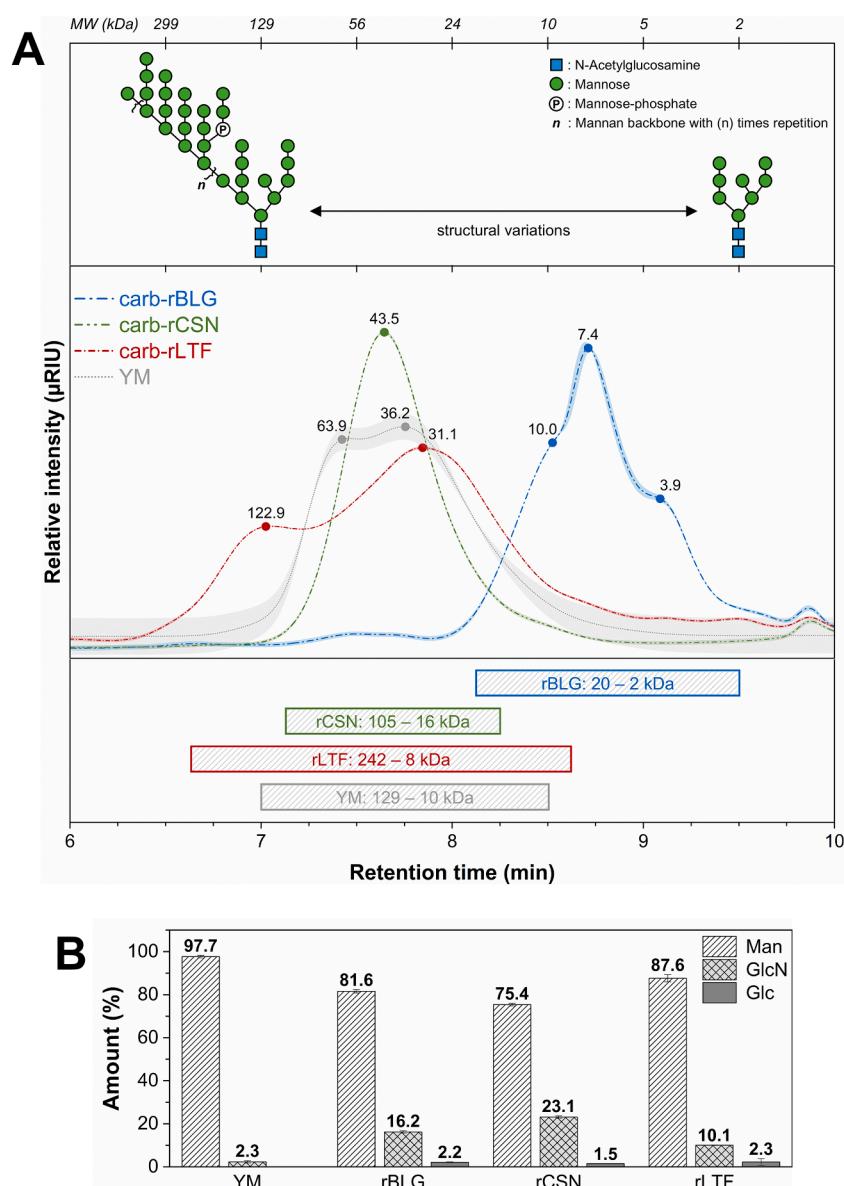


Fig. 3. A) SEC chromatogram of carbohydrates from crude recombinant milk protein fractions compared to yeast mannan as reference, the number above the peaks corresponds to the calculated molecular weight detected for each peak (in kDa), the bar below the graph corresponds to the molecular weight range of the mannans, the illustration above the graph represents the structural variations of yeast mannans adapted from literature (Cuskin et al., 2015; Orlean, 2012). B) Approximated sugar composition of carbohydrates, identified as mannose (Man), glucosamine (GlcN), and glucose (Glc).

separation, the protein molecular weight distribution of the crude recombinant protein fractions was compared to that of animal-derived reference proteins by SEC. For β -lactoglobulin and lactoferrin, their molecular weights were close to their molecular weight in a dimeric state (Fig. 4A), as they were measured with an acidic elution buffer (pH ~2.5). Since the analysis is based on the hydrodynamic volume, and globular proteins were used as the molecular weight standards, it was difficult to interpret the result for the randomly coiled, self-associating α -casein.

The protein molecular weight distributions of the recombinant and bovine β -lactoglobulin and lactoferrin were roughly comparable. Still, the rBLG sample gives a double peak. One of the peaks (36.2 kDa) is aligned with bBLG dimers (35.8 kDa), while the other peak has a slightly larger molecular weight of 43.1 kDa. The rCSN is significantly larger (57.9 kDa) than the bCSN (47.1 kDa). The molecular weight of caseins cannot be interpreted straightforwardly, as it is not a globular protein, and it tends to self-associate.

The results of SDS-PAGE in Fig. 4B indicate the protein size and state of glycosylation. The rCSN and rLTF have comparable molecular weights as their bovine counterparts (α -casein at ~30 kDa and lactoferrin at ~80 kDa), whereas rBLG showed a double band in the range of 17–20 kDa, unlike its bovine counterpart, which has only a single band. The latter observation aligns with the double peak seen in rBLG using SEC (Fig. 4A). Still, the difference in molecular weight between SDS-PAGE and SEC is because proteins elute as non-covalent dimers in SEC. The size difference between bCSN and rCSN in the SEC measurement was not observed in SDS-PAGE. Native-PAGE and non-reduced

SDS-PAGE could be added to further investigate these dimerisation or aggregation states in future work.

Glycans were detected in the vicinity of the protein bands in rCSN and rLTF but not in rBLG. This indicates that rCSN and rLTF were mannosylated (glycosylated with mannans). The rCSN sample also contained mannoprotein, resulting in a smeared band at around 100 kDa. Lastly, all recombinant milk protein fractions contained residual free mannans, which did not penetrate the gel beyond a certain depth of approximately 250 kDa, with rLTF having the highest free mannan content (aligned with Table 1).

LC-MS was performed on the purified recombinant milk protein fractions and their animal-derived references to determine their intact molecular mass, complementing the result of SEC and SDS-PAGE, as shown in Fig. 4C. The bBLG contained two masses of 18,362 and 18,277 Da, which correspond to bBLG genetic variants A and B, respectively. In the purified rBLG (variant A), larger masses than bBLG variants A were discovered at 19,277, 19,406, and 19,477 Da. The additional mass of 915–1115 Da was expected from the incomplete cleaving of the signal peptide. As reported by Hoppenreijns et al. (2024a) alteration in the leucine residue in the N-terminus occurred, from LIV... to AKEEGV-SLEKRIV... or EKGVSLERKV....

The bCSN showed three masses of 23,542, 23,614, and 23,694 Da, equivalent to α_{s1} -casein genetic variant B with 7P, 8P, and 9P (Farrell et al., 2004; Nadugala et al., 2022) with the mass of α_{s1} -casein-8P (23,614 Da) being the most abundant, while the purified (unphosphorylated) rCSN showed three masses at 20,989, 21,242, and 21,175 Da, a mass of 21,429 Da (equivalent to α_{s1} -casein-OP genetic variant A), and

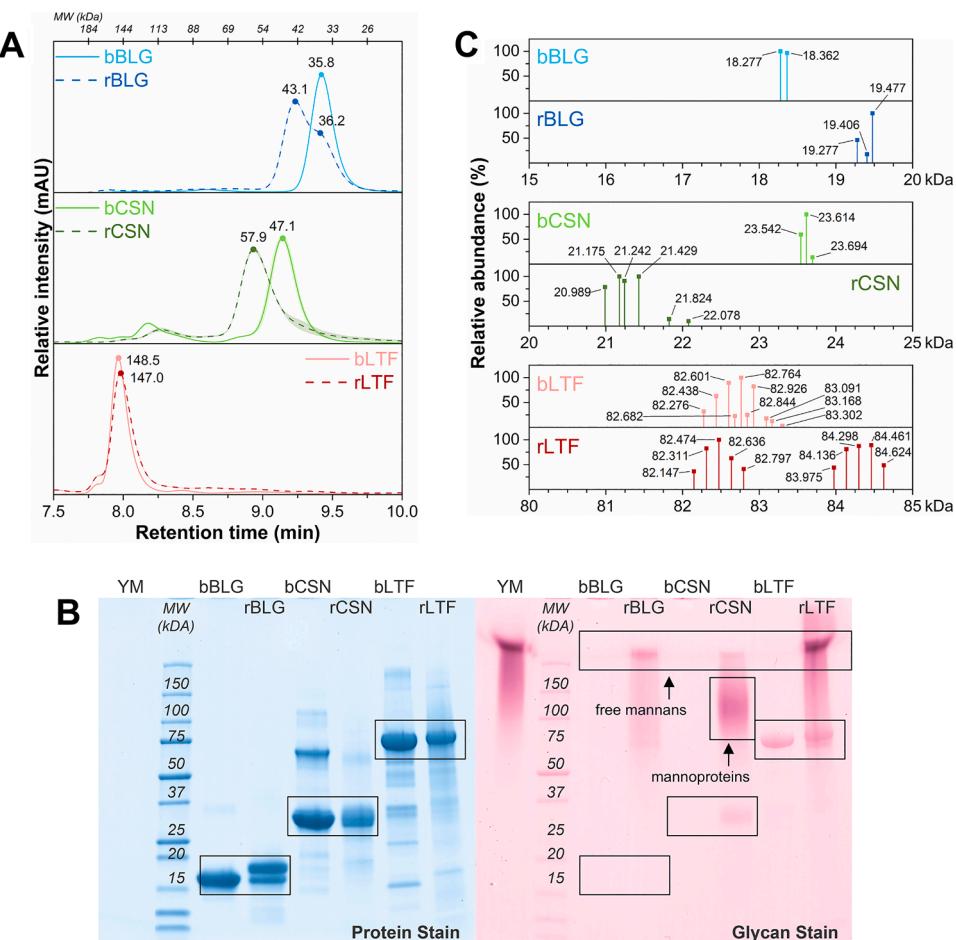


Fig. 4. A) SEC chromatogram of crude recombinant milk protein fractions compared to the target proteins, the number above the peaks corresponds to the estimated molecular weight for each peak (in kDa). B) Reducing SDS-PAGE results in protein stain (blue-left) and glycan stain (magenta-right). C) Deconvoluted mass spectra depicting proteoforms that were identified, the numbers represent the average masses in kDa.

two masses at 21,824 and 22,078 Da. The mass of 21,429 Da corresponds to full-size α_{s1} -casein variant A without glycosylation or phosphorylation (Farrell et al., 2004). The mass of 21,175 Da corresponds to α_{s1} -casein variant A (21,429 Da) without N-terminal Arg-Pro; 21,242 Da corresponds to α_{s1} -casein variant A (21,429 Da) without C-terminal Trp; and 20,989 Da corresponds to α_{s1} -casein variant A (21,429 Da) without N-terminal Arg-Pro and C-terminal Trp. The mass of 22,078 Da corresponds to α_{s1} -casein variant A (21,429 Da) with an additional four hexoses (mannoses), while the 21,824 Da corresponds to the mass of 21,175 Da (the protein without N-terminal Arg-Pro) with four hexoses. The α_{s1} -casein genetic variants A and B differ in 13 amino acids, in which residues 14–26 (EVLNENLLRFFVA) were present in variant B and deleted in variant A (Farrell et al., 2004; Nadugala et al., 2022).

Lactoferrin exhibited mass variations, ranging from 82,276 to 83,302 Da for bLTF and from 82,147 to 82,797 Da and 83,975 to 84,624 Da for purified rLTF. These variations can be attributed to differences in the glycosylation patterns of lactoferrin (Mu et al., 2024). The purified rLTF had larger masses than bLTF by 673–1322 Da, which shows that it has more glycosylation than bLTF by 4 to 8 additional mannose units (Skoog

et al., 2025).

Drawing upon SEC, SDS-PAGE, and intact LC-MS analyses, the collective data suggest that most of the recombinant milk proteins exhibited varying degrees of glycosylation, depending on their type, even when the underlying amino acid sequence is presumed to be largely correct.

3.4. Surface charge

The electrokinetic charge of the proteins was assessed using ζ -potential measurements, expressed in millivolts (mV) and shown in Fig. 5A. This electrokinetic potential value reflects the difference in electrical potential between the ion-rich layer surrounding the surface and the bulk solution (Hayat et al., 2022; Kallay et al., 2006; Selvamani, 2019). The pH where the electrokinetic potential equals zero (0 mV) is commonly recognised as the isoelectric point (IEP) (Kallay et al., 2006), but it does not necessarily reflect the true IEP of the crude recombinant milk protein fractions since the presence of the mannan masks the net charge of the protein in the system (Hoppenreijis et al., 2024a).

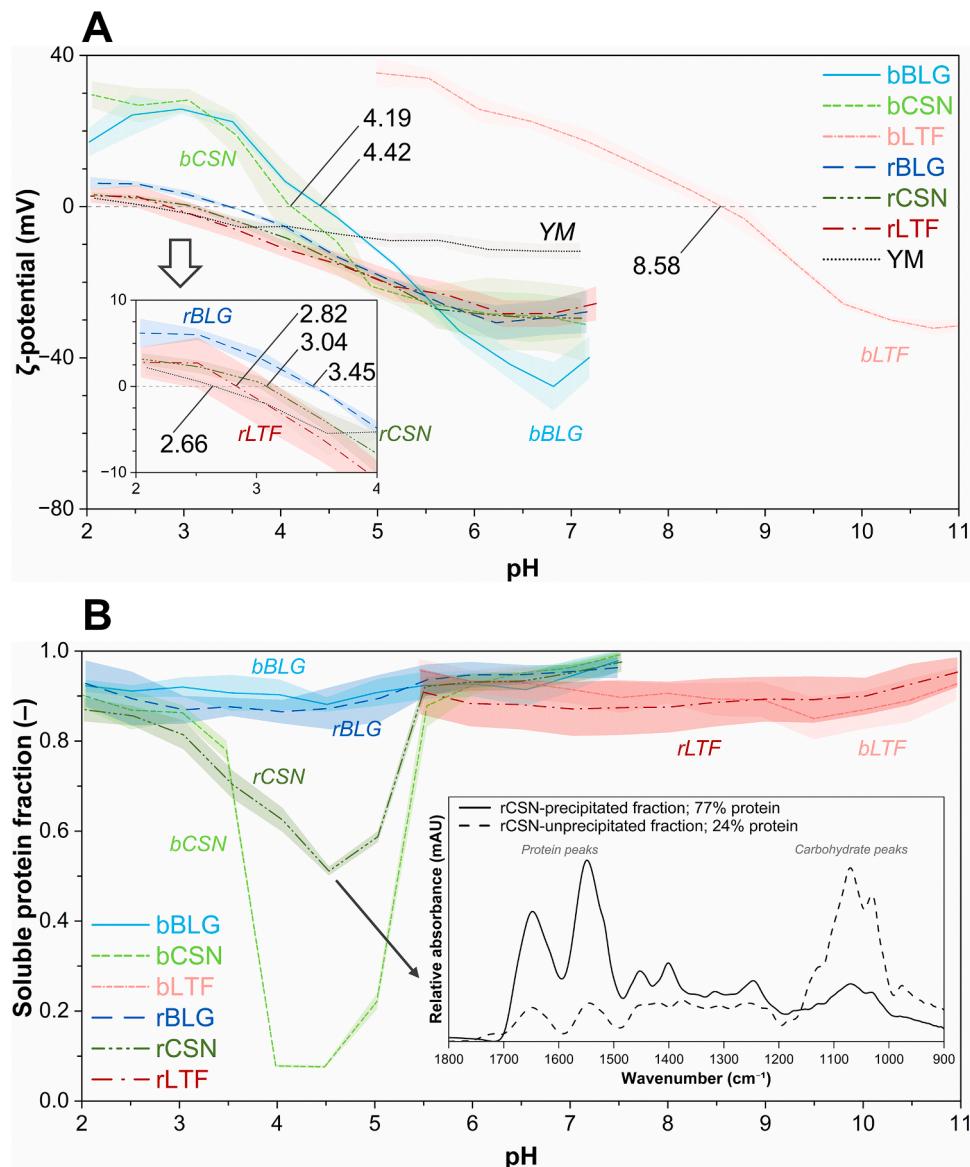


Fig. 5. A) ζ -potential profile of crude recombinant milk protein fractions compared to the references, the label showed pH value at 0 mV. B) Protein solubility profile of crude recombinant milk protein fractions compared to the references. Inset graph: FTIR spectra of the soluble and insoluble fractions obtained from the precipitation of rCSN at pH 4.5, showing peaks in the protein region (1700–1600 cm^{-1}) and carbohydrate region (1200–950 cm^{-1}).

In Fig. 5A, the electrokinetic potential profiles of all crude recombinant milk protein fractions and the animal-derived references were measured from pH 2 to 7, except for bLTF which was measured at pH 5 to 11, due to its high IEP. The IEPs of all bovine milk proteins (bBLG: 4.42, bCSN: 4.19, and bLTF: 8.58) were close to the values mentioned in literature (bBLG: 5.13, bCSN: 4.60, and bLTF: 8.81) (Farrell et al., 2004; Walstra et al., 2005). However, all crude recombinant milk protein fractions containing mannans showed a relatively similar potential profile, distinct from the references. The zero potential value, in this case, reflected the net charge of the particles in the system, including the proteins and mannans. The zero-charge values ranged between 3.45 for rBLG to 2.82 for rLTF. Meanwhile, the YM alone only has a stable, slightly negative charge of approximately 3 to -10 mV in that pH range, which would be consistent with carboxyl groups.

The results for rBLG were similar to a previous report, which showed a shift of the potential profile to a lower value in the crude protein

compared to the purified one (Hoppenreijns et al., 2024a). The shift of rLTF is extensive and might also be caused by differences in the degree of iron complexation, glycosylation level, and the level of free mannan (Table 1). Purified recombinant lactoferrin was reported earlier to have an IEP ranging from 8.25 to 9.63 (Skoog et al., 2025).

3.5. Precipitation behaviour at the IEP

Fig. 5B shows that only bCSN and rCSN precipitate at their IEP, at pH ~4.5. However, the precipitated fraction of rCSN was 1.9 times less than that of bCSN, with 51.1 % of the proteins remaining soluble. The difference in genetic variant is not likely to cause this difference. The precipitated (77 % protein) and unprecipitated (24 % protein) fractions of rCSN were analysed with FTIR, as shown in the inset of Fig. 5B. The precipitated fraction consisted mainly of protein (1700–1500 cm⁻¹) with a small peak in the carbohydrate region (1200–950 cm⁻¹), consistent

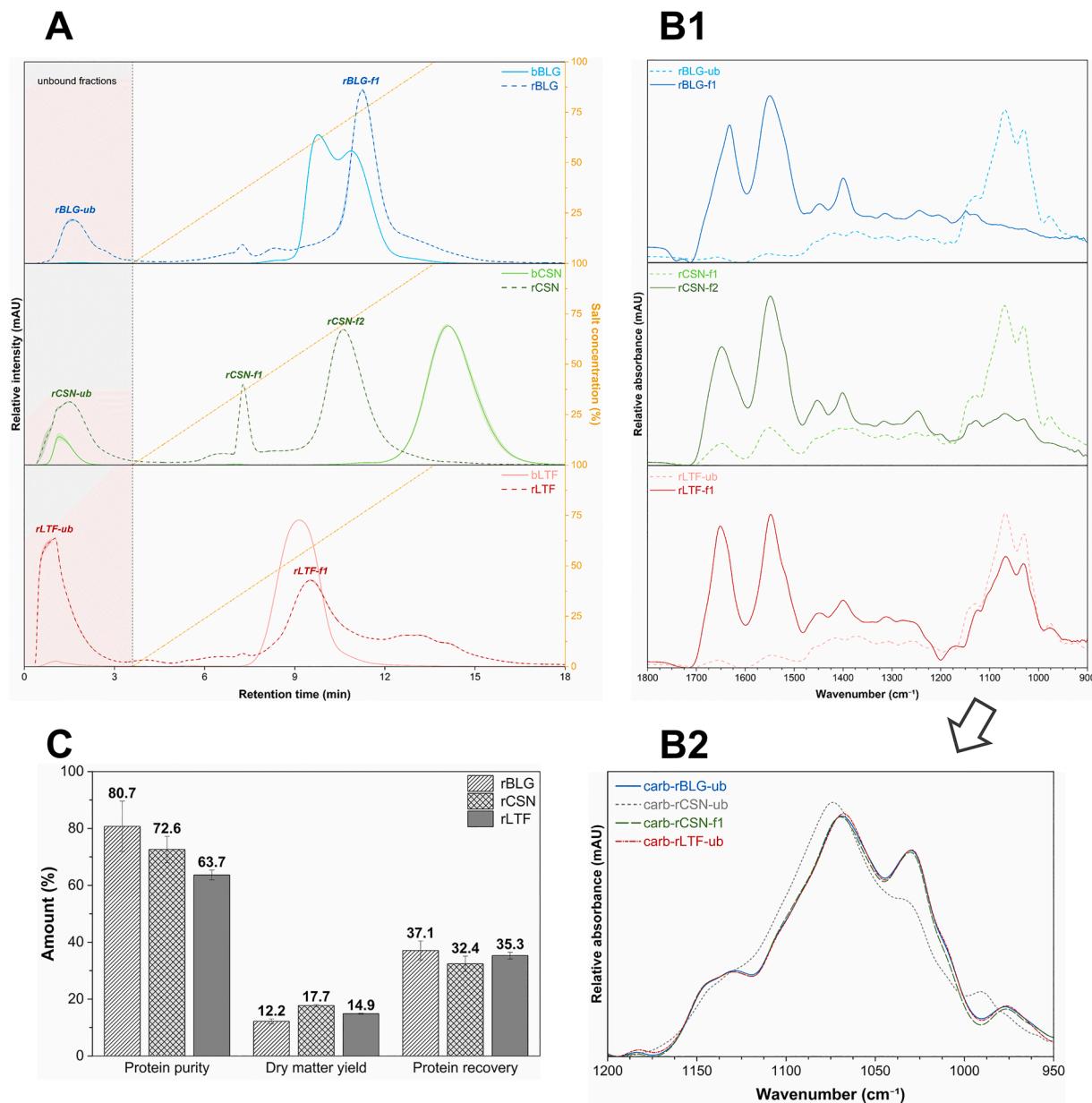


Fig. 6. A) AEX-LC chromatograms of crude recombinant milk protein fractions compared to the references, the main fractions are labelled: -ub: unbound fraction, -f1: protein fraction #1, -f2: protein fraction #2. FTIR spectra of the main fractions: B1) fingerprint region, showing peaks in the protein region (1700–1600 cm⁻¹) and carbohydrate region (1200–950 cm⁻¹), and B2) zooming into the carbohydrate region, to compare the mannan-rich fractions. C) Calculated purity, yield, and recovery of AEX-LC.

with some minor glycosylation of rCSN. The unprecipitated fraction features a carbohydrate peak that is significantly larger than the protein peak, indicating the presence of mannoproteins. Additionally, this fraction was identified to contain a trace (7 %) of casein (data not shown).

3.6. Anion-exchange chromatography

Fig. 6A shows that all crude recombinant milk protein fractions had noticeable concentrations of material that did not interact with the column (unbound fraction), with the rLTF fraction having the highest amount. As shown in **Fig. 6B1**, the unbound fractions (rBLG-ub and rLTF-ub) were confirmed as free carbohydrates, likely mannans (with very little protein), the main impurities in the rBLG and rLTF samples. However, in rCSN, the rCSN-f1 fraction had the same spectral profile in the carbohydrate range as rBLG-ub and rLTF-ub ($1200\text{--}950\text{ cm}^{-1}$), while rCSN-ub did not (**Fig. 6B2**). The rCSN-f1 fraction is likely mannoprotein as it also had notable protein content (shown as two peaks between 1700 and 1500 cm^{-1} ; **Fig. 6B1**). This confirms our earlier speculation that the protein fraction that did not precipitate at the IEP, is indeed predominantly mannoproteins. The rCSN-f1 fraction eluted after the salt concentration reached around 30–40 % (**Fig. 6A**), which means that rCSN-f1 has a slightly net negative charge, which indicates the presence of protein in this fraction, unlike the unbound fraction.

The main peaks of rBLG and rLTF align with those from the animal-based references. The double peak shown in bBLG (**Fig. 6A**) indicates the presence of two genetic variants, A and B, while the rBLG only contained variant A (Hoppenreijns et al., 2024a). Besides, the main peak of rLTF (**Fig. 6B1**) showed a three times higher absorbance in the carbohydrate range compared to its animal-based reference (data not shown), a sign of stronger glycosylation of the protein. This aligns with the intact LC-MS (**Fig. 4C**), as well as the N-glycosylation analysis by Skoog et al. (2025), who concluded that yeast-based recombinant lactoferrin has a high-mannose structure that was not present in the animal-based lactoferrin.

Fig. 6C suggests that anion-exchange chromatography can achieve a high final protein purity of 64–81 %, but has a low yield and recovery of 12–18 % and 32–37 %, respectively.

3.7. Reflection on size-based separation and enzymatic hydrolysis of mannan

Understanding the molecular sizes of the target protein and its associated mannan impurities is crucial for assessing the feasibility of size-based separation techniques, such as membrane filtration. Therefore, we summarise the carbohydrate size, as determined by SEC (**Fig. 3A**), alongside the protein size obtained from both SEC and LC-MS (**Fig. 4**) in **Fig. 7A**. The masses of multimers (dimer, tetramer, hexamer, and octamer) from rBLG were also calculated based on the LC-MS results.

A simple concentration mode dead-end filtration was attempted on a binary mixture of bBLG and mannan fraction from rBLG (rBLG-ub; **Fig. 6A**) to compare rejection profiles for mannans and proteins. The results (**Fig. S. 1**) show that with membranes having MWCO values of 50 or 100 kDa, full rejection was observed for both proteins and mannans. This contrasts our expectation that the proteins would be retained and the mannans would pass through the membrane. With a 200 kDa membrane, both proteins and mannans permeated. These results indicate that the size-based separation of proteins and mannans is relatively complex. Possible explanations could be an association between the proteins and the mannans, or the formation of a selective cake layer on the membrane, formed by retained components.

An attempt was made to degrade the mannans enzymatically to reduce their size, thus widening the size difference with the target protein. The result in **Fig. 7B** shows that the release of reducing ends was insignificant during the first two hours, but increased to 18.6 % after overnight incubation (~21 h). The poor result is suspected to be caused by the complexity of the yeast mannan (Baek et al., 2024), while the available enzymes were insufficient to degrade this complex structure.

4. Discussion

4.1. Mannans as the main impurity

The recombinant milk proteins produced by *K. phaffii* share the presence of mannan as the main impurity, regardless of the target protein. As seen from FTIR (**Fig. 2A**), these carbohydrates were similar (~99 %; **Table S. 2**) among the recombinant milk proteins and were similar in

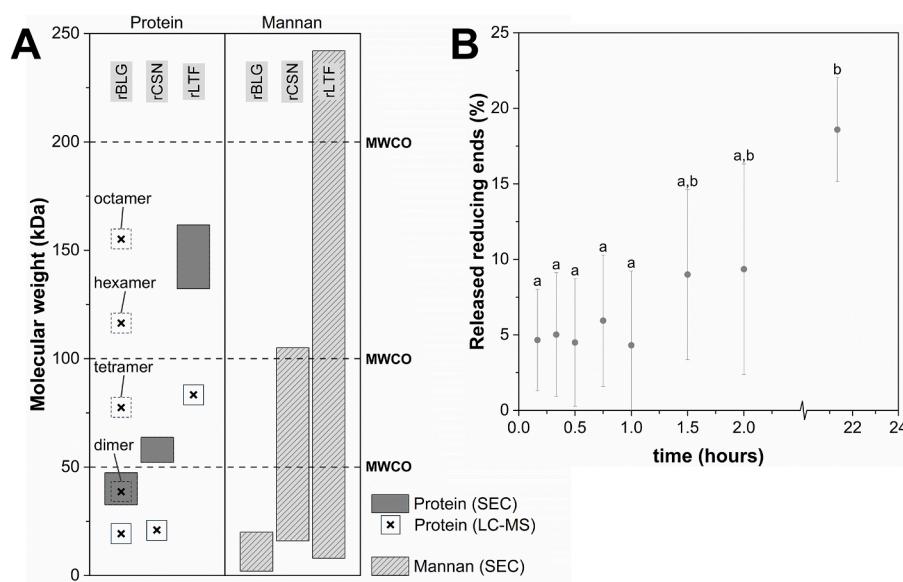


Fig. 7. A) Size comparison between protein and carbohydrate fractions in the crude recombinant milk protein; the protein size represented by the hydrodynamic volume (by SEC) and by the averaged mass (by LC-MS), including calculated mass from multimers of rBLG. The dashed lines represent the molecular weight cut-off (MWCO) of the common membrane filters. B) Released reducing ends at specific time points during enzymatic hydrolysis of mannan, significant differences at $p < 0.05$ is indicated by the letters.

FTIR profile to the yeast mannan reference (>95 %). They were confirmed as mannans upon hydrolysis (Fig. 3B). However, the mannans ranged between 2 and 242 kDa with respect to their molecular weight distributions (Fig. 3A) and showed slightly different sugar compositions (Fig. 3B). The molecular weight and the type of yeast mannan can vary strongly (from 2 to 620 kDa), and its size range depends on several factors, like the number of mannose residues in the main α 1,6-linked mannose backbone and the degree of branching and phosphorylation (Baek et al., 2024; Cuskin et al., 2015; Li et al., 2020; Orlean, 2012). This variation was also affected by the different attachments of the mannan on CWP, whether O- or N-glycosylation. In O-glycosylation, the mannan can only have up to 6 mannose residues, but in N-glycosylation, the mannan can be extensively branched with hundreds of mannose residues (Klis et al., 2006). Furthermore, the drying method did not affect the molecular weight of yeast mannan (Faustino et al., 2023), so it could not be related to the material processing history.

After purification of protein using AEX-LC (Fig. 6), the mannans could be identified as free (unattached) mannans (rBLG-ub and rLTF-ub), as mannoprotein (rCSN-f1), or attached to the protein by glycosylation (rCSN-f2 and rLTF-f1). The downstream processing should be able to separate the unattached mannan or mannoprotein from the target protein.

Researchers have studied genetic methods for optimising the release and production of yeast mannan (Gonzalez-Ramos et al., 2008; Tanaka et al., 2012). If this method can be reversed so that mannan synthesis during upstream processing and fermentation can be suppressed, downstream processing could become less complicated.

4.2. Characteristics of recombinant milk proteins

The recombinant milk proteins produced by *K. phaffii* fermentation closely resembled the target proteins (Table S. 3) with a degree of similarity of ~95 % in their secondary structure. The mannans mask the charge of the protein (Fig. 5A), affecting IEP. This effect was notably stronger for lactoferrin, as it has a higher isoelectric point, which means that it has more basic amino acids that carry positive charges at neutral and acidic pH (Farrell et al., 2004), making it more suitable to have stronger interaction with a negatively charged carbohydrate.

For rBLG, no glycosylation was found (Fig. 6B1). The incomplete cleavage of signal peptides, as indicated by the larger intact mass (Fig. 4C) could alter the technological properties, such as gelling properties (Pan et al., 2025), even though elongated N-termini seemed not to affect its emulsion properties (Hoppenreijns et al., 2024a, 2024b; Keppler et al., 2021), and no significant differences in solubility (Fig. 5B) or charge (in AEX; Fig. 6A) were evident.

In rCSN, minor glycosylation was detected (Fig. 4C) which increased its hydrodynamic volume (Fig. 4A). Furthermore, the difference in retention times of the main peaks between the recombinant and bovine casein in AEX (Fig. 6A) indicates that rCSN has a significantly lower net negative charge than bCSN since the proteins are eluted earlier. The difference in genetic variation is probably not the cause, but the presence of minor glycosylation is expected to reduce the net negative charge. The analysis of the rCSN-f2 fraction with FTIR confirmed this glycosylation through the large peak between 1100 and 1000 cm⁻¹. This carbohydrate peak corresponds to free mannan, but since it eluted together with the protein, it is likely that the sugar is (covalently) attached to the protein. This is confirmed by SDS-PAGE (Fig. 4B). It is reasonable that glycosylation lowers the net negative charge: a similar decrease was observed previously for glycosylated caseins (Huppertz, 2013).

The glycosylation of rCSN aligns with previous studies showing that *K. phaffii* may add glycosylation to proteins that are typically non-glycosylated, whether intended or accidentally (Breithauer and Castellino, 2010; Kalidas et al., 2001; O'Leary et al., 2004; Radoman et al., 2021; Saito et al., 2002). The recombinant β -casein produced in yeast was N-glycosylated with mannan, but contained some phosphorylation

sites as well, which were not further quantified (Choi and Jiménez-Flores, 2001). Choi and Jiménez-Flores (2001) also mentioned that the glycosylation was suggested to improve the solubility, water-holding capacity, and the interfacial activity of the casein; however, it remains unclear whether such an altered casein would be incorporated into a casein micelle. Furthermore, the rCSN did not contain any phosphorylations, which will influence its behavior in casein micelle assembly (Antuma et al., 2023) and other applications, such as acid gelation and emulsification (McCarthy et al., 2013).

In contrast to rBLG and rCSN, rLTF was similar to the reference protein regarding size, charge, and elution time on SEC (Fig. 4A) and AEX-LC (Fig. 6A). Nonetheless, as mentioned before, rLTF has a different glycosylation pattern than its animal-derived reference (Fig. 4C and 6B1), which indicates different glycan attachment or different glycosylation sites (Skog et al., 2025) and might potentially affect its digestibility. It was reported that a higher glycosylation degree in κ -casein slows the gastric digestion rate due to the increased steric hindrance for proteases (Sheng et al., 2021). Similar effects may also be observed in the case of lactoferrin.

To conclude, recombinant technology enables the production of milk proteins that are highly similar to the target protein, even though attaining the exact molecular analogy remains challenging. More investigations are needed to determine whether the structural similarity in these proteins is sufficient for attaining comparable technological and biological functional properties. But this also depends on the impurities present.

4.3. Purification efforts of the proteins

Anion-exchange chromatography is generally used to purify recombinant proteins to 80–95 % purity (Aro et al., 2023; Lojewska et al., 2016; Saraswat et al., 2013). As mentioned in Fig. 6C, the method gives low dry matter yield and protein recovery on a lab scale. Furthermore, the process requires a large amount of buffer, and the purified product is highly diluted (with about a 20-fold dilution factor), requiring extensive dialysis (desalting), concentration, and drying afterward.

On a pilot-scale, Aro et al. (2023) reported a single-step anion-exchange chromatography used to purify recombinant β -lactoglobulin produced by fungi *Trichoderma reesei*, yielding approximately 1 g/L purified protein with 95 % purity, but the protein recovery was only 19 %. These inefficiencies make anion-exchange chromatography less suitable for large-scale purification in the food industry. The complexity of chromatography significantly impacts its overall economic feasibility for food components. At the same time, high purity levels for recombinant milk protein may not be necessary to attain good functionality for application in food (Hettinga and Bijl, 2022; Hoppenreijns et al., 2024b; Lie-Piang et al., 2024).

Alternatively, among the tested crude recombinant milk protein fractions, isoelectric precipitation only works for rCSN (Fig. 5B) to a final protein purity of up to 77 %. This method can precipitate almost all of the casein and separate the mannoprotein in the supernatant, with only 7 % of the casein remaining unprecipitated. As literature suggests, caseins precipitate at their IEPs, while whey proteins like β -lactoglobulin and lactoferrin require denaturation to do so (Anema, 2020; O'Mahony and Fox, 2013). Previous research also demonstrated that precipitation at IEP was possible with recombinant β -casein to concentrate the protein before further purification (Clegg and Holt, 2009).

The overlapping molecular sizes of the recombinant milk proteins and the mannan impurities (Fig. 7A) complicate size-based separation methods, such as membrane separation. The size of the mannan fraction from crude recombinant milk proteins varies widely (Figs. 3A and 7A), with the mannan from crude rBLG having the smallest molecular weight (2–20 kDa). The size of the protein fraction of the rBLG can vary based on its state (19 kDa as a monomer to 155 kDa as an octamer based on simple addition, or, considering hydrodynamically closed-packed spheres, an octamer with an effective radius of 3.4 nm, roughly

corresponding to 140 kDa (Erickson, 2009; Gottschalk et al., 2003)). Thus, it is assumed that only rBLG could potentially be separated from the mannan using membrane filtration since it has a significant size difference between the protein and carbohydrate fractions, particularly when the protein is in its multimeric states (tetramer–octameric). In rCSN and rLTF, the overlapping size of protein and mannans makes the size-based separation impractical.

Nevertheless, the assumption holds only without protein–carbohydrate interaction and membrane fouling. The quaternary structure of rBLG as monomers or multimers depends on pH, temperature, and salt concentration (Gottschalk et al., 2003; Sakurai et al., 2001), and the membrane separation efficiency depends on multiple factors, including filtration mode, membrane material, and operating parameters (Chen et al., 2019; Cowan and Ritchie, 2007; Ebersold and Zydny, 2004; Espina et al., 2009). Thus, numerous variables must be considered beyond the assumption that the separation is based on size exclusion alone. So that only rBLG may be potentially purified using this method if variables are carefully optimised.

Enzymatic hydrolysis of mannan does not provide a straightforward solution (Fig. 7B). A more sophisticated enzyme cocktail would be required to digest the complex molecular structure of the mannan (Cuskin et al., 2015). Cuskin et al. (2015) mentioned that at least three more enzymes need to be used, including endo- α -mannosidase, surface endo-mannannase, and mannose-phosphatase, which is produced by the fermentation of *Bacteroides thetaiotaomicron*. Even though it could be applied at the industrial level by immobilising the enzyme, for example, this processing will still be cost-ineffective and thus not preferable for food production.

An alternative purification method is necessary to make the production of these recombinant milk proteins affordable for the food industry. Electrostatic interactions seem to be important, as demonstrated by the ability to precipitate the rCSN from a mixture, separating it from the mannoproteins. This finding opens up chances of applying this method on a larger scale in the food industry, as it only needs a decantation step and several chemical uses to adjust the pH. So, overall, this alternative is applicable for scaling up to the industrial level.

Further exploitation of electrostatic interactions can also be examined in the future. For example, the use of an additional food-grade precipitating agent, such as phosphate salt or another polyelectrolyte, could also be an option (Hoppenreijis et al., 2024a). Another way would be to use electrophoresis as separating field, as was suggested by Ayan et al. (2023).

Finally, high purity may not be necessary for good functionality. Food are almost never pure substances, but basically always mixtures. Mannans from brewer yeasts are sold as a high-value commodity, recognised for its good oenological properties and positive health effects (Bastos et al., 2022; Domizio et al., 2014; Dupin et al., 2000; Gonzalez-Ramos et al., 2008; Yadav et al., 2020). Recent studies have indicated that the removal of mannan from rBLG might not be strictly required from a technological functionality perspective. Specifically, the emulsifying properties of unpurified yeast-based β -lactoglobulin were found to be comparable to those of the purified fraction and bovine β -lactoglobulin (Hoppenreijis et al., 2024a). However, the presence of mannan was shown to impair the gelation and foaming properties of the protein substantially (Hoppenreijis et al., 2024b). The effect of mannan on the functionality of the other two recombinant milk proteins is unknown for now, but can be expected to be similar. High purity is most likely required to some degree, especially for recombinant lactoferrin, as it is mainly used as a fortified bioactive compound in highly controlled foods, such as infant formula.

5. Conclusions

Three different recombinant dairy proteins were investigated: β -lactoglobulin (rBLG), α -casein (rCSN), and lactoferrin (rLTF). All recombinant milk proteins had a secondary structure comparable (~95 %)

to their animal analogs (bBLG, bCSN, and bLTF), rBLG and rLTF had molecular weights, net charge, and solubility profiles similar to those of bBLG and bLTF. In contrast, rCSN has a larger hydrodynamic volume and smaller net charge, which may be due to minor glycosylation and missing phosphorylation.

Mannan was found to be the major impurity of all recombinant milk proteins produced by *K. phaffii* fermentation, regardless of the target protein. The mannan can be in the form of free (unbound) mannan or as mannoprotein, but also as glycosylation of the proteins. While its charge can mask the protein charge, it does not affect the solubility profile of the proteins unless covalently attached, as in the case of rCSN.

Separation by anion-exchange chromatography is possible and gives 63.7–80.7 % purity but with low recoveries of up to 40 %. Size-based separation is complex, as the proteins and mannans have overlapping molecular weight ranges. Precipitation at the IEP is commonly applied with bCSN but is not an option for bovine whey proteins. Of the recombinant proteins, only rCSN is susceptible to precipitation. Further research is needed to investigate this precipitation behaviour of rCSN as well as other potential electrostatic-based separation methods for rBLG and rLTF.

Funding sources

This work is part of the Protein Transition 2.0 project (KIC1. ST01.20.013, Knowledge and Innovation Covenant—MISSION 2020), financed by the Dutch Research Council (NWO).

Ethical statement

The research presented does not involve any animal or human study.

CRediT authorship contribution statement

Aryo D. Nugroho: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Rensong Ji:** Writing – original draft, Investigation, Formal analysis. **Yi Ling Chin:** Writing – review & editing, Project administration, Conceptualization. **Albert J.R. Heck:** Writing – review & editing, Supervision, Funding acquisition. **Karli R. Reiding:** Writing – review & editing, Supervision. **Remko M. Boom:** Writing – review & editing, Supervision. **Julia K. Kepler:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All authors report that the Dutch Research Council provided financial support. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Acknowledgements

We want to greatly acknowledge Marisol Calderon, Hannes Rehrl, Ellen Wright, Ching Yan Char, and Lara Fischer (all from Food Process Engineering, Wageningen University) for helping with the preliminary experiments, and Carolina Pandeirada and Margaret Bosveld (both from Food Chemistry, Wageningen University) for helping with the sugar composition analysis. We would also like to thank the consortium partners for their feedback.

Supplementary materials

Supplementary material associated with this article can be found, in

the online version, at [doi:10.1016/j.fufo.2025.100735](https://doi.org/10.1016/j.fufo.2025.100735).

Data availability

Data will be made available on request.

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