Review

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Review

Advances in the Purification of Lactoferrin and Lactoperoxidase from Dairy Whey

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Abstract

The development of strategies and processes to valorize cheese whey is an area of constant advancement and growth, primarily due to the large volume generated daily and inherent technical difficulties in cost-effectively processing this material. In this regard, the present review, with a critical perspective from the authors, will discuss and analyze different strategies based on chromatographic innovations to develop processes that enable the direct and selective capture and purification of bovine lactoferrin (LF) and lactoperoxidase (LP) proteins from cheese whey. These proteins possess interesting commercial value and represent minor components of the whey protein fraction, with whey being their main source. The advancements achieved in terms of efficiency in the selectivity and purity obtained for the target proteins through the development of affinity chromatographic matrices and processes will be discussed, as well as the progress made in terms of productivity and whey processability.

Keywords: dairy whey; lactoferrin; lactoperoxidase; purification

1. Introduction

Whey is a primary by-product resulting from the manufacture of cheese, casein, and yogurt. The type of whey produced depends on the method used for casein precipitation [1]. Sweet whey is the most common type, produced when casein is precipitated with rennet at a pH of 6.5 during the making of semi-hard and hard cheeses. Acid whey is a by-product of cream cheese, quark, paneer, and Greek yogurt manufacture, resulting from casein precipitation using lactic acid or mineral acids at a pH of 4.6. Whey is often considered a waste product despite its rich composition of valuable biomolecules [1]. Historically, a significant portion of whey was discarded, leading to environmental concerns. However, recognition of the nutritional and functional properties of whey proteins has led to increasing efforts in its valorization [2]. Whey contains a complex mixture of proteins that require isolation and purification to fully exploit their nutritional and functional characteristics. The subsequent fractionation of these proteins yields valuable ingredients that are used in many important food and pharmaceutical applications [1,3,4]. Moreover, whey serves as an excellent model mixture for investigating the chromatographic separation of proteins [5].

Whey contains approximately 50% of milk solids and 20% of the total proteins from milk, serving as a source of lactose, proteins, vitamins, and dairy minerals [1,6]. Lactose constitutes over 70% of the total dry matter in whey, whereas proteins make up 7.5–14%. The main protein components are β-lactoglobulin (BLG, 40-50% w/w, α-lactalbumin (ALA, 12-15% w/w), and glycomacropeptide (GMP, 12% w/w, present only in rennet whey). Minor proteins and peptides include immunoglobulins (Igs, 8.0% w/w), bovine serum albumin (BSA, 5.0% w/w), lactoferrin (1.0% w/w), lactoperoxidase (0.5% w/w), and proteose-peptone [1]. Whey proteins are considered one of the most valuable food proteins available due to their richness in all essential amino acids, which are integral to human health, and their unique functional and nutraceutical properties [7]. Proteins like BLG are used as emulsifiers,

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foaming, and gelling agents. ALA, as other example, is a component in infant formula and has antimicrobial and antiulcer properties.

Historically, untreated whey was a waste product with a high organic load. Currently, strict environmental regulations limit their disposal, making its valorization necessary. Modern use focuses on biological/chemical conversion (e.g., biogas or ethanol) and, most importantly, fractionation of its components. Fractionation, typically involving ultrafiltration, leads to the recovery and concentration of whey proteins into high-value ingredients such as Whey Protein Concentrate (WPC, up to 80% protein), Whey Protein Isolate (WPI, up to 90% protein) and pure proteins. Among these, LF and LP have garnered significant attention due to their diverse biological activities [5,7,8].

Lactoferrin is a glycoprotein found in external secretions in mammals with iron-chelating capacity and with antimicrobial, antiviral, anti-inflammatory, and immunomodulatory properties [9]. In addition, abnormal levels of LF in the human body have been implicated in serious diseases like inflammatory bowel disease, Alzheimer's disease, and dry eye disease [9]. Recent studies suggest that LF can be used as a biomarker for the diagnosis of various diseases. LF has a molecular weight of around 80 kDa and a basic isoelectric point (pI) of approximately 8.0. Its concentration in sweet whey is typically low, around 0.08–0.20 g/L [10,11]. LF has considerable commercial value due to its potential applications in the food, pharmaceutical, and cosmetic industries [6]. Consequently, the isolation and purification of LF from whey are important goals [12].

Lactoperoxidase is also a glycoprotein present in milk, whey, and colostrum. The molecular weight of LP is similar to that of LF, approximately 78 kDa, and has a high pI of approximately 9.6. Its concentration in sweet whey is also low, about 0.03–0.06 g/L [11,13]. LP is an enzyme that belongs to the lactoperoxidase system and has potent antimicrobial activity, making it valuable in various areas like food, cosmetics, pharmaceuticals, veterinary medicine, and agriculture [2,8]. LP oxidizes certain substances, like SCN-, in the presence of hydrogen peroxide to form strong antimicrobial substances. LP plays a significant role in protecting the lactating mammary gland and intestinal tract of newborn infants against pathogenic microorganisms. Also, LP has been implicated in the degradation of various carcinogens and in protecting animal cells against peroxidative effects. Because of its value, studies focus on the efficient separation and optimization of LP recovery, often investigating its separation concurrently with lactoferrin, using techniques like ion-exchange chromatography [8,12].

The isolation and purification of these valuable proteins from the complex matrix of cheese whey remain a challenge, demanding the development of efficient and cost-effective purification strategies [14,15].

Scaling up laboratory-developed purification protocols to industrial levels presents the following practical challenges [16]:

- Cost-effectiveness: minimizing the cost of adsorbents, reagents, and energy;
- Process efficiency: achieving high yields and purity while maintaining high throughput;
- Fouling: the problem of non-specific binding and fouling of membranes or resins by other whey components, which can reduce the life and efficiency;
- Protein stability: maintaining the biological activity and integrity of whey proteins throughout the purification process.

2. Whey Protein Purification

Extensive pre-treatments, such as centrifugation, precipitation, calcium chelation, and/or filtration, are typically used in the traditional methods for separating high-value proteins from milk or whey in order to eliminate residual fat and caseins [6,17]. Even though these pre-treatment shown to be efficient steps, they can lead to a drop in protein yield or activity, which ends up making the process more expensive and complicated. Therefore, the development of more effective and straightforward techniques for capturing these proteins is highly important [6,17].

Chromatography plays a pivotal role in the downstream processing of proteins, offering high selectivity and resolution. Whey proteins have been purified using a variety of chromatographic

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methods, such as affinity chromatography, size exclusion chromatography, hydrophobic interaction chromatography, and ion exchange chromatography [14,15,18–25].

Ion exchange chromatography, a fundamental technique, is widely used because of its ability to separate proteins based on their charge, which is highly dependent on medium pH. It is often employed for major whey proteins purification and can also be adapted for the minor proteins [16]. A critical challenge when processing raw milk is the presence of fat and casein, which can clog chromatographic columns and lead to excessive backpressure [17]. It was demonstrated that maintaining the processing temperature around milking temperature (35−37ºC) is crucial to prevent fat solidification and minimize fouling, allowing significant quantities of raw milk to pass through the column without exceeding the maximum allowable backpressure [17]. The choice of chromatographic resin is paramount. The particle size of the resin influences both the adsorption kinetics and allowable flow rates/backpressure. Larger beads, like those in SP Sepharose Big Beads, generally offer lower backpressure, which is advantageous for processing viscous and particle-laden raw materials [17]. Pioneering work in chromatographic techniques, particularly cation exchange chromatography, have shown promise for the direct capture of LF and LP from milk or whey [17,26]. Fee and Chand (2005) developed two ion exchange chromatography methods for the direct purification of proteins from unprocessed whole milk immediately after milking. The first approach utilized a single-stage batch process with two types of cation exchangers: SP Sepharose Big Beads (average diameter 155 μm) and SP Sepharose Fast Flow (average diameter 90 μm). The second method employed a packed column specifically using SP Sepharose Big Beads, which, due to its large particle diameter, allows for operation at significantly lower column backpressures. Unlike traditional techniques, both methods leverage the natural temperature of the milk as it leaves the animal (35–37 °C). This strategic temperature control prevents milk fat from solidifying and blocking the system while simultaneously favoring faster adsorption kinetics. By using raw whole milk directly from the farm, these processes eliminate the need for extensive pre-treatments to remove fat and caseins. The authors concluded that these robust and rapid techniques are ideal for on-farm implementation; as they avoid the loss of protein activity associated with conventional steps and do not significantly alter the macroscopic properties of the milk. Therefore, this innovative approach aims to bypass the pre-treatment steps, simplifying the purification process, reducing operational costs, and potentially increasing the overall yield and purity of the target proteins. The ability to directly process raw milk or whey for the isolation of these valuable proteins opens up new possibilities for on-farm processing and enhances the sustainability of dairy operations [6,26].

Affinity chromatography stands out due to its highly specific interaction between a target molecule and a complementary ligand immobilized on a stationary phase [27]. It is a desirable choice for complex biological mixtures like whey because of its specificity, which frequently results in high purity and recovery in a single step. However, the nature of the ligand often limits the applications of this technology to be used only in products of very high commercial value. In this context, dye-affinity chromatography has become a viable and affordable substitute for traditional affinity ligands. Through a combination of ionic, hydrophobic, and hydrogen bonding interactions, textile dyes, often triazine-based, can mimic natural ligands and bind to a variety of proteins, including LF and LP.

The choice of the support for ligand immobilization is also crucial because it affects how much can be adsorbed, the hydrodynamic characteristics, how productive the process is, and the overall efficiency of purification [24,28]. Membrane chromatography is a promising technology that offers faster processing and lower pressure drops compared to packed columns. This can be especially useful for large-scale processing of whey [16,29].

On the other hand, the batch process, using adequate diffusive resins, is highly suitable for scalability implementation due to its simplicity, effective protein extraction in a single step, and potential for batch traceability. One of the main benefits of batch purification is that raw, untreated milk can be directly processed [17].

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3. Purification of Lactoferrin and Lactoperoxidase

Traditionally, a variety of chromatographic techniques, such as hydrophobic interaction, ion exchange, and affinity chromatography, have been used to purify LF from natural sources such as milk, whey, and colostrum [24]. Methods for LP purification include ion exchange and affinity chromatography [25].

Ion exchange is mainly used in large-scale processes, based on LF and LP pI [24]. However, processing becomes more difficult due to the low concentration of both proteins in cheese whey, which frequently necessitates conditioning and concentration of the starting material to increase the yield of the chromatographic process. These processes achieve yields between 50 and 96%, and LF is often co-purified with LP [24]. Examples of co-purification include yields of 73% for LP and 50% for LF using commercial sulfonic membrane adsorbent [30] and a yield of 84% for LF and 10% of the initial LP content purified from a sweet whey concentrate using a similar cation exchange system [31].

Similarly, ion exchange processes achieve variable LP yields between 40 and 90% and are often co-purified with significant levels of LF [25].

As was previously mentioned, triazine dye affinity chromatography is a cost effective approach. These dyes are low-cost pseudo-bioaffinity ligands and are widely used for chromatography. They are easy to immobilize on different supports and are chemically and thermally stable [28]. All these advantages make them suitable for protein purification on an industrial scale.

3.1. Triazine Dye Chromatography

Since 1996, our group has been dedicated to the development of efficient LF and LP purification processes using dye-affinity chromatography, moving beyond traditional column chromatography [32].

3.1.1. Membrane Based Supports

In the initial stage of this research, which is detailed below, a screening of eight triazine dyes immobilized on Sepharose were tested, and it was demonstrated that the triazine dye Red HE-3B exhibited acceptable chromatographic performance for the direct purification of LF from colostrum and sweet whey.

Given the large volumes of whey required to produce marketable quantities of LF, membrane-based supports appear to be a suitable choice for industrial-scale operations. In this membrane based adsorptive systems, solute transport limitations are significatively reduced allowing high speed chromatographic processing. However, a significant limitation of these systems used to be their low adsorptive capacity. To overcome this limitation and enhance the productivity of the membrane system used, the strategy implemented in our work focused on increasing the available surface area by grafting acrylic copolymer blends prior to dye immobilization [33]. By employing a direct gamma radiation grafting process with equal proportions of glycidyl methacrylate (GMA) and dimethyl acrylamide (DMAA) monomers, the adsorptive capacity of material was significantly enhanced compared to that achieved using GMA alone. In this sense, productivity can be defined as the mass of purified protein per unit of matrix volume per unit of processing time, and the quantity of purified protein is contingent upon the efficiency of the adsorption and elution steps. Therefore, by increasing the adsorptive capacity, the productivity of the system can be increased. On the other hand, hollow-fibers were selected as the best-suited membrane configuration for processing raw material samples as those of dairy industry. The membranes used (polysulfone hollow-fibre microfiltration membranes, GE Healthcare Bio-Sciences) featured an internal surface of 15 m^2/g, a nominal internal pore diameter of 0.65 um and a nominal porosity of 80%. As mentioned, hollow fibers were synthesized by grafting a glycidyl methacrylate/dimethyl acrylamide copolymer onto the polysulfone membranes, followed by the immobilization of Red HE-3B dye. This innovative approach exhibited a significantly higher maximum LF adsorption capacity (111 mg LF/mL

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membrane) than obtained with the same ligand immobilized on agarose beads (9.3 mg/mL) (Figure 1). The cartridge assembled with these membranes, operated at a flow rate of 5 mL/min, achieved a productivity of 501.5 µg/h*mL (Figure 1). This represents a 500% increase compared to the Sepharose matrix, which yielded 82.8 µg/h*mL at a flow rate of 0.4 mL/min (Figure 2), which was the highest allowed flow to avoid resin compression. Furthermore, when tested with colostrum in a batch system, the dye-membranes adsorbed 91% of the LF, compared to 56% adsorbed by dye-agarose beads. Optimal desorption was achieved with 2 M NaCl in 25% ethylene glycol, indicating the strong binding nature and efficient recovery potential of this membrane system. The LF purification yield was 90.1% for the membranes and 44.8% for beads. A purity of 94% was reached in a single step using the dyed membranes, with casein and immunoglobulin as the only remaining contaminants. Despite its high performance and purity, the system encountered scale-up challenges. These were likely due to the membrane's pore size and concentration polarization effects, which hindered the system's viability at larger operational scales.

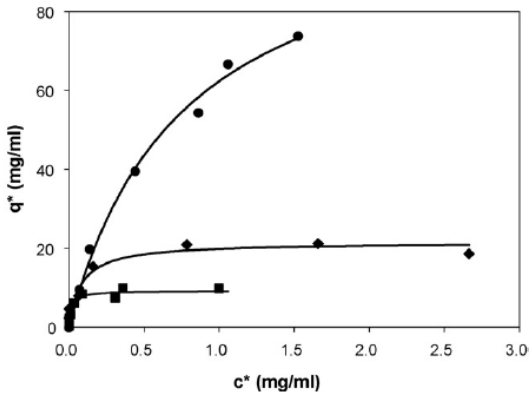

Figure 1. Isotherms for LF adsorption on Red HE-3B/Sepharose 4B (■), Red HE-3B/GMA membranes (♦) and Red HE-3B/GMA-DMAA membranes (●). c*: LF equilibrium concentration in the supernatant. q*: LF equilibrium concentration bound to the membrane per unit of total membrane volume. From [33].

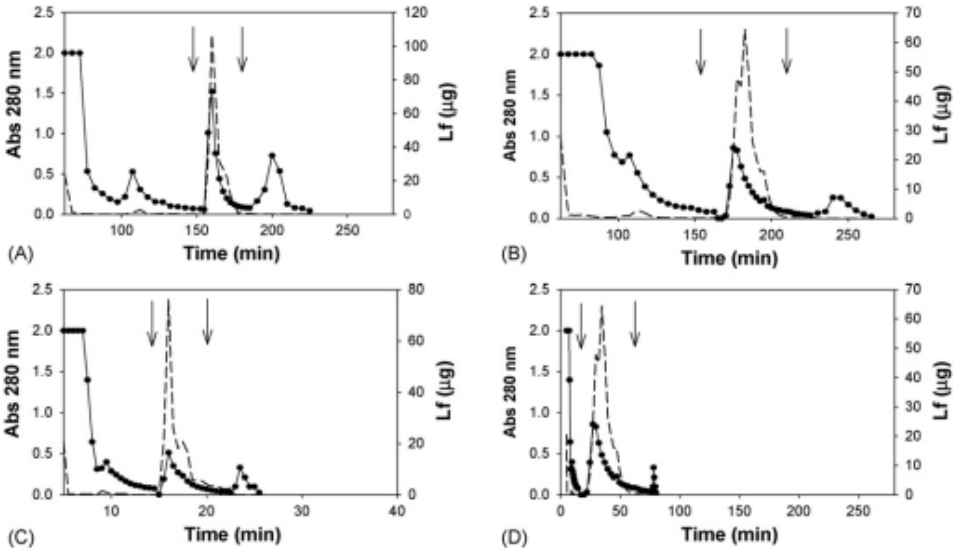

Figure 2. LF purification pattern obtained with Red HE-3B/Sepharose 4B at a flow rate of 0.4 mL/min (A) and with Red HE-3B/GMA-DMAA membranes at 0.4 mL/min (B); at 5.0 mL/min (C); at 5 mL/min (D) for the load, wash and regeneration steps and 0.4 mL/min for the elution step. The first arrow indicates the change to elution buffer; second arrow indicates the change to regeneration buffer. (- - -) LF concentration determined by ELISA. (-●-) Absorbance at 280 nm. From [33].

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3.1.2. Particle-Based Supports

As mentioned, a Sepharose matrix functionalized with eight different triazine dyes as ligands was evaluated for the affinity chromatography purification of LF from rennet whey [32]. While three of the dyes showed no adsorption across the pH range tested, the remaining five adsorbed varying amounts of LF depending on the pH of whey. Based on gradient elution data, a batch purification technique was developed using Red HE-3B as an affinity ligand, initially immobilized on the agarose based matrix. The optimized procedure achieved an 82% recovery of the LF content with a high purity of about 90%.

An advantage of using triazine dyes as ligands is that expanding the number of dyes evaluated during the screening process increases the likelihood of identifying superior ligands for a target protein. Consequently, a new screening revealed that the previously untested Yellow HE-4R dye showed similar performance to Red HE-3B. Therefore, a comparative study was conducted using commercial Sepharose resins as the initial chromatographic support. The density of the dyes immobilized in the Sepharose 4B matrix was 1.46 ± 0.02 μmol/g for Red HE-3B and 2.27 ± 0.14 μmol/g for Yellow HE-4R. Adsorption isotherm analysis showed that the Yellow HE-4R immobilized on Sepharose 4B (S-Y matrix) had a higher maximum capacity (Qmax = 40.24 mg/g) than the Red HE-3B immobilized on Sepharose 4B (S-R matrix) (Qmax = 27.82 mg/g). Elution tests showed that the S-Y matrix had higher elution and yield than S-R. The matrix was tested during three consecutive cycles without matrices regeneration obtaining similar purification factors along the three cycles. The same behavior and pattern of contaminants were observed in the analysis of the eluates by SDS-PAGE (Figure 3). The purity of the recovered LF was over 90% for both matrices. This approach, based on a low-cost ligand, showed an efficient performance for the recovery and purification of LF directly from whey, with a yield of 71% and a purification factor of 61 [28].

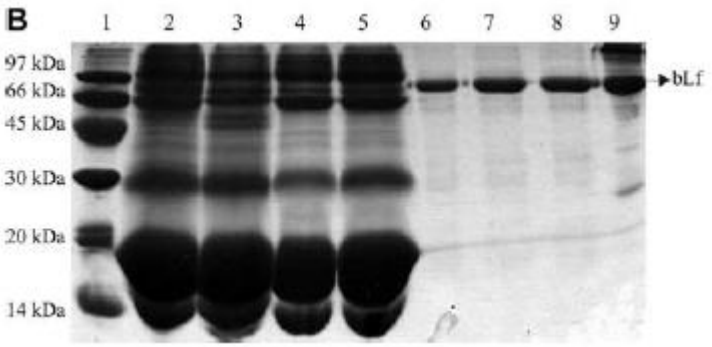

Figure 3. SDS-PAGE analysis of the purification process during three consecutive cycles using Sepharose-Yellow HE-4R matrix. Lane 1, molecular weight markers; lane 2, bovine whey; lane 3-5, supernatant after adsorption in cycle 1, 2 and 3, respectively; lane 6-8, eluate in cycle 1, 2 and 3, respectively; lane 9, standard bovine Lactoferrin (2 mg/mL). From [28].

Based on these results and the previously mentioned limitations of membrane systems, and considering the observations of Fee and Chand [17], our group opted for chitosan mini-sphere-based systems as chromatographic support to use for direct protein purification from raw materials. Chitosan mini-spheres are low-cost, widely available, biocompatible, biodegradable, and environmentally friendly [34,35]. Previous research in our laboratory had already demonstrated the chromatographic utility of these chitosan supports, specifically in the batch purification of wheat germ lectin from crude plant extracts [36]. That study showed that these mini-spheres facilitate the direct processing of crude samples, as they can be easily recovered from the medium through a simple sieving process, eliminating the need for centrifugation, due to its large size (approximately 1.76 mm). Furthermore, due to their differential density relative to the medium, the resins exhibit rapid and spontaneous sedimentation. Additionally, through simple chemical modifications, such as

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crosslinked with epichlorohydrin, we have enhanced their mechanical strength to resist multiple operational cycles and standard sanitation conditions.

Based on these results, further research evaluated the performance of a different dye immobilized on chitosan mini-spheres, compared to Red HE-3B to identify superior ligands [24]. To this end, chitosan mini-spheres were synthesized by the acid-base coacervation technique and chemically cross-linked with epichlorohydrin to enhance their mechanical and chemical stability, followed by the covalent immobilization of the two dyes (Red HE-3B and Yellow HE-4R). The synthesized matrices showed a homogeneous interconnected macroporous structure with an absence of agglomerations (Figure 4). During the optimization process, these chitosan mini-spheres were evaluated against commercial agarose supports modified with the same dyes (Sepharose 4B). The results demonstrated that Yellow HE-4R immobilized on the chitosan matrix exhibited the highest adsorption capacity for LF (51.1-58.3 mg LF/g matrix). The mini-spheres effectively adsorbed around 96% of the LF present in sweet whey within 1 hour of contact, and over 80% of the adsorbed LF was eluted. A notable achievement was a yield of 77% with purity greater than 90% in a single purification step (Figure 5). Furthermore, the process proved efficient for multiple consecutive cycles without regeneration, highlighting its robustness and potential for industrial application. An important characteristic of this matrix is that it can be used without the need to pre-condition the whey (modification of ionic strength, diafiltration) [24].

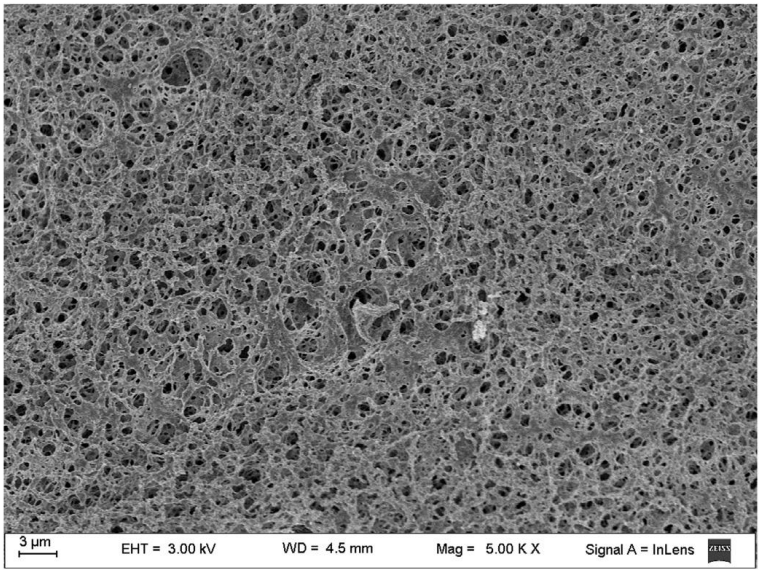

Figure 4. Scanning electron microscopy image of chitosan matrix before cross-linking and dye coupling. From [24].

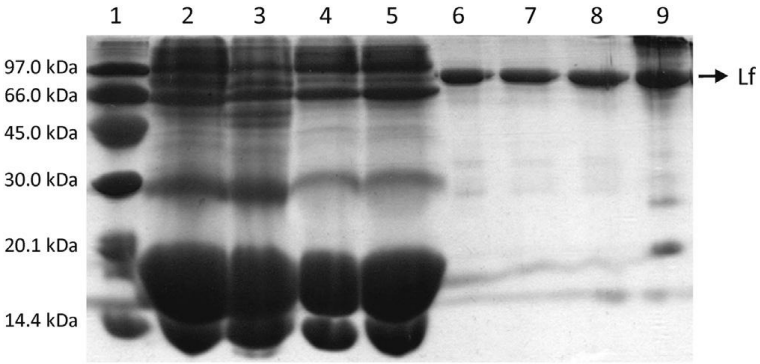

Figure 5. SDS-PAGE of the lactoferrin (Lf) purification process using a chitosan matrix cross-linked with 500 mM epichlorohydrin coupled to Yellow HE-4R during three purification cycles: lane 1, protein ladder; lane 2,

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whey; lane 3-5, supernatant after adsorption from cycle 1, 2 and 3, respectively; lane 6-8, eluate from cycle 1, 2 and 3, respectively; lane 9, commercial Lf (2 mg/mL). From [24].

As a noteworthy extension of this study, Urtasun et al. (2013) described a study focused on the selection of triazine dyes as affinity ligands for the purification of recombinant bovine lactoferricin (Lfcin B) [37]. Lfcin is a cationic peptide naturally generated through the acid hydrolysis of LF by gastric pepsin, which releases an N-terminal domain fragment with significantly greater biological activity than the intact protein. Due to its potent antimicrobial, antifungal, antiviral, and antitumor properties, this peptide has gained increasing biotechnological relevance as a high-value therapeutic agent. The challenge lies in efficiently purifying Lfcin B, often expressed at low concentrations, from complex fermentation cultures. The study identified specific dyes that exhibited strong and selective binding interactions with the target peptide using the SPR (surface plasmon resonance) technology, as screening methodology. These findings were then applied to demonstrate the effectiveness of the selected dye-ligands in purifying recombinant Lfcin B using affinity chromatography. The results indicated that the chosen dyes, specifically Yellow HE-4R, could efficiently capture Lfcin B, leading to its successful isolation and purification.

On the other hand, our group has also made significant strides in the purification of lactoperoxidase, often using similar dye-affinity approaches. A novel process for bovine lactoperoxidase purification from sweet whey was described using dye-affinity chromatography [25]. This study involved screening 18 different triazine dyes immobilized on Sepharose 6B. Five dye-Sepharose matrices showed over 90% LP adsorption directly from whey (Reactive Red 4, Orange R-HE, Blue R-HE, Yellow HE-4R, and Red HE-3B) [25]. Whey processed using the reactive Red 4-sepharose matrix in batch mode showed the highest LP purification yield (87%) and relative purity over 80% according to SDS-PAGE gel densitometry. Under these conditions, low levels of LF (<0.5% of the original LF concentration) were co-purified [25]. This systematic approach to ligand selection is crucial for optimizing purification performance. Otherwise, purification of LP from whey using packed bed column mode with the reactive Red 4-sepharose matrix showed lower yields (64%), and additional whey pretreatments were needed for dynamic processing. These pretreatments (thermocalcic precipitation, centrifugation and filtration) were necessary to remove fat and prevent column clogging and increased backpressure.

In a subsequent phase, the simultaneous adsorption of LF and LP proteins onto the same dye-affinity matrix, followed by their differential elution, was investigated [38]. This approach is similar to ion-exchange chromatography but aims to improve purity and yield by minimizing cross-contamination between the two target proteins. This research investigated the use of chitosan mini-spheres with an immobilized dye, as an affinity ligand, to purify LP and LF from dairy whey. Through the synthesis and characterization of these materials, this study focused on establish a foundation for the development of an integrated and highly efficient process for the simultaneous separation of both high-value proteins.

This could lower operating costs and improve overall product recovery. A screening of eleven immobilized dyes on chitosan mini-spheres identified Orange R-HE as the most effective ligand for the co-adsorption of both proteins. This matrix was characterized and utilized for LP and LF purification using a single-step direct adsorption and a two-step elution process, with minimal cross-contamination [38]. Moreover, mechanical characterizations were also conducted to comprehend and im-prove the usage cycles of the mini spheres. Compressive strength and stiffness were measured in order to evaluate the changes in material hardness after the addition of the cross-linking agent (epichlorohydrin) and the dye as ligand (Figure 6 A). Stiffness increased in direct proportion to the concentration of epichlorohydrin added, but when Orange R-HE was immobilized, stiffness decreased, suggesting that the dye alters the three-dimensional network of the hydrogel. Despite this reduction, the stiffness remains sufficient to ensure that the matrix does not undergo excessive deformation during processing. Furthermore, the elastic behavior of the matrices was evaluated (Figure 6 B). The matrices showed primarily elastic/viscoelastic behavior at low cross-linker

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concentrations, regaining their shape without damage after ten cycles. In contrast, at the highest cross-linker concentration, the high rigidity led to irreversible plastic deformation. However, this behavior was improved by the dye addition, enabling the structural network to reorganize following compression. On the other hand, the Langmuir isotherm model was used to characterize the interaction between LP and LF and the matrix, yielding Qmax values of 56 mg/g for LF and 77 mg/g for LP. Adsorption studies showed that the interaction of LP with chitosan mini-spheres-Orange R-HE was partially influenced by ionic strength, whereas LF adsorption was not significantly affected. These results revealed the importance and differences in both electrostatic and hydrophobic interactions between LP and LF and the matrix [38]. Direct adsorption was possible even in the presence of 0.15 M NaCl. To maximize differential elution conditions and reduce cross-contamination between LP and LF, Response Surface Methodology (RSM) was employed [38]. The elution process also confirmed the multimodal nature of the interactions. A higher salt concentration (2 M NaCl) supplemented with 50% propylene glycol was needed for the elution of LF, whereas LP was successfully recovered by raising the pH and ionic strength (1 M NaCl). After three consecutive purification cycles, two separate products (LP and LF) with good yields (≈70% for LP and ≈60% for LF) and high purity, with minimal cross-contamination, could be obtained using a single-step direct adsorption process from sweet whey and a two-step differential elution process [38]. As previously mentioned, if these two high-value proteins could be directly recovered without the need for sweet whey pretreatments, yield could be raised and operating costs could be reduced. The impact of chemical modifications on the mechanical properties of chitosan was another important topic covered in that study. It was demonstrated that the covalent binding of the dye to the chitosan matrix increases both its stiffness, as measured by Young's modulus, and its elastic recovery. These findings are critical when evaluating the material's suitability for large-scale production and its durability over multiple operational cycles.

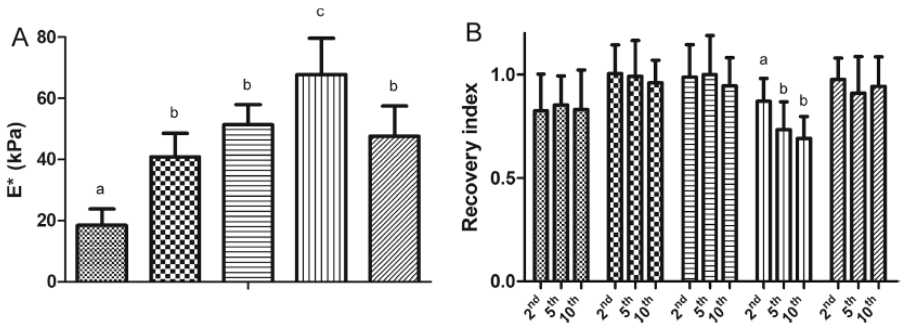

Figure 6. Mechanical characterization of chitosan mini-spheres. Mini-spheres cross-linking with different ratio of epichlorohydrin (equivalents): 0.0 ( ), 1.0 ( ), 2.5 ( ), 5.0 ( ) and 5.0 + Orange R-HE ( ). (A) Determination of compression modulus and (B) Compression-relaxation cycles. Different letters represent significant differences among media ($p < 0.05$). From [38].

3.2. Co-Purification of Lactoferrin and Lactoperoxidase Using Ion Exchange Chromatography

Given the regulatory constraints regarding the use of triazine dyes as chromatographic ligands for some specific uses of the proteins, our group also explored cation-exchange chromatography for LF and LP purification. To this end, a strategy was developed utilizing sulfanilic acid as a functional ligand, immobilized onto the previously described chitosan mini-spheres. Both proteins have similar molecular weights and a basic isoelectric point. Due to these similar physicochemical properties, LP and LF are traditionally purified using ion exchange chromatography. However, this often results in cross-contamination, especially for the LP fraction, where part of the LF co-elutes.

The work presented in Hirsch et al. (2020) also builds upon previous work with chitosan mini-spheres [39]. The platform shows the potential for integrated processes, even though its primary focus is on LF purification and WPI recovery. In this study, an integrated process for cheese whey

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valorization was developed. It uses an anion-exchange matrix with glycidyltrimethylammonium as a ligand to recover WPI after direct capture of LF and LP using a cation-exchange matrix functionalized with sulfanilic acid.

A more comprehensive approach to whey valorization is suggested by the capacity to recover WPI in addition to LF. The utility and adaptability of the synthesized chitosan-based resins for multi-product recovery were shown, despite the fact that effective separation of LP was not accomplished with these matrices [39]. The method successfully separated LF with a purity of 94% and a recovery yield of 81%, demonstrating that chitosan-based materials are a practical and cost-effective alternative to traditional commercial resins in LF purification. The stability of this developed matrix is one of its main benefits. Because the mini-spheres can be regenerated and used again for multiple adsorption-desorption cycles without experiencing a significant loss of capacity, they are a low-cost choice for industrial scale-up.

4. Conclusions

Through the years, our group has focused on providing cost-effective solutions for the valorization of cheese whey, demonstrating the technical feasibility of recovering and isolating high-value bioactive proteins. Furthermore, our contribution extends beyond process development to the design of the associated chromatographic matrices, the most expensive and critical consumables in the purification cycle. The exploration of novel matrices, such as chitosan mini-spheres and grafted polysulfone hollow fiber membranes has led to improved adsorption capacities and purification efficiencies compared to traditional matrices like agarose beads. The successful utilization of various triazine dyes (Yellow HE-4R, Red HE-3B, reactive Red 4) as affinity ligands demonstrates a viable and economical alternative to more expensive affinity biological ligands.

The achieved yields and purification factors highlight the effectiveness of the developed methods in isolating target proteins directly from complex whey mixtures in a single step.

Future directions could focus on further optimizing the binding and elution conditions for simultaneous purification of LF and LP, exploring continuous chromatographic systems for industrial scale-up. The continued innovation in affinity chromatography, particularly with cost-effective and versatile ligands and matrices, holds immense promise for maximizing the value of dairy whey and contributing to a more sustainable dairy industry.

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Abbreviations

The following abbreviations are used in this manuscript:

LF	Lactoferrin
LP	Lactoperoxidase
BLG	β-lactoglobulin
ALA	α-lactalbumin
GMP	Glycomacropeptide
Igs	Immunoglobulins
BSA	Bovine serum albumin
WPC	Whey Protein Concentrate
WPI	Whey Protein Isolate
pI	Isoelectric point
Qmax	Maximum capacity
Lfcin B	Recombinant bovine lactoferricin
CMS	Chitosan mini-spheres
RSM	Response Surface Methodology

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