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β-Lactoglobulin Separation from Whey Protein: A Comprehensive Review of Isolation and Purification Techniques and Future Perspectives

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ABSTRACT

Cow milk, although rich in essential nutrients, is a well-known allergic food that can cause allergic reactions in infants and young children. β-Lactoglobulin accounts for 10% of the total protein in milk and 50% of the whey protein, which has high nutritional value and excellent functional properties but is also the main allergen leading to milk protein allergy. Exploring the mechanism of milk allergy and selecting suitable separation and purification methods to obtain high-purity β-Lactoglobulin is the premise of research on reducing allergenicity. In this review, the research progress in membrane technology, gel filtration chromatography, ion exchange chromatography, affinity chromatography, precipitation and aqueous 2-phase system separation for the separation and purification of milk β-Lactoglobulin is reviewed in detail to promote the further development of milk β-Lactoglobulin separation and purification methods and provide a new method for the development of hypoallergenic dairy products in the future. Among these methods, ion exchange chromatography and gel chromatography are widely used, precipitation is generally used as a crude purification step, and high-performance liquid chromatography and membrane technology are used for further purification to improve the purity of allergens.
Keywords: milk allergy, allergy mechanism, β-lactoglobulin, separation and purification techniques, hypoallergenic dairy

INTRODUCTION

Cow milk (CM) contains protein, fat, carbohydrates, vitamins, minerals and probiotics, making it an excellent source of nutrients. It is easy to digest and absorb and plays a crucial role in human nutrition and health. However, CM is also one of the 8 major common food groups that cause food allergies. Since CM is a high-quality protein source, when breast-feeding is not possible, milk formula becomes the first choice for supplementing infants and young children with nutrients. As a result, milk becomes one of the first allergens to which newborns are exposed, and milk allergies are extremely common in infants and young children because of the immature development of the gastrointestinal tract (Pessato et al., 2016). Several studies have indicated that approximately 8% of children are allergic to food, and the incidence rate is increasing (Sicherer and Sampson, 2018).

Cow milk allergy (CMA) is usually a type of immune disease caused by milk protein; its pathogenesis is relatively complex, and the most common is an IgE-mediated type I hypersensitivity reaction (Keith, 2020). This type of reaction is also known as a rapid hypersensitivity reaction; it begins within an hour after the ingestion of allergens; is the most serious type of food allergy; often involves multiple systems of the body; leads to complex clinical symptoms, such as acute urticaria, eczema, allergic rhinitis or asthma; and, in severe cases, can lead to anaphylactic shock symptoms, even those that are life-threatening (Venter et al., 2018). The pathogenesis includes 2 stages. The first stage is the sensitization stage, in which the immune system abnormally programs and stimulates B lymphocytes to produce IgE antibodies after exposure to milk allergens. The second stage is the allergy stage, in which exposure to the same milk allergen again induces IgE binding to epitopes on the milk allergen and promotes the release of bioactive mediators, leading to severe allergic symptoms (Crittenden and Bennett, 2005). Currently, with the increasing diversity of food materials, milk is frequently used as a component or processing aid in the food industry and is widely added to various foods. Therefore, milk protein components are inevitably present during food processing, transporta-

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

Table 1 Composition and classification of common proteins in milk

Milk protein	Allergen	Molecular mass (kDa)	Amino acid number	Isoelectric point	Concentration (mg/ml)	Proportion of total protein (%)
CN	Bos d 8				30	80
α_{s1}-CN	Bos d 9	23.6	199	4.9–5	12–15	29
α_{s2}-CN	Bos d 10	25.2	207	5.2–5.4	3–4	8
β-CN	Bos d 11	24.0	209	5.1–5.4	9–11	27
κ-CN	Bos d 12	19.0	169	5.4–5.6	3–4	10
γ_1-CN	—	20.5	180	5.5	1–2	6
γ_2-CN		11.8	104	6.4		
γ_3-CN		11.6	102	5.8		
WP					5	20
α-LA	Bos d 4	14.2	123	4.8	1–1.5	5
β-LG	Bos d 5	18.3	162	5.3	3–4	10
BSA	Bos d 6	66.4	582	4.9–5.1	0.1–0.4	1
Ig	Bos d 7	160	—	—	0.6–1	3
LF	—	76.2	703	8.7	0.09	/

Note: "-" indicates not found in literature; "/" indicates negligible.

tion, storage and other processes. This is undoubtedly a significant health risk for patients with CMA.

The vast majority of milk allergens are milk proteins, and according to the report of Monaci et al. (Monaci et al., 2006), there are approximately 30 kinds of potentially allergic proteins in milk. These proteins are generally divided into 2 parts: casein (CN) and whey protein (WP). WP has an intramolecular disulfide bond, and the structure is relatively stable and contains the essential amino acids necessary for human metabolism. It is mainly composed of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), as well as many secondary proteins, such as immunoglobulin (Ig), bovine serum albumin (BSA) and lactoferrin (LF) (Aguero et al., 2017). In breast milk, whey protein is the predominant component; in contrast, CM mainly consists of casein (Yu et al., 2021). The difference in composition between cow milk and breast milk makes the immune system of sensitive people think that most of the proteins in cow milk are foreign proteins, and once they are absorbed, the body can induce an allergic reaction. The composition and main physicochemical properties of the proteins in milk are shown in Table 1 (Huang et al., 2023; Monaci et al., 2006; Somma et al., 2008). At present, CN, α-LA and β-LG are generally considered the main allergens in milk (Hochwallner et al., 2014). Owing to the absence of β-LG in breast milk, this protein is the first foreign protein encountered by infants and young children. According to surveys, 82% of milk allergy patients are allergic to β-LG, making it the main allergen in milk (X. Li et al., 2022).

β-LG is the main protein in ruminant whey, accounting for 10% of the total milk protein and 50% of the whey protein. Each monomer weighs approximately 18.3 kDa, exists naturally as a dimer (Figure 1) and consists of 162 amino acid residues, including 2 disulfide bonds (Cys^{66}–Cys^{160} and Cys^{106}–Cys^{199}) and one free cysteine residue (Cys^{121}) (C. Li et al., 2022), with an isoelectric point of 5.3. β-LG has more than 2 genetic variants, of which β-LG A and β-LG B are the most common and have sensitizing potential. The 2 differ only at positions 64 and 118, with type A being Asp and Val and type B being Gly and Ala, respectively (Farrell et al., 2004). In fresh milk (pH 6.6–6.8), β-LG exists in the form of dimers. When the pH is less than 3.5, the dimers dissociate into monomers. When the pH is between 3.5 and 5.2, the dimers tetramer to form octamers. When the pH is above 7.5, the dimers dissociate and undergo conformational changes to form swollen monomers. β-LG belongs to the highly allergenic lipocalin protein family; the N terminus of β-LG has a similar sequence that is not easily destroyed, and the 19th amino acid is Trp (Indyk et al., 2017). The spatial structure of β-LG is a bucket-like structure formed by 8 antiparallel β-folds, with a hydrophobic cavity in the center, an α-helix and a β-fold outside the bucket (Kontopidis et al., 2004). This stable structure makes β-LG relatively resistant to acids and proteases, and some of the protein can remain intact after digestion, thus causing allergic reactions. High-purity and immunoactive milk allergens are the material basis of milk allergy research, so the research and development of isolation and purification technology has become active and strengthened with increasing milk allergy research. Since the proportion of patients allergic to β-LG is the highest among milk allergic people, the development of isolation and purification methods without loss of β-LG activity has attracted increasing attention (Neyestani et al., 2003).

Owing to the physical and chemical properties of the major allergens in milk, such as the isoelectric point, ligand binding, and amphiphilic properties, various milk allergens have been successfully isolated and purified via selective conditional precipitation, chromatographic separation, ultrafiltration and membrane separation, meeting the requirements of many studies (Hoffmann-Sommergruber et al., 2008). In this work, milk allergies and their hazards, as well as the related properties of β-LG, are introduced, and the research progress in the isolation and purification of β-LG in milk is described to lay a foundation for expanding the application of β-LG in CM in the food industry.

Isolation and purification of β-LG from milk

At present, many methods have been developed for the separation of the main allergens in milk: according to the different solubilities of proteins in a single solution, separation methods include isoelectric point precipitation and the salting out method; separate methods are based on different solubilities in 2 compatible solvents, such as aqueous 2-phase extraction; separate methods according to the different charging properties in the chromatographic column, including anion exchange chromatography and cation exchange chromatography; separation methods according to the different adsorption capacities of proteins and adsorption materials, including hydrophobic interaction chromatography, affinity chromatography and hydroxyapatite chromatography; and separation methods according to protein molecular size, including membrane technology and coagulation chromatography. The isolation and purification of β-LG can be achieved by the above methods under different conditions. In addition, the use of a variety of separation methods combined with the purification of β-LG has also become a new direction for the development of isolation and purification technology.

Membrane separation method

A membrane is a polymer material with a special selective separation function that can separate a fluid into 2 different parts, allowing one or several substances to pass through and intercept and separate other substances. Membrane separation is a method of concentrating or separating mixed liquids into 2 different liquid components via specific semipermeable membranes on the basis of their selected permeability, which is driven by pressure differences, potential differences, concentration differences, and other driving forces (Nunes et al., 2020). Separation technology has the advantages of high separation and purification efficiency, no secondary pollution, low energy consumption, a small footprint, simple operation and easy automatic control (Li et al., 2020). Membrane technology can be divided into microfiltration (MF) (removal of impurities such as starch, pectin, and cell walls), ultrafiltration (UF) (retention of proteins and polysaccharides), nanofiltration (NF), reverse osmosis (RO) (concentration of active ingredients), and electrodialysis (ED) (Figure 2) methods on the basis of different membrane pore sizes (Al Aani et al., 2020; Anis et al., 2019; Charcosset, 2021). Among them, RO technology is only used for concentration, whereas other membrane technologies are widely used in dairy production, including the separation of β-LG from whey, creating many new products with high added value (Chen et al., 2019). Bhattacharjee et al. (Bhattacharjee et al., 2006) used a combination of ultrafiltration and ion exchange membrane methods to isolate β-LG with a purity of 87.6% from milk whey. Goodall et al. (Goodall et al., 2008) used ion exchange membranes to selectively separate whey protein and reported that the anion exchange membrane had higher selectivity for β-LG than other whey proteins and could separate relatively pure β-LG. After further UF, the recovery was greater than 90%, and the purity was greater than 99%. Arunkumar and Etzel (Arunkumar and Etzel, 2014) utilized the advantage of whey permeate not containing GMP, casein, and fat to place a positive charge on a regenerated cellulose UF membrane with a molecular weight of 300 kDa, and after classifying the positive charge, they separated β-LG from the whey infiltrate via the tangent flow UF method. Compared with that of the uncharged UF membrane, the selectivity of β-LG was significantly improved, and the results show that the 3-stage UF membrane system can separate 99% of β-LG from the rest of the whey protein, with a purity of 83%. Hanušová et al. conducted 3 methods to separate β-LG from whey under low pH conditions (Hanušová et al., 2014). Research has shown that after adding 7% NaCl to whey, some whey proteins precipitate, whereas β-LG and casein macropeptides remain in the supernatant. Minerals were subsequently removed from the supernatant via ED, and ethanol was added to the mixture to precipitate casein peptides, resulting in β-LG with yields and purities of 91% and 97%, respectively. The bipolar membrane formed by the anion exchange layer, cation

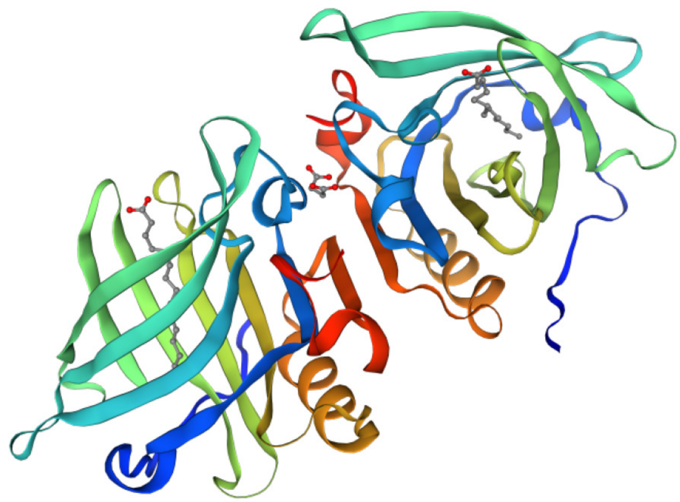

Figure 1. 3D structure diagram of bovine β-lactoglobulin

exchange layer, and mesophilic layer is a special type of ion exchange membrane that has been incorporated into the ED system for acidification and separation of milk proteins. Bazinet et al. (Bazinet et al., 2004) reported that β-LG could be isolated with a recovery rate of 44.0% and a purity of 98% at an initial concentration of 5% whey protein when whey protein was separated via bipolar membrane electroacidification. This demonstrates the feasibility of bipolar membrane electroacidification for separating milk proteins.

With the progress of science and technology, membrane separation technology has developed rapidly, and its application has become increasingly extensive, realizing the transformation from laboratory to large-scale industrial application, which is called the high-tech with the greatest development potential in the 21st century by the Western scientific and technological community. However, after the separation of protein via membrane technology, the membrane surface is prone to pollution; that is, the interceptors accumulate on the membrane surface, and the permeants block the membrane holes, resulting in reduced membrane separation performance (Chew et al., 2020), so it is necessary to use a membrane surface cleaning method that is suitable for this process.

Gel filtration chromatography method

Gel chromatography, also known as size exclusion chromatography (SEC), uses a separation medium composed of gel particles with a specific pore size distribution. When the sample mixture enters the column, large-molecule proteins cannot easily enter the gel particles and flow out quickly, resulting in a short retention time. Small-molecule proteins, however, diffuse into the micropores of the particles, leading to increased flow volume and extended retention time due to the obstructive effect of the porous gel structure. Medium-sized proteins partially enter the particles, with retention times falling between those of large and small molecules. Ultimately, each component exits the column sequentially from largest to smallest, achieving separation on the basis of molecular size (Kahle and Wätzig, 2018). It is a simple and rapid separation and analysis technique that has been widely used in the separation of milk protein allergens. Dextran and agarose gels are commonly used as gel media in gel chromatography (Chen et al., 2023). Aich et al. used Sephacryl S-200 (dextran gel S-200) via gel filtration chromatography to separate β-LG from the whey protein fraction in the supernatant. Research has shown that the final isolated pure β-LG has a molecular weight of 17.44 kDa. The monomeric molecular weight of β-LG is approximately 18 kDa, so the obtained product is almost pure (Aich et al., 2015). Neyestani et al. (Neyestani et al., 2003) used DEAE-C anion exchange chromatography and Sephadex G-50 gel filtration chromatography to separate β-LG from bovine whey, with a purity of more than 95%. Golebiowski et al. (Golebiowski et al., 2020) isolated β-LG from whey protein isolate solution via SP Sephadex column chromatography. During the process, coelution of α-LA and β-LG was observed, but

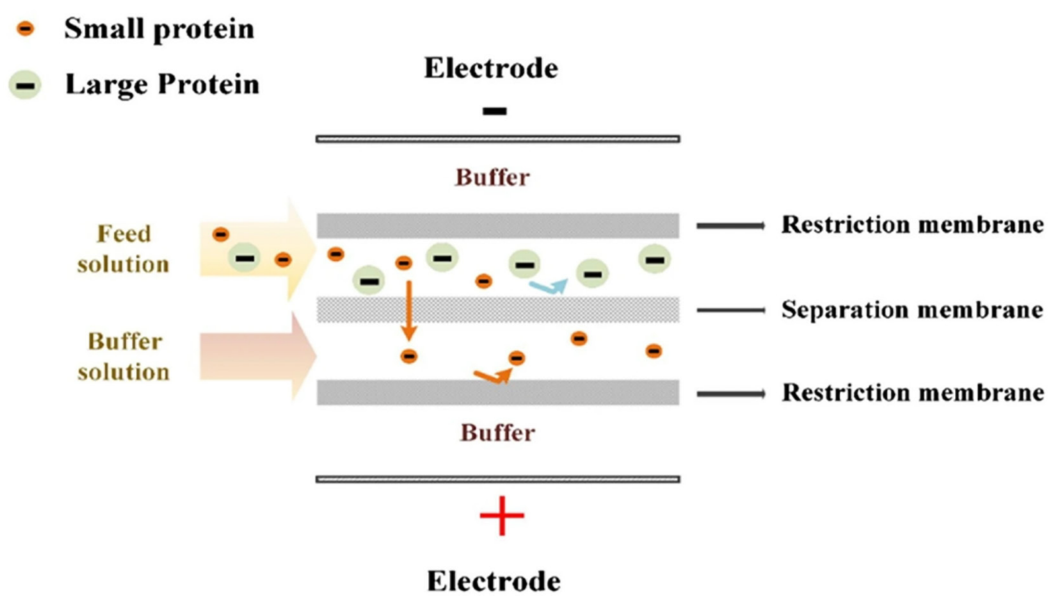

Figure 2. The protein separation process used an UF membrane as the separation membrane for ED. The UF membrane intercepts the large molecular proteins in the passing feed solution, allowing only small molecular proteins to pass through, thus achieving separation. Reproduced with permission from (Wang et al., 2020).

there was a concentration effect. The isoelectric points of these 2 proteins are tightly localized within the pH range, and optimal separation selectivity is achieved by slightly increasing the pH value (from 4.0 to 4.2) to elute α-LA and slightly decreasing the pH value (from 5.0 to 4.8) to obtain the elution of β-LG. The yield of the separated β-LG was 56%, and the purity was greater than 95%. When separating whey protein, it is difficult to separate α-LA and β-LG by one gel because their molecular weights are similar. Therefore, gel filtration chromatography is often used in combination with some crude purification methods for further purification to obtain higher-purity protein. Owing to its simple equipment and minimal investment, this method is often combined with ion exchange chromatography and is widely used both domestically and internationally.

Ion exchange chromatography method

Ion exchange chromatography (IEC) is a widely used technique for protein separation and purification. In solution, proteins generally have charged surfaces. When the sample mixture enters the column, the different proteins exhibit varying charges due to their distinct isoelectric points. This results in different degrees of adsorption to the ion exchange chromatography medium. By adjusting the pH, the surface charges of the proteins are altered, leading to differences in their adsorption to the exchange medium. This method leverages these differences in adsorption to achieve protein separation (Abd El-Salam and El-Shibiny, 2017). The use of ion exchange agents as fillers can be divided into anion exchange chromatography and cation exchange chromatography. The primary ion exchange agent for separating β-LG from whey via ion exchange chromatography is an anion exchange resin. Under suitable pH conditions, β-LG and other proteins in whey, which have different charges, dissolve and interact with the ion exchanger, facilitating separation. For proteins with similar charge properties to β-LG but differing in charge quantity, varying concentrations of salt solutions can be employed for further separation. A high-capacity ion exchange method for the single-column separation of β-LG from whey was described by Ng et al. (Ng and Snyder, 2013). As a result, the separation purity was greater than 90%. This study demonstrated the feasibility of preparing purified lactoglobulin using a single high-capacity anion exchange resin. Santos et al. (Santos et al., 2012) demonstrated that the Mono Q5/50 GL anion exchange column in a rapid protein–liquid chromatography system can separate and recover 60.5% pure β-LG from WPC 80. Stojadinovic et al. (Stojadinovic et al., 2012) studied the separation and purification of β-LG from the skim whey of raw cow milk via anion exchange chromatography, and pure β-LG with a total yield of 80% was ultimately isolated. Some cation exchange resins can also be used for the separation of β-LG. For example, in a one-step study in which α-LA and β-LG were separated from CM while retaining their antigenicity, Mao et al. obtained 95% pure β-LG accurately through cation exchange chromatography (Mao et al., 2017). El-Sayed et al. (El-Sayed and Chase, 2010) obtained β-LG with a purity of 95% and a recovery rate of 78% through cation exchange chromatography separation. Ion exchange chromatography is one of the most commonly used methods for separating and purifying milk allergens because of its good separation efficiency, high yield, short time consumption, and simple operation (Wang et al., 2019). It is widely used in laboratories and industrial production. However, ion exchange resins are expensive, have a certain service life, and require frequent replacement. In addition, in industrial production, the salt concentration in the ion exchanger is difficult to control accurately, so it can only be eluted step by step rather than continuously increasing the salt gradient, and the separation effect will decline. Therefore, it is necessary to combine other separation and purification technologies, such as gel chromatography, to improve the separation purity.

Affinity chromatography methods

Affinity chromatography involves the process of using one of 2 substances with high specificity affinity between each other as a stationary phase and utilizing the affinity with the stationary phase to separate and purify the other in a mixture. This technology can be used to select substances that can selectively bind to specific proteins as stationary phases; these substances include enzyme compounds and beads such as agarose and have good chemical stability and few nonspecific interactions to achieve selective adsorption of individual proteins (Chen et al., 2023). Affinity chromatography is a commonly used method for separating charged or uncharged molecules and is suitable for whey protein separation. When whey protein is separated, the ligands fixed on the matrix include inorganic ions, small organic compounds, hydrophobic molecules, and antibodies. Because β-LG has the unique ability to bind certain small hydrophobic molecules, it can also be separated and purified from whey through affinity chromatography (Figure 3). Vyas et al. (Vyas et al., 2002) successfully isolated β-LG from whey via an affinity separation method by immobilizing the natural form of β-LG with all trans retinas on biocalcite. The material was then moved from a packed bed column to a stirred tank and fluidized bed column to improve purity, resulting in a recovery rate of approximately 46% and a purity of over 95% for β-LG. Heddleson et al. (Heddleson et al., 1997) isolated β-LG from whey via an N-retinol-diatomaceous earth bioaffinity column on the basis of previous studies. The median purity of β-LG isolated by bioselective chromatography under normal elution conditions (0.4 M sodium phosphate, pH 7.0) was 94.5%, with a recovery rate of approximately 71%. Ceramic hydroxyapatite, a mineral form of calcium apatite calcium used as a filler in affinity columns, has been found to recover up to 55% of β-LG from acid whey with a purity of 96%. Notably, the adsorbed β-LG was eluted with trace amounts of IgG, BSA, and LF. Then, SEC (Superdex 75 column) can be used to further increase the purity to an appropriate 99% (Schlatterer et al., 2004). Affinity chromatography technology has high specificity and can separate and produce specific proteins with very high purity.

Isoelectric point precipitation method

Because proteins are amphoteric electrolytes with the lowest solubility at the isoelectric point, the method of separating various allergen-containing proteins in milk on the basis of their different isoelectric points is called isoelectric precipitation. It is commonly used to remove miscellaneous proteins after extraction and make some miscellaneous proteins that are far away from electric points, such as the protein to be purified (target), sink out of the solution by adjusting the pH value of the extraction solution. Owing to the good solubility and tolerance of β-LG under low-pH and high-salt conditions, β-LG was isolated from whey under low-pH and high-salt conditions (Mailliart and Ribadeau-Dumas, 1988; Mate and Krochta, 1994). β-LG exhibits zwitterionic ionization in solution. When the pH of the solution is equal to its isoelectric point (pI = 5.3), β-LG is in a facultative molecular state with zero net charge. The electrostatic repulsion between molecules is small, making it easier for them to collide and condense with each other, resulting in precipitation. Haller and Kulozik adjusted the pH value to 3.4, selectively precipitated α-LA, and then separated high dry matter α-LA deposits and fully clarified β-LG supernatant under the action of a co-flow decanter centrifuge, and the purity of natural β-LG in the supernatant reached 99.7% (Haller and Kulozik, 2020). Bonnaillie and Tomasula (Bonnaillie and Tomasula, 2012) used HCl to regulate pH in the range of 3.0–5.5 at moderate temperatures, so that α-LA precipitated and formed aggregates, while β-LG enriched in the liquid. The best enriched solid and liquid components contained 58% α-LA and 76% β-LG, and the recoveries were 99% and 74%, respectively. However, the purity of β-LG was not very high. Alomira et al. (Alomirah and Alli, 2004) successfully separated α-LA and β-LG from whey by adjusting the pH with the salt concentration (sodium citrate), resulting in purities of 90% and 95% and recovery rates of 89% and 69%, respectively. Chitosan is a polysaccharide copolymer of glucosamine and N-acetylglucosamine. It is positively charged at acidic pH, allowing it to interact with the anionic portion of β-LG (Casal et al., 2005) and causing β-LG to aggregate and precipitate (Figure 4). Montilla et al. (Montilla et al., 2007) explored the conditions for maximum extraction of β-LG from β-Lg-chitosan complexes to recover high-purity β-LG. The effects of pH, ionic strength and the volume ratio of sodium acetate solution to whey on the separation of β-LG and β-LG-chitosan complexes were studied. First, the pH was adjusted so that chitosan and β-LG formed a polymer precipitate. Then, 10 mL of 0.1 M sodium acetate solution at pH 9 was added to the β-LG-chitosan precipitate obtained from the whey. The recovery rate of the dissolved β-LG was 90%, and the purity was 95%. Sodium acetate is a safe and nontoxic food additive recognized by the United States Food and Drug Administration (FDA) and the European Union. The isoelectric point precipitation method is easy to perform, low in cost, and does not require high equipment. The purity of the isolated allergen meets the requirements for further research, making it a commonly used crude extraction method. However, the isoelectric points of α-LA and β-LG are similar, so when separating β-LG from whey, several methods, such as salt precipitation, chromatography, or other methods, are generally used in combination for separation and purification.

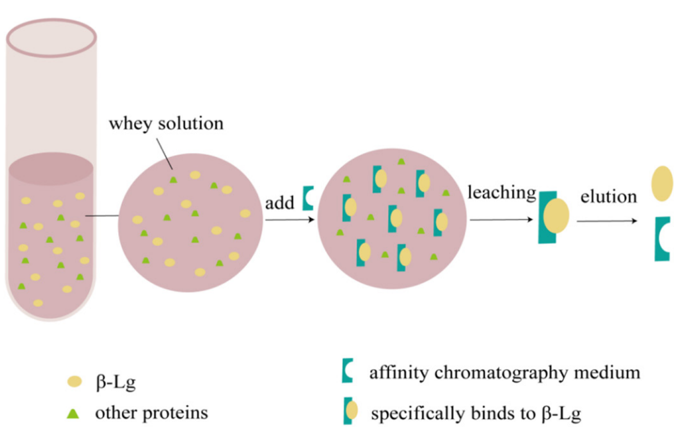

Figure 3. Principle of affinity chromatography for separating β-lactoglobulin. Affinity chromatography medium with specific binding to β-LG was added to the whey solution as the stationary phase, and then β-LG was separated from the solution. Reproduced with permission from (Rodriguez et al., 2020) and modification.

Salting-out precipitation method

The solubility of proteins at low salt concentrations increases with increasing salt solution concentration, which is referred to as salt solubility. When the salt concentration continues to rise, by affecting the electrostatic and hydrophobic interactions between proteins, the surface charge of the protein is neutralized, and the solubility of the protein is reduced to different degrees, resulting in precipitation, which is called salting out, to achieve separation and purification (Dahal and Schmit, 2018). Salting-out precipitation is a common chemical method used for protein separation and extraction. It can precipitate target proteins and play a role in concentration, preliminary separation, and purification of proteins. Ammonium sulfate is commonly used in the separation and purification of major allergens in milk to isolate these allergenic proteins. As early as 1997, Caessens et al. used this method to isolate β-LG with a recovery rate of 68% and a purity of 86% (Caessens et al., 1997). The salt-out precipitation method is simple to perform, can remove more impure proteins, and can protect volatile proteins; however, the resolution of this method is poor, and the purification capacity is not strong. Therefore, only as a pretreatment step for the crude purification of allergens, if further purification is needed, still needs to be combined with ion exchange chromatography, coagulation chromatography and other methods. A combination of precipitation and UF was used by Gésan-Guiziou et al. (Gésan-Guiziou et al., 1999) to separate β-LG from whey, ultimately obtaining a purity of 85–94% for the separated β-LG. Compared with other methods for the separation and purification of β-LG, the purity obtained ranged from 82.5% to 94.1%, indicating good separation efficiency. Lozano et al. (Lozano et al., 2008) used ammonium sulfate differential precipitation combined with chromatography to separate β-LG. First, β-LG was separated from other whey proteins via 50% ammonium sulfate. The precipitate was subsequently dissolved and separated again using 70% ammonium sulfate, leaving a supernatant rich in β-LG. After dialysis and freeze-drying, the proteins were separated via ion exchange chromatography to obtain β-LG with a purity greater than 95%. Mao et al. (Mao et al., 2017) established a method for separating and purifying β-LG from fresh milk via ammonium sulfate combined with anion exchange chromatography. Research has shown that during the precipitation process of 10% to 50% ammonium sulfate, β-LG can reach a high concentration, and 50% ammonium sulfate is the best choice for precipitating β-LG. The purity of the β-LG obtained was 95%, with a recovery rate of over 89%. The combination of ammonium sulfate precipitation and chromatography or other separation methods can achieve a high content of homogeneous protein production, greatly retaining the natural structure of β-LG, with the advantages of fast separation speed, high efficiency, low cost, high purity, and simple posttreatment process, which is widely used in the dairy industry.

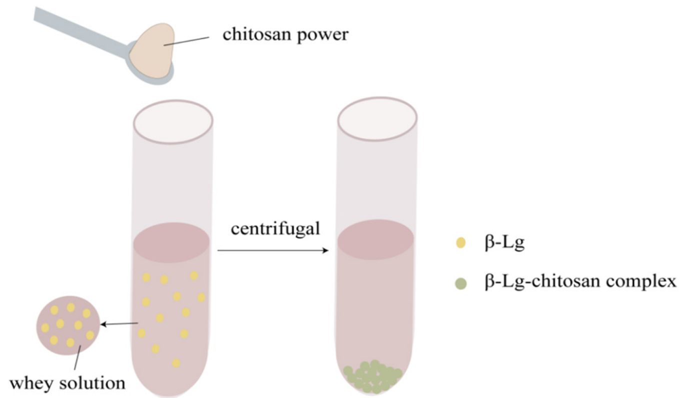

Figure 4. Illustration of β-LG in whey isolated from chitosan. Chitosan forms a complex precipitate with β-LG and is separated from the whey solution.

Aqueous 2-phase extraction method

In 1896, Beijerinck reported that when gelatin is mixed with agar or soluble starch at a certain concentration in water, it forms a turbid and opaque solution, which then separates into 2 phases. When 2 substances (such as polymers and salts or polymers and polymers) are dissolved in water, the phenomenon of forming 2 phases that do not dissolve each other due to incompatibility is called a 2-phase aqueous extraction system (ATPS). The ATPS has received widespread attention since the 1960s and has been widely applied in the separation and purification of various protein products over the past few decades (Capezio et al., 2005). ATPS is a separation method that is simple to perform, easy to scale up, has a high water content, has a mild extraction environment, has high biocompatibility and can be used continuously (Hamta and Dehghani, 2017). When ATPS is used to separate β-LG, 2 incompatible substances are mixed in water under certain conditions and ratios to form a 2-phase system, after which a solution containing the target substance (β-LG) is introduced into the 2-phase system and thoroughly stirred. β-LG preferentially distributes to one of the aqueous phases on the basis of its solubility in the 2 aqueous phases and then undergoes treatment to obtain purified β-LG from the aqueous phase containing β-LG. Alcântara et al. (Alcântara et al., 2011) studied the separation of α-LA, β-LG, and glycomacropeptides in ATPS formed by polyethylene glycol (PEG) and potassium phosphate and investigated the effects of pH and NaCl concentration on separation. The system was optimized by a central composite rotation design and response surface method. Finally, α-La and glycomacropeptide were recovered from the polymer-rich phase, and β-LG was recovered from the salt-rich phase, with an extraction rate of 97.3%. Kalaivani et al. (Kalaivani and

Regupathi, 2015) treated whey protein with acid and then separated β-LG from whey via ATPS. They reported that the remaining proteins were in the polymer-rich phase, whereas β-LG was in the salt-rich phase, with recovery rates and purities of 96% and 76%, respectively. Freire et al. (Freire and Pereira, 2016) used a biphasic system to investigate the separation of α-La and β-LG at 25°C, pH 7.0, and different polymer molecular weights. The results showed that the separation efficiency was best in the PEG 1500 (14–50 wt%) and potassium phosphate (18–30 wt%) systems, in which α-La was enriched in the upper phase, whereas β-LG was enriched in the lower phase, with more than 90% yield and purity. Although the separation of β-LG by ATPS has many advantages, the polymer commonly used in the system is PEG, and the acceptable daily intake of PEG recommended by the World Health Organization is less than 25 mg per kilogram of body weight; thus, the elimination of PEG from the final protein produced by dual aqueous extraction needs to be demonstrated later.

Multitechnology combination method

At present, there are many ways to separate and purify β-LG from whey. Each method of separation and purification has advantages, but it also has certain limitations. In practical applications, when a separate separation method is used to purify β-LG, its purity and biological properties may not meet subsequent needs. Therefore, it is necessary to explore methods and technologies that can improve the separation and purification efficiency of β-LG in milk whey. There may be synergistic effects between different separation and purification methods, resulting in complementary advantages, so the combined application of 2 or more separation and purification technologies to separate β-LG in milk whey can significantly improve its separation effect, which is an excellent commercial production method. For example, when β-LG is separated from whey, the first use of membrane technology for crude extraction, followed by further purification via chromatography, cannot only improve the selectivity of the membrane binding capacity and protein recovery and purity but also reduce the cost and reduce the impact of membrane contamination, overcoming limitations such as long chromatographic separation times. Toro Cirra et al. (Toro-Sierra et al., 2013) utilized a combination of multiple methods to achieve the separation of β-LG and α-LA in whey, providing the possibility for pilot scale and industrial applications to produce pure natural β-LG or α-LA fractions. First, α-LA was selectively precipitated by heating and pH was adjusted to 3.4, then β-LG was separated by microfiltration and ultrafiltration in turn and further purified. Finally, β-LG protein components with a purity of 97.2% were obtained, and the total yield was 80.2%~97.3%. Yang et al. (Yang et al., 2022) first removed foreign proteins via ultracentrifugation, isoelectric point precipitation and ammonium sulfate precipitation and then purified β-LG via gel chromatography and ion exchange chromatography. The purity of β-LG ultimately reached 100%, and the yield was 54.89%, which was higher than that in most reports. Compared with a single method for separating and purifying β-LG, combining multiple separation methods is more competitive.

CONCLUSION AND FUTURE PERSPECTIVES

Milk, a natural dairy product, provides a balanced array of nutrients essential for our daily diet, making it a valuable source of nutrition. Whey protein, derived from milk whey, offers significant nutritional benefits to the human body and has become widely used as a raw food material due to technological advancements. β-LG is the key protein of milk whey, which is rich in nutrients; can reduce human blood cholesterol concentrations; provides amino acids needed for the human body, as well as antibacterial, immune regulatory and other functions; and has good application prospects in the food industry and health product industry. However, it also has a high degree of sensitization, which can cause milk allergic immune reactions, resulting in health risks. Therefore, research on the main allergens of milk is highly important, and solving the separation technology of these allergens has become an important part of its development and utilization. The effective isolation of high-purity β-LG from whey is the basis of research on milk allergies and is essential for reducing milk allergies. This paper introduces the common separation and purification methods of β-LG from milk whey. Although there are currently many technologies for the separation and purification of β-LG from milk, there are still some problems that need to be solved. The purity of β-LG separated by simple and low-cost methods makes it difficult to meet the needs of subsequent experiments, and separation and purification technology with high separation purity requires more precise experimental equipment and high cost. In addition, the traditional single separation method may also have several disadvantages, such as pollution risk, low extraction content and reduced purity. Several studies have shown that the combination of multiple separation and purification methods can effectively alleviate these problems and improve the recovery and purity of proteins. For example, combining the ammonium sulfate precipitation method with chromatography effectively separated β-LG from whey. The precipitation method initially separates visible impurities in whey protein, whereas chromatography enables finer separation in later stages, removing persistent impurities to obtain high-purity β-LG. This integrated method preserves the integrity of whey protein components without the use of organic reagents, maintains the natural activity of β-LG, and cost-effectively improves recovery and separation purity.

With the advancement of separation technology and the increase in market demand, the use of multiple separation methods combined with the purification of β-LG will become the main development trend in the future. With increasing attention given to cow milk allergies, the separation and purification technology of the cow milk allergen β-LG, which is both economical and effective for the separation and purification of β-LG in CM, will also be further studied. In addition to improving existing technologies, new separation techniques must be explored and developed to achieve automation of the entire process of β-LG separation and subsequent proteomic analysis. Future separation methods are expected to focus on achieving high speeds, improving extraction rates, increasing flux, increasing cost-effectiveness and environmental sustainability, and incorporating new identification elements.

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Abbreviations: β-LG = β-Lactoglobulin, α-LA = α-Lactalbumin, ATPS = aqueous two-phase system, BSA = bovine serum albumin, CM = cow's milk, CMA = cow's milk allergy, ED = electrodialysis, Ig = immunoglobulin, IEC = ion exchange chromatography, LF = Lactoferrin, MF = microfiltration, NF = nanofiltration, PEG = polyethylene glycol, RO = reverse osmosis, SEC = size exclusion chromatography, UF = ultrafiltration, WP = whey protein

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