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Isolation and Purification of Bioactive Proteins from Bovine Colostrum

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1. Introduction

Bovine colostrum is the milk secreted by cows during the first few days after parturition. It contains many essential nutrients and bioactive components, including growth factors, immunoglobulins (Igs), lactoperoxidase (Lp), lysozyme (Lys), lactoferrin (Lf), cytokines, nucleosides, vitamins, peptides and oligosaccharides, which are of increasing relevance to human health. Much research work has been done on the structure and function of bovine colostrum proteins. IgG was widely utilised in the immunological supplementation of foods, specifically in infant formulate, and yielded sales of approximately US$100 million in 2007 (Gapper, *et al*., 2007). In the highly competitive and valuable international market for IgG-containing products, some of the products are usually priced based on IgG content. Another important protein from bovine colostrum is lactoferrin. Its diverse range of biological activities such as anti-infective activities toward a broad spectrum of species, antioxidant activities and promotion of iron transfer are expanding the demand in the market. It also exhibits the potential for chemoprevention of colon and other cancers as a natural gradient. Apart from the two kinds of bovine colostrum proteins, α-lactalbumin has been claimed as an important food additive in infant formula due to its high content in tryptophan and as a protective against ethanol and stress-induced gastric mucosal injury. β-Lactoglobulin is commonly used to stabilize food emulsions for its surface-active properties. Bovine serum albumin (BSA) has gelation properties and it is of interest in a number of food and therapeutic applications (Almecija, *et al*., 2007). Therefore, fractionation for the recovery and isolation of these proteins has a great scientific and commercial interest.

As a result of this growth in the commercial use of bovine colostrum proteins, there is great interest in establishing more efficient, robust and low cost processes to purify them. Although great deals of studies have been done for the separation and purification of colostrum proteins due to their wide application in food industry, medicine and as supplements, large scale production system for the downstream processing of recombinant antibodies still represents the major issue. Lu (Lu, *et al*., 2007) designed a two-step ultrafiltration process followed by a fast flow strong cation exchange chromatography to isolate LF from bovine colostrum in a production scale. A stepwise procedure for purification of the crude LF was conducted using a preparative-scale strong cation exchange

chromatography. The purity and the recovery of the final LF product were 94.20% and 82.46%, respectively. The process developed in Lu's work was a significant improvement over the commercial practice for the fractionation of LF from bovine colostrum. Recently, Saufi et al. developed a cationic mixed matrix membrane for the recovery of LF from bovine whey, the absorbent was developed by embedding ground SP Sepharose cation exchange resin into an ethylene vinyl alcohol polymer base membrane (Saufi & Fee, 2011). The static LF binding capacity of the cationic Mixed Matrix Membrane (MMM) was 384 mg/mLmembrane or 155 mg/mL membrane, exceeding the capacity of several commercial adsorptive membranes. The membrane chromatography system was operated in cross-flow mode to minimize fouling and enhance LF binding, resulting in an LF recovery as high as of 91%, with high purity. The system was operated at a constant permeate flux rate of 100 Lm^{-2} h^{-1}, except during the whey loading step, which was run at 50 Lm^{-2} h^{-1}. This is the first time a cross-flow MMM process has been reported for LF recovery from whey.

The traditional protein fraction process usually included initial processes such as centrifugalization and membrane treatment, and polishing steps such as chromatographic procedures. To further utilize bioactive substance such as bovine colostrum sIgA and IgG, a procedure including salting out, ultra-filtration and gel chromatography in proper sequence on isolation and purification of bovine colostrum sIgA and IgG was reported (LIU& Y.Y.X.G.a.X. 2007). The purity and yield of bovine colostrum sIgA were 85.3% and 42.8%, respectively. The purity and yield of bovine colostrum IgG were respectively 97.2% and 64.4%.This preparative method provided theoretical and experimental foundation for sIgA and IgG industrial production. Depending on the market requirement, other procedures may be employed as the suitable steps for the products' commerciality, such as freeze-drying and crystallization. Therefore, the protocols for the purification of proteins should be designed according to the feed stock and final requirement.

Although a wide variety of protocols can be used to separate bioactive proteins from complex food stock, chromatographic procedure is the most prevalent form as high-resolution fractionation technique. In this section, we will discuss the use of chromatographic procedures and other techniques as high-resolution techniques for the fraction of bovine colostrum proteins. Special attention will be paid to the amount of bio-product denaturation or activity loss that occurs. Particular attention will also be paid to the quality of the separated bio-product. The understanding about processes that lead to these activity losses would then assist in minimizing these activity losses.

2. Precondition of bovine colostrum

2.1 Preparation of acid whey

In order to avoid the problems caused by high viscosity of bovine colostrum, researchers usually employ acid whey as the beginning feed stock. The method is as follows. Bovine colostrum samples were collected within the first day after cow parturition from the dairy plant and were immediately frozen and stored at −18°C. The frozen samples were thawed and the lipid fraction were removed by centrifugation at 8,000 r/min for 15~20 min at 4°C. Acid colostral whey was prepared by precipitation of the casein from skimmed colostrum with 1 mol/L HCl at pH 4.2 and the precipitated casein was removed by microfiltration. The whey was then adjusted to pH 6.8 with 1 mol/L NaOH and then went through centrifugation.

2.2 Membrane filtration

Membrane filtration provides promising results for the fractionation of whey proteins and it has traditionally been based solely on differences in molecular mass. Until recently, membranes were thought to achieve separation only between proteins differing in size by at least a factor of 10. Almecija (Almecija, *et al*., 2007) investigated the potential of ceramic membrane ultrafiltration for the fractionation of clarified whey. They employed a 300 kDa tubular ceramic membrane in a continuous diafiltration mode. The effect of working pH was evaluated by measuring the flux-time profiles and the retentate and permeate yields of α-lactalbumin, β-lactoglobulin, BSA, IgG and lactoferrin. The study results showed that at pH 3, 9 and 10 permeate fluxes ranged from 68 to 85, 91 to 87 and 89 to 125 L/(m^{2}h), respectively. On the other hand, around the isoelectric points of the major proteins (at pH 4 and 5), permeate fluxes varied from 40 to 25 and from 51 to 25 L/(m^2 h), respectively. For α-lactalbumin and β-lactoglobulin, the sum of retentate and permeate yields was around 100% in all cases, which indicates that no loss of these proteins occurred. After 4 diavolumes, retentate yield for alpha-lactalbumin ranged from 43% at pH 9 to 100% at pH 4, while for β-lactoglobulin, was from 67% at pH 3 to 100% at pH 4. In contrast, BSA, IgG and lactoferrin were mostly retained, with improvements up to 60% in purity at pH 9 with respect to the original whey. The results of this paper obtained were explained in terms of membrane–protein and protein–protein interactions.

2.3 Precipitation

Precipitation method is an effective way to concentrate the proteins due to their different pI, sensitivity to the ionic strength and other properties. Salting-out is widely used for the pretreatment of bovine whey to selectively precipitate the protein of interest or impurities. Lozano (Lozano, *et al*., 2008) used an improved method successfully and rapidly separated β-lactoglobulin from bovine whey. Firstly, differential precipitation with ammonium sulfate was used to isolate β-lactoglobulin from other whey proteins using 50% ammonium sulfate. The precipitate was dissolved and separated again using 70% ammonium sulfate, leaving a supernatant liquid enriched in β -lactoglobulin. After dialysis and lyophilization, isolation of the protein was performed by ion-exchange chromatography. Comparison of physicochemical and immunochemical analysis showed that the identity and purity of the isolated protein was comparable with that of the Sigma standard. Spectroscopic results showed that the method used for protein isolation did not induce any changes in the protein native structural properties. Ammonium sulfate precipitation method played a vital role for this rapid, efficient and inexpensive two-step process that allowed high homogeneous protein yield.

3. Chromatographic procedures for the separation of bovine colostrum proteins

3.1 Ion exchange chromatography

3.1.1 Introduction

Proteins contain charged groups on their surfaces that enhance their interactions with solvent water and hence their solubility. Charged residues can be cationic or anionic and it is noteworthy that even polar residues can also be charged under certain pH conditions. These charged and polar groups are responsible for maintaining the protein in solution at physiological pH. Because proteins have unique amino acid sequences, the net charge on a

protein at physiological pH is determined ultimately by the balance between these charges. This also underlies differing isoelectric points (pIs) of proteins (Himmelhoch, 1971). Therefore, bioactive proteins can be absorbed by different ion-exchange chromatography [Fig. 1] due to the different charge type and pI. The ion-exchange resins are then selectively eluted by slowly increasing the ionic strength (this disrupts ionic interactions between the protein and column matrix competitively) or by altering the pH (the reactive groups on the proteins lose their charge) (Dolman, *et al*., 2002)

Fig. 1. a) Anionic (negatively charged) proteins exchange. b) Cationic (positively charged) proteins exchange.

3.1.2 Applications in Isolation and purification of bioactive proteins from bovine colostrum

The whey proteins can be fractionated and separated by different ion exchange chromatography. A water-jacketed chromatography column (XK 26/40, Amersham Biosciences) packed with SP Sepharose Big Beads cation exchanger was used to recover and fractionate whey proteins (Doultani, *et al*., 2004). The chromatographic procedure involved sequentially pumping different solutions into the column: (1) equilibration (EQ) buffer to adjust column pH; (2) whey; (3) EQ buffer to rinse unbound material from the column; and (4) different elution buffers to selectively desorbed different bound proteins.

The optimum conditions for initially separating the proteins such as α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulin G and lactose from a sweet dairy whey mixture could be determined by a commercial anion-exchange resin (Gerberding & Byers, 1998). The separation was accomplished with simultaneous step elution changes in salt concentration and pH. It was found that the anion-exchange step was most effective in separating β-lactoglobulin from the feed mixture. Followed by the anion-exchange separation, the breakthrough curve was processed using a commercial cation-exchange resin to further recover the valuable immunoglobulin G.

A simple and useful method for β-lactoglobulin isolation from bovine whey was presented recently (Lozano, *et al*., 2008). Differential precipitation with ammonium sulfate was used to isolate β-lactoglobulin from other whey proteins using 50% ammonium sulfate. The precipitate was dissolved and separated again using 70% ammonium sulfate, leaving a supernatant liquid enriched in β-lactoglobulin. After dialysis and lyophilization, isolation of the protein was performed by ion-exchange chromatography. This was a rapid, efficient and

inexpensive two step method that allows high homogeneous protein yield and has advantages over other methods since it preserves the native structure of β-lactoglobulin.
In 2006, Andrews reported a simple, rapid and cost-effective preparation of two milk peptide components in a high degree of purity, and in gramme quantities, for evaluation of such properties (Andrews, *et al*., 2006). The purification process was more efficient if β-casein was used as starting material. In this work, we prepared 46 g of β-casein from sodium caseinate in a simple rapid DEAE-cellulose ion-exchange chromatography stage. This was followed by in vitro hydrolysis with plasmin and precipitation and gel filtration steps.
R. Hahn (Hahn, *et al*., 1998) investigated a fractionation scheme for the economically interesting proteins, such as IgG, lactoferrin and lactoperoxidase, based on cation exchangers. In his work, S-Sepharose 2 FF, S-Hyper D-F and Fractogel EMD SO 650 (S) were considered as successful candidates for the large-scale purification of 3 bovine whey proteins.
Fweja (Fweja, *et al*., 2010) isolated Lactoperoxidase (LP) from whey protein by cation-exchange using Carboxymethyl resin (CM-25C) and Sulphopropyl Toyopearl resin (SP-650C). The recovery was much greater with column procedures and the purity was higher than batch column.
Xiuyun Ye (Ye, *et al*., 2002) described a mild and rapid method for isolating various milk proteins from bovine rennet whey. β-Lactoglobulin from bovine rennet whey was easily adsorbed on and desorbed from a weak anion exchanger, diethylaminoethyl-Toyopearl. However, α-lactalbumin could not be adsorbed onto the resin. α-Lactalbumin and β-lactoglobulin from rennet whey could also be adsorbed and separated using a strong anion exchanger, quaternary aminoethyl-Toyopearl. The rennet whey was passed through a strong cation exchanger, sulphopropyl-Toyopearl, to separate lactoperoxidase and lactoferrin. α-Lactalbumin and β -lactoglobulin were adsorbed onto quaternary aminoethyl-Toyopearl. α-Lactalbumin was eluted using a linear (0–0.15 M) concentration gradient of NaCl in 0.05 M Tris–HCl buffer (pH 8.5). Subsequently, β-lactoglobulin B and β-lactoglobulin A were eluted from the column with 0.05 M Tris–HCl (pH 6.8), using a linear (0.1–0.25 M) concentration gradient of NaCl. The disadvantage of this system may be the disappearance of Ig and bovine serum albumin (BSA).

3.1.3 New ion-exchange process and technology

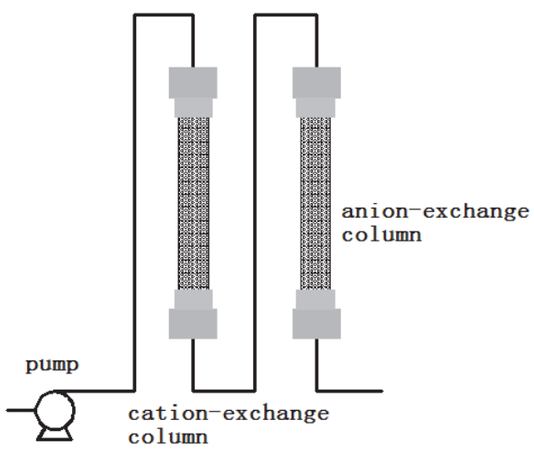


Fig. 2. The process of two ion-exchange columns in series for the isolation of Lf and IgG

Recently, the ion-exchange chromatography was improved to adapt the requirement of separation. It was combined with other ion exchange steps and with affinity chromatography to achieve complete purity in a wide range of biological systems and a wide variety of protein classes. Wu and Xu developed a novel process which could separate LF and IgG simultaneously from bovine colostrum by combining cation (CM-sepharose FF) and anion (DEAE-sepharose FF) ion exchange chromatography which showed in Fig.2.

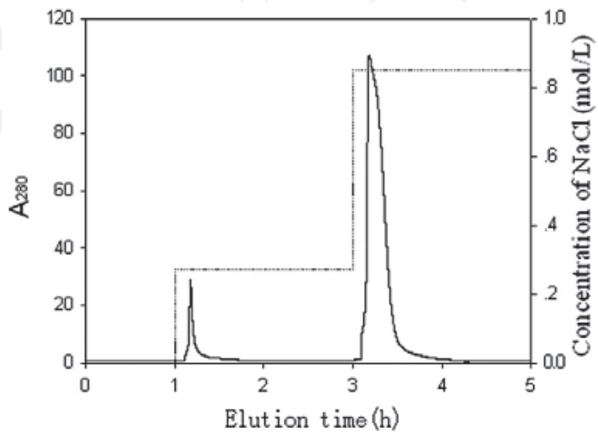


Fig. 3. Isolation of LF from of the ultrafiltrated colostrum whey by cation-exchange chromatography using CM-sepharose FF column (1.6 × 25 cm). Adsorption phase, 500 mL ultra-filtrated colostrum whey (pH 6.8); washing phase, 200 mL de-ironed water; eluting phase, 200 mL 0.27 mol/L and 200 mL 0.85 mol/L NaCl solution with sequential saline gradient.

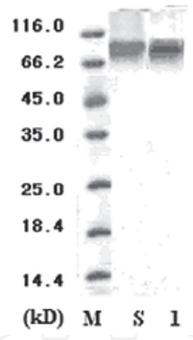


Fig. 4. SDS-PAGE profile of fractions obtained in ultrafiltrated whey by cation-exchange column using saline gradient. Lane M, protein markers; lane S, Lf standard; lane 1, elution peak with 0.85 mol/L NaCl.

After dilution, the ultra-filtrated whey was passed though a cation-exchange column of CM-sepharose FF followed by an anion-exchange column of DEAE-sepharose in series. When the whey (pH = 6.8) was passed through the CM-sepharose column, proteins with pI above 6.8 were adsorbed on the resin. Figure 3 showed the results of CM-sepharose FF cation-exchange chromatography. After the unabsorbed proteins were eluted from the column, the column was washed with sodium chloride solutions of increasing molarities (0.27 and 0.85 mol/L) in a stepwise manner. The fraction in the first peak (P1) was weakly adsorbed

proteins which could not be retained on the resin during washing with 0.27 mol/L NaCl solution. The more strongly adsorbed proteins were eluted and formed the second peak (P2). The fraction in P2 was identified as Lactoferrin (LF) by SDS-PAGE (Fig. 4, Lane 1) and the purity of LF analysised by HPLC was 96.6%.

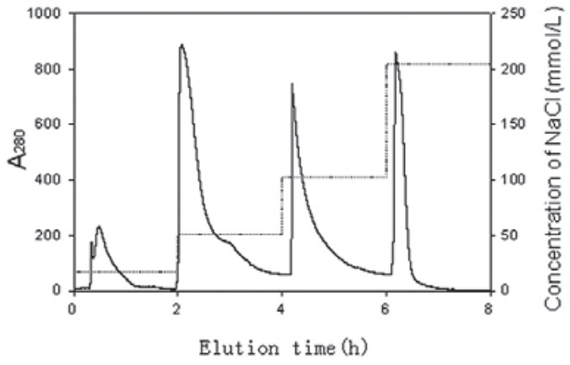


Fig. 5. Isolation of LF from the ultrafiltrated colostrum whey by an ion-exchange chromatography using DEAE-sepharose FF column (1.6 × 75 cm). Adsorption phase, 500 mL ultra-filtrated colostrum whey (pH 6.8); washing phase, 300 mL de-ironed water; eluting phase, 600 mL 17 mmol/L, 600 mL 51 mmol/L, 600 mL 103 mmol/L, and 600 mL 205 mmol/L NaCl solution in a stepwise manner.

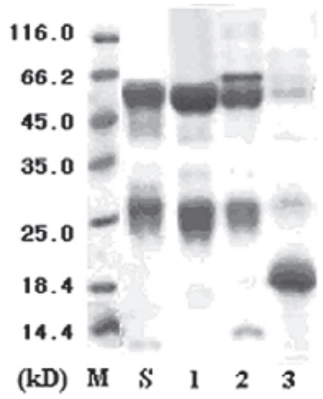


Fig. 6. SDS-PAGE profile of fractions obtained in ultrafiltrated whey by anion-exchange chromatography using saline gradient. Lane M, proteins marker; lane S, IgG standard; lane 1, elu-tion peak with 51 mmol/L NaCl; lane 2, elution peak with 103 mmol/L NaCl; lane 3, elution peak with 205 mmol/L NaCl with stepwise saline gradient.

When the colostrum whey was passed though the DEAE-Sepharose FF column, the proteins with pI below 6.8, including IgG were exchanged on the resin. After washed by de-ionized water, the column was eluted by sequential stepwise gradients with 17, 51, 103, and 205 mmol/L NaCl. The elution profiles were shown in Fig. 5. The second peak in Fig. 5, which was eluted by 51 mmol/L NaCl, was identified as IgG by SDS-PAGE (Fig. 6, lane 1) and it showed high IgG immune activity as measured by ELISA method. IgG was also detected in the third peak of Fig. 5, which was eluted with 103 mmol/L NaCl (Fig. 6, lane 2). Both SDS-

PAGE and ELISA methods shown that the fraction in the second peak had higher purity and IgG activity than that in the third peak.

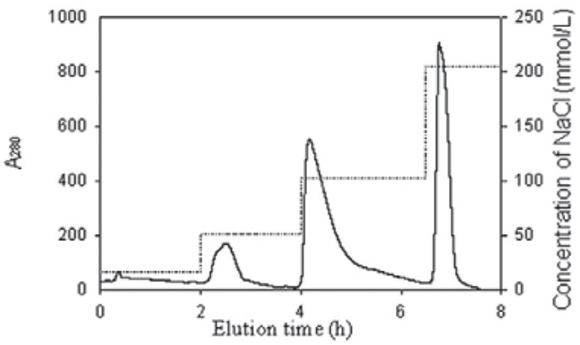


Fig. 7. Isolation of LF from of the un-ultrafiltrated colotrum whey by anion-exchange chromatography using DEAE-sepharose FF column (1.6 × 75 cm). Adsorption phase, 500 mL ultra-filtrated colostrum whey (pH 6.8); washing phase, 300 mL deironed water; eluting phase, 600 mL 17 mmol/L, 600 mL 51 mmol/L, 600 mL 103 mmol/L, and 600 mL 205 mmol/L NaCl solution in a stepwise manner.

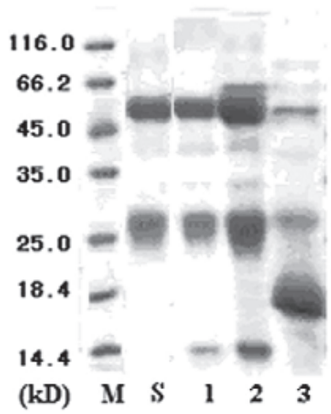


Fig. 8. SDS-PAGE profile of fractions obtained in un-ultrafiltrated whey by anion-exchange chromatography using saline gra-dient. Lane M, proteins marker; lane S, IgG standard; lane 1, elution peak with 51 mmol/L NaCl; lane 2, elution peak with 103 mmol/L NaCl; lane 3, elution peak with 205 mmol/L NaCl with sequential saline gradient.

Elution curves (Fig. 5 and Fig. 6) and SDS-PAGE profiles (Fig. 7 and Fig. 8) showed that four protein fractions could be separated by anion-exchange chromatography with the same saline gradient using both un-ultrafiltrated and ultrafil-trated samples. Compared Fig. 5 with Fig. 7, elution with 103 mmol/L and 205 mmol/L NaCl produced relatively both the same broad peaks with tailing, but the peaks washed by 17 mmol/L and 51 mmol/L NaCl showed that the fractions from ultrafiltrated whey sample had the higher protein concentration than those from the un-ultrafiltrated. Proteins in whey can be agglomerated and denaturized within ultrafiltra-tion process and the SDS-PAGE profiles also indicated that the peak contained other proteins in colostrum whey. From the results, it could be deduced that the higher concentration of the other proteins in the ultrafil-trated whey than that in un-

ultrafiltrated whey was by reason that the other proteins which were exchanged nonspecifically on the resin could be desorbed at relatively low saline solu-tion such as 17 mmol/L NaCl.

The majority of IgG could be eluted with 51 mmol/L NaCl with both un-ultrafiltrated and ultrafiltrated colostrum whey and both fractions had the same IgG purity about 95% (w/w) by HPLC analysis, but the peak obtained in ultrafiltrated whey had higher IgG concentration than that obtained in un-ultrafiltrated whey. Small molecule such as salts (ions), sugars, and amino acids could be easily adsorbed on the sorbent and reduced IgG adsorption capacity of the resin. The ultrafiltrated whey, due to the fact that low molecular mate rials in the original whey were removed by ultrafiltration, showed higher ion exchange capacity for IgG and resulted in a higher concentration of IgG in the fraction. According to SDS-PAGE profiles (Fig. 6 and Fig. 8), the proteins eluted with 103 mmol/L contained IgG, BSA and α-lactalbumin. The concentration of α-lactalbumin in Fig. 6 was lower than in Fig. 8 for the reason that α-lactalbumin was mostly removed by ultrafiltration process. The major protein in the fractions eluted by 205 mmol/L NaCl for both ultrafiltrated and un-ultrafiltrated colostrum whey was β-lactoglobulin. Furthermore, there was no clear difference in the β-lactoglobulin concentration for both ultrafiltrated and un-ultrafiltrated colostrum whey samples. Although the molecular weight of β-lactoglobulin was 28 kD, under pH 7.0 the major portion of β-lactoglobulin could be polymerized into dimmer. Therefore, the major portion of β-lactoglobulin was retained, while the colostrum whey was ultrafiltrated with a 50 kD molecular weight cut-off polyethersulfone membrane.

	Bovine colostrum	Acid whey	Step 1[a]	Step 2[b]	Step 3[c]	Step 4[d]	Step 5[e]
Concentration of Lf (mg/mL)	1.02	0.94	0.8	0.03	0.01	0.7	-
Recovery (%)	100	92.16	78.43	-	-	68.63	-
Concentration of IgG (mg/mL)	25.00	18.25	17.75	17.24	0.11	-	11.34
Recovery (%)	100	73.00	71.00	68.96	-	-	45.38

[a]Acid whey was ultrafitrated with 50 kD molecular weight cut-off membrane.
[b]Whey was passed through cation- exchange column.
[c]Whey was passed through anion- exchange column.
[d]Fraction was eluted with 0.85 mol/L NaCl on cation exchange column.
[e]Fraction was eluted with 51 mmol/L NaCl on anion exchange column.

Table 1. Concentration and recovery yield of LF and IgG at each step of the over all separation process

Concentrations of LF and IgG at every step of the separation process were analyzed by ELISA method (Table 1). According to the results shown in Table 1, the activity of LF was only decreased by a little (about 7%), but the activity of IgG was lost severely (about 25%) during preparation of the acid colostrum whey. During ultrafiltration process, the activity of LF was lost a lot (about 14%), whereas that of IgG was lost a little (only 2%). On the other hand, 9.8% of LF activity and 23.6% of IgG activity were lost during cation-exchange chromatography and anion-exchange chromatog-raphy, respectively. In summary, the recovery yields for LF and IgG in the overall separation process were 68.83% and 45.38%, respectively.

In summary, a novel process for the isolation of the high value bovine LF and IgG from colostrum whey was developed. The LF and IgG were purified by two ion-exchange columns in series. The two resins had opposite polarity. Results showed that the proposed procedures were fast, reliable, and effective. Additionally, ultrafiltration can be used as a pretreatment method to remove small molecules and to increase both the product purity and recovery rate of LF and IgG. Furthermore, the serial ion-exchange chromatography need not use buffers to maintain pH of the whey samples and can be operated at high flow rates. In general, the purities of 96.6% (w/w) LF and 95.0% (w/w) IgG were obtained with respective recovery rates of 68.83% and 45.38% by serial cation-anion exchange chromatography from ultrafiltrated bovine colostrum. (Wu & Xu, 2009)

Isidra Recio (Recio & Visser, 1999) reported a membrane method for the rapid isolation of antibacterial peptides from lactoferrin (LF) which was more rapid and offers several economic advantages than exchange chromatography. Cheese whey was filtered through a cation-exchange membrane, and the selectively bound LF was directly hydrolysed in situ with pepsin. Inactive LF fragments were washed off the membrane with ammonia, and a fraction enriched in LFcin-B was obtained by further elution with 2 M NaCl.

Ulber (Ulber, *et al*., 2001) discussed the application of several membrane types for a crossflow filtration of sweet whey to remove insoluble particles and lipids from the whey with the aim of obtaining permeate which could be directly used for down-streaming the minor component via ion exchange membrane adsorber systems. Using a two-step downstream process consisting of a cross-flow filtration and a membrane adsorbent was possible to isolate bLF from sweet whey in a very suitable manner. The advantages of a membrane adsorbent system in direct comparison with ion exchange chromatographic support were to be found in its higher flow rates and, therefore, shorter cycle times as well as in easier handling and upscaling.

Saufi (Saufi & Fee, 2009) described the application of Mixed Matrix Membrane (MMM) chromatography for fractionation of β-Lactoglobulin from bovine whey. MMM chromatography was prepared using ethylene vinyl alcohol polymer and lewatit anion exchange resin to form a flat sheet membrane. The membrane was characterized in terms of structure and its static and dynamic binding capacities were measured. The optimum binding for β-Lactoglobulin was found to be at pH 6.0 using 20 mM sodium phosphate buffer. The MMM had a static binding capacity of 120 mg/g membrane (36 mg/mL membrane) and 90 mg/g membrane (27 mg/mL membrane) for β-Lactoglobulin and α-Lactalbumin, respectively. In batch fractionation of whey, the MMM showed selective binding towards β-Lactoglobulin compared to other proteins. The dynamic binding capacity of β -Lactoglobulin in whey solution was about 80 mg/g membrane (24 mg β-Lac/mL of MMM), which was promising for whey fractionation using this technology. The mixed matrix membrane showed excellent potential for a whey protein fractionation application, particularly for selective binding of β-Lac. The membrane had a defect-free structure and provided a high binding capacity for β-Lac in whey solution, compared with other proteins. The MMM had maximum equilibrium binding capacities of 150 mg β-Lac/g membrane and 90 mg α-Lac/g membrane in individual pure protein experiments. In batch fractionation of whey, the MMM had almost the same binding capacity for β-Lac as it did for pure β-Lac.

Anders Heebøll-Nielsen (Anders, *et al*., 2004) described the design, preparation and testing of superparamagnetic anion-exchangers, and their use together with cation-exchangers in the fractionation of bovine whey proteins as a model study for high-gradient magnetic fishing. Crude bovine whey was treated with a superparamagnetic cation-exchanger to

adsorb basic protein species, and the supernatant arising from this treatment was then contacted with the anion-exchanger. In the initial cation-exchange step quantitative removal of lactoferrin (LF) and lactoperoxidase (LPO) was achieved with some simultaneous binding of immunoglobulins (Igs). The immunoglobulins were separated from the other two proteins by desorbing with a low concentration of NaCl (≤0.4 mol/L), whereas lactoferrin and lactoperoxidase were co-eluted in significantly purer form when the NaCl concentration was increased to 0.4-1 mol/L. The anion-exchanger adsorbed β-lactoglobulin selectively allowing separation from the remaining protein.
Compared with the other chromatographic methods, ion-exchanger chromatography has the advantages of low cost, reduced steps, continuous feed-in, and easy to scale-up. It has shown potential for commercial applications.

3.2 Affinity chromatography

Affinity chromatography is a prevailing procedure to isolate and purify the active substances. This technique is based on molecular recognition or bio-recognition which is widespread in many professional disciplines, such as biology, molecular biology and chemistry (Wilchek & Chaiken, 1968; Wilchek & Miron, 1999; Scopes, 1999)

3.2.1 Principles of affinity chromatography

Affinity chromatography primarily requires a group of proteins to have a reversible interaction with a specific ligand attached to a solid matrix; in addition, the effectiveness of affinity purifications relies on the ability of the protein to recognize specifically an affinity adsorbent. As for the procedure of affinity chromatography, when the compound is passed through the affinity column at a certain flow velocity, the desired active substances will be attached to an affinity adsorbent immobilized to the chromatography matrix. With the different solution passing through the affinity column, the binding between the absorbent and the active substances can be loosened by a change in buffer conditions, such as the pH, ionic strength or polarity, consequently the desired component are eluted relatively free of contaminants. Virtually, affinity chromatography always result in high selectivity, high resolution and high capacity for the proteins of interest. The key stages in an affinity chromatography are shown in Figure 9.

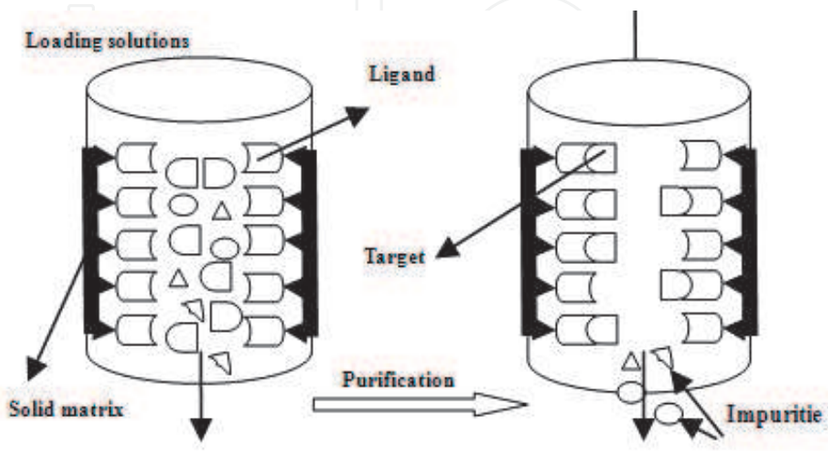


Fig. 9. The basic principle of affinity chromatography

The first protein which was purified by affinity chromatography was α-amylase in 1910. From then on, affinity chromatography was applied extensively. There are lots of various applications derived from affinity chromatography; we can see a series of those in table 2

1	Immunoaffinity chromatography	11	Centrifuged affinity chromatography
2	Lectin affinity chromatography	12	Affinity repulsion chromatography
3	Metal-chelate affinity chromatography	13	Theophilic chromatography
4	Covalent affinity chromatography	14	Membrane-based affinity chromatography
5	Perfusion affinity chromatography	15	Weak affinity chromatography
6	High performance affinity chromatography	16	Receptor affinity chromatography
7	Affinity precipitation	17	Molecular imprinting affinity
8	Filter affinity transfer chromatography	18	Library-derived affinity ligands
9	Dye-ligand affinity chromatography	19	Affinity partitioning
10	Affinity electrophoresis	20	Affinity capillary electrophoresis

Table 2. Various Techniques stemmed from Affinity Chromatography (Wilchek & Chaiken, 1968)

3.2.2 Application of affinity chromatography

In 1988, Lee applied the Cu-loaded immobilized metal affinity chromatography to separate of immunoglobulins from bovine blood which were pre-retreated by polyphosphate precipitation. The IgG gained by this procedure were almost pure after the residual polyphosphate (Lee, *et al*., 1966). In 1990, Timothy immobilized a DNA-aptamer specific for human L-selectin to a chromatography matrix to create an affinity column; meanwhile, they used this column to purify a recombinant human L-selectin-Ig fusion protein from Chinese hamster ovary cell-conditioned medium. A 1500-fold purification with an 83% single step recovery came out by the first-step purification, and this demonstrated that oligonucleotide aptamers could be effective affinity purification reagents (Romig *et al*., 1999). Roque immobilized the ligand 8/7 on to hexanediamine-modified agarose as affinity media, and applied this media to purify the immunoglobulins and Fab fragments by affinity chromatography. The finding shows the ligand 8/7 hibits the interaction of PpL with IgG and Fab by competitive ELISA and has negligible binding to Fc. The ligand 8/7 adsorbent is better than an artificial protein L to bind to immunoglobulins from different sources, in short, all this reflects the efficient isolation immunoglobulins from raw samples (Roque, *et al*., 2005)

In 1995, Bottomley isolated human IgG by using immobilized analogues of protein A for affinity chromatography. They applied a linear gradient from pH 5.0 to pH 3.0 of 0.5 M acetate buffer to elute the loaded column. In this study, the problems related to low pH elution could be decreased while the pH range for elution increased (Bottomley *et al*., 1995).

In 2001, Tu conducted a research of preparing LF bound sepharose 4B gel which was used as an affinity ligand. After the crude IgY or rabbit serum was loaded to the Lactoferrin bound sepharose 4B column, the column was washed and eluted by two kinds of buffer. All the collected fractions were treated and analyzed. This study revealed antibody specific against LF for affinity chromatography from crude IgY was more reliable than that from rabbit serum (Tu *et al*., 2001). Analogously, Chen made a preparation of Lysozyme bound sepharose 4 fast flow gel which was applied to isolate IgY in the affinity chromatrography. This research showed the binding capacity was lower and the dissociation constant was higher than both of the monoclonal antibody immunoaffinity column chromatography; in addition, this Lysozyme bound sepharose 4 immunoaffinity column was competent in sparating IgY specific against Lysozyme from yolk (Chen *et al*., 2002)

3.2.3 Novel affinity chromatography process for the purification of bioactive protein from Bovine colostrum

Ounis once used heparin affinity chromatography to separate the protein components from two whey protein solutions which produced by ion-exchange chromatography (IEC-WPI) and microfiltration / ultrafiltration (MF/UF-WPI) respectively (Ounis, *et al*., 2008). After the column was equilibrated, WPI solution was passed through the column at a flow rate of 1 mL/min, then the column was washed by 0.01 M phosphate buffer , in the wake of this, sequential elution steps were executed with 0.01 M phosphate buffer containing 0.5, 1.0 or 2.0 M NaCl. The passed solutions were collected every step and determined by the bicinchoninic acid (BCA) protein assay, Enzyme-Linked ImmunoSorbent Assay, reversed-phase high-performance liquid chromatography and 2-dimensional gel electrophoresis respectively. The results from these determinations revealed that heparin affinity chromatography had not only the capacity to separate the major proteins contained in WPIs, but also the ability of concentrating the minor cationic proteins and some growth factors.

Affinity membrane chromatography is a technique which combines membrane chromatography with affinity interaction; the membranes contain biospecific ligands on their inner pore surface. As a result of convective flow of the solution through the pores, the mass transfer resistance is tremendously reduced, and binding kinetics dominates the adsorption process. Affinity membrane chromatography provides high selectivity and fast processing for the isolation and purification of proteins. In 2007, Wolman applied affinity membrane chromatography to purify lactoferrin from whey and colostrum in only one step. The study used a hollow fibres synthesized by grafting a glycidyl methacrylate or dimethyl acrylamide copolymer to polysulfone membranes and attaching the Red HE-3B dye to them. According to the comparison between the productivity produced by Red HE-3B hollow-fibre membranes and d-Sepharose, Red HE-3B hollow-fibre membranes showed a more acceptable chromatographic performance for Lf purification from bovine colostrum than the obtained with d-Sepharose. In addition, the Lf obtained from bovine colostrum by this one-step procedure contained the casein and immunoglobulin as the only contaminants, so it could be treated as a final product practically (F.J. Wolman, *et al*., 2007; Dimartino, *et al*., 2011; Zou, *et al*., 2001)

Akita made an immunoaffinity column with specific egg yolk immunoglobulin (Ig) Y against bovine IgG1 and IgG2 and used this column to isolate the IgG1 and IgG2 from cheddar cheese whey of colostrun. The study revealed that the potential binding capacity of

IgY could come up to 38% after the immobilization by reductive amination. Meanwhile, this immunoaffinity column with specific egg yolk immunoglobulin (Ig) Y could be used to isolate the bovine immunoglobulin G subclasses from whey and colostrum specificly. (Akita & Chan, 1998)

In 1998, a study by Kim was based on application of affinity chromatography to separate the immunoglobulin G from Cheddar cheese whey. Initially, they make a preparation of IgY which is specific to IgG, then, biotinylation of IgY and immobilization of avidin columns were performed, after that, they coupled each other together and determined the binding capacity of avidin-biotinylated IgYIgG columns, finally the cheddar cheese whey was loaded and IgG was isolated. This study showed that IgG from Cheddar cheese whey could be isolated one step by the avidin biotinylated IgYIgG column chromatography. It's notable that the IgG binding capacity of this study was 50-55% and purity of the recovered IgG was 99%. There is possible for this avidin biotinylated IgYIgG column to be applied in high-purity IgG (Kim & Chan, 1998).

In 2007, Chen synthesized a micron-sized monodisperse superparamagnetic polyglycidyl methacrylate (PGMA) particles coupled with heparin (PGMA-heparin) and they isolated lactoferrin from bovine whey. In the main procedure, they made a preparation of magnetic affinity adsorbents and the whey which was going to be isolate firstly, then whey was incubated with magnetic affinity adsorbents at a certain proportion. After that, the adsorbents were eluted with the same butter respectively in different concentration sequentially. The results from analysis and determination indicate the potential application of magnetic PGMA-heparin particles for production of high purified LF from whey (Chen, *et al*., 2007).

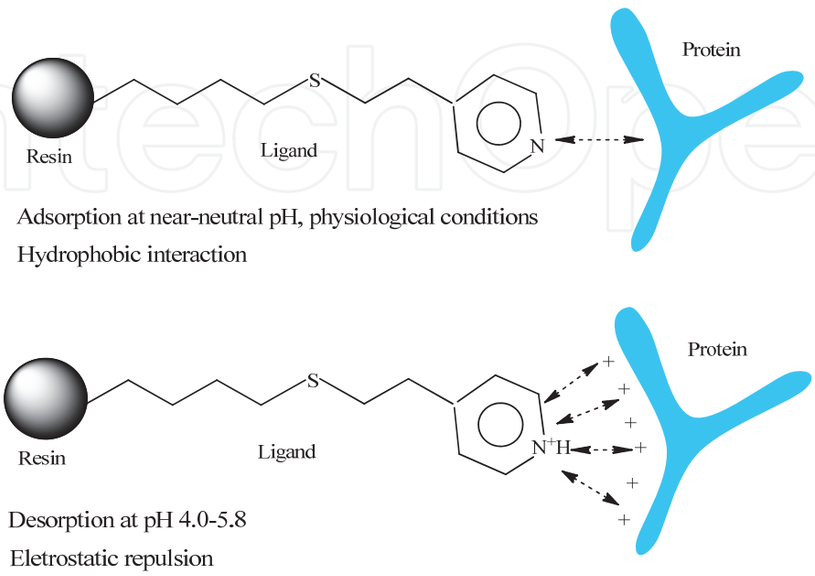
3.3 Hydrophobic Charge Induction Chromatography (HCIC)

The nutritional values and physiological benefits of Igs, a major whey protein in bovine colostrum, have received more and more attention in the last two decades. As a result, developing low cost and high efficiency purification process to fulfil the growing demand of Igs is significantly necessary. Traditionally, by taking the advantage of different isoelectric points of whey proteins, various kinds of ion-exchange sorbents have been synthesized for the purification of immunoglobulins. In practice, however, single or merely several ion-exchange chromate-graphic procedures can hardly obtain high purity protein of interest from acid whey of bovine colostrum. Hydrophobic charge-induction chromatography, or HCIC, is a novel chromatographic technique for separation of biological macromolecules, based on the pH-dependent behavior of ionizable, dual-mode ligands. Selectivity is orthogonal to ion exchange and other commonly employed chromatographic modes (Boschetti, *et al*., 2000).

3.3.1 The mechanism of HCIC

HCIC binding is based on mild hydrophobic interaction and is achieved under near-physiological conditions, without the addition of lyotropic or other salts. Desorption is based on electrostatic charge repulsion and is accomplished by reducing the pH of the mobile phase. Under mild acidic conditions (pH4.0–4.5), the ligand and target molecule take on a net positive charge; binding is thus disrupted and elution occurs. Elution is conducted using dilute buffer (e.g., 50mM acetate). The new BioSepra MEP HyperCel sorbent from Life Technologies, Inc. (LTI; Rockville, MD) has been optimized for capture and purification of monoclonal and polyclonal IgG. The heterocyclic ligand, derived from 4-

mercaptoethylpyridine (4-MEP), provides efficient capture and purification of antibodies from a broad range of sources, such as animal sera, ascites fluid and a variety of cell culture supernatants, including protein-free, chemically defined, protein-supplemented and serum-supplemented media.



At neutral pH, (top) the ligand is uncharged and binds molecules through mild hydrophobic interaction. As the pH is reduced (bottom), the ligand becomes positively charged and hydrophobic binding is disrupted by electrostatic charge repulsion.

Fig. 10. Mechanism for hydrophobic charge-induction chromatography

3.3.2 The advantage of HCIC

a. Independent of ionic strength

Compared with hydrophobic interaction chromatography (HIC), HCIC is also typified by adsorption of proteins to a moderately hydrophobic surface. However, HCIC could adsorb proteins without the presence of high concentrations of a lyotropic salt such as ammonium sulphate. HCIC matrices have a higher ligand density than HIC, therefore, it could bind proteins at low ionic strength. High ligand density (80 mmol/mL) matrices have been used for mixed mode hydrophobic ionic chromatography for the purification of chymosin, which resulted in high capacity (Burton, 1997). Furthermore, chymosin could be adsorbed at high and low ionic strength, therefore, a pretreatment step of salt addition, or removal by dialysis, dilution or ultrafiltration was not required. HCIC reduced sample preparation requirements. This method was simple, efficient, inexpensive and provided very good resolution of chymosin from a crude recombinant source.

b. pH-dependent binding

At the beginning, the matrices of HCIC absorbents contained amine linkages or carboxyl groups, therefore, the absorbents were charged at pH 4–9 range. Adsorption to an uncharged surface was only possible at pH extremes. Nonspecific electrostatic interactions could result in lower capacity and product purity and/or matrix fouling problems. Furthermore, charged groups could interfere with adsorption of target proteins. If

carboxyl/amine groups were replaced with weaker acids or bases such as imidazole, uncharged matrix form could be obtained within the pH 4–10 range. In its preferred form, adsorption is carried out under conditions which do not cause electrostatic repulsion between the protein and the matrix. However, by reducing the pH of the mobile phase, like charge are established on both ligand and protein. When pH of the mobile is reduced, the magnitude of the opposing charges depends on the pI of the target protein and the pKa of the ligand. Desorption is prompted by electrostatic charge repulsion by reducing the pH of the mobile phase.

3.3.3 Research progress and application of HCIC for protein purification

Recent efforts to improve hydrophobic interaction chromatography (HIC) for use in monoclonal antibody (mAb) purification have focused on two approaches: optimization of resin pore size to facilitate mAb mass transport, and use of novel hydrophobic charge induction (HCIC) mixed mode ligands that allow capture of mAbs under low salt conditions. Hydrophobic charge induction chromatography (HCIC), as a mixed-mode chromatography, achieves high adsorption capacity by hydrophobic interaction and facile elution by pH-induced charge repulsion between the Solute and ligand. In 2008, Chen (Chen, 2008) evaluated standard HIC and new generation HIC and HIC-related chromatography resins for mAb purification process efficiency and product quality both as isolated chromatography steps and in purification process trains. They found that the HCIC Mercapto-Ethyl-Pyridine (MEP) resin, which shows a different salt impact trend and impurity resolution pattern from standard HIC resin, can not only capture mAb from crude CHO fermentation supernatant but also substantially enhance mAb purification process flow efficiency when serving as a polishing role. Under the condition of 0.4 M NaCl, the binding capacity of MEP resin for IgG reached 30 mg/g resin near pH=7, higher than Butyl-650M resin 20.5 mg/g resin.

Large amount of study on the mechanism and optimization of HCIC resins have been conducted. Sun (Sun, 2008) reported a new medium, 5-aminoindole-modified Sepharose (Al-Sepharose) for HCIC. The adsorption equilibrium and kinetics of lysozyme and bovine serum albumin (BSA) to Al-Sepharose were determined by batch adsorption experiments at different conditions to provide insight into the adsorption properties of the medium. The results showed that the influence of salt type on protein adsorption to Al-Sepharose was corresponded with the trend for other hydrophobicity-related properties in literature. Both ligand density and salt concentration had positive influences on the adsorption of the two proteins investigated. The adsorption capacity of lysozyme decreased rapidly when pH decreased from 7 to 3 due to the increase of electrostatic repulsion, while BSA, an acidic protein, achieved maximum adsorption capacity around its isoelectric point. Dynamic adsorption experiments showed that the effective pore diffusion coefficient of lysozyme remained constant at different salt concentrations, while that of BSA decreased with increased salt concentration due to its greater steric hindrance in pore diffusion. High protein recovery by adsorption at pH 7.10 elution at pH 3.0 was obtained at a number of NaCl concentrations, indicating that the adsorbent has typical characteristics of HCIC and potentials for applications in protein purification.

In 2010, Wang (Wang, 2010) introduced the methods of molecular simulation to study the interactions between MEP and IgG. Firstly, molecular docking is used to identify the potential binding sites around the protein surface of Fc Chain A of IgG, and 12 potential binding sites were found. Then 6 sites were further studied using the molecular dynamics

simulations. The results indicated that MEP ligand tends to bind on the hydrophobic area of Fc Chain A surface. At neutral conditions, MEP can bind stably on the site around TYR319 and LEU309 of Fc Chain A, which showed obviously a pocket structure with strong hydrophobicity. The analysis of trajectory revealed that hydrogen bonds exist between MEP and the former two amino acids around the simulation period. The binding of MEP to other sites were relatively unstable, and depends on the initial binding modes of MEP. When the pH lowered to 4.0, it could be found that MEP bound formerly on the Fe Chain A departed quickly due to the electrostatic repulsion, weaker hydrophobic interaction and the disappearance of hydrogen bonds. With the aids of molecular simulations, the separation mechanism of HCIC was verified from the view of molecular interactions-the binding with hydrophobic interactions at neutral condition and the desorption with electrostatic repulsion at acid condition.

Lin (Lin, 2010) used the immunoglobulin of egg yolk (IgY) to investigate the effects of salt on HCIC. The adsorption behavior of antibody IgY on several HCIC adsorbents as a function of salt concentration was studied using adsorption isotherms and adsorption kinetics. The hydrodynamic diameters and potentials of IgY at various salt concentrations were also determined. It was found that the saturated adsorption capacities increased linearly with increasing salt concentration because of the improvement of hydrophobic interactions between IgY and the HCIC ligands. The pore diffusion model was used to evaluate the dynamic adsorption process. The total effective diffusivity showed a maximum value at an ammonium sulfate concentration of 0.2 M. The results indicate salt-promoted adsorption under the appropriate concentration due to a reduction of protein size and the enhancement of hydrophobic interactions between IgY and the HCIC ligand. Therefore, the addition of a proper amount of salt is beneficial for antibody adsorption in the HCIC process. Although certain progress has been achieved in recent years, advanced study is still necessary for the wide and mature application of HCIC.

4. Conclusion

Bovine colostrum or whey is a mixture of lactose, protein, fat and minerals. Therefore, the isolation of specific bioactive proteins such as LF and Igs is still a challenge. The application of bovine active proteins should be considered when designing the isolation protocol. With the development of application scope in food industry and biomedicine, isolation of high purity bovine proteins has attracted more and more attentions. The criteria for separation of proteins from bovine colostrum and milk or their by-products should be 1) bioactive proteins retain a reasonable recovery rate and purity; 2) utilization of organic solvents and other non-food grade chemicals is avoided because of the potential application as nutraceutical and functional foods; and 3) the separation procedures have a potential for commercialization.

To get high purity bioactive proteins from bovine colostrum in commercial scale, chromatographic procedures are essential in the process. Compared with the other chromatography separation processes, IEC, HCIC and affinity chromatography have the potential to be utilized in purification of proteins from bovine colostrum in commercial scale. The protocol of selecting a certain chromatographic procedure is based on the characteristics of the proteins in the bovine colostrum, such as their size, shape, charge, hydrophobicity, solubility and biological activity.

IEC is one of the liquid chromatography techniques which based on electrostatic interactions. Different proteins in bovine colostrum have different charges and interact

differently in ion exchange chromatography. As a main kind of bioactive protein, LF which has relative high isoelectric point (pI) compared with other milk proteins and is suitable to be isolated by this method. Many sorts of cation ion exchangers, such as CM and SP resins can be selected in purification of LF. Affinity chromatography which is based on molecular recognition or bio-recognition can be used in separation of antibodys in bovine colostrum or bovine whey with high purity, such as IgG and IgA. However, considering the production scale and cost, this technology is limited to be applied in commercial scale. Compared with affinity chromatography, hydrophobic charge-induction chromatography (HCIC) based on the pH-dependent behavior of ionizable, dual-mode ligands is a hopeful chromatographic technique for separation of biological macromolecules, especially antibodies in bovine colostrum with relative low cost and high efficiency such as high purity achieved in a single step, high protein capacity, and easy cleaning. Moreover, the small molecular substances in bovine colostrum or bovine whey, such as lactose, vitamins, and oligosaccharides, can be isolated by applying membrane filtration, especially nanofiltration and ultrafiltration.

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