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Separation technologies to produce dairy ingredients

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17.1 Introduction

Milk, the first source of nutrition for newly born mammals, is rich in components essential for life-maintaining functions and growth. These components include fat, proteins, enzymes, lactose and minerals. During many centuries humans have used the nutritional value of bovine milk through the production and consumption of bulk products, such as fluid milk and cheese. Whey, produced in large quantities during cheese manufacturing, was originally discharged to the environment. In the course of the twentieth century, human behaviour with respect to feeding their newly born and regarding their perception of the discharge of waste streams changed drastically. Tightened environmental regulations and new economic threats, such as increased discharge costs, forced the dairy industry to look for alternative outlets for their waste streams. Whey was concentrated and spray-dried, and the whey powders produced were sold to the animal feed industry at relatively low prices. As the nutrition of human infants started to divert from human to bovine milk, gradually different bovine milk-based infant formulas were demanded for newly born humans.

The dairy industry realised the value of ingredients present in whey (see Table 17.1) in that respect. Processes to separate milk and especially whey into different fractions and to isolate specific components from milk or whey to produce speciality products with a higher value were developed and implemented in the industry.

The most important separation technologies that are currently used for the production of these speciality products – crystallisation, membrane filtration and chromatography – will be discussed in the following section. Since

Table 17.1 Examples of ingredients in whey and their (potential) application

Ingredient	(Potential) application
Lactose	Drug carrier in pharmaceutical applications. Component in infant formulas (de Wit, 2001). Feedstock for production of lactulose, galacto-oligosaccharides, lactitol and other health-improving ingredients (Strohmaier, 1998, Timmermans, 1998)
Sialyllactose/sialic acid	Nutraceutical, in medical and health products (Brian *et al*., 1994; Shimatani *et al*., 1990; Horton, 1998)
Proteins:	
α-Lactalbumin	Rich in essential amino acids for nutritional requirements of growing infants. Used for enriching current infant formulas (Chatterton, 2001)
β-Lactoglobulin	Stabilises emulsions and foams. Alternative for caseinate, egg-white and gelling agents in food products (Visser and Paulsson, 2001).
Glycomacropeptide (GMP)	Therapeutic uses (Coolbear *et al*., 1998)
Immunoglobulins (IgG1, IgG2, IgA, IgM)	Defence protein. Anti-microbial function and protection of neonates against infections (Mallée and Steijns, 2001)
Lactoferrin	Defence function. Anti-microbial, antiviral, iron absorption, anti-oxidant, anti-inflammatory immune modulation, anticancer (Mallée and Steijns, 2001; Li-Chan *et al*., 1995). Application in infant formula, cosmetics, oral care products, functional foods and as additive for the prevention of *E. coli* and Salmonella growth on fresh meat in the USA
Lactoperoxidase	Anti-microbial and anti-oxidant protein (Perraudin and Reiter, 1998). Application in toothpaste and mouthwash (Horton, 1998).
Growth factors (TGF-beta, IGF-1, IGF-2, FGF-1, FGF-2,PDGF-BB)	Growth-promoting proteins (Mallée *et al*., 2001)

chromatography is a relatively new technology in dairy processing, it will be outlined in slightly more detail. The production of whey protein concentrates (WPC) and isolates (WPI) and the separation of lactose, α-lactalbumin (α-lac), β-lactoglobulin (β-lg) and defence proteins will then be discussed. Many of these dairy proteins contain fragments (bioactive peptides and individual amino acids) with specific functional properties (Meisel and Schlimme, 1996). Enzymatic hydrolysis is used to produce these fragments. This has boosted their use in infant formula and sports drinks. Recently, the isolation of these fragments has received increasing attention. The last section of this chapter will

deal with the isolation of these components and the development of alternative separation technologies.

17.2 Separation technologies

For the isolation of ingredients from milk or milk derivatives several technologies are applied commercially. The most important and generally applied ones are membrane filtration and chromatography. The principles of these technologies are outlined in this section. Crystallisation, which is used mainly for lactose production, is described briefly. Apart from these separation technologies other mature technologies, such as centrifugation, evaporation, spray-drying and precipitation, are frequently used. Examples of the use of these technologies for bulk processing in the dairy industry are:

- The removal of micro-organisms, fat and other solidified components by centrifugation
- The concentration of milk or whey and subsequent powder production using evaporation and spray-drying
- The production of cheese and the isolation and separation of proteins (e.g. α-lac or β-lg) by precipitation.

Since these technologies are common practice in dairy processing (Walstra *et al*., 1999), they will not be discussed in this chapter.

17.2.1 Crystallisation

Crystallisation features nucleation and growth of small crystals from a solution (Van der Heijden and Van Rosmalen, 1994). The formation of the crystals occurs as a consequence of supersaturation of the solution. The driving force for crystallisation is the difference in chemical potential between liquid (μ_l) and solid (μ_s) phase ($\Delta\mu_l - \mu_s$). The relative supersaturation σ gives an indication of the difference from the equilibrium state. It is defined as:

$$\sigma = \exp\ (\Delta\mu/kT) - 1$$

where k and T are Boltzmann's constant and absolute temperature, respectively.

In industrial crystallisation supersaturation of the isolated crystalline component is generally achieved via (flash) evaporation, salting-out, precipitation or cooling. For evaporation and flash evaporation, supersaturation is a consequence of the increase of the concentration as a result of evaporation of the solvent. During flash evaporation, the temperature drop further enhances the driving force for crystallisation and also contributes to the formation of the crystals. During salting-out crystallisation, the formation of crystals is achieved by adding an anti-solvent to the liquid, thereby creating supersaturation. Precipitation is usually achieved by mixing of two feeds containing the separate ingredients of the desired product. After mixing of these feeds the nucleation starts as

a consequence of the high degree of supersaturation. During cooling crystallisation, the supersaturation is a result of the temperature reduction imposed.

Even when a situation is created where supersaturation of the liquid occurs, the formation of crystals can still be absent. In this case a metastable situation is reached. Higher supersaturation (for primary or spontaneous nucleation) or initiation of crystal nucleation due to external factors (for secondary nucleation, the addition of small product crystals) is required to enter the unstable region and initiate the crystallisation process. For (flash) evaporative and cooling crystallisation generally the secondary nucleation mechanism is used and the primary mechanism can be neglected completely (Van der Heijden and Van Rosmalen, 1994). In those situations, conditions just in excess of supersaturation are required to start the crystallisation process. For precipitation crystallisation, primary nucleation is the overriding mechanism. Consequently, a large number of very small particles with sizes between 1 and 10 μm are formed. Aggregation of these particles results in the formation of larger particles. The particle size distribution is hard to control for precipitation crystallisation.

The disadvantage of crystallisation is the slow growth rate of the crystals, leading to large residence times (typically between 0.5 and 5 hours) and consequently big crystallisation vessels. Usually one crystallisation step is sufficient for the required purity of the product, since contaminants, e.g. salt ions, are not easily incorporated in the crystals. The separation of the crystals from the mother liquid is usually achieved by sedimentation, centrifugation, filtration or using a washing column.

17.2.2 Membrane filtration

Membrane filtration was introduced in the dairy industry around 1970. At present it is considered to be a mature technology with an estimated installed total membrane surface area of more than 500 000 m^2 in the dairy industry worldwide. Approximately 70% of this area is being used for treatment of whey (Timmer and Van der Horst, 1998).

Membrane filtration is a separation process that makes use of semi-permeable polymeric or ceramic materials (membranes). During the separation process, part of the feedstock will be rejected or retained by the membrane (the concentrate or retentate), whereas the other part will flow through the membrane (the permeate). The main driving force in most dairy applications is the difference in operating pressure between the concentrate (retentate) and the permeate side of the membrane (ΔP) minus the difference in osmotic pressure ($\Delta\Pi$) between the solutions at the concentrate and permeate side of the membrane. The osmotic pressure difference is a result of the difference in chemical potential between the solutions on either side of the membrane. Important characteristics of the membranes are the flow per membrane surface area (the flux, J) or the permeability (the flux per unit pressure applied, $J/\Delta P$) and the retention, defined as $1-c_p/c_r$ (c_p = concentration in the permeate; c_r = concentration in the retentate). The flux and the permeability play an important

role in the required capital investment for the membrane filtration installation, whereas the retention describes the separation efficiency of the process.

For pressure-driven membrane filtration four categories are recognised. These categories are, going from tight to more open membranes (Van der Horst, 2000):

1. Reverse osmosis (RO, typical operating pressures 3–4 MPa)
2. Nanofiltration (NF, typical pore sizes around 1 nm and operating pressures 1.5–3 mPa)
3. Ultrafiltration (UF, typical operating pressures 0.3–0.8 MPa)
4. Microfiltration (MF, typical pore sizes 0.2–2 μm and operating pressures of 0.05–0.2 MPa).

The permeation of typical components from dairy liquids through RO, NF, UF and MF membranes is presented schematically in Fig. 17.1. Generally, the flux through the membrane decreases when the concentration factor (the ratio of the feed volume to the retentate volume) is increased. When the removal of certain components from the feed by concentration alone becomes uneconomic due to too low a flux, a combination of concentration and subsequent diafiltration (DF) is used. During DF, water is added to the feed of the membrane module and a similar amount of permeate is removed.

Mechanisms for the separation of components during membrane filtration are:

- Pore size exclusion
- Donnan exclusion (based on charge interactions between the charged surface layer of the membrane and the charged components)

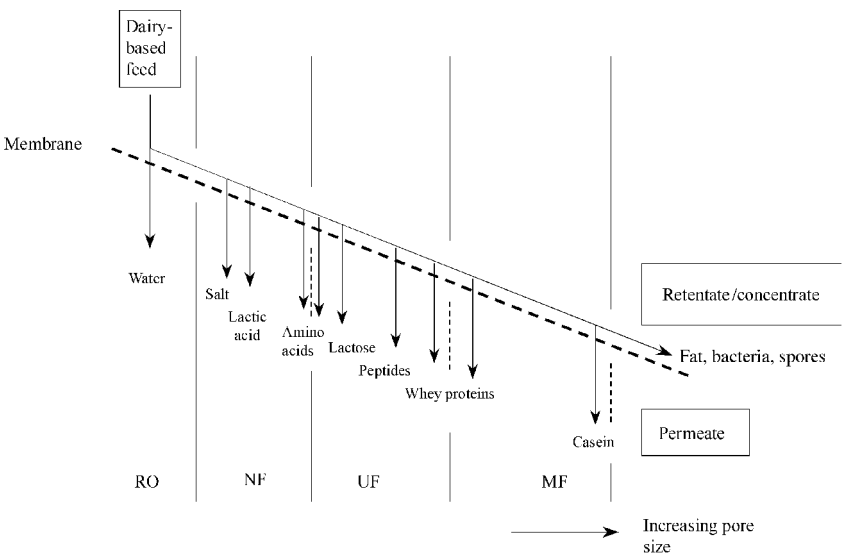

Fig. 17.1 Permeation of typical components from dairy or dairy-based liquids for RO, NF, UF and MF membranes (adapted from Van der Horst, 2000).

- Dielectric exclusion (due to a difference in static electric energy between the retentate and the liquid in membrane pores)
- Hydrophobicity/hydrophilicity of the membrane.

The membrane filtration process can be described by different models, e.g. the Extended Nernst-Planck (Schlögl, 1966) and Maxwell-Stefan (Straatsma *et al*., 2002) transport models.

To avoid or limit concentration polarisation (formation of a layer at the membrane surface, where components accumulate and the concentrations are higher than in the bulk of the retentate) and fouling of the membranes, a considerable part of the retentate is recycled to the inlet of the membrane system. This type of operation, commonly used in the dairy industry, is called cross-flow operation. The following membrane module configurations are used commercially:

- Spiral-wound modules
- Tubular modules
- Capillary or hollow-fibre modules
- Plate and frame modules.

Further information on membrane filtration can amongst others be found in Walstra *et al*. (1999) and Van der Horst (2000).

17.2.3 Chromatography

Solid/liquid chromatography is based on the adsorption/desorption of components, present in a feedstock, to/from specific (functional) groups present on the pore surface of a resin. The component targeted for isolation in a so-called capture step should preferably have the highest adsorption and lowest desorption kinetics compared to the other components under the resin loading conditions used. The retardation time of the target component is thus much higher than that of the other components present in the feed solution. The operation mode and the adsorption mechanism used during chromatography determine the success of this process for component isolation.

Operation mode

During chromatography the following operation modes are applied:

- Stirred-tank operation
- Fixed-bed operation
- Expanded-bed operation
- Radial-flow operation
- Simulated moving-bed (SMB) operation.

During stirred-tank chromatography the feed solution is brought into contact with resin particles which are kept in suspension by mechanical mixing. An advantage of this type of operation is that relatively small resin particles can be

(and have to be) used. This results in relatively low internal mass-transfer limitations in the resin particles and consequently a high particle utilisation. The main disadvantage compared to fixed-bed chromatography is the relatively low driving force for adsorption as a consequence of mixing of the liquid (ideally mixed versus plug-flow operation).

In most operation modes the resin particles are loaded into a cylindrical vessel, the chromatographic column. The feedstock is supplied to the column in a buffer solution to create optimal conditions for maximum adsorption of the target component. The column is usually operated in the traditional fixed-bed mode, where the particles are fixed between distinct borders. The column can be operated in up-flow (liquid supply at the bottom and withdrawal at the top) or in down-flow (liquid supply at the top and withdrawal at the bottom) mode. In both cases the column is operated liquid-full at relatively low liquid superficial velocity. The selection of the particle size is usually a compromise between a low pressure-drop (large particles) and a low mass-transfer limitation in the particle (small particles). The selection of the pore size of the particles is a compromise between sufficiently effective diffusion of the target component in the pores, sufficiently large pores, and a large uptake capacity of the resin, usually relatively small pores. The maximum uptake capacity of the resin, which is resin- and component-specific, can be determined by measuring a break-through curve (the amount of the target component which can be adsorbed before the concentration of the target component at the outlet starts to increase to the inlet concentration).

In industrial applications the feedstock is switched off or switched to another column (swing mode) when a certain fraction of the maximum uptake capacity is reached. The column is washed and subsequently a different buffer solution is supplied to the loaded column to desorb the components adsorbed to the resin (elution process) and to regenerate the bed. This change in buffer solution may be done stepwise or using a gradient going from the adsorption buffer to the final desorption buffer. The main advantage of chromatography is the high separation selectivity, especially compared to membrane filtration. Disadvantages of fixed-bed chromatography are the relatively high costs and possible plugging during processing of feeds with a high fouling tendency. To avoid plugging, often a pre-treatment step, e.g. MF, is required.

This latter disadvantage can be avoided by using expanded-bed operation, where the column is operated in up-flow mode. The superficial liquid velocity must be sufficiently high to exceed the minimum fluidisation velocity of the particles (the solid particles experience a drag force that exceeds the gravitational force and the interaction forces between the particles) to maintain bed expansion. Furthermore the liquid velocity should be sufficiently low to avoid strong mixing of the particles and liquid in the column, thus approaching plug-flow conditions for the liquid phase. The volume of the expanded bed may be two to three times the initial bed volume. In this operation mode generally relatively high-density resin particles are used to allow a sufficiently high superficial velocity and liquid throughput. This can be achieved by the use of an

inert high-density core in the resin particle (Olander *et al*., 2001). Desorption of the captured component takes place in fixed-bed mode.

Recently, radial-flow chromatography was introduced in the dairy industry as an alternative to fixed-bed columns (Nielsen, 2000). In this chromatographic process the resin particles are confined between two concentric cylindrical tubes 11–15 cm apart. The feed is supplied to the top of the outer tube and withdrawn from the column at the bottom of the inner tube. The main advantage of this patented Sepralac® technology is the high utilisation of the resin in combination with a low pressure-drop over the system.

SMB chromatography was also recently introduced (Horton, 1998) for the separation of valuable proteins from dairy streams. This technology features a series of chromatographic columns and a periodic change of the introduction points of feed, washing liquid and regenerate or a frequent rotation of the columns placed on a carousel (see Fig. 17.2) using fixed introduction points of feed, washing liquid and regenerate. For the latter operation type, the carousel is rotated such that each column changes one position (see Fig. 17.2). In both cases counter-current operation of the liquid and resin is obtained.

SMB chromatography allows for continuous operation and leads to the use of less resin and buffer volume. The main disadvantage of this system is the relatively high capital investment costs. However, for applications where resin

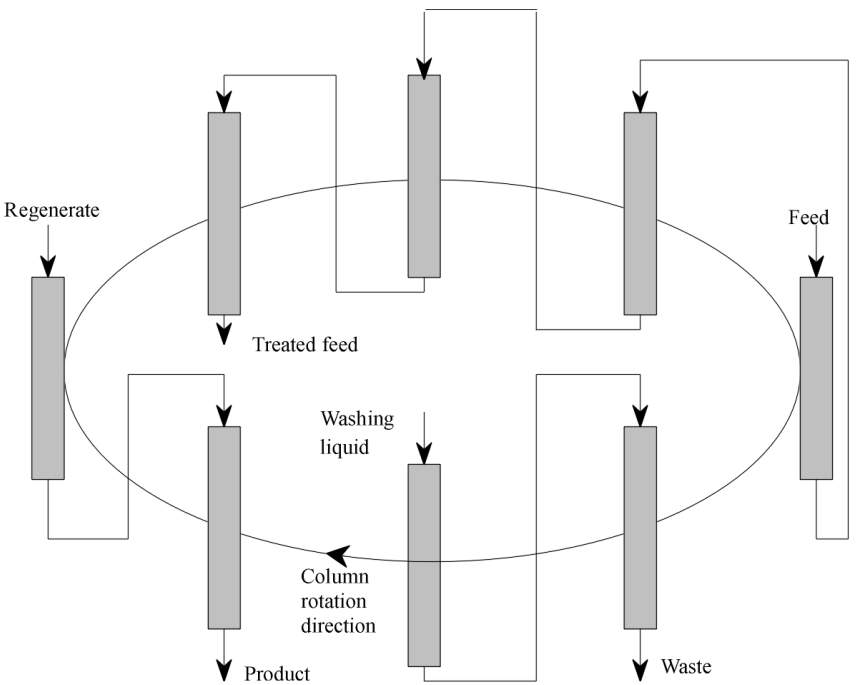

Fig. 17.2 Schematic representation of SMB operation.

replacement costs are the most important cost factor, this system may be an attractive alternative to the fixed-bed contactor.

Adsorption mechanisms
During chromatography the following types of adsorption can be applied:

- Ion-exchange chromatography (IEC)
- Hydrophobic interaction chromatography (HIC)
- Affinity chromatography.

IEC is the most widely applied within the dairy industry. The adsorbent is usually a polymeric resin with ionogenic functional groups. Anion-exchange resins contain positively charged surface groups such as NH_3^+ or NR_3^+, whereas in cation-exchange resins negatively charged surface groups such as CO_2^- or SO_3^- are present. The counter-ions of the charged surface groups present in fresh or regenerated resins to maintain electroneutrality are exchanged with the charged target components during the adsorption (capture) step. The charge of target proteins or protein fragments can be manipulated by adjusting the pH of the solution sufficiently in excess (for a negatively charged target component) or below (to obtain a positively charged target component) the iso-electric point (pI) of the target component. Desorption of the target component can be induced by changing the pH or the ionic strength of the buffer solution, thereby changing the net charge of the adsorbed component and the adsorption/desorption kinetics of the component to the surface of the resin.

In HIC, adsorption of the molecules to the resin matrix (usually sugar polymers) is based on the hydrophobic interaction between the functional (e.g. ethyl, octyl or phenyl) ligand on the surface of the resin pores and a hydrophobic group present in the component targeted for isolation (Marx, 1999). The adsorption activity of hydrophobic components to the matrix is generally high at high salt concentrations. To induce desorption of captured molecules from the HIC resins, eluents with decreased salt concentration are used.

Affinity chromatography is based on the interaction of a special component from the feed solution to a ligand (receptor). An example is the use of Protein A as ligand for the isolation of immunoglobulin G (IgG). Generally resin investment and replacement costs for affinity chromatography are much higher than for IEC, as a consequence of the high costs for production, isolation and binding of the ligands. However, in specific situations, when much higher binding capacities can be reached or binding using other technologies is not feasible, affinity chromatography is used.

17.3 Isolation of ingredients

17.3.1 Production of whey protein concentrates (WPC) and isolates (WPI)

Whey contains, apart from water, mostly lactose (72% of the total solids), minerals (8% of the total solids) and whey proteins, e.g. α-lac, β-lg, bovine

serum albumin (BSA), Ig and lactoferrin (LF). Whey powders produced through evaporation and spray-drying are, due to their unbalanced composition, not attractive for human consumption and are mainly used in animal feed (De Wit, 2001). The introduction of membrane filtration, especially UF, and in a later stage NF, has resulted in the production of whey powders with a higher value. The simultaneous concentration and demineralisation of acid, salty or sweet whey by NF creates products which are used in human foods, e.g. chocolate. More information about NF for the production of whey powders and advantages of NF over a process comprising concentration by RO and demineralisation by electrodialysis (ED) is described by Bargeman *et al.* (2003).

UF of whey results in simultaneous concentration, demineralisation and lactose removal from this feedstock (see Section 17.2.2) and consequently a whey concentrate with a protein/total solids ratio higher than originally present in whey. After further concentration and spray-drying whey powders with protein percentages, based on total solids, of 35 (WPC35), 50 (WPC50), 60 (WPC60), 70 (WPC70) or 80 (WPC80) are produced (Van der Horst, 2000). Functional properties and applications of these different WPCs have been reported by De Wit (2001). The quality and composition of the WPC powder produced depend on (Van der Horst, 2000):

- Whey quality and history
- Pre-treatment prior to UF
- UF process conditions
- Further processing of UF retentate
- Storage.

Usually, spiral-wound UF membranes with a molecular weight cut-off of around 10 000 daltons are used to minimise lactose and maximise the protein retention. It is crucial to avoid the loss of whey proteins as much as possible. The retentions for monovalent cations and anions during UF are close to zero. However, since the anions, e.g. phosphates and carbonates, present in whey are slightly larger than the cations, the retention for the latter is usually slightly lower. To maintain electroneutrality in the permeate, the lower anion salt transport is compensated for by a slightly higher OH^- transport. Consequently, the pH of the UF permeate is typically 0.04–0.10 higher than that of the feed (Walstra *et al.*, 1999).

The concentration factor typically required for the production of different WPCs and the typical protein contents of these WPCs are shown in Fig. 17.3. The membrane flux during UF is reduced when the concentration factor is increased, as a consequence of the strongly increasing protein concentration in the retentate and consequently an increasing viscosity (Van der Horst *et al.*, 1992; De Wit, 2001). For protein percentages in the retentate as high as 60%, the flux is reduced so much that DF operation becomes required to further increase the protein concentration if desired. Using DF, protein percentages as high as 80% can be obtained (De Wit, 2001).

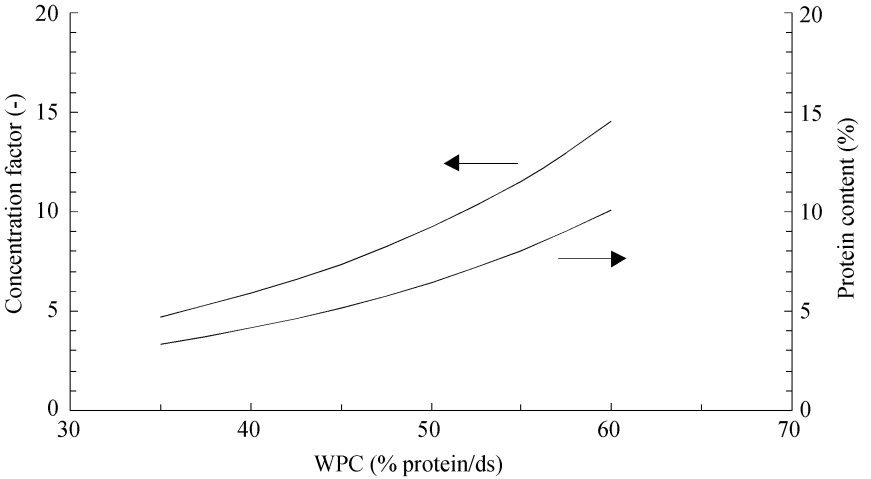

Fig. 17.3 Typical protein contents and required concentration factors for WPC.

Originally, UF was carried out at temperatures between 50ºC and 60ºC. During concentration at these temperatures precipitation of calcium phosphate in the pores and gel or concentration polarisation layer of the membrane may occur, resulting in membrane fouling and a strong flux reduction. This can be avoided when the whey is pre-treated prior to UF by one of the following methods (Walstra *et al*., 1999):

- Increasing temperature or pH to allow precipitation and removal of calcium phosphate by dead-end filtration before UF
- Reducing pH to avoid precipitation during UF
- Selective removal of calcium before UF.

The solubility of calcium phosphate is much higher at lower operating temperatures. To avoid membrane fouling, more and more UF installations for WPC production are operated at temperatures between 10ºC and 15ºC. Thus the pre-treatment steps required for high temperature operation can be avoided. A disadvantage of low-temperature operation is the considerably higher viscosity of the permeate. A reduction of the temperature from 50ºC to 10ºC increases the permeate viscosity by a factor of 2, resulting in a 50% lower flux. However, due to the strongly reduced membrane prices, this is not regarded as an important drawback. Furthermore, microbial growth at temperatures below 15ºC is substantially slower than at temperatures above 50ºC and especially at temperatures between 15ºC and 50ºC. Low-temperature operation consequently results in a decreased cleaning frequency. It should be realised that the actual flux and retentions, and consequently the compositions of the permeate and retentate, strongly depend on the membrane used and the history of the whey treatment.

WPIs have a protein content of 90% or higher. The main non-protein component of WPC80 is fat (De Wit, 2001), which is retained in the concentrate

during UF/DF. Consequently, for the production of WPI a separate fat-removal step using MF is required, prior to UF/DF and subsequent concentration. Alternative processes, featuring stirred or fixed-bed cation-exchange, were introduced for production of WPI from acid whey and acid casein whey (Etzel *et al*., 1998; De Wit, 2001). The composition and functional properties of the WPIs produced from these different sources and using these different processes differ substantially (De Wit, 2001).

17.3.2 Separation of lactose and sialyllactose

Lactose, a disaccharide, is unique to milk. Its content in whey is typically 4.6–5.2%. Several grades of lactose, varying from industrial via edible to pharmaceutical grade, are produced commercially (see Table 17.2). The yellowish colour of some of the lactose grades is a result of the presence of riboflavin, also known as vitamin B2. Generally tomahawk-shaped α-lactose crystals are produced. However, for use in direct compression of pills, anhydrous lactose, β-lactose or spray-dried lactose (a combination of monohydrate and amorphous lactose) are manufactured (De Boer and Dijksterhuis, 1998).

The traditional industrial process for the isolation of lactose involves pasteurisation and subsequent concentration of whey, using conventional evaporation (Strohmaier, 1998). In some cases the whey is pre-concentrated using NF or RO. The degree of concentration that can be achieved is limited by the onset of salt precipitation, resulting in fouling of the evaporator tubes. When the salts in the concentrated whey are allowed to crystallise in a separate vessel prior to further concentration of the whey, this problem is largely avoided. Lactose crystallisation occurs at the same time (Walstra *et al*., 1999). The required crystallisation time is reduced, and the fraction of lactose crystallised (the lactose yield) is increased, when higher concentration factors in the evaporator are used (see Table 17.3). Riboflavin is one of the crystal growth inhibitors present in whey. To avoid the production of very large lactose crystals as a consequence of slow nucleation and slow crystal growth, seed lactose crystals are supplied to the crystallisation vessel to enhance the secondary nucleation and the amount of crystals formed.

Table 17.2 Lactose grades

Lactose grade	Minimum lactose content (%)	Typical application	Colour
Industrial grade	98.0	Feed, fermentation, technical applications	Light yellow
Food grade	99.0	Bakery, confectionery, baby food, infant formula	White to pale yellow
Pharmaceutical grade	99.8	Excipient in pharmaceuticals	Pure white

Table 17.3 Effect of dry solids content and crystallisation time on lactose yield (based on Roetman, 1982)

Dry solids content in whey (%)	Fraction crystallised after 2 h	Fraction crystallised after 4 h
42.6	8	16
54.0	43	58
59.5	76	78
63.4	85	87

After the lactose crystallisation the crystals are separated and dried using a special lactose dryer (Strohmaier, 1998). Depending on the required lactose quality further refining may be required. During purification of the α-lactose hydrate crystals by recrystallisation, lower crystal growth rates are obtained as a consequence of the presence of a mixture of lactose monophosphates. These can be removed by ion exchange (Walstra *et al*., 1999).

Since the introduction of the WPC processes, lactose is also separated from the whey proteins using UF (see Section 17.3.1). This is a process option widely used in the dairy industry with more than 160 000 m^2 UF membrane surface area implemented worldwide (Timmer and Van der Horst, 1998). The UF permeate contains, apart from lactose, significant amounts of minerals. NF is frequently used for the demineralisation of UF permeate prior to lactose crystallisation, to increase lactose yield and reduce crystallisation costs as a result of reduced washwater requirements. The NF installation is at present usually operated in cross-flow operation at temperatures below 15ºC. This process is described in more detail by Bargeman *et al*. (2003).

Milk and whey furthermore contain minor amounts of sialyloligosaccharides such as 3′-sialyllactose, 6′-sialyllactose and sialyllactoseamine (Brian *et al*., 1994). These sialic acid-containing lactose molecules are anti-adhesives and anti-infectives. These valuable components (typically $60 000–100 000/kg) are used for infant formulas and as effective ingredients for drugs and foods (Shimatani *et al*., 1990). Snow Brand Milk Products Company (Shimatani *et al*., 1990) and Neose Pharmaceuticals Inc. (Brian *et al*., 1994), amongst others, have patented processes for the isolation of these components.

In the Snow Brand process (Shimatani *et al*., 1990), ED or ion exchange using a cation and subsequently a strongly basic anion-exchange resin or a combination of these processes is used to remove remaining proteins and ions from deproteinised whey or skim milk. Seeding, e.g. by addition of α-lactose, and drying follow these process steps, to produce a sialic acid-containing lactose product. Products containing typically 50 mg sialic acid per 100 g lactose are thus obtained.

In the Neose process preferably mother liquid from lactose crystallisation is used as process feed. Positively charged proteins and ions are removed from the mother liquid using, e.g., cation-exchange chromatography, prior to the use of

anion-exchange chromatography for the adsorption of the sialyloligosaccharides. Typical superficial velocities used are in the range of 2–15 cm/min. Elution of the adsorbed sialyloligosaccharides is achieved using a lithium or sodium salt buffer. The elution procedure using lithium salts is claimed to be the key factor in the success of this process. The elute can be concentrated and dried. When sodium salts are used the elute has to be desalted, using, for instance, NF/DF prior to drying. Lithium salts can be removed from the lithium sialyloligosaccharides by washing the obtained solids using organic solvents at temperatures between 0ºC and 5ºC. Isolation of individual sialyloligosaccharides from the mixture of lithium sialyloligosaccharides obtained can be achieved using anion-exchange chromatography (Brian *et al*., 1994). Ion-exchange steps are performed at room temperature. An alternative to this isolation procedure is a solvent extraction process using, e.g., packed columns of lactose or using supercritical CO_2 (Brian *et al*., 1994).

17.3.3 Separation of *α*-lactalbumin and *β*-lactoglobulin

A further step in the separation of whey proteins is the isolation of individual whey proteins. Applications of purified α-lac and β-lg are listed in Table 17.1. Several processes have been developed and reported for the isolation of α-lac and β-lg. In some cases only one purified fraction is produced. This section focuses mainly on simultaneous production of purified α-lac and β-lg fractions.

Two production process categories can be distinguished. One uses a phase-transition step (precipitation of α-lac or β-lg), whereas the other is carried out without phase transition (Timmer and Van der Horst, 1998). An example of the first category, starting from a WPI, features the following steps (De Wit and Bronts, 1995):

1. Cation-exchange chromatography to capture calcium and to destabilise α-lac
2. Separation of the resin and the liquid phase
3. pH adjustment of the product solution to 4.3–4.8
4. Incubation of the product at a temperature between 10ºC and 50ºC to promote α-lac flocculation
5. Separation of an α-lac-rich fraction and a β-lg-rich fraction using, e.g., centrifugation or MF
6. Increasing the pH of the α-lac-rich fraction to redissolve the fraction.

α-Lac-rich (62% pure at 80% yield) and β-lg-rich (85% pure at 85% yield) fractions are thus produced simultaneously. The purity of the redissolved α-lac-rich fraction can be increased further to 74% at the expense of yield (reduced to 70%) using 0.1μm MF.

The process described by Stack *et al*. (1995) uses similar steps. However, in their process the demineralisation using ED and IEC is done on raw whey. Subsequently a heat treatment is carried out and lactose crystallisation is allowed before the pH is adjusted and precipitation of α-lac is induced. After the

separation of the fractions, UF or MF and subsequent spray-drying is used to produce powders rich in α-lac and β-lg, the latter with 94.6% purity, respectively. The purity of the β-lg fraction is mainly limited by the presence of IgG and caseinomacropeptide (CMP), which do not precipitate (Timmer and Van der Horst, 1998). Consequently, the production of β-lg from acid casein whey results in higher purity as compared to sweet whey, as a result of the absence of CMP in this whey type (Gésan-Guiziou *et al*., 1999).

The separation of α-lac and β-lg without phase transition has the advantage that the likelihood of protein denaturation is reduced considerably. The use of membrane filtration for this process has been studied extensively (Timmer and Van der Horst, 1998). However, membrane filtration alone does not result in production of very pure products. This is due to the relatively small difference in the apparent molecular weight between α-lac and β-lg (approximately 14.000 and 36.000 g/mol, respectively). Furthermore the difference in iso-electric points (pI) between α-lac and β-lg is small (4.2–4.5 and 5.1, respectively). Therefore the difference in size exclusion and Donnan exclusion for these components is too small to obtain sufficient separation efficiency and production of α-lac and β-lg fractions with sufficient purity simultaneously. Production of enriched α-lac fractions, with purity as high as 90%, using membrane filtration is reported by, e.g., Timmer and Van der Horst (1998). Furthermore, membrane filtration can be used as part of the separation process, sometimes in combination with a precipitation process (Timmer and Van der Horst, 1998; Van der Horst, 2000).

Several processes without phase transition use IEC for the purification of α-lac and β-lg. In these processes ion-exchange is not used for removal of calcium, leading to destabilisation and precipitation of α-lac, but to capture proteins and elute them selectively. Outinen *et al*. (1995) use anion-exchange chromatography for the separation between α-lac and β-lg. Their adsorption process is carried out at a pH between 6 and 7, where both components have a net negative charge. The majority of the α-lac flows through the anion-exchange column, whereas most of the β-lg is adsorbed to the resin, despite the higher pI of β-lg. The water used for washing the loaded column is added to the run-through material for production of a purified α-lac fraction. The adsorbed β-lg is eluted using a 2–5% wt NaCl solution. The ratio of α-lac over β-lg for the α-lac-rich and β-lg-rich fraction was typically 2.2 and 0.07, respectively, for Diaion HPA 75 (Resindion, Mitsubishi Kasei Corp., Japan), a strong anion-exchange resin. The reported yields for α-lac in the α-lac-rich fraction and for β-lg in the β-lg-rich fraction were typically 78% and 87%, respectively. Contaminants were Ig, BSA and orotic acid in the α-lac-rich, and Ig and BSA in the β-lg-rich fraction. Glycomacropeptides (GMP) were removed from the whey prior to the anion-exchange process through precipitation and removal of the precipitate. The flow rate used during this process was relatively low, 3–5 column volumes per hour.

Alternatively, Etzel (1999) proposes the use of a single cation-exchange step. Prior to ion-exchange the pH of the whey is reduced below 4.5. At this pH, α-lac and β-lg are captured by the cationic resin as a consequence of their net positive charge. The adsorbed α-lac and β-lg are eluted as different fractions using

eluents with different pH. Although the pI for α-lac is lower than for β-lg, surprisingly a β-lg-rich fraction is obtained at pH 4.9. Subsequent elution at pH 6.5 produces an α-lac-rich fraction.

17.3.4 Isolation of defence proteins and growth factors

During the last decade special attention has been paid to the development and implementation of industrial production processes for purified defence proteins such as lactoferrin (LF), lactoperoxidase (LP), immunoglobulins (Ig) and growth factors. These valuable proteins are present in bovine milk and whey in low concentrations, e.g. 10–30 mg LP/l whey and 30–100 mg/l whey (Chiu and Etzel, 1997; Kussendrager *et al*., 1997). LF, a salmon-coloured powder, is used to enrich infant formulas (Burling, 1994), since bovine milk is deficient in LF as compared to human milk. The latter has a 20-fold higher concentration (Chiu and Etzel, 1997). LP is used in toothpaste to reduce caries. Several other applications of these proteins are listed in Table 17.1.

The pI values of LF and LP are 7.8–8.0 and 9.2–9.9, respectively (Groves, 1971). Other proteins present in milk or whey have much lower pI. Consequently, LF and LP are positively charged at pH 6.5, whereas the other proteins are negatively charged. Industrial processes (see Fig. 17.4) to isolate these minor proteins from especially whey, a relatively low-cost feed, as developed by Burling (1989; 1994), Kussendrager *et al*. (1997) and Sato *et al*. (1996), consequently use cation- exchange chromatography. In these processes generally a pre-treatment step is used to remove micro-organisms, fat and protein aggregates and to avoid clogging the fixed-bed cation-exchange chromatography column. MF using 1.4 μm pore diameter membranes is often used, but also other technologies like bactofugation (centrifugation), UF or coarse filters can be applied (Kussendrager *et al*., 1997). The use of pasteurisation is avoided to prevent inactivation of these very heat-sensitive defence proteins as a consequence of protein denaturation. Depending on the procedure used to elute the proteins adsorbed in the resin pores, LP and LF can be obtained as a mixture or as separate products (Kussendrager *et al*., 1997). During elution LP is desorbed first, while LF remains adsorbed to the resin and is eluted later.

The Sato process also produces secretory immunoglobulin A (SIgA, secretory component associated with immunoglobulin), a component preventing

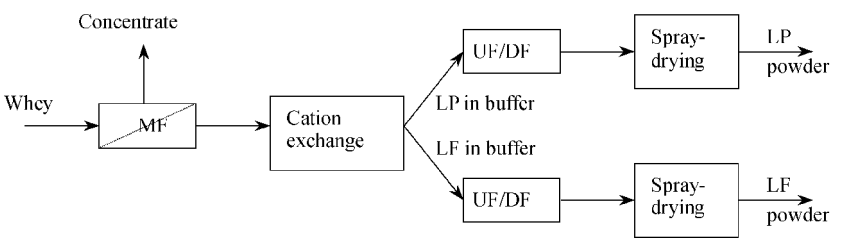

Fig. 17.4 Typical industrial process for the isolation of lactoferrin and lactoperoxidase.

infectious diseases for infants. The following elution procedure is used, after the adsorption and washing step, to obtain the components separately (Sato *et al*., 1996):

1. Selective elution of LP using a buffer with an ionic strength between 0.2 and 0.5 and a pH $\leq$ 5
2. Selective elution of secretory component using a buffer with an ionic strength of 0.1–0.5 and pH > 5
3, Selective elution of LF using a buffer with an ionic strength in excess of 0.5 and pH > 5.

During the Sato process product purities of about 80% or higher are achieved in a single-stage cation-exchange process. Different types of adsorbers, e.g. fixed-bed and slurry adsorbers, can be used for the isolation of LP, SIgA and LF. A rotary-column adsorber is mentioned as the preferred option for efficient mass production (Sato *et al*., 1996). A serious disadvantage of this adsorber compared to the other adsorber equipment mentioned is the presence of rotary parts.

The Burling and Kussendrager processes use similar ionic strength buffers for the elution of LP and LF, but no variation in pH is applied. The Burling process provides products with purities in excess of 95%, as commercially proven in the Kristianstad dairy plant (Burling, 1994). Furthermore, the cation-exchange step results in a more than 500-fold concentration of the product. In the process described by Burling and Sato for fixed-bed cation-exchange, low flow rates are used (typically 1.25 bed volumes/min) resulting in very long loading times (15–20 h). The low flow rates used are a consequence of the small particle size of the resins used in these processes and the pressure drop limitations of the system. The long loading times are clearly a disadvantage of these processes.

In the fixed-bed cation-exchange process of Kussendrager *et al*. (1997) the use of high superficial velocities (in excess of 500 cm/h) and high liquid loads (100–600 bed volumes/h) is achieved by the use of SP Sepharose Big Beads. This resin has particle sizes in excess of 100 μm. Pressure-drops in excess of 10 bar/m and even as high as 40 bar/m can be applied. More than 80% of the LF and LP present in the whey feedstock is captured in this process.

Apart from conventional fixed-bed chromatography, SMB chromatography is also used for the isolation of LF from whey, e.g. at Agri-mark Vermont. As an alternative capture step, affinity chromatography is mentioned in several publications and patents. However, this capture step is usually much more expensive than IEC and consequently not preferred for the isolation of LF and LP.

In practically all cases the eluted fractions have to be demineralised using UF/DF or ion exchange, and dried to produce the desired products. Spray-drying and freeze-drying are used for the production of LP and LF powders. The advantage of freeze-drying is the low heat treatment of the valuable products. The main disadvantage of this technology is its high process costs. Especially, the investment, energy and operating costs for spray-drying are lower than for freeze drying.

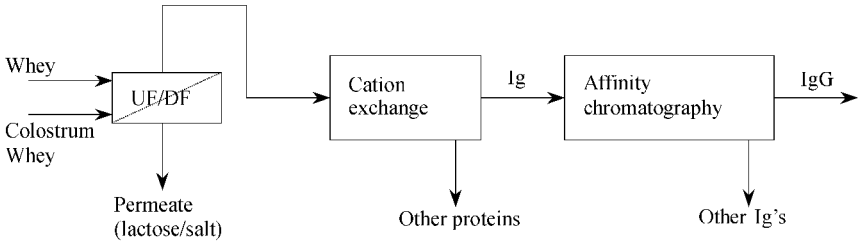

Fig. 17.5 Isolation of Ig and IgG (adapted from Mallée and Steijns, 2001).

Immunoglobulins are the best-known antimicrobial proteins (Mallée and Steijns, 2001). Bovine milk, but especially colostrum, contains significant amounts of immunoglobulins (especially IgG). Obviously, colostrum is a good source of Ig, but the availability of this feed is limited. Furthermore, the concentration of Ig in colostrum drops relatively fast during the first days after the birth of the calf. Commercial processes use whey from colostrum and milk, to produce enriched fractions, which may contain 80% Ig after UF/DF. Further purified Ig and IgG products may be obtained by the use of IEC and affinity chromatography (Mallée and Steijns, 2001). The isolation of Ig and IgG is schematically shown in Fig. 17.5.

Apart from the traditional fixed-bed chromatography process, expanded-bed chromatography can also be used for the simultaneous isolation of LP, LF and IgG from whey and subsequent production of WPC or WPI from the remaining proteins in the whey (Olander *et al.*, 2001). For the use of this expanded-bed adsorption pre-filtration is not required.

An alternative process for the isolation of Ig from whey or whey derivatives features precipitation of other proteins by supplying a cationic polymer (e.g. chitosan) and fatty acids to the feedstock simultaneously. The precipitate can be removed by low-speed centrifugation. The remaining supernatant contains more than 60% Ig. This supernatant can be further concentrated, diafiltered and dried (Acker *et al.*, 1997).

During the mid-1990s, the isolation of growth factors from whey received more attention. Goddard *et al.* (1998) describe the production of whey growth factor extract (WGFE) using:

1. MF for de-fatting and clarification of whey
2. Cation-exchange chromatography of MF permeate to capture the growth factors
3. Concentration and desalting of the elute containing the growth factors using UF
4. Sterilising and packaging of the WGFE.

The WGFE contains a mixture of the growth factors IGF-1 (22 μg/l), IGF-2 (24 μg/l), PDGF-BB (4 μg/l), TGF-beta (3 μg/l), FGF-1 (0.2 μg/l) and FGF-2 (0.2 μg/l). Since some growth factors can have a negative effect on the activity

of others, there is a clear incentive to produce separate fractions containing individual growth factors (Mallée *et al*., 2001). Several of these processes have been patented. Most of these require many downstream processing steps or use a LP denaturation step, both of which are economically undesirable. A simpler process for the isolation of individual fractions of TGF-beta (transforming growth factor) and IGF-1 (insulin-like growth factor) from a milk product is presented by Mallée *et al*. (2001). This process resembles that proposed by Goddard *et al*. (1998) but has a second adsorption step for the isolation of the individual growth factor fractions. The process features the following steps:

1. Minimal heat treatment for sufficient reduction of the micro-organism content of the feed and minimum protein denaturation
2. MF for fat removal
3. Capture of growth factors from feed using a cation-exchange column
4. Elution
5. Capture of growth factors from elute using a hydroxyapatite column
6. Elution.

After the elution step, the following fractions are obtained:

- IGF-1 (between 50 and 500 μg/g peptide) practically free of TGF-beta
- TGF-beta (more than 200 pg/g peptide) practically free of IGF-1
- Optionally LP.

The IGF-1 and TGF-beta fractions furthermore contain approximately 30–50% Ig on a protein basis.

17.3.5 Production and isolation of bioactive peptides and amino acids from dairy proteins

Apart from the isolation of ingredients with high value like α-lac and β-lg and defence proteins, the production and isolation of active fragments from these and other dairy-based proteins such as amino acids, e.g. tryptophan and arginine, and functional peptides, e.g. phosphopeptides, antimicrobial peptides and opioid peptides, are becoming increasingly important. The active fragments are usually produced through enzymatic hydrolysis of the proteins (Visser and Floris, 2000). The hydrolysates are used directly in infant formula, and skin care and hair conditioning products. The active fragments are attractive alternatives to ingredients produced from non-natural sources using fermentation with genetically modified or pathogenic micro-organisms. Isolation of ingredients can be done using chromatography, in some cases preceded by membrane filtration as first separation step. Due to the variety of ingredients and consequently the variety in production processes, these are not discussed in further detail here.

17.4 Developments in separation technology

17.4.1 Membrane adsorbers

To overcome the long adsorption cycles and low liquid velocities required for most fixed-bed chromatography processes, membrane adsorbers using microporous ion-exchange and affinity membranes have been and are being developed. Several studies report the isolation of LF and LP using commercially available cation-exchange membranes (e.g. Sartobind^{TM} Membrane Adsorber). Chiu and Etzel (1997) used a microporous membrane with a pore size of 3–5 μm in laboratory-scale tests. This membrane contained R–CH_2–SO_3^- groups covalently bonded to the internal pore surface. LF and LP recoveries were 50% and 73%, respectively. Loading times with respect to traditional ion-exchange chromatography could be reduced significantly. Pilot-scale experiments using modules of 1 m^2 confirmed the feasibility of membrane adsorbers for the isolation of LF and LP (Ulber *et al*., 2001). A loading capacity of 2 g LF/m^2 was reported. A product purity of 85% and 95% was achieved for LP and LF, respectively. On the basis of the results a process for the isolation of LF and LP from whey is proposed similar to the traditional process proposed for ion-exchange, but replacing the fixed-bed resin column by a membrane absorber. Recently, production of lactoferrin using cation-exchange membranes was started on a commercial scale.

Etzel (1999) reported the feasibility of the isolation of enriched α-lac and β-lg fractions from whey using cation-exchange membrane adsorbers. For the isolation of IgG, affinity membranes with immobilised Protein A are being developed. These membranes are based on, e.g., composite chitosan–cellulose membranes (Yang *et al*., 2002) or nylon membranes coated with dextrans–polyvinyl alcohol (Castilho *et al*., 2000).

Since the production and isolation of biologically active peptide fragments from proteins (e.g. antimicrobial peptides) is becoming more and more important, membrane adsorbers provide new opportunities for the industry. Apart from the isolation of peptides from hydrolysates produced after enzymatic hydrolysis in stirred vessels, this technology can also be used in a different way (Recio and Visser, 1999). Prior to enzymatic hydrolysis the target protein is adsorbed by the membrane (see Fig. 17.6). Following the loading procedure, the feed is switched to a different membrane adsorber and an enzyme solution is supplied to the loaded membrane to perform the enzymatic hydrolyses of the adsorbed protein. After this hydrolysis procedure the bioactive peptides are eluted at high purity. Very pure lactoferricin, a proven bioactive peptide originating from LF, could be produced by feeding whey directly to the adsorber, following the procedure described. Furthermore the feasibility of the process for several other applications was proven. The main advantage of this procedure over the traditional one is that the entire feed is not hydrolysed, but only the adsorbed protein. The feed can therefore practically retain its initial value.

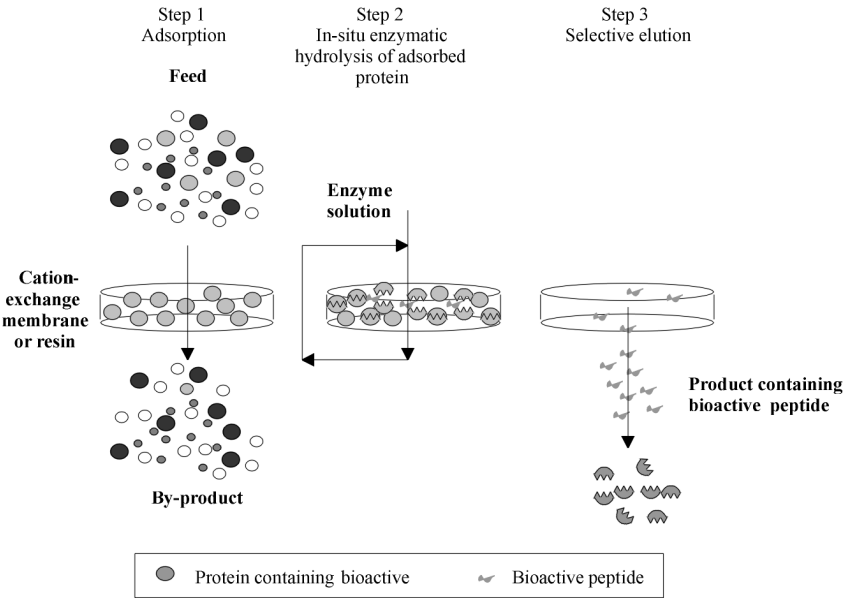

Fig. 17.6 Production of bioactive peptides by adsorption of milk proteins followed by in-situ hydrolysis of the adsorbed protein (adapted from Recio and Visser, 1999).

17.4.2 Electro-membrane filtration

Electro-membrane filtration (EMF) is currently being developed for the isolation of valuable charged ingredients such as proteins or protein fragments. This technology combines electrophoresis and conventional membrane filtration. A conventional MF, UF or NF membrane separates the feedstock from the permeate (see Fig. 17.7). The selection of this membrane depends on the size of the component to be isolated. Ion-exchange membranes are used to shield the feedstock and the permeate from the electrodes, thereby preventing degradation of these solutions and fouling of the electrodes. To obtain a selective isolation of the charged target components, the electrical field strength in the feedstock compartment is maximised in relation to convection (a function of the transmembrane pressure) and diffusion. Examples of successful separations are the isolation of lysine from casein hydrolysate (Bargeman *et al*., 2000), of antimicrobial peptides from a LF hydrolysate (Bargeman *et al*., 2000) and an α_{s2}-casein hydrolysate (Bargeman *et al*., 2002a, 2002b). EMF is very selective in the isolation of a mixture of charged components, but less selective in the isolation of individual charged components. However, for many applications in the food industry the product purities are sufficiently high. On the basis of first cost evaluations, the processing costs of EMF will be considerably lower than those for chromatography (Bargeman *et al*., 2002c). Further technology development and scale-up of EMF will be required before this technology can be implemented in the industry.

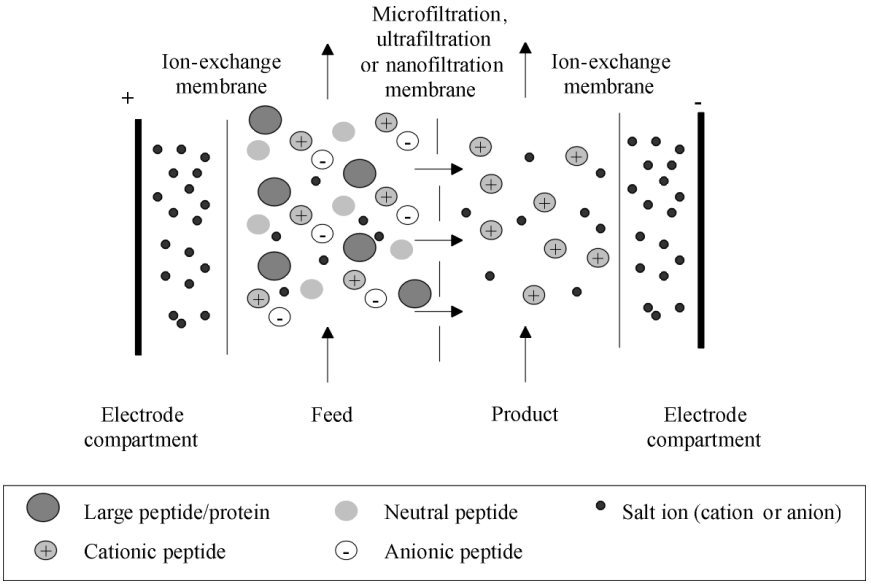

Fig. 17.7 Principle of electro-membrane filtration (EMF).

17.5 Sources of further information and advice

More information about crystallisation, membrane filtration and chromatography for protein separation can be found in the book *Basic Principles of Membrane Technology* (Mulder, 1991), in the chapter on 'Industrial mass crystallisation' in Van der Heijden and Van Rosmalen (1994) and in the book *Protein Purification, Principles, High-Resolution Methods, and Applications* (Janson and Rydén, 1998), respectively.

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