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Fractionation of major and minor Whey Proteins using Membrane-based Ion-exchange Chromatography

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Contents

Abbreviations

a	isotherm parameter
A	coefficient in van Deemter equation, m
AEC	anion exchange chromatography
Asp	aspartic acid
b	isotherm parameter
B	coefficient in van Deemter equation, $m^2 \cdot s^{-1}$
BSA	bovine serum albumin
C	coefficient in van Deemter equation, s, and conductivity, $S \cdot m^{-1}$
CEC	cation exchange chromatography
CV	column volume
c_S	concentration in liquid phase, $kg \cdot m^{-3}$
d	column diameter, m
D_0	molecular diffusivity in mobile phase, $m^2 \cdot s^{-1}$
D_{eff}	effective pore diffusivity, $m^2 \cdot s^{-1}$
D_p	molecular diffusivity in pore, $m^2 \cdot s^{-1}$
DSC	differential scanning calorimetry
F	Faraday constant, 96485 $C \cdot mol^{-1}$
G	Gibbs free energy, $J \cdot mol^{-1}$
H	enthalpy, J, and height equivalent to a theoretical plate (HETP)
His	histidine
I	ionic strength, M
IEC	ion exchange chromatography
IgG	bovine immunoglobulin G
J	flux, $kg \cdot m^{-2} \cdot s^{-1}$
K	adsorption equilibrium constant, $m^3 \cdot kg^{-1}$
L	column length, m
α-La	α-lactalbumin
β-Lg	β-lactoglobulin
Lf	lactoferrin
LPO	lactoperoxidase
M_W	molecular weight, $g \cdot mol^{-1}$, Da
MF	microfiltration
N	number of theoretical plates

pI	isoelectric point
Δp	column pressure drop, Pa
q	concentration in stationary phase, $kg \cdot m^{-3}$
Q	quaternary ammonium ligand
S	entropy, $J \cdot K^{-1}$, and sulfonic acid ligand
T	temperature, K
T_d	denaturation temperature, K
TFMC	tangential flow membrane chromatography
Tyr	tyrosine
u	linear velocity, $m \cdot s^{-1}$
V	volume, m^3
$\dot{V}$	volumetric flow rate, $m^3 \cdot s^{-1}$
WP	whey protein
WPI	whey protein isolate
x	distance, m
Y	yield

Greek symbols

δ	film thickness, m
ε_0	permittivity of vacuum, $F \cdot m^{-1}$
ε_p	intraparticle porosity
ε_r	dielectric constant
ζ	zeta potential, V
η	mobile phase viscosity, $Pa \cdot s$
κ	Debye-Hueckel parameter, inverse Debye length, m^{-1}
λ	Debye length, m, steric hindrance factor, -, wavelength, nm
σ	surface charge density, $C \cdot m^{-2}$
Ψ	electric surface potential, V
τ	tortuosity factor, -

Abstract

The present work describes a process developed for the fractionation of α-lactalbumin (α-La), β-lactoglobulin (β-Lg), BSA, immunoglobulin G (IgG), lactoperoxidase (LPO) and lactoferrin (Lf) from native whey consisting of an anion- followed by a cation-exchanger step. Ion-exchange chromatography is used with a porous membrane as the support matrix for the functional groups. These are covalently bound to the membrane surface as well as in the interior of the membrane structure. The pores inside the membrane are reached by convection. Thus, a faster mass transfer and a full utilization of binding sites is possible in comparison to conventional packed-bead columns with slow diffusional mass transfer. Furthermore, tangentially overflown membrane adsorber were evaluated for the whey protein fractionation without prefiltration of whey and the mass transfer properties were investigated. The most important difference is that in the tangential flow device the flow is not forced through the membrane pores. It is led through a gap between two membrane layers by which pore blockage due to particles and protein aggregates in whey is prevented. However, it is assumed that only the functional groups close to the membrane surface are reached convectively, whereas the inner membrane pores are accessed by diffusion.

The developed process uses food grade buffers in order to equilibrate the functional groups. The same buffers with an increased sodium chloride concentration elute the bound protein fractions one after the other at a specific ionic strength. In the first step, β-Lg und BSA are isolated from the whey on an anion-exchanger. In the second step, LPO, IgG und Lf are purified on a cation-exchanger. α-La remained in the whey serum with a recovery of 94-97%. Although the surface of α-La is negatively charged at the process conditions, α-La does not adsorb to the anion exchanger. Counter ions present in the whey serum screen the surface charges. The other target proteins are not affected by this effect, because their surface charge density is higher compared to that of α-la.

In contrast to theoretical expectations it was found that Lf does not bind completely to the cation-exchanger, although it has a strong positive net charge. Further investigations revealed, that there is a correlation between the iron saturation of Lf and the ability to adsorb to the sulfonic acid ligands. Iron-saturated holo-lactoferrin adsorbs almost completely independently from the pH in the acidic and neutral

region. In contrast, the adsorption of iron-free apo-lactoferrin decreased with decreasing pH-value. This effect was most probably the result of intermolecular interactions between the strong cationic patches on the protein surface and the negative charges in the iron binding site.

The process was transferred from lab scale to pilot scale using a customized pilot plant. The purity and yield of the protein fractions as well as the binding capacity of the membrane were highly similar at both scales. Repeated bind and elute cycles without intermediate cleaning revealed an initial drop in binding capacity which then remained constant. A cleaning-in-place with acid and base fully recovered the initial binding capacity.

Furthermore, the process temperature was varied between 10 and 50°C. Processing at ambient temperature is avoided in the dairy industry in order to reduce microbial growth. It should be determined whether hot or cold temperature has an effect on the process performance. It was expected that elevated temperatures might harm the protein structure if combined with mechanical processing. Cold temperature is assumed to decrease binding capacity. Additionally, in case of the tangential flow membrane adsorbers, slower diffusion at low temperature is expected to affect the binding capacity as function of contact time.

Contrary to the assumptions the binding capacity of β-Lg decreased with increasing temperature. The effect was observed in the different tested flow systems, but was more pronounced for the radial flow adsorber with convective transport. From the obtained results it was deduced that the temperature dependent binding capacity also correlated with the mass transfer mechanisms. The reason for the increased binding capacity at low temperature is most probably the dimerization of β-Lg below 40°C because the thickness of the adsorbed layer changes in dependence of the quaternary structure. The adsorbed layer thickness would consist of one protein molecule per ligand for monomers and two molecules for the prolate dimers provided that they adsorb to their short end. But if many ligands are available, the dimer might adsorb to the long end. At this point the transport kinetics come into play. The slower the dimers are transported to the binding sites the more ligands are available and side-on adsorption is likely to occur. For proteins adsorbed in a horizontal position, the number of surface charges interacting with the ligands is higher than in vertical position. That leads to a strong adsorption and other β-Lg molecules cannot displace the bound dimers. Thus, end-on adsorption is more likely to occur when the mass

transfer to the binding sites is fast. Hence, the binding capacity of dimeric β-Lg correlates with the kinetics of ligand occupation.

Finally, the radial and tangential flow systems were directly compared in terms of binding capacity and processing time at low temperature. With the same load ratio of whey to membrane area, the maximum binding capacity is 20% less in the tangential flow system compared to convective transport. Besides that, the ligand saturation takes more than 120 minutes in the tangential flow device and not more than four minutes using the radial flow adsorber. Hence, for the fractionation of whey proteins from native whey the radial flow adsorber is preferable to the tangential flow membranes.

Kurzfassung

In der vorliegenden Arbeit wird ein zweistufiges Verfahren zur Fraktionierung der Molkenproteine α-Lactalbumin (α-La), β-Lactoglobulin (β-Lg), BSA, Immunglobulin G (IgG), Lactoperoxidase (LPO) und Lactoferrin (Lf) aus nativer Molke beschrieben. Die Trennmethode beruht auf der Ionenaustauschchromatographie mit porösen Membranen als Trägermatrix. Funktionelle Gruppen sind auf der Oberfläche der Membran und in der Porenoberfläche kovalent gebunden und dienen der selektiven Ad- und Desorption der Zielproteine. Die inneren Membranporen werden konvektiv angeströmt. Dadurch ist, im Gegensatz zu gepackten Säulen mit diffusivem Stofftransport, ein schneller Stofftransport und eine vollständige Nutzung der Bindungskapazität möglich.

Zusätzlich wurden tangential überströmte Membranadsorber zur Fraktionierung von Molke ohne vorherige Mikrofiltration sowie deren Stofftransporteigenschaften untersucht. Der größte Unterschied zum konvektiv durchströmten Modul liegt darin, dass der Fluss nicht durch die poröse Membran gezwungen wird, sondern durch einen Spalt zwischen zwei Membranschichten entlang der Membranoberfläche fließt. Dadurch wird eine Porenverblockung durch Partikel und Proteinaggregate vermieden. Es wurde vermutet, dass der Stofftransport nur in den Poren nahe der Membranoberfläche konvektiv ist, jedoch die Poren im Inneren der Membran durch Diffusion erreicht werden.

Der Fraktionierungsprozess beginnt mit der Konditionierung der Liganden mittels lebensmitteltauglicher Puffersalze. Nach der Proteinadsorption werden mit denselben

Puffern und einem erhöhten NaCl-Gehalt diese nacheinander bei einer spezifischen Ionenstärke eluiert. Im ersten Schritt werden β-Lg und BSA mittels Anionentauscher und im zweiten Schritt werden LPO, IgG und Lf mitttels Kationentauscher getrennt. α-La verbleibt im Molkeserum mit einer Ausbeute von 94-97%. Obwohl die Oberflächenladung bei den verwendeten Prozessbedingungen negativ ist, adsorbiert α-La nicht am Anionentauscher. Der Grund ist die Ladungsabschirmung durch im Molkeserum vorliegenden Gegenionen. Bei den übrigen Zielproteinen ist dies nicht der Fall, weil die Ladungsintensität höher ist im Vergleich zum α-La.

Entgegen der theoretischen Erwartung hat sich während der Prozessentwicklung gezeigt, dass Lactoferrin nur unvollständig am Kationentauscher bindet, obwohl es stark positiv geladen ist. Untersuchungen haben ergeben, dass dies mit der Eisensättigung des Metalloproteins zusammenhängt. Während eisengesättigtes Holo-Lactoferrin im sauren bis neutralen pH-Bereich nahezu vollständig adsorbiert, bleibt bei eisenfreiem Apo-Lactoferrin mit sinkendem pH-Wert zunehmend mehr Protein ungebunden. Dieser Effekt wurde auf intermolekulare Wechselwirkungen zwischen den stark kationischen Bereichen auf der Proteinoberfläche und der anionischen Eisenbindungstasche zurückgeführt.

Nach der Prozessentwicklung im Labormaßstab wurde ein Prozesstransfer zum Pilotmaßstab an einer speziell angefertigten Pilotanlage durchgefürt. Es wurden vergleichbare Ergebnisse bzgl. Reinheit und Ausbeute der einzelnen Fraktionen sowie Bindungskapazität der Membran erzielt. Ein Belasungstest mit wiederholten Trennzyklen ohne zwischengeschaltete Reinigung hat gezeigt, dass die Bindungskapazität nur initial marginal abnimmt und im weiteren Verlauf konstant ist. Ein Cleaning-in-place mit Säure und Lauge konnte die urpsrüngliche Bindungskapatität wieder nahezu vollständig regenerieren.

Des Weiteren wurde der Einfluss der Prozesstemperatur zwischen 10 und 50°C untersucht. Der Grund ist, dass das mikrobielle Wachstum bei Raumtemperatur durch die Verarbeitung bei hoher oder niedriger Temperatur reduziert wird. Es sollte gezeigt werden, ob eine hohe oder niedrige Temperatur die Prozesseigenschaften beeinflusst. Beispielsweise könnten erhöhte Temperaturen zusammen mit mechanischer Beanspruchung die Proteinstruktur verändern. Niedrige Temperaturen hingegen könnten die Bindungskapazität negativ beeinflussen. Im Fall der tangential überströmten Membranen könnte die verringerte Diffusionsgeschwindigkeit zudem die Bindungskapazität verringern.

Die Ergebnisse haben gezeigt, dass, entgegen der Erwartungen, mit steigender Temperatur die Bindungskapazität erheblich sank. Dieser Effekt wurde in allen untersuchten Adsorbersystemen festgestellt, jedoch war er bei dem konvektiv durchströmten System besonders ausgeprägt. Es wurde gefolgert, dass die temperaturabhängige Bindungskapazität mit dem Stofftransportmechanismus korreliert. Als Ursache für die erhöhte Bindungskapazität bei niedriger Temperatur wurde die Dimerisierung von β-Lg unterhalb von 40°C vermutet. Bei maximaler Besetzung der verfügbaren Bindungsstellen würden Dimere die zweifache Höhe der adsorbierten Schicht ergeben im Vergleich zu Monomeren. Das setzt allerdings voraus, dass sich die Dimere vertikal anordnen. Aus diesem Grund hat der Transportmechanismus einen Einfluss auf die Dimeradsorption und somit auf die Bindungskapazität. Je schneller der Transport von Dimeren zu den Liganden ist, desto wahrscheinlicher ist eine vertikale, dichte Besetzung der Oberfläche. Ist der Transport langsam, stehen den Dimeren viele Bindungsplätze zur Verfügung und sie adsorbieren horizontal. Ist dies der Fall, ist die Adsorptionsstärke aufgrund der größeren Anzahl an elektrostatischen Wechselwirkungen pro Proteinmolekül erhöht. Eine Verdrängung durch weitere Dimere ist unwahrscheinlich. Somit korreliert die Bindungskapazität von dimerem β-Lg mit der Kinetik der Oberflächenbesetzung.

Schließlich wurde das tangential überströmte Membranadsorbermodul mit dem radial angeströmten Modul in Bezug auf Bindungskapazität und Prozessdauer bei niedriger Temperatur verglichen. Bei gleichem Beladungsverhältnis war die maximale Bindungskapazität im tangential überströmten Modul 20% geringer als im konvektiv durchströmten Adsorber. Zudem dauert die Sättigung aller Liganden bei der tangential überströmten Membran mehr als 120 min und bei der konvektiv durchströmten Membran vier Minuten. Somit ist für die Molkenproteinfraktionierung die radiale Bauweise den tangential überströmten Membranen vorzuziehen.

1. Introduction

Whey, from bovine milk, when conventionally used, is a low cost byproduct of cheese manufacturing. In contrast, whey with an altered composition can be a highly valuable product. For example, the price for demineralized whey (WPI) is approx. 10 times higher than for whey protein concentrates. The reason is that WPI is the basis for infant formula and is used for sports nutrition (Driskell, 2007). Even higher prices can be charged, if single proteins are isolated (Smithers, 2008). Either the purified protein gains an added value compared to the native protein mixture, because it can be used as functional food additive or the depletion of undesired proteins increases the value of whey. Therefore, the separation of whey proteins (WP) has been investigated for the last decades for the purpose of isolation or removal of single compounds. A highly selective separation technique is usually required, since WP have similar physico-chemical properties. Hence, chromatographic methods are most frequently used for this purpose (El-Sayed & Chase, 2009; Fee & Chand, 2006; Gerberding & Byers, 1998; Konecny, Brown, & Scouten, 1994; Lu, Xu, Wang, & Yang, 2007; Turhan & Etzel, 2004; Ye, Yoshida, & Ng, 2000; Yoshida, Wei, Shinmura, & Fukunaga, 2000).

The most important proteins for health beneficial applications are α-lactalbumin (α-La), lactoferrin and β-lactoglobulin (β-Lg). Lactoferrin and α-La are demanded, because of their high abundance in human breast milk, whereas β-Lg is not contained at all (Armaforte et al., 2010). α-La and lactoferrin are rich in essential amino acids. Therefore, an increased ratio of α-La to β-Lg in whey protein based infant nutrition is desired (Lien, 2003). The reason is that the protein density is limited to 2.8-4.5 g per 100 kcal and, at the same time, a given profile of essential amino acids has to be fulfilled. Both requirements lead to the necessity of depleting those protein fractions which are low in essential amino acids such as β-Lg, or to enrich the whey protein with α-La or lactoferrin. Detailed numbers and the legislative context were described by Jost, Maire, Maynard, & Secretin (1999). Lactoferrin is also a highly demanded functional ingredient. In 2012, isolated lactoferrin has obtained GRAS status and it was accepted as safe novel food by EFSA (EFSA Journal, 2012). Another reason for the removal of β-Lg from bovine whey, besides its low essential amino acid content, is its allergenic potential. It is the most allergenic protein in whey from cow's milk. In order to prevent the development of a cow's milk

allergy in infants, who cannot be breastfed, hypoallergenic food can be fed. This must not contain any epitopes of β-Lg. This is currently produced by complete hydrolysis in order to ensure the elimination of any epitopes (Bu et al., 2013). The depletion of β-Lg would allow for a native protein composition without the need for masking bitter peptides that occur in whey protein hydrolysates.

1.1 Liquid chromatography

Chromatography is a separation technique, where a solute in the mobile phase, which can be liquid or gaseous, interacts with the stationary phase. The stationary phase can be liquid or solid. It is placed in a column and, the mobile phase is passed through. The affinity of the solutes to the stationary phase is characterized by the partition coefficient K (-), which is described by the Henry law (1):

$$q = K \cdot c \qquad (1)$$

with q as the concentration in the stationary phase ($mg \cdot mL^{-1}$) and c as the concentration in the liquid phase ($mg \cdot mL^{-1}$). The linear correlation of the Henry law is valid for dilute conditions.

In the following, liquid chromatography is referred to a system containing a liquid mobile phase, which can be polar or apolar, and a solid stationary phase. In general, a chromatographic separation is governed by thermodynamic and mass transfer phenomena. The thermodynamic contributions determine the retention behavior of the target molecules and hence, the retention time. If a complex mixture of target molecules is to be separated, the separation efficiency increases with increasing difference in K. Mass transfer phenomena do not directly affect the adsorption and desorption processes but dispersion effects in the column and the kinetics of a separation process.

Another important aspect is the separation task. If it is a preparative purification, the native structure has to be maintained. High throughput and binding capacity are crucial factors. Furthermore, the process has to be robust since fluids in food technology and biotechnology are crude mixture, and the composition can vary from batch to batch. In contrast, in analytical chromatography, the aim is to identify or quantify a molecule or to obtain knowledge on the structure. The molecule is often decomposed during the process.

Adsorption equilibria

Isotherms are used to describe the adsorption equilibrium, where the concentration in the adsorbed phase is in equilibrium with the concentration in the solution. Affinity constants and maximum binding capacities can be derived from functions which describe the experimental data. The adsorption equilibrium between the liquid and adsorbed phase, as described by the linear correlation in (1), is not valid in preparative chromatography, because the sample concentration is as high as possible for an efficient process (Yamamoto, Nakanishi, & Matsuno, 1988). Protein adsorption depends on many external factors such as mobile phase composition, temperature, solute concentrations and protein-protein-interactions. Therefore, it is mandatory to measure isotherms under representative environmental and processing conditions. Due to the number of influencing factors protein adsorption in complex mixtures is almost impossible to predict. Hence, it is required to use empirical approaches. Empirical adsorption isotherms, that are exponential functions with or without a maximum, are frequently used in the design of protein chromatography. One of the most important ones is the Langmuir isotherm (Langmuir, 1918) (2):

$$q = q_{max} \cdot (K \cdot c)/(1+K \cdot c) \qquad (2)$$

where q_{max} is the maximum binding capacity ($mg \cdot mL^{-1}$). Langmuir assumed that molecules adsorb in a monolayer, that there are no interactions between the solutes, and all adsorption sites are equally accessible. In protein adsorption and undiluted systems these assumptions are not true, but the isotherm is often a good and simple approximation. Another important model is the Freundlich isotherm (Freundlich, 1906) (3) which does not have these assumptions; there is no q_{max} and a non-linear range at low protein concentration:

$$q = a \cdot c^{1/b} \qquad (3)$$

where a and b are empirical constants, with b > 1. The courses of the Henry-, Langmuir- and Freundlich-isotherms are shown in Fig. 1.

The practical applicability of these two fundamental models was investigated in numerous studies. It is known that these simple equations are not able to describe the complex adsorption process in detail, because phenomena such as protein-protein-interactions, changes of protein conformation or rate-limiting factors are not considered.
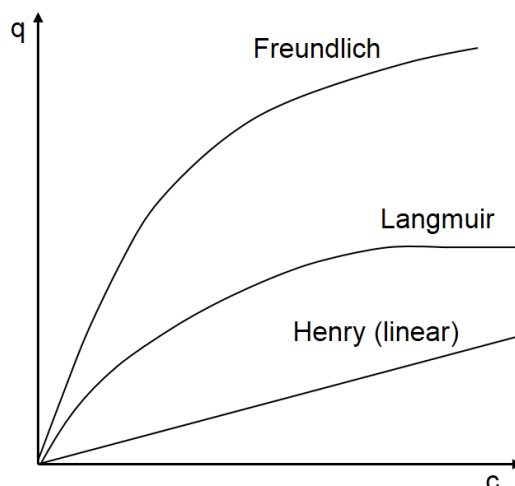
Fig. 1: Courses of adsorption isotherms: solid phase concentration q as a function of liquid phase concentration c.

However, due to their simplicity they are often applied and adequate for the determination of adsorption affinities and binding capacities, as for example Anirudhan & Senan (2011), Carta, Ubiera, & Pabst (2005) and Suen & Etzel (1994) showed. There are many other mathematical approaches for the description of the complex protein adsorption, but they are not further discussed in the present work. Recommended review papers on this topic are Foo & Hameed (2010) and Rabe, Verdes, & Seeger (2011).

Ion-exchange chromatography and thermodynamic considerations

In general, adsorption takes place when the Gibbs free energy ΔG (4) has a negative value:

$$\Delta G = \Delta H - T \cdot \Delta S \qquad (4)$$

where H is the enthalpy and S is the entropy in a given system.

The entropy increases with temperature as molecules tend to freely move in space. The release of ions and hydration water molecules, which are adsorbed to protein surfaces, is a gain in entropy. This is the driving force for hydrophobic interactions. Hydrophobic surfaces, which adsorb to each other, allow the release of hydration water and ions which is thermodynamically favorable compared to a highly ordered structure of water molecules close to a hydrophobic surface.

The enthalpic term includes electrostatic interactions, Van-der-Waals forces, hydrogen bonds and other interactions between charged species. In ion exchange

chromatography electrostatic interactions dominate and, hence, hydrophobic effects and interactions can be neglected (Ståhlberg, 1999).

In ion-exchange chromatography the stationary phase has covalently attached functional groups that are positively or negatively charged. The ligands can be further classified into weak and strong ion-exchangers. The latter are charged independently from the surrounding pH-value, whereas the weak ion-exchanger are charged across a limited pH-range. The most commonly used ligands, their chemistry and charge properties are listed in Tab. 1.

Tab. 1: Chemistry of the functional groups of common ion-exchanger types (Pharmacia Biotech AB, 1995)

Anion-exchangers	Chemistry	Type
Diethylaminoethyl	$-O-CH_2-CH_2-N^+H(CH_2CH_3)_2$	Weak, pH 2-9
Quaternary ammonium	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$	strong
Cation-exchangers		
Carboxymethyl	$-O-CH_2-COO-$	Weak, pH 6-10
Methyl sulphonate	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2SO_3^-$	strong

The main impact factors on the adsorption process in ion-exchange chromatography are discussed in the following section.

Effect of pH-value

The net charge of a protein depends on the environmental pH-value due to the dissociation of carboxyl and amino groups of the amino acids at the protein surface. If the pH is above the pI of a protein, it is negatively charged. If the pH is below the pI, it is positively charged. As a rule of thumb, an electrostatic interaction takes place when the difference between pI and pH is approx. one pH-unit. At zero net charge the protein does not interact with the charged ligands of the stationary phase.

Effect of ionic strength

The concentration of ions in the solution surrounding a protein influences the surface charge. Although it will not change the positive or negative charge sign, the ionic strength has a strong impact on the surface potential. Two factors are important for the electrostatic interaction: The surface charge density and the electrostatic surface potential Ψ. Ψ_0 at the surface of the protein can be calculated by the Debye-Hueckel-approximation (5):

$$\Psi_0 = \sigma \cdot (\kappa \cdot \varepsilon_0 \cdot \varepsilon_r)^{-1} \qquad (5)$$

where σ is the surface charge density ($C \cdot m^{-2}$), κ the Debye-Hueckel parameter (m^{-1}), which is the inverse of the Debye-length λ (m), ε_0 is the permittivity of vacuum ($C^2 \cdot N^{-1} \cdot m^{-2}$) and ε_r the dielectric constant of the solvent. The Debye-length is the thickness of the electric double layer also known as Stern-layer. Its value is simply a function of the ionic strength of the surrounding fluid and is not influenced by the surface charge density. For example, for monovalent ions it is calculated according to (6).

$$1/\kappa = 0.304 \cdot (M_{NaCl})^{-1/2} \text{ [nm]} \qquad (6)$$

Furthermore, the surface potential at any distance x from the protein surface can be calculated by the linearized Poisson-Boltzmann-equation (7).

$$\Psi(x) = \Psi_0 \cdot e^{-\kappa \cdot x} \qquad (7)$$

The net charge of a protein is measured as the potential at the Stern-layer and is known as zeta-potential (ζ). The zeta-potential can be measured experimentally and at zero net charge it is defined as the isoelectric point of a protein. From the previous equations it can be summarized that the surface potential decreases with distance from the protein surface and with increasing ionic strength. This correlation is illustrated in Fig. 2.
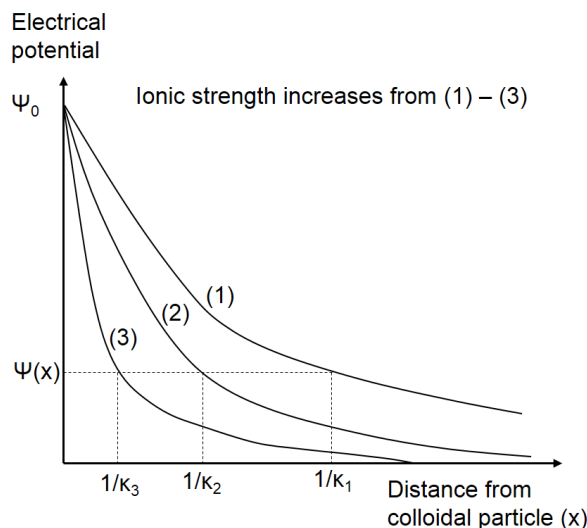
Fig. 2: Electrostatic surface potential Ψ as function of distance x from the particle surface at different ionic strengths.

An electrostatic interaction occurs when two oppositely charged species, e.g. the charged stationary phase and the protein, are in a certain distance to each other, where the attractive forces are large and the repulsive forces are small. Repulsive forces occur close to the surface as a result of sterically displaced counterions of

both, the protein and the stationary phase. However, ionic interactions are long range forces, and the effective interaction distance is around a few nanometers. At a distance larger than the Debye-length λ, ΔG becomes small and asymptotically approaches zero, even if it is still negative. It should be mentioned that the theories described in this section were developed for ideal systems and have some assumptions that are not close to reality. However, calculated and experimentally obtained values for adsorption correlate well (Ståhlberg, 1999). But proteins often do not have a homogeneous surface charge distribution and are not ideal spheres. This can lead to unexpected results, such as adsorption of a protein to like-charged surfaces. This occurs if charge patches with opposite charge to the net charge of the protein are large enough not to be compensated by neighboring charges and if the Debye-length at the patch allows an interaction (Chen et al., 2011; Xu et al., 2011). This is not necessarily the case as described by Seyrek, Dubin, Tribet, & Gamble (2003), since proteins are not ideal spheres.

Other effects of the ionic strength affect the stability of proteins, which are known as salting-in and salting-out effects. Briefly, the protein has a maximum stability at a certain ionic strength and above and below this value the protein tends to aggregate. The salting-out effect is a hydrophobic effect at high salt concentration because of a lack in hydration water. The salt molecules occupy the hydration water molecules and the proteins aggregate in order to reduce the surface area exposed to the polar liquid phase. At low ionic strength λ is very large, e.g. in 0.1 mM NaCl the Debye-length λ ($=1/\kappa$) is 30.4 nm (see eq. (6)). This is approx. ten times the diameter of a globular protein in whey. The electrostatic attraction between protein molecules has a larger radius when the ionic strength is low. This leads to aggregation which can be avoided by an increase in ionic strength. This effect is known as salting-in effect.

Effects of temperature

The temperature has a minor effect in ion-exchange chromatography since the major contribution to adsorption is of enthalpic nature. In contrast, in hydrophobic interaction chromatography temperature is crucial for the adsorption efficiency. However, temperature changes can lead to an altered binding capacity of the stationary phase and process efficiency, because of the effect on the viscosity of the mobile phase, changes in the material of the stationary phase, the temperature dependence of the buffer pH-value, etc. The latter problem can be avoided if the pH is set under final temperature conditions. Effects of the stationary and mobile phase

have been reported for packed bed chromatography (Yamamoto et al., 1988). Although enthalpic driven reactions can also depend on temperature, the enthalpy change needs to be large in order to have a significant impact on the thermodynamic equilibrium. This is generally not the case for ion-exchange interactions with $\Delta H \leq 2$ kcal·mol^{-1}. Therefore, temperature effects in IEC are normally considered negligible (Helfferich, 1995).

Mass transfer phenomena and adsorption kinetics

The mass transfer of a solute from the bulk mobile phase to the porous stationary phase, where it adsorbs, can be divided in several steps, as depicted in Fig. 3. In the bulk phase the transport is convective (blue arrow). The solid-liquid-interphase is a laminar layer, which is passed by film diffusion (black solid arrow), then the solute enters the porous stationary phase where the transport is molecular diffusion in the pores (black dotted arrow) and, finally, diffusion into the adsorbed layer towards the ligands (not shown) occurs. The convective transport is fast since it has the same velocity as the flow rate.

Convective flow

Pore diffusion
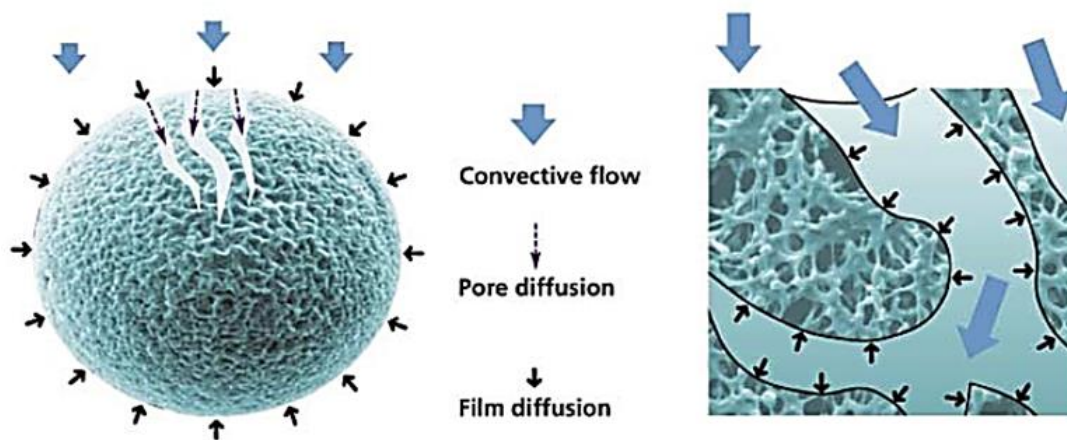
Film diffusion

Fig. 3: Schematics of the mass transfer regimes in a conventional bead (left) and a membrane adsorber (right) (Fischer-Frühholz, 2005)

Diffusion is the rate limiting step, where pore diffusion is slower than film diffusion, which is described by the following correlations. The mass transfer rate can be described by the first Fick's law (8), where the flux (J) depends on the diffusion coefficient (D), the stagnant film thickness (δ) and the concentration gradient between the bulk (c_L) and the stationary phase (c_S).

$$J = -D \cdot (c_L - c_S) / \delta \tag{8}$$

Inside the pore, the effective pore diffusion is described by eq. (9), which describes that the pore diffusion D_e is decreased by the intra-particle porosity ε_p, the tortuosity factor τ_p and the steric hindrance coefficient λ.

$$D_e = D \cdot \varepsilon_p \cdot \lambda / \tau_p \qquad (9)$$

τ_p is the ratio between the distance in a real porous matrix and the straight route between two spots in a pore. The steric hindrance coefficient λ is the ratio between the molecule diameter and the pore diameter. The larger the ratio the smaller is λ, and hence, the slower is the intraparticle diffusion. When the ratio is < 0.01, then λ = 1 and has no impact.

The separation efficiency is not only governed by the mass transfer into the porous stationary phase, but also by dispersion effects along the column length. Due to axial dispersion the injected solute is not transported as a plug flow but has a parabolic profile. This is the major reason for peak broadening, which is undesired. The narrower an elution peak the higher the probability for baseline separation, which implies that impurities of neighboring peaks are avoided.

The previously mentioned effects by mass transfer phenomena are summarized by the Van-Deemter-equation (10) which describes the height equivalent to a theoretical plate (HETP). The smaller the HETP, the higher is the number of theoretical plates N in a column with bed height L and the better is the separation efficiency of a chromatographic column.

$$H = A + B/u + C \cdot u \qquad (10)$$

where H is HETP, A the eddy term, B the axial dispersion term, C the mass transfer term and u the linear velocity ($cm \cdot s^{-1}$). The different terms of the Van-Deemter-equation show that several factors have an influence on the separation efficiency and that the flow rate has positive and negative contributions. The A-term describes that eddies lead to a mixing in the interstitial volume between the packed beads which is independent of the flow rate. The axial dispersion (B-term) becomes smaller with higher flow rate, whereas the C-term increases with the flow rate because the time for diffusion into the pores is limiting. The HETP as function of flow rate v is shown in Fig. 4. The resulting HETP-curve is the sum of the three terms A, B and C.
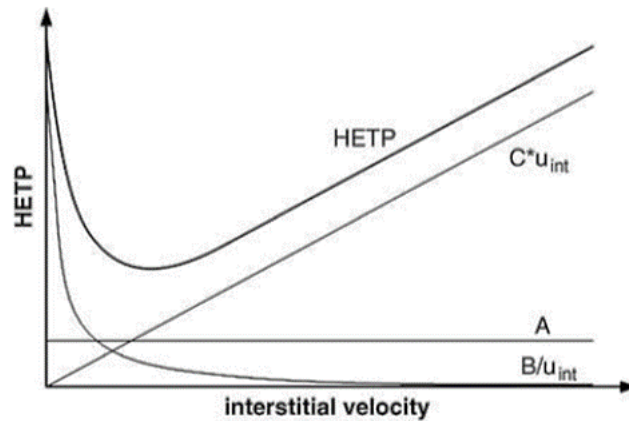
Fig. 4: Relationship between HETP and interstitial phase velocity, i.e. the flow rate in the bulk phase between packed beads (Schmidt-Traub, Schulte, & Seidel-Morgenstern, 2012)

In preparative chromatography biomolecules are separated, which are often antibodies, enzymes or other proteins. Those molecules are large compared to the pores of the stationary phase, which is why the C-term dominates. Hence, a low flow rate is favorable for the dynamic binding capacity. The flow rate also influences the pressure drop (Δp) along the column which is described by the Blake-Kozeny equation (11):

$$\Delta p = 150 \cdot \eta \cdot L \cdot v_0 / d_P^2 \cdot (1-\varepsilon)^2 / \varepsilon^3 \tag{11}$$

Where η is the viscosity of the mobile phase (Pa·s), L is the bed height (m), v_0 is the linear velocity (m·s^{-1}), d_P is the particle diameter (m) and ε the porosity (-). The pressure drop is often the limiting factor in large scale chromatography because of several factors. For the scale-up it is important to keep the bed height and the flow rate constant so that Δp is constant as well. It increases proportionally with the column length why it is important to preferably increase the diameter rather than the height of the column. The porosity and particle size cannot be varied in a large extent in order to maintain a good resolution. From the facts discussed in this section it can be concluded that the chromatographic process at industrial scale is limited to low flow rates. This is a general bottleneck in the purification of proteins by IEX. Therefore, other types of stationary phases and flow properties have been developed in the recent decades. Amongst those attempts are continuous processes, radial flow chromatography and convectively dominated stationary phases, such as membranes and monoliths. In the latter case, the process efficiency is optimized by avoiding pore diffusion. The properties, advantages and disadvantages of membrane based chromatography are discussed in the next section.

Time-dependency of adsorption processes

The maximum amount of adsorbed proteins on a surface does not only depend on the protein adsorption affinity but also on the adsorption history as elucidated by Norde, Buijs, & Lyklema (2005). It is known that (electrostatic) interactions influence the protein structure, because interaction with a surface affects the thermodynamic equilibrium of the folded protein. Hence, it can be favorable for a protein to rearrange its structure, e.g. to unfold and then to occupy more space as it would be possible if the surface is already filled. Consequently, the number of interactive sites between a surface and the protein depends on the availability of the surface for interaction. The faster a surface is filled with adsorbed proteins the less a protein tends to unfold and increase its footprint on the surface. Of course, the strength of interaction and the stability of a protein play an important role. The random sequential adsorption (RSA) is another model, also known as car parking model, which considers the inequality of adsorption sites and the history of the adsorption process (Evans, 1993). In conclusion, the transport kinetics can influence the adsorption kinetics and the maximum binding capacity of stationary phases.

Membrane based stationary phases

In membrane based chromatography a cellulosic membrane with a pore size of 1-5 µm is used instead of porous beads. In contrast to beads which are usually used for bioprocessing purposes, where pores are in a range between 30-400 nm, the membrane pores are between 800 nm and 5 µm. The development of such columns started with stacked layers and axial flow. The next generation had a coiled membrane around a solid core with radial flow (Fig. 5). The main advantages of the radial flow device are a more homogenous flow profile and a linear scalability because the number of membrane layers was kept constant while only the diameter d of the core and the length of the membrane h were increased.

With membrane chromatography flow rates of up to 5 column volumes·min^{-1} can be realized at low Δp because the porosity is large compared to packed beds (see eq. (11)). At the same time the binding capacity is independent from the flow rate because there is no limiting pore diffusion but only convection through the membrane pores, as shown in Fig. 3. The relevance of the C-term in eq. (10), which describes

the mass transfer limitation by e.g. pore diffusion, becomes negligible. This means that the contact time does not influence the binding capacity.
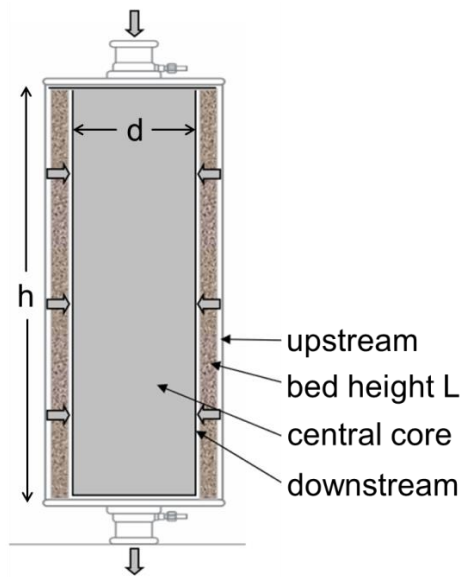

Fig. 5: Flow geometry in a radial flow membrane adsorber device, adapted from (Eibl & Eibl, 2010, p. 303)

The disadvantage of the high porosity is a small specific surface area and hence, the specific binding capacity is lower than in porous beads. In radial flow membrane adsorbers the flow is forced through the pores of the membrane. Although the pore size is relatively large, particles or lipoid substances can cause pore blocking and deposit layer formation. A filtration or centrifugation step for particle removal is mandatory.

In order to overcome this additional downstream unit operation, another chromatographic approach is the increase of the interstitial volume. One well known example is the expanded bed adsorption and another rather new technique is the tangential flow membrane chromatography (TFMC). The latter was invented by Sartorius Stedim Biotech and the production series is named Sartobind® Direct Capture. The setup is described in Fig. 6 which shows a membrane coiled around a solid core similar to the radial flow system, but between the membrane layers is a spacer screen. The spacer generates a 250 µm gap between the membranes, which are tangentially overflown along the column length h. The difference to radial flow columns is that the flow is not forced through the pores of the membranes and, hence, the pressure drop is negligible. Volumetric flow rates up to 20 CV·min⁻¹ are applied which is four times faster than in radial flow columns. It has to be considered that with increasing flow rate the contact time between target molecule and functional groups decreases and, that might result in low ligand utilization. Since the membrane material is the same as for radial flow columns a significant number of ligands is attached to the pore surface inside the membrane. Hence, the adsorption time in a chromatographic process with TFMC is usually enhanced by a recirculation of the feed solution.
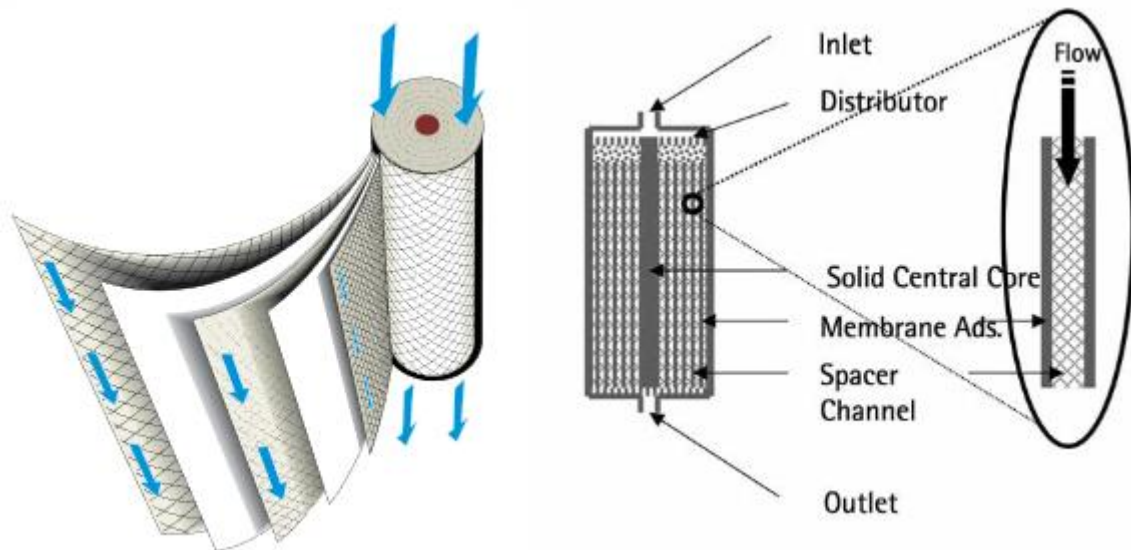
Fig. 6: Scheme of a tangential flow membrane adsorber device depicting the flow channel between two membrane layers realized by a spacer screen (microsite.sartorius.com)

First studies on the application of TFMC were described by Schwarz (2006) who isolated Lf from unfiltered whey. However, the loading time of 770 min and a yield of 63% showed that the construction of the different tested adsorber types was not yet optimal. The successful application of TFMC for the isolation of valuable compounds from crude feed stock, such as potato juice and whey, has been published later by Kreuß & Kulozik (2009) and Schoenbeck et al. (2013). Both working groups applied a loading time of 10 min. Kreuß & Kulozik (2009) recirculated the feed until a complete depletion of the target molecule was obtained. Schoenbeck et al. (2013) loaded in single-pass mode, the ratio of bound to unbound target proteins was not mentioned. However, the elongated loading time compared to radial flow adsorber devices indicates that mass transfer is not convective only, but diffusional transport into the inner membrane pores also plays a major role. The mass transfer properties were not investigated in detail in the studies mentioned above.

1.2 Whey from bovine milk

Whey is the serum phase of milk which contains solutes such as lactose, minerals, vitamins and the whey protein fraction. Casein is the major part of milk proteins with approx. 80% and does not belong to the whey proteins. It precipitates at pH 4.6 or upon the enzymatic cleavage of κ-casein by rennet. The whey protein fraction which is 20% of the total milk protein is soluble at pH 4.6 and is not affected by rennet (Belitz, Grosch, & Schieberle, 2001). Different types of whey are obtained in dependence of the casein precipitation process. Whey from renneted milk is known as sweet or cheese whey. If the casein precipitated upon acidification, it is referred to as acid whey. The decrease of the pH-value is usually the result of fermentation with lactic acid bacteria. Whey can also be a byproduct of sodium caseinate production by acid precipitation. This type of whey is known as technical whey. Whey is often separated from caseins by cross flow membrane filtration where the casein is removed via microfiltration (MF) (Etzel, 2004; Onwulata & Huth, 2008). For infant nutrition this whey protein production process is advantageous, because no carryover of contaminating genetic material from lactic acid bacteria or rennet enzyme occurs.

The whey composition depends strongly on the production process, for major constituents see Tab. 2. Cheese whey has a higher protein concentration than acid whey, since it contains the cleaved part of κ-casein, which is called caseinomacro-peptide. There are also differences in the mineral composition and pH-value. While acidified whey has a pH of approx. 4.6, the pH of cheese whey and milk MF-permeate is close to that of milk (pH > 5.6). A distinct difference in the calcium concentration between cheese and acid whey is the result of released calcium ions from calcium phosphate bridges at low pH. The calcium phosphate bridges stabilize the casein micelle and are not affected by the renneting process. The conductivity of whey is around 5 mS·cm^{-1}. The high conductivity can influence the surface charge of proteins by shielding charged amino acid side chains. The phospholipids represent the lipoid part of whey which remains after skimming and can lead to fouling of membranes. In order to remove the phospholipid fraction, a precipitation step can be applied (Maubois, Pierre, Fauquant, & Piot, 1987).

Tab. 2: Typical composition of sweet and acid whey (adapted from Jelen, 2003 and Thompson, Boland, & Harjinder Singh, 2009, p. 465)

Components	Sweet whey (g·L^{-1})	Acid whey (g·L^{-1})
Total solids	63–70	63–70
Lactose	46–52	44–46
Protein	6–10	6–8
Caseinomacropeptide	1.0	-
Lipids	0.6	0.1
Calcium	0.4–0.6	1.2–1.6
Phosphate	1–3	2–4.5
Lactate	2.0	6.4
Chloride	1.1	1.1

Whey proteins

The physico-chemical properties of whey proteins are described in the context of their relevance for the preparative chromatographic separation. The molecular structure is discussed in terms of surface charge and surface charge distribution as well as its stability in dependence of environmental conditions such as temperature, pH and conductivity. The techno-functional and bio-functional properties of the single proteins are not in the focus of the present work. A detailed description thereof can be found e.g. in Mine, Li-Chan, & Jiang (2010).

β-lactoglobulin

Bovine β-Lg is the most abundant whey protein (approx. 3.3 g·L^{-1}) and has been extensively studied with regard to all kinds of whey processing. In the present context only environmental conditions below 70°C, at ambient pressure and without chemical denaturants will be summarized.

β-Lg is a globular protein with a molecular weight of 18.3 kDa (Sawyer & Kontopidis, 2000). Different genetic variants occur in ruminant species. In bovine milk the variants A and B are present, differing in two amino acids; Asp64 and Gly64, and Val118 and Ala118 in variant A and B, respectively. Different values for the pI can be found in literature, ranging from 5.2-5.4. It belongs to the lipocalin family what implies that it has a barrel-like structure with a hydrophilic surface and a hydrophobic pocket where apolar molecules can be bound (Pervaiz & Brew, 1987). At physiological conditions the protein is dimeric and has a size of 3.6x7.0 nm (Norde et al., 2005) and at pH 2-3 and > 9 it dissociates into monomers (see Fig. 7). Between pH 3.5 and 5.5 octamers form at cool temperatures consisting of two tetramers (Sawyer & Kontopidis, 2000).
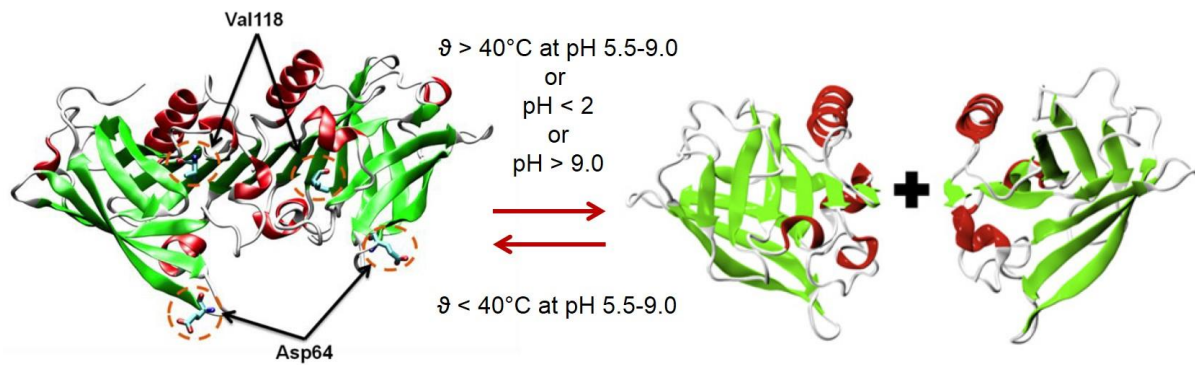
Fig. 7: Illustration of dimeric (left) and monomeric (right) β-lactoglobulin A. The dissociation of the dimer into monomers as function of temperature and pH-value is described by the red arrows. β-sheets are green and α-helices are red. The β-sheets form a barrel-like structure with a hydrophobic pocket. In the dimeric molecule the Asp64 and Val118, which are replaced by Gly64 and Ala118 in β-lactoglobulin B, are indicated by black arrows (Figures adapted from Mercadente et al. (2012)).

Besides the pH, also the temperature has a significant effect on the protein structure. The so-called Tanford transition describes the non-native state of the protein at a temperature treatment up to 55°C, where the dimer dissociates into monomers at physiological pH, and further denaturates upon heating.

The structure is stabilized via two disulfide bonds and one thiol group is free but buried in the inner part of the native molecule. In non-native states of the protein the sulfhydryl becomes reactive and can lead to intermolecular interactions by disulfide interchange reactions. Additionally, the carboxyl group of Glu89 becomes reactive. This happens in a temperature range between 40 and 65°C, where structural changes are still reversible (Dunnill & Green, 1966; Iametti et al., 1996; Manderson, Hardman, & Creamer, 1998; Tanford & Taggart, 1961). In most cases the free thiol is at site 121, but has been determined at 119 as well (McKenzie, Ralston, & Shaw, 1972).

Several working groups investigated the stability in dependence of the ionic strength and found a tendency of aggregation at low conductivity. Treece, Sheinson, & McMeekin (1964) investigated the solubility of β-Lg in water and NaCl, without pH adjustment by buffering salts. Solubility was significantly higher for β-Lg B compared to β-Lg A in a range of 6.25-25.00 mM NaCl with an average maximum value of 0.61 g·L^{-1} for β-Lg A and 3.1 g·L^{-1} for β-Lg B. The maximum solubility for the mixture of both was found at 1.0 g·L^{-1} at 25°C. Majhi et al. (2006) observed aggregation of β-Lg dimers in a pH range between 3.8-5.2 and a maximum at pH 4.3-4.8 at 4.5 mM NaCl. The reasons are electrostratic attractions between oppositely charged patches on the dimer surface. At pH 5.0 and at 4.5-500 mM NaCl they found a linear

decrease in aggregation with increasing ionic strength. De Wit & van Kessel (1996) found that β-Lg from different whey protein concentrates and isolates becomes insoluble at pH 4.6-5.2, which is close to its pI and in agreement with the results of Majhi et al. (2006). Furthermore, it was observed that at ionic strength > 0.1 M the protein remained soluble.

In terms of electrostatic interactions the protein has been thoroughly investigated. The charge density of the anionic molecule is high (-17.6 and -15.6 mV for β-Lg A and B, resp.) (Lucas et al., 1998). The number of surface charges is 27 negative charges and 21 positive charges taken from crystal structures by Vries (2003). He also identified the distribution of charge patches and summarized them in a plot shown in Fig. 9 (right). The charge distribution was also shown in three dimensional molecule structures in dependence of the pH, e.g. by Majhi et al. (2006), displayed in Fig. 8. Chen et al. (2011) and Xu et al. (2011) found electrostatic interactions between positively charged β-Lg and like-charged molecules and explained this behavior by large charge patches of the opposite charge sign as the net charge.
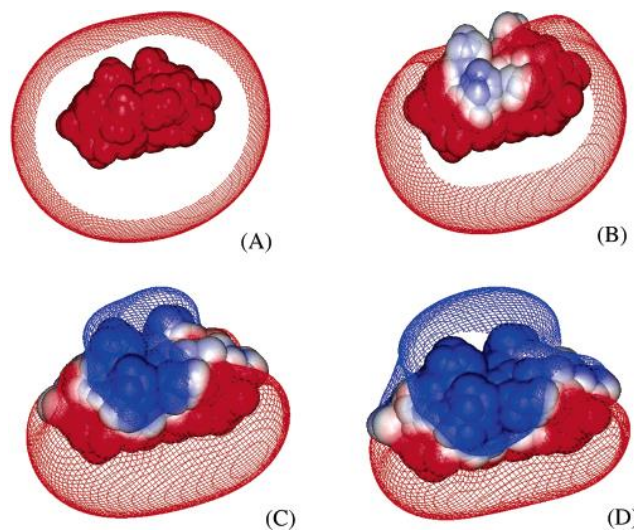
Fig. 8: Electrostatic potential contours (positive in red and negative in blue) around a β-Lg dimer at an ionic strength of 4.5 mM and at pH 4.03 (A), 4.59 (B), 4.98 (C) and 5.22 (D) (Majhi et al., 2006).

α-lactalbumin

The second major whey protein with a concentration of approx. 1 g·L^{-1} is α-La which has a molecular weight of 14.2 kDa and its pI is between 4.2-4.5 (Belitz et al., 2001). The dimensions are 4.3x3.2x2.8 nm (Galisteo & Norde, 1995a). The globular protein structure is stabilized by four disulfide bonds and it does not contain any free thiol groups. The prevalent part in milk is stabilized by a Ca^{2+}-ion bound in the center of

the molecule. The calcium containing molecule is named holo-α-La, the calcium free protein is known as apo-α-La. The Ca^{2+}-ion can be removed from the protein at acidic pH together with chelating reagents such as citrate. In the apo-form, the protein is more temperature sensitive. While the holo-form denatures at 65°C, the melting temperature of apo-α-La is 20-40°C below (Zhong, Gilmanshin, & Callender, 1999). In any case, aggregation occurs only when β-Lg or BSA are present, which have a free thiol-group. The paired cysteine residues of α-La are unreactive in a wide temperature range (Havea, Singh, & Creamer, 2001). Structural changes between the native and the unfolded state involve the molten globule state in which different part of the protein, the α-helices and β-sheets, unfold differently dependent on the presence of calcium. If denatured, the protein refolding can be facilitated by excess of calcium at neutral pH.

Lucas et al. (1998) calculated the surface charge from the amino acid content which is -3.7 mV. They further investigated the transmission of α-La through cationic ultrafiltration membranes. As expected, the retention decreased with increasing ionic strength due to charge screening. This is, because charged patches with several likely charged amino acid residues on the protein surface and the charged membrane repulse each other. Vries (2003) illustrated the frequency of positive and negative patches on α-La and β-Lg with the according patch size, i.e. the number of charged amino acid residues per patch (Fig. 9 (left)). In sum the positive and negative surface charges are 17 and 20, respectively, which are distributed over several charged patches.

α-La is known to be able to bind to like-charged surfaces via the oppositely charged patches similar to β-Lg. This was shown for sulfonate ligands and negatively charged α-La by Galisteo & Norde (1995a). Another study by Galisteo & Norde (1995c) showed similar effects with an AgI matrix and thermodynamic data revealed electrostatic interactions between the like charged partners. Anyway, the binding capacity was significantly decreased in comparison to pH values where the protein was positively charged. The impact of the ionic strength was not pronounced at any pH above the pI. But at the adsorption maximum around pH 4, the binding capacity was remarkably higher at 5 mM KCl compared to 50 mM KCl indicating a charge screening effect. Galisteo & Norde (1995a) further investigated the impact of protein rigidity on the maximum binding capacity in a hydrophobic stationary phase with charged sulfonic acid ligands. They used apo- and holo-α-La as a less stable and a

rigid protein, respectively. No difference between the two proteins was observed, but they found that the calcium ion of the holo-α-La was released during the adsorption process. This was later confirmed by titration studies (Galisteo & Norde, 1995b).
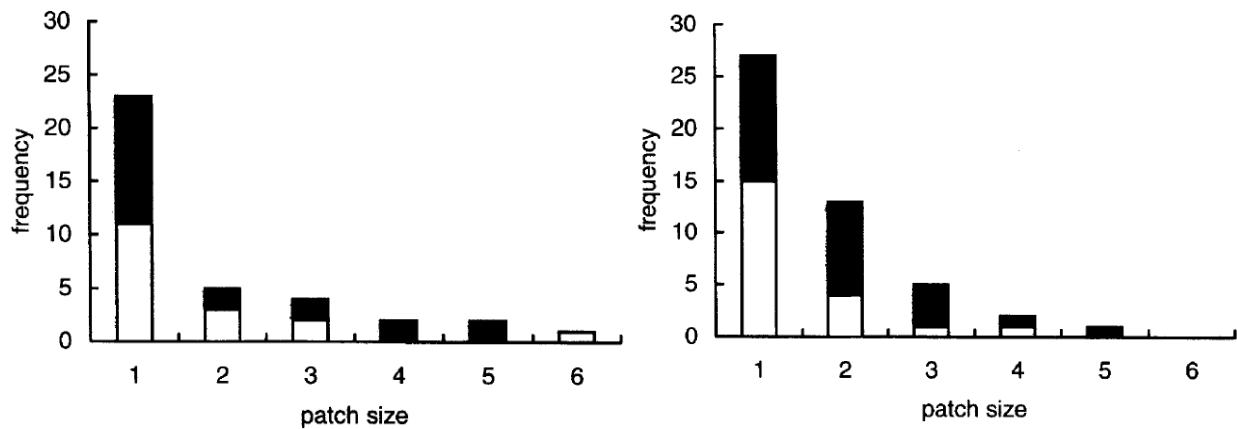

Fig. 9: Frequency of positively (white) and negatively (black) charged patches with different patch size, i.e. the number of charged amino acid residues, on the surface of α-La (left) and β-Lg (right) (Vries, 2003).

It has to be mentioned that the studies by Galisteo and Norde (1992) were done with hydrophobic matrices and the impact of hydrophobic interactions additional to the electrostatic interactions cannot be neglected. For example, when using hydrophilic stationary phases, the holo-α-La was less subject to structural changes and the loss of calcium (Norde & Anusiem, 1992).

Bovine serum albumin

Bovine serum albumin (BSA) is a globular whey protein which has a concentration of 0.3-0.6 g·L^{-1} in whey and a molecular weight of 66-69 kDa (Belitz et al., 2001; Hahn, Schulz, Schaupp, & Jungbauer, 1998). It has an ellipsoidal shape and the dimensions are 9.0x6.0x5.0 nm (Saikia, Saha, & Das, 2014). The protein has 17 disulfide bridges and one unpaired cysteine residue in position 34 (Giancola et al., 1997). Although the structure is stabilized via several disulfide bonds, BSA is known as a soft protein. That means the structure is prone to changes upon adsorption (Saikia et al., 2014). The pI is between 4.9 and 5.1 (Belitz et al., 2001; Norde et al., 2005), the zeta potential is -14.6 mV (Saikia et al., 2014). The high surface potential is caused by the high content of charged amino acids such as aspartic acid, glutamic acid, lysine and arginine (Giancola et al., 1997).
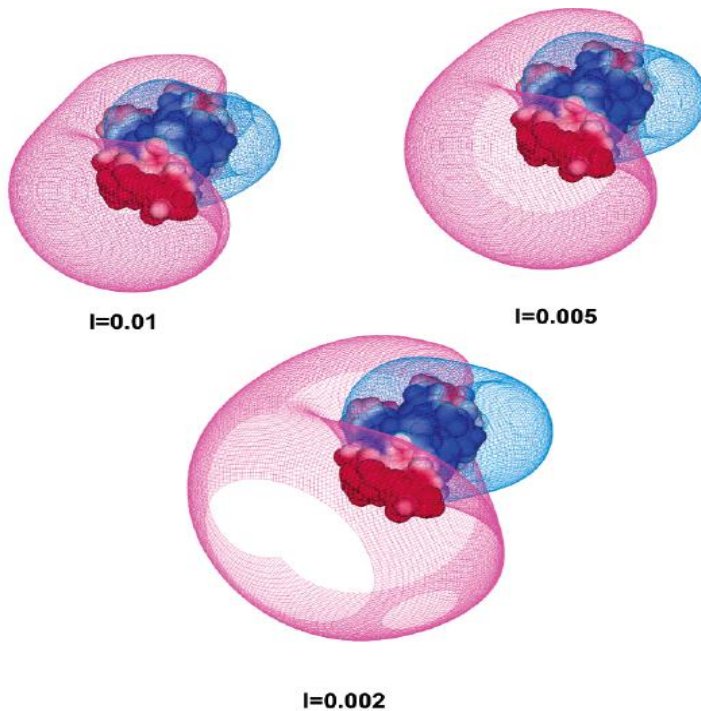
Fig. 10: Electrostatic potential contours (negative in red and positive in blue) around BSA at I=0.002-0.01 at pH 6.5 (Seyrek et al., 2003).

The electrostatic potential contours were investigated and visualized at various pH and ionic strength conditions. At a pH between 6.5 and 7.0 it was shown that the major part of the surface is negatively charged and a small region is positively charged (Antonov, Mazzawi, & Dubin, 2010; Seyrek et al., 2003). A similar result was obtained by (Saikia et al., 2014) although the positive patch was rather hidden in the inner part of the molecule. The effect of the ionic strength on the surface potential at pH 6.5 is significant, as can be seen in Fig. 10.

At pH 5.6 the positive and negative charges are homogenously distributed. The surface potential is displayed for 5 and 150 mM NaCl in Fig. 11. Several works have been published on the adsorption of BSA to like-charged surfaces, both cationic and anionic. It was found that BSA did not interact with cationic counterparts below its pI, but at very low ionic strength close to its pI. At 5 mM, the critical pH is at the pI of BSA. However, a significant adsorption occurs at pH > 6.25, where negative domains are present (Xu et al., 2011). Adsorption of BSA to negatively charged ZnS particles was investigated by Saikia et al. (2014) who observed interactions at pH 7 where BSA is clearly negatively charged. However, this effect was not dedicated to the positive patch, but rather to the ability of a soft protein to adapt the molecular structure in order to favor adsorption. Structural changes have been described for BSA in dependence of pH, ionic strength and temperature.

Between pH 4.3 and 8.0 the protein structure remains unchanged with 185 ionized groups. Below this pH range, BSA has a higher surface charge and is known as form F because it moves fast upon gel electrophoresis. Above pH 8.0 the molecule is in its basic form (B). The structural changes are reversible (Giancola et al., 1997). The pH has also an effect on the
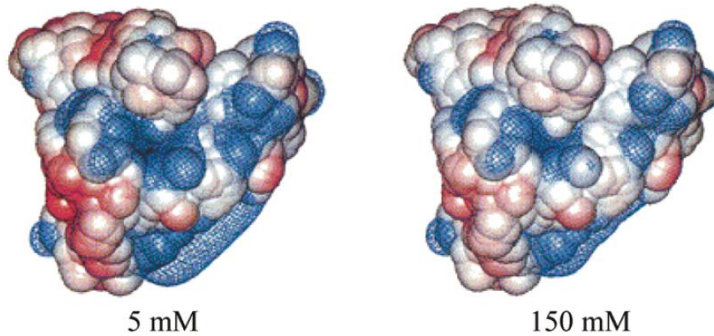

Fig. 11: Electrostatic potential contours (negative in red and positive in blue) around BSA at pH 5.6 (Grymonpré, Staggemeier, Dubin, & Mattison, 2001).

denaturation temperature (T_d) as shown by DSC measurements (Boye, Alli, & Ismail, 1996). The highest stability was observed at pH 5 and the lowest at pH 9 where denaturation occurs at 60.8°C. The T_d strongly depends on the ionic strength. In presence of 0.5 M NaCl T_d is 70.7°C and at 1 M NaCl T_d is 72.9°C, both measured at pH 6.8.

Lactoferrin

Bovine Lactoferrin (bLf) is an iron-binding glyco-protein which consists of a single polypeptide chain of 689 amino acids and has a molecular weight of approx. 80 kDa. The molecular weight varies with the number of glycosylated sites with a maximum of five. The tertiary structure is stabilized via 17 disulfide bonds, no unpaired thiol group is present (Pierce et al., 1991).

The polypeptide chain folds into two globular lobes, the N- and C-lobe, with highly similar structures which are connected via a α-helix. Each lobe can bind one iron atom together with a carbonate ion (Anderson et al., 1989) (see Fig. 12). The amino acids involved in the iron binding are two Tyr, one Asp and one His. The iron binding site is anionic, thus Fe^{3+} has a higher binding affinity than Fe^{2+} (Sánchez et al., 1992). 15-20% of native bLf are saturated with iron. The iron-depleted protein is known as apo-form and the iron saturated molecule is referred to as holo-form. The calculated pI for bLf is 9.5, whereas experimental values between 7.8 and 9.5, depending on the applied method, have been determined (Steijns & van Hooijdonk, 2000). The high pI is the result of positive charge patches in the N-terminus and in the interlobe region (Baker, Baker, & Kidd, 2002). Bokkhim et al. (2013) have

investigated the pI of apo- and holo-bLf by zeta potential measurements and found a pI-range of 5.5-6.5 for apo-bLf and 8.0-9.0 for holo-bLf. In contrast, Brisson, Britten, & Pouliot (2007b) have investigated the electrophoretic mobility of native and holo-bLf between pH 6-8 and did not observe significant differences and thus, no difference in the net surface charges.
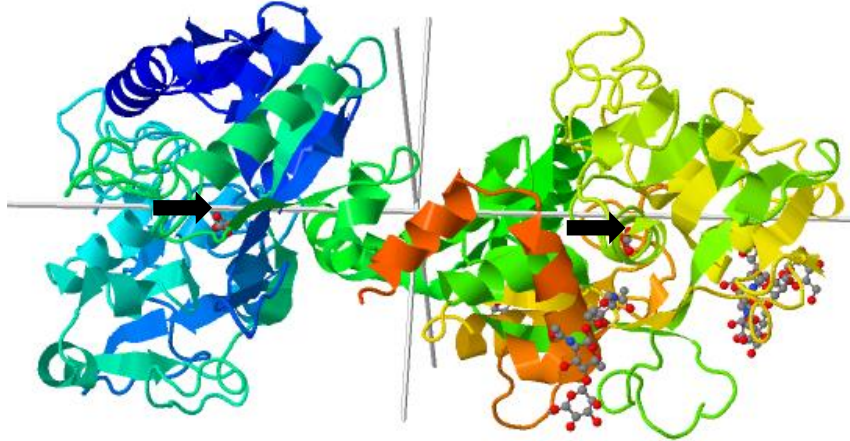

Fig. 12 Crystal structure of bLf (pdb-entry 1BLF) shows the N- und C-lobe. The iron and carbonate ligands are indicated by an arrow.

The state of iron binding strongly influences the stability of bLf. With regard to temperature, DSC measurements revealed that the T_d for apobLf is approx. 60°C and for holo-bLf 90°C in aqueous solution without pH adjustment (Bokkhim et al., 2013) as well as at pH 7 (Bengoechea, Peinado, & McClements, 2011). The lower heat stability of apo-bLf compared to holo-bLf in milk and other model systems was also shown by Brisson, Britten, & Pouliot (2007c) and Brisson, Britten, & Pouliot (2007a). Sánchez et al. (1992) also showed that bLf was more stable in phosphate buffer than in the native environment milk. Furthermore, the ionic strength and pH-value influence the nativity of bLf. Kawakami et al. (1992) found a higher protein stability during thermal treatment at low ionic strength (< 0.01 M NaCl), which was further increased by an acidic environment. They suggested that repulsion between the molecules with positive surface potential decreased intermolecular interactions. The same effect was observed by Abe et al. (1991) who studied the heat treatment of apo-Lf at pH 2-11.

Lactoperoxidase

Lactoperoxidase (LPO) is a peroxidizing enzyme (EC 1.11.1.7). Together with hydrogen peroxide and thiocyanate it constitutes the antimicrobial LPO system in

milk. LPO contains 612 amino acid residues and its molecular weight is approx. 78.4 kDa (Paul, Ohlsson, & Henriksson, 1980). It has a positive surface charge and a high isoelectric point of approx. 9 (Carlström et al., 1969). Besides a large number of basic amino acid residues, the surface also exhibits five potential N-glycosylation sites resulting in a carbohydrate content of about 10 % (Kussendrager & van Hooijdonk, 2000). The protein structure is very complex. The catalytic center of LPO contains a heme group, which is covalently bound to the polypeptide chain through a disulfide bridge (Thanabal & La Mar, 1989). Six additional disulfide bonds tightly connect an α-helix rich region around the heme that cause a high temperature resistance. The absence of free thiol groups enhances the heat stability (Dillon & Board, 1994) as well as a calcium ion that is bound via an Asp in the heme region (Booth et al., 1989; Cals et al., 1991; Shin, Hayasawa, & Lönnerdal, 2001). The peripheral region is high in β-sheets and more flexible than the center of the molecule.

Thermal denaturation is an irreversible two-step process. Around 40°C the outer region unfolds without an effect on the enzymatic activity whereas around 70°C the catalytic core denatures and the enzyme becomes inactive (Boscolo, Leal, Ghibaudi, & Gomes, 2007). The impact of pH and calcium concentration on the molecular integrity is significant at elevated temperature. LPO is more heat stable at neutral to basic pH and the presence of calcium is beneficial for the protein stability. Thus, in whey and milk LPO exhibits a higher conformational stability as in buffer systems. Also at ambient temperature, it was shown that pH values below pH 5.4 were shown to be detrimental (Kussendrager & van Hooijdonk, 2000).

Due to the heme group LPO absorbs light at a wavelength of 414 nm, which no other whey protein can absorb (Pruitt & Tenovuo, 1985). This can be used for the quantification of smallest amounts of LPO in a mixture of whey proteins.

Immunoglobulins

Immunoglobulins (Ig) are antibodies and are present in bovine milk to deliver passive immune defense to the neonates. IgG is the major immunoglobulin in bovine milk. It is a monomer with a molecular weight of approx. 150 kDa. Two variants of IgG exist, IgG1 with a concentration of 0.3-0.6 $g \cdot L^{-1}$ and IgG2 with approx. 0.05 $g \cdot L^{-1}$. The pI of IgG1 is 5.5-6.8 and of IgG2 it is 7.5-8.3 (Farrell Jr. et al., 2004). The secretory IgA (sIgA) comprises two Ig monomers, connected via a joining chain and a secretory

component surrounds the protein. It has a M_w of 385-430 kDa and a concentration of 0.01-0.1 $g \cdot L^{-1}$ (Farrell Jr. et al., 2004; Mehra, Marnila, & Korhonen, 2006). IgM consists of 5 Ig monomers and has a Mw of 900-1000 kDa and a concentration of 0.04-0.1 $g \cdot L^{-1}$ (Mehra et al., 2006).

Due to the low concentration of IgA and IgM in bovine milk and whey only IgG is described in detail. The concentration of IgG in whey is approx. 0.5 $g \cdot L^{-1}$ (deWit & Klarenbeek, 1984), as in milk. The protein has a typical structure for monomeric immunoglobulins. It contains two heavy and two light chains that are connected via disulfide bonds (for a schematic illustration see Fig. 13). The amino acid sequence between IgG variants is conserved in the constant region (Fc), whereas the variable region (Fab) has a specific amino acid constitution. The Fab region is responsible for the high specificity of an antibody for one antigen. The conformational and functional stability of immunoglobulins in milk and whey was investigated by several research groups. Vermeer & Norde (2000) studied the sensitivity of IgG towards temperature by DSC measurements. The results revealed that the Fab region denatures at 61°C and the Fc region at 71°C, both reactions are irreversible. The Fc fragment was found to be sensitive to low pH. When DSC analysis was run at pH 3.5 there was only the Fab denaturation peak at 61°C but no denaturation occured at 71°C. Hence, unfolding was already induced by the low pH. However, the extent of heat denaturation was quite different ranging from 1 to 40 % at high-temperature short-time of milk (HTST, 72°C for 15 s) (Li-Chan et al., 1995; Mainer, Sanchez, Ena, & Calvo, 1997). The reason might be different analytical techniques.
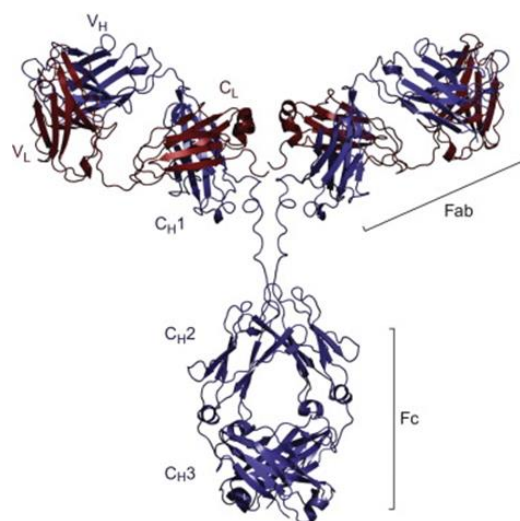

Fig. 13: Structure of an immunoglobulin G (IgG) molecule, light chain in red, heavy chain in blue (Lowe et al., 2011).

1.3 Motivation

Whey contains several high-value proteins due to techno- and bio-functional properties. The isolation of these proteins is of interest for the dairy industry, because in its pure form each protein can be applied to food and infant formula according to its specific properties (Smithers, 2008). In the last few decades, various attempts of whey protein isolation using chromatography, membrane filtration or combinations thereof have been reported in literature. Reviews by Ghosh (2002) and El-Sayed & Chase (2011) present a good overview. Frequently used stationary phases are membrane based ion-exchangers, because their handling is relatively simple, the investment costs are comparably low and processes are fast. Most of the processes are developed at lab scale and a transfer to larger scales is not investigated except for Gerberding & Byers (1998), Kristiansenet al., (1998), Lu et al. (2007) and Kreuß & Kulozik (2009). However, only Gerberding & Byers (1998) isolated four proteins in a two-step process at large scale whereas the others focused only on one protein. Also at lab scale the isolation of more than four proteins in one process is not described.

Therefore, the major aim of the present work is the development of a two-step process for the simultaneous separation of α-La, β-Lg, IgG, BSA, LPO and Lf using membrane based ion-exchange chromatography. A schematic depiction of the two-step process is shown in Fig. 14. One anion- and one cation-exchanger membrane step are the basis for the fractionation process using food grade buffers and an ionic strength gradient for the stepwise elution of adsorbed proteins. Investigated impact factors are pH-value of buffers and whey, ionic strength of buffers and the elution gradient as well as protein load ratio. These factors are
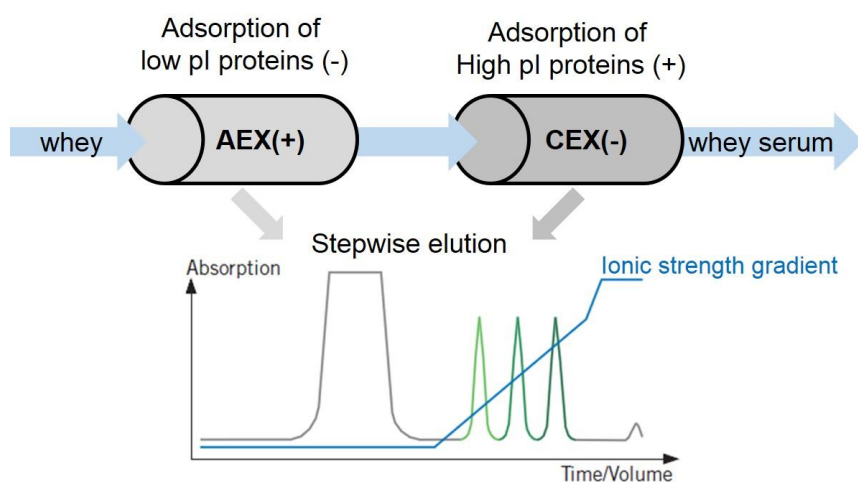

Fig. 14: Schematic presentation of the two-step process: proteins with low pI are negatively charged and adsorb to the cationic ligands of the AEX, proteins with high pI are positively charged and bind to the anionic ligands. Sequential elution allows complete separation of the six target proteins.

used to optimize purity, yield and concentration of obtained protein fractions. A high purity can be obtained, if baseline separation between eluted proteins is achieved. A high yield is possible, if complete adsorption of one protein and its complete elution without simultaneous elution of other adsorbed proteins is realized.

Based on available knowledge on the protein properties and published separation processes, the following challenges are expected:

The properties of whey and whey proteins play a major role for the ion-exchange process. Protein concentrations differ by a factor of 100, and that makes it difficult to reach a high yield for minor proteins. The selective ad- and desorption depends on the net surface charge and the surface charge distribution. A large difference in the pI of the proteins facilitates their separation. However, Lf and LPO, both have pI 9. The pIs of BSA and β-Lg are also very close together. Hence, the shape of the elution gradient and possibly displacement effects can be used to optimize the separation success. The stationary phase also plays a significant role in the process development. Membrane adsorbers are known to have a low resolution, because of unavoidable dispersion effects. This impedes baseline separation and sharp peaks are needed for high purity, yield and concentration.

The second main task of this work is the process transfer to pilot scale at minimized bioburden conditions. The scale-up will be investigated using a customized pilot plant and key figures will be compared between both scales. The microbial growth is intended to be reduced via a microfiltration step installed upstream and processing at cold or warm temperature.

Finally, the application of tangential flow membrane chromatography will be investigated, because this type of stationary phase allows the load of unfiltered whey. However, mass transfer and rheological properties are assumed to be significantly different compared to the radial flow adsorber used for process development and scale-up. Therefore, mass transfer phenomena in both adsorber devices will be characterized and evaluated in order to propose the most efficient process conditions.

A comprehensive understanding of the thermodynamics in protein adsorption from a complex mixture and the mass transfer properties of different membrane adsorber types will be used to optimize the separation and process efficiency.

2. Summary of Results

Publications as basis for the thesis are summarized in this chapter. The full length papers are attached in appendix 6.1. The main focus of the publications and their context is briefly summarized.

Publication 1:

- An analytical RP-HPLC method for the simultaneous quantification of the six target proteins was developed.
- Development of preparative fractionation process at lab scale: ion-exchange sequence, adsorption and elution conditions, load ratio.
- Purity and yield were around 90% and higher except for the yield of Lf. Not more than 63% of Lf could be obtained in one pure fraction.

Publication 2:

- Process from Publication 1 transferred to pilot scale at a 50 fold increased membrane area.
- Key factors (binding capacity, yield and purity) compared between both scales, with highly similar results. The yield of Lf was even lower at pilot scale with approx. 39%.
- Separation performance without intermediate cleaning between cycles and the regenerability of the initial binding capacity were investigated.

Publication 3:

- Problem of the low recovery of Lf observed in Publication 1 and 2 adressed
- Adsorption studies with holo- and apo-Lf were conducted in order to determine the impact of iron-binding on the adsorption behavior.
- Isoelectric points of apo- and holo-Lf determined via polyacrylamide-gelelectrophoresis based isoelectric focusing (PAGE-IEF), effect of pI and protein net charge on the adsorption behavior in cation-exchangers investigated.
- Isotherms at pH 4.8 using holo- and apo-Lf in different buffer systems and native whey serum for determination of impact of protein concentration and media properties on adsorption affinity and maximum binding capacity in cation-exchange membranes.

- Impact of process temperature on adsorption of β-Lg between 10 and 50°C investigated in order to find optimal processing conditions at reduced microbial growth.

- Isotherms at 10-50°C should deliver information on adsorption affinities and maximum binding capacities.

- Investigation of impact of flow geometry on mass transfer effects and binding kinetics, convective transport in radial flow membrane adsorbers, diffusional transport in batch adsorption studies.

- Determination of effect of convective contributions on mass transfer rates in tangential flow membrane adsorbers.

Publication 1

Fractionation of whey proteins by means of membrane adsorption chromatography

A two-step process for the separation of major and minor proteins from acid whey with ion-exchange chromatography was developed. The aim was to establish a robust process applicable in the dairy industry. The used stationary phase is a membrane adsorber functionalized with anion or cation-exchanger groups (AEX, CEX) for the selective ad- and desorption of six whey proteins. The chromatographic device is ready to use and does not require specific knowhow and costly equipment. The matrix is a porous membrane with a pore size of 3-5 µm allowing flow rates of up to 5 column volumes per minute. This is fast compared to packed columns with typical flow rates of less than one column volume per minute.

The target proteins are α-La and β-Lg as well as the minor proteins BSA, IgG, LPO and Lf. Ion-exchange chromatography is the separation technique of choice, because the isoelectric points (pI) of the target proteins range between pH 4.2-9.0. Thus, surface net charges can be reversed by adjusting the pH of the whey. Once a protein adsorbed, desorption is possible using an elution buffer with increased ionic strength. During process development different types of aqueous, food grade buffers, adsorption pH and elution gradients were screened. Tested buffers were sodium

acetate at pH 4.0-5.7 and sodium phosphate at pH 6.4-7.4. The elution gradient had 0-1000 mM NaCl which was either linearly or stepwise increased.

The final process begins with the adsorption of β-Lg and BSA to an AEX, 0.03 M phosphate buffer, pH 7.0. BSA is eluted at 0.1 M NaCl and β-Lg at 1 M NaCl. The flowthrough which contains the unbound protein is adjusted to pH 4.8. A CEX step follows, equilibrated with 0.1 M sodium acetate, pH 4.8. Lf, LPO and IgG bind whereas α-La remains widely unbound because the pH is close to its pI. The adsorbed protein was separated by a stepwise gradient: IgG elutes at 0.25 M NaCl, LPO at 0.35 M NaCl and Lf at 1.00 M NaCl. The purity of the isolated minor proteins and β-Lg was nearly 100 %, α-La was 88% pure. The recovery of most proteins was close to 90% and higher except for Lf which had a yield of approx. 63%.

Publication 2

Fractionation of all major and minor whey proteins with radial flow membrane adsorption chromatography at lab and pilot scale

A chromatographic separation process for the isolation of six proteins from acid whey has been previously developed at lab scale and was described in publication 1. The process comprised two steps: AEX is used to purify BSA and β-Lg, and CEX binds the minor proteins Lf, LPO and IgG. A stepwise gradient with increasing ionic strength separately elutes the bound proteins. α-La remains unbound in the whey serum phase.

In the present paper the scalability and robustness of the process was investigated. For the scale-up, a 50-fold increased membrane area was used and a customized pilot plant was designed.

The AEX step is critical in terms of binding capacity, because in this step β-Lg is depleted which represents 80% of the total whey proteins. The dynamic binding capacity (DBC) was compared at lab and pilot scale and was consistent at approx. 0.46 mg β-Lg·cm^{-2} membrane area. The robustness of this step was examined in five repeated bind and elute cycles at maximum load ratio without intermediate cleaning. At lab scale the β-Lg depletion was 99%. At pilot scale an initial drop in binding

capacity was observed, but then the β-Lg depletion was constant at 96% of the initial capacity.

After cleaning with acidic and alkaline solution the maximum binding capacity could be regained. In the CEX step α-La, Lf, LPO and IgG were purified. Due to the partly very low protein concentration of 0.01 mg·mL^{-1} high yields and concentration factors were aimed at for an efficient process.

Additionally, the purity of each fraction was determined which is important for their individual application in infant food and food supplements. α-La, which remained in the serum phase, had a purity of 91% at both scales and a yield of 94 and 97% at lab and pilot scale, respectively. The purification of Lf was more successful at lab scale with a purity of 97% and a yield of 66% vs. 66% purity and 39% yield at pilot scale. LPO isolation was good at lab scale in terms of purity (86 vs. 57%) and more efficient at pilot scale in terms of yield (75 vs. 81%). The purification of IgG was 95% and 88% at lab and pilot scale, respectively and the yield was 75% at lab scale and 86% at pilot scale. The concentration factors determined for Lf was 5.6 ± 1.9, for LPO 13.7 ± 2.4 and for IgG 14.1 ± 0.5.

The process was successfully transferred to pilot scale and linear scalability was demonstrated. The results in terms of binding capacity, repeatability, purity and yield showed that the process is industrially applicable. A full recovery of the initial binding capacity after five repeated separation cycles was possible with an acidic and alkaline cleaning-in-place process.

Publication 3

Impact of the iron saturation of bovine lactoferrin on the adsorption to a strong cation-exchanger membrane

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The isolation of Lf from bovine whey with cation-exchange chromatography was investigated and described by several research groups and high purity and concentration of the Lf fraction could be achieved. However, the yield was only between 25-88%. A low yield was also observed in publications 1 and 2. No correlation between process conditions (pH-value, buffer type, ion-exchanger type) and the recovery of Lf could be found during process development or based on

literature results. Since Lf is an iron-binding protein, different isoforms are present in whey. The iron-status might influence the adsorption behavior in cation-exchange membranes, which was not yet described in literature.

Therefore, the aim of this study was to investigate the impact of iron-binding of Lf on the adsorption affinity in cation-exchanger as affected by the pH-value.

In native whey, approx. 20% of Lf is iron saturated, known as holo-Lf and approx. 80% has no iron bound (apo-Lf). The protein structure depends on the iron binding status. The protein has two highly similar lobes with an iron binding pocket each. The lobe is closed when iron is bound and otherwise it is open. The latter form has a negative charge that binds Fe^{3+}. The surface has two pronounced positive charge patches resulting in a high pI of approx. pH 9. Due to the high pI the isolation using CEX is frequently applied. However, the impact of iron binding on the adsorption and desorption behavior of Lf in CEX has not been studied so far. This paper used apo- and holo-Lf for adsorption studies with a strong cation-exchanger membrane. Different buffers in a range of pH 4.8-7.0 were investigated. The maximum binding capacity of native Lf was determined followed by adsorption studies with apo-Lf and holo-Lf. Furthermore, isoelectric focusing (IEF) was used to determine the pI of native, holo- and apo-Lf in order to evaluate the impact of pI on the adsorption behavior. Finally, isotherm studies were conducted in order to gain information on adsorption affinities of Lf isoforms in dependence of buffer type and pH-value.

The pI determined by IEF was approx. 9.3-9.5 for all isoforms. Hence, apo- and holo-Lf have a positive surface net potential in the pH range of pH 4.8-7.0. The pH dependent adsorption study showed a remarkable impact of pH and iron binding state on the binding behavior. At near neutral pH, 100% of apo- and holo-Lf bound, whereas with decreasing pH less apo-Lf adsorbed. At pH 4.8 only 45-70% apo-Lf bound, depending on the buffer type. In contrast, more than 85% holo-Lf adsorbed independently of the buffer type. The adsorption affinities determined by isotherm studies at pH 4.8 confirmed the higher binding affinity of holo-Lf compared to apo-Lf.

Publication 4

Adsorption of beta-lactoglobulin in anion exchange membrane chromatography versus the contacting mode and temperature

The effect of temperature and flow geometry in membrane adsorber devices on the adsorption kinetics and binding capacity of β-Lg in AEX was investigated.

The process development and scale-up, which was described in publications 1 and 2, was done at ambient temperature. But the processing of milk and whey at industrial scale needs to be at high (≥ 50°C) or low temperature (≤ 10°C) in order to avoid microbial growth. Although temperature has in general a negligible effect in ion-exchange chromatography it has a significant impact on the structure and reactivity of β-Lg. Hence, it is assumed that the protein adsorption to AEX is affected by temperature.

The different flow geometries allow the investigation of convective and diffusive mass transfer in dependence of temperature. In radial flow adsorber devices fast convection dominates the mass transfer but pore blocking can occur. When the feed solution is passed tangentially over the membrane surface pore blocking is reduced. However, an overall slower adsorption compared to radial flow devices is assumed because convective transport is assumed to access only the pores close to the surface and transport in the inner membrane pores is diffusive. Isotherm studies were used to determine maximum binding capacity vs. temperature and batch adsorption studies gave reference values for pure diffusion.

Batch adsorption experiments showed that diffusion rates increase with temperature. Not only has the viscosity contributed to the faster diffusion but also the monomer-dimer conversion that occurs when temperature comes below 40°C.

The binding capacity decreased with increasing temperature in radial flow adsorber devices and in isotherm studies. The suggested reason was the adsorbed layer thickness which is smaller for monomers than for dimers. At low temperature the binding capacity was around 0.8 mg·cm^{-2} in both experiments. Using tangentially overflown membrane devices, the mass transfer was 10 times faster compared to pure diffusion. The total adsorption time was 120 min compared to 300 min in batch

adsorption. In radial flow devices the adsorption was complete after 4 min at similar maximum binding capacities.

Based on these results, a low process temperature of 10°C is recommended for all types of membrane adsorber devices. Since pore blocking did not seem to be a limiting factor under the investigated conditions, the use of radial flow adsorbers with convective transport is favorable since processing times are significantly shorter.

3. Discussion

The aim of the present work was the development of a complete separation process for six major and minor whey proteins in fresh whey using ion-exchange chromatography. This process is intended for application in the dairy industry. This led to some engineering requirements, which are different from established chromatographic separations typically performed in the pharmaceutical and biotechnological field. The installation and execution of chromatographic processes in dairy industry needs to as uncomplicated as possible since specifically skilled employees with scientific and analytical background are not affordable and so is the purchase and maintenance of sophisticated equipment. An additional constraint for the process development was the choice of chemicals for buffer solutions, which had to be food grade in order to avoid further removal of residual chemicals by diafiltration or evaporation.

The core of any chromatographic process is the column and its adsorption mechanism. For preparative protein purification ion-exchange chromatography is conventionally used. One reason is that aqueous protein-protecting buffers can be used and another reason is that both the selectivity and binding capacity are high. The support matrix of chromatographic columns plays an important role for the resolution between separated proteins and also the operability. The stationary phase was chosen according to the following demands: Fast flow rates, no specific knowledge for packing and quality testing required, generally easy handling, cleaning in place applicable, high cycle numbers without loss of performance and relatively low investment costs since the expected return on investment is low compared to pharmaceutical products. Ready to use membrane adsorber devices were chosen since they fulfill the requirements to a large extent. They are constructed such that the flow is forced through several layers of porous membranes functionalized with

cation- or anion-exchanger groups. For a linear scale-up, the total membrane area is increased but the number of layers, thus, the bed height, is the same at all scales. While membrane adsorbers have several advantages compared to packed columns, they are also known for a poorer resolution due to dispersion effects. As a result, elution peaks are broader with membrane chromatography compared to packed beads and, hence, baseline separation is more difficult to obtain.

With the ability to bind either positively or negatively charged proteins the bind and elute conditions on CEX and AEX had to be screened to develop a process as simple as possible. This means that the number of process steps has to be minimized, natural fluctuation of whey properties must not affect the process, and the adjustment of load material pH, conductivity and concentration should be avoided if possible. For example, the load material might have a conductivity which is too high for ion-exchange chromatography. But the decrease of conductivity is undesired because desalting is expensive and dilution of whey increases the volume to be processed and therefore, the overall processing time. The pIs of the target proteins are approx. between pH 4 and 9. Hence, buffer systems in this pH range were investigated, so that positive and negative net charge of each protein can be investigated for selective adsorption. Once several proteins are bound to an ion-exchanger the stepwise elution had to be realized by increasing ionic strength of the elution buffer.

The different properties of the six target proteins held some challenges. Their concentration in whey ranges from $ng \cdot mL^{-1}$ to $mg \cdot mL^{-1}$ so that a high yield particularly for the minor fractions Lf and LPO was a major focus. Selectivity was crucial because both impurities in target fractions and the loss of valuable protein due to overlapping elution peaks should be avoided. Some proteins have similar isoelectric points, so that a separate elution was not expected.

The process development at lab scale screened the process parameters pH and type of buffer salts, elution gradient shape and load ratio. Flow rate was kept constant according to manufacturer's recommendation and all experiments were run at ambient temperature. The final process started with AEX equilibrated with sodium phosphate buffer at pH 7 to bind β-Lg and BSA. Although α-La is also negatively charged at pH 7, its binding affinity was low. This was observed by an incomplete binding and some early eluting α-La in the wash phase. The low adsorption affinity is a consequence of the low net potential of α-La due to a high conductivity of whey. The charged amino acids at the protein surface are easily screened by counter ions

in the whey serum phase. It is well known that ion-exchange processes are sensitive towards high conductivity, which is recommended to be < 2 mS·cm^{-1}, as a rule of thumb. The conductivity of native whey is around 5 mS·cm^{-1}. The process was further optimized by an increased load ratio which resulted in the displacement of weakly adsorbed α-La by β-Lg which, in contrast, has a strong binding affinity. Thus, the complete α-La fraction was found in the flowthrough to be further purified. In the second separation step, CEX was equilibrated with sodium acetate at pH 4.8. The flowthrough phase from the AEX step was adjusted to pH 4.8 so that LPO, Lf and IgG are positively charged. pH 4.8 is close to the pI of α-La which remained again unbound. The adsorbed minor proteins could be separately eluted at different conductivities. However, it was observed that the recovery of Lf in the eluted phase was only around 60%. The other part did not bind to the cation-exchanger ligands and was detected in the flowthrough phase. Similar results have been reported in literature for Lf isolation tasks using IEC. But these results were not further discussed or investigated. During process development some improvements were reached by an adaptation of the load ratio. Some α-La bound weakly to the sulfonic acid ligands since the net charge is slightly positive at pH 4.8. It was assumed that competition for binding sites might occur between the large amount of α-La and the minor protein fractions. An increased load volume indeed resulted in higher purity of both the unbound α-La and the eluted Lf. Nevertheless, not more than 63% of Lf was recovered in the eluted Lf fraction. Finally, the flowthrough of three AEX cycles is required for the load of one CEX cycle.

The process developed at lab scale was transferred to pilot scale using the same type of radial membrane adsorber device, so that the scale-up was linear. This is possible because the bed height, which is the main limiting factor in chromatography for high flow rates at large scales, is constant at all scales. The membrane area was 50 fold increased from 110 cm² to 5500 cm² and the column volume from 3 mL to 150 mL. The load ratio and volumetric flow rate were linearly increased and it was found that pressure drop was as low as at lab scale. The binding capacity was highly similar over repeated cycles at both scales. Differences were observed between the separation efficiency with regards to purity and yield of the minor protein fractions. While the purity was higher at lab scale the yield was better at pilot scale (see Tab. 3). As discussed earlier, baseline separation is difficult to obtain with membrane adsorber devices. The consequence of overlapping elution peaks is that either the

yield or the purity are high or both are not satisfactory. For example, when the yield is to be maximized, the whole eluted fraction is collected and the part which overlaps with another peak reduces the purity. If the purity has to be high that part of a peak is collected which does not overlap with another elution peak. Hence, the overlapping fractions are lost for the yield.

Tab. 3: Purity and yield of fractions from cation-exchanger at lab and pilot scale (means ± standard deviation, n = 2).

	α-La	LF	LPO	IgG
Lab scale				
Purity (%)	90.6 ± 0.7	97.3 ± 1.1	85.6 ± 2.8	94.8 ± 0.4
Yield (%)	94.1 ± 0.5	65.7 ± 6.1	74.7 ± 2.3	74.6 ± 1.5
Pilot scale				
Purity (%)	90.7 ± 0.6	89.0 ± 4.4	56.5 ± 4.9	88.3 ± 2.3
Yield (%)	96.7 ± 1.3	38.6 ± 13.1	80.8 ± 14.1	86.1 ± 2.0

Repeated separation cycles without intermediate cleaning-in-place revealed that an initial drop in binding capacity after the first loading step occurs, but then is stagnant over the following four cycles. A typical CIP procedure with acidic and alkaline solution nearly fully recovered the initial binding capacity at both scales.

One unsolved problem that occurred during the process development was the inhomogeneous binding behavior of lactoferrin which resulted in a poor yield thereof. Within the possible variations in adsorption conditions such as pH-value, buffer type and ligand occupancy as a function of load ratio the problem could not be overcome. A separate study should deepen process understanding for the interaction between Lf and sulfonic acid ligands. Lf is an iron-binding protein that can accommodate up to two Fe^{3+} atoms. In native whey, approx. 20% of total Lf are saturated with iron and the remaining approx. 80% do not contain any iron. The iron binding induces remarkable changes in the protein structure, especially in the conformation of the two iron binding lobes. The apo-form is more open and exposes the negatively charged amino acid residues for the interaction with Fe^{3+}. The lobes of iron saturated holo-Lf are closed. The protein has an overall positive surface potential and therefore, Lf has a high pI around pH 9. Two regions on the protein surface have a high density of positively charged amino acids. It was suggested that the inhomogeneous binding behavior is somehow connected to the different isoforms of Lf. Adsorption studies with holo- and apo-Lf were conducted at pH 4.8 – 7.0 at non-overloading conditions

in order to avoid displacement effects. It was found that at near neutral pH both isoforms adsorbed completely. The adsorption of holo-Lf was less susceptible to acidic pH than apo-Lf. More than 85% holo-Lf adsorbed even at pH 4.8, whereas only 45-70% apo-Lf bound at pH 4.8. Isotherm studies confirmed the higher adsorption affinity of holo-Lf towards sulfonic acid ligands at pH 4.8 compared to apo-Lf. In the same study, the pI of both isoforms was determined via isoelectric focusing and revealed that both are in the range between pH 9.3-9.5. Thus, the surface net charge of apo-Lf cannot explain different adsorption behavior to CEX since the positive surface charge increases with decreasing pH. Hence, even better adsorption is expected at acidic pH for holo- and apo-Lf. A more likely reason is an intermolecular interaction due to electrostatic attraction between positively and negatively charged regions on the protein surface. This would explain why apo-Lf, which has a negative domain in the lobe, binds significantly worse at lower pH than holo-Lf, which does not have the negative patch, because Fe^{3+} is bound. However, the process understanding cannot be used to optimize the present separation process. A change of adsorption conditions to pH 7 would result in unbound IgG, because the pH is close to its pI. An additional separation step would be required. If apo-Lf would be saturated with iron it would have the advantageous adsorption properties of holo-Lf but that would require an extensive pretreatment of the whey as described in Bokkhim et al. (2013). This is not feasible, neither from an economical point of view nor for due to a loss of nativity of the other whey proteins. Additionally, apo-Lf is more valuable in terms of biofunctionality, e.g. antimicrobial properties, than holo-Lf because it can sequester iron.

It can be concluded that a pocess for the separation of several proteins with some expected difficulties could be developed. The membrane based stationary phase exhibited good resolution at both scales. Although packed beds have less axial dispersion than porous membranes and therefore, narrower peaks, the resolution between the minor protein fractions was adequate. When packed columns are run at large scale using comparable flow rates, the large beads lead to similar peak broadening (Gerberding & Byers, 1998; Hahn et al., 1998; Kristiansen et al., 1998; Wu & Xu, 2009). On the other hand packed beds are more susceptible towards blocking and a more extensive particle removal would be necessary. No problems with packing quality, gap building and back pressure occurred with membrane chromatography, which proves the applicability for untrained personnel.

However, the radial flow membrane device requires the microfiltration of whey in order to remove cheese debris and other particles that might block the 3-5 μm membrane pores. The crossflow filtration step implies the installation of further equipment. To eliminate the prefiltration step, a tangential flow membrane chromatography (TFMC) device was investigated. The flow is not forced through the porous membrane but flows more or less pressureless in the channels between two membrane layers realized by a spacer screen with a thickness of 250 μm. In contrast to spiral wound membranes for crossflow filtration, there is no transmembrane pressure. Hence, pore blocking is not to be expected, because there is no drag force towards the membrane pores and eventual particles can pass the 250 μm gap without clogging them. The drawback of the TFMC device is the mass transfer regime, which is only convection at the membrane surface and dominantly diffusion in the inner membrane pores (see Fig. 15). The selectivity known from packed beds and radial flow membrane chromatography cannot be reached with TFMC, because dispersion effects in the large channels between the membranes do not allow a sharp step gradient.
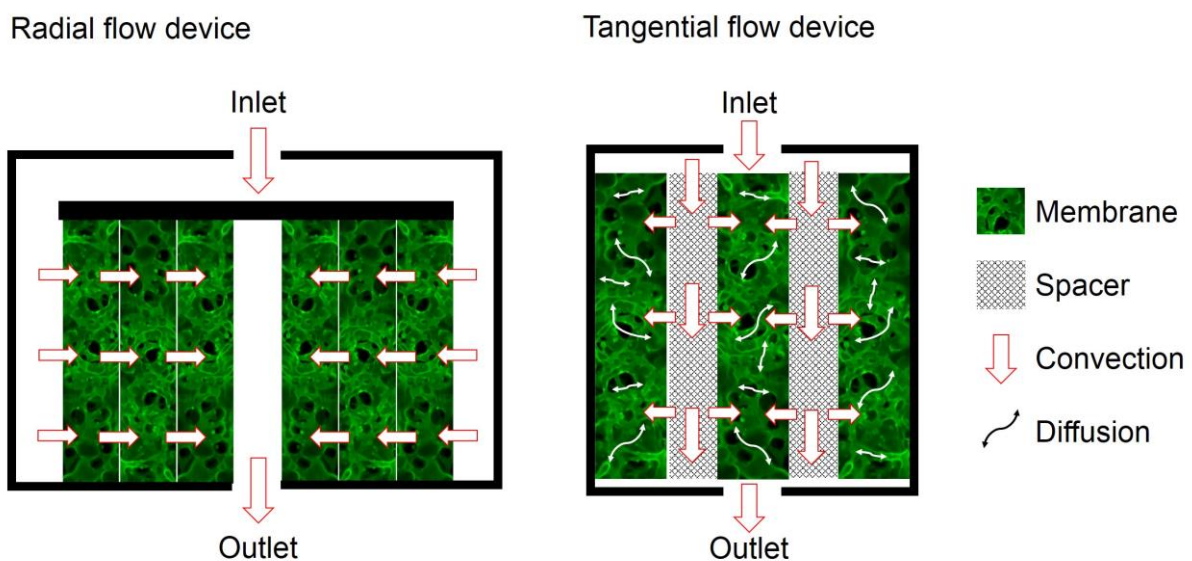
Fig. 15: Schematical illustration of the radial flow adsorber type (left) and the tangential flow adsorber type (right). In radial flow adsorbers mass transfer is convective through the membrane layers. In tangential flow adsorbers, convection takes place in the channels between mambrane layers and in the pores close to the channels. Inside the membranes mass transfer is diffusive. In the channels, where the spacer net separates the membarne layers, dispersion occurs.

However, the selective adsorption of only one target protein without gradient elution is applicable. For that purpose, the effect of temperature has been studied in order to evaluate, whether diffusion is significantly accelerated at higher temperature. The

used model protein was β-Lg. On the one hand, because the selective removal of β-Lg is of high interest for infant formula and on the other hand, because it is most sensitive towards temperature changes. The reactivity significantly increases at temperatures around 40°C. In batch experiments under pure diffusional conditions the effective diffusivity increased with increasing temperature overproportional to the viscosity effect of the mobile phase. Additionally, the dimer-monomer conversion around 40°C contributed to a higher diffusion rate. However, it was shown that the time until saturation of the binding sites takes more than two hours even at the fastest diffusion rate. In comparison to the loading time of not more than 5 min in radial flow membrane devices the application of TFMC does not appear to be suitable for industrial applications. In order to avoid an additional microfiltration step prior to the radial flow chromatography, other methods for particle removal such as high-performance centrifuges could be installed upstream.

The effect of temperature was further considered, because the processing of fresh whey at ambient temperature involves the risk of microbial growth even if it was microfiltered. Apart from the transfer kinetics also adsorption affinity and undesired interactions between proteins or with the stationary phase might occur at elevated temperature. Therefore, several studies were done in a temperature range between 10 and 50 °C. Isotherm studies showed that the equilibrium binding capacity increased with decreasing temperature and the same effect was observed for breakthrough experiments in radial flow membrane devices. This result was unexpected, because temperature so far is known to be negligible in IEC in contrast to hydrophobic interaction chromatography or reversed phase chromatography. This is also the reason why only very few studies deal with protein separations using IEC in dependence of temperature. It is reported that the effect was either negligible or the adsorption increased with increasing temperature (Finette, Mao, & Hearn, 1997; Mihelič, Podgornik, & Koloini, 2003). In the present case, it was considered that the reason must be directly related to the properties of β-Lg other than thermodynamics in IEC in general. At native pH, at which β-Lg is adsorbed to the AEX, it is known to be dimeric at cold and ambient temperature and dissociates into monomers around 40°C. Dimeric β-Lg is assumed to build a thicker adsorbed layer than the monomer at 50 °Cso that more protein molecules can be accommodated with the same number of binding sites (see Fig. 16, left and right).
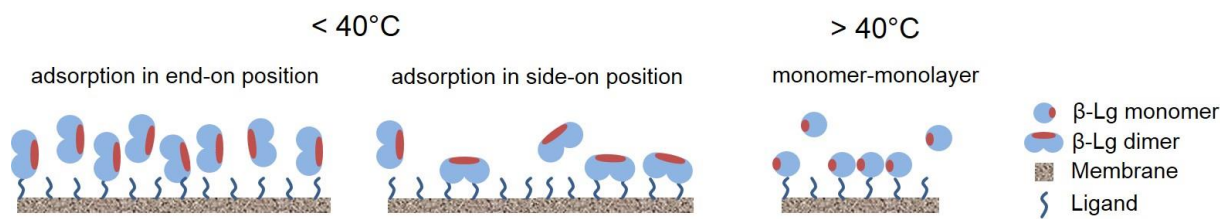
Fig. 16: Illustration of adsorbed layers of β-Lg in dependence of temperature. Dimeric β-Lg below 40°C can adsorb in end-on position (left) or in side-on position (middle). Above 40°C β-Lg is monomeric (right).

However, the extent of the temperature effect depended on the membrane adsorber setups. Batch adsorption experiments with pure diffusion were compared to radial flow adorbers with convective transport. Additionally, isotherm experiments were done at 10-50°C. When mass transfer was fast, which is the case for convection and high protein concentration in isotherm experiments, the maximum binding capacity was strongly dependent on temperature. When diffusion was slow, as in the batch adsorption experiments, the binding capacity was approx. the same between 10-50°C. It was assumed, that the orientation of adsorbed β-Lg dimers is the reason for this effect. If β-Lg adsorbs in end-on position (Fig. 16, left), the thickness of the adsorbed layer equals two monomers. This happens, if the transport is fast and dimers compete for binding sites. If the transport is slow, many ligands are available and dimers in side-on position (Fig. 16, middle). Once they adsorbed at several sites, the adsorption is strong and a displacement by other dimers is most probably very slow or even unlikely to occur.

4. Conclusions and Outlook

The overall aim of this work was the development and scale-up of a simultaneous separation process for six major and minor whey proteins using membrane based ion-exchange chromatography. This is the first study reporting the purification of more than three proteins in a complex mixture, such as whey, in two simple steps. It can be concluded that the application of membrane adsorber devices is well suited for the loading of microfiltered whey without pore blocking over repeated cycles. Although it was expected that the purity will be low due to dispersion effects in membrane adsorber devices and that some proteins will not be separable, the opposite was found. Purity, yield and concentration factors of resulting fractions are similar or even better in comparison to other whey separation attempts described in literature. However, it has to be taken into consideration that the process was

optimized for the separation of six proteins in two steps and does not deliver the best practice for each single protein. Although for most proteins the adsorption conditions are very good, lactoferrin had a low binding affinity. It was found that a complete adsorption of lactoferrin is only possible at near neutral pH, whereas the acidic pH region is not favorable. Hence, if lactoferrin was the only target protein, pH conditions should differ from those suggested in the present process.

Furthermore, it was found that temperature plays a significant role in whey protein separation using ion-exchange chromatography. Despite the fact that temperature is known to be negligible in electrostatic interactions compared to hydrophobic interactions, adsorption of the major whey protein β-Lg was found to be highly susceptible to temperature. The binding capacity for β-Lg at pH 7 in sodium phosphate buffer doubled when the process was run at 10°C instead of 50°C. This result leads to the conclusion that processing at low temperature is favorable.

Finally, the applicability of tangential flow membrane chromatography (TFMC) was investigated and evaluated. From the results obtained with β-Lg in acid whey at pH 7 it could be demonstrated that the isolation of a single protein is feasible. TFMC has the claimed advantage that fluids with high particle load can be processed without prefiltration. Such load material cannot be used for conventional chromatographic processes under any circumstances except in expanded bed technology (Du, Lin, Xiong, & Yao, 2013; Du, Lin, Zhang, & Yao, 2014) where no densely packed bed is used. However, TFMC has several disadvantages such as low binding capacity, slow adsorption, and increased dispersion and dilution effects in the column. Therefore, it is not recommended for load materials that can be processed with radial flow membrane devices. The advantage of radial flow membrane devices is the convective transport of target molecules to the functional groups throughout a membrane cross section. A bind and elute process takes only a few minutes independently from the scale. In contrast, in TFMC fast adsorption takes place only at the membrane surface whereas it takes hours to reach the inner membrane pores. Hence, the process is rather run below ligand saturation and, thus, binding capacity is very low. Additionally, the elution process is subject to dilution and dispersion in the large channels between the membranes. This is also the reason why the simultaneous adsorption of several proteins and the stepwise elution thereof is not efficiently applicable with TFMC compared to radial flow membrane devices.

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6. Appendix

6.1 Non-reviewed papers

Voswinkel, L., Kulozik, U.: Membranbasierte Chromatographie zur Wertsteigerung von Süßmolke. DMW – Die Milchwirtschaft 4 (7) 225 - 227, 2013

Voswinkel, L., Kulozik, U.: Membrane based Chromatography creates Added Value in Sweet Whey. EDM – European Dairy Magazine 25 (2) 4 – 6, 2013

Voswinkel, L., Kulozik, U.: Fraktionierung von Proteinen aus Molke mit adsorptiven Membranen. Abschlussbericht zu AiF/FEI 16540 N, TU München

6.2 Oral presentations with first authorship

Voswinkel, L., Kulozik, U.: Cyclodextrine und Lipocaline als Träger für hydrophobe Substanzen. Technologieseminar Weihenstephan „Mikroverkapselung und Mikropartikulierung: Native und neue Strukturen aus Proteinen und Polysacchariden", Freising, Weihenstephan, Germany, 28. - 29.10.2010

Voswinkel, L., Kulozik, U.: Fractionation of whey proteins by means of membrane adsorption chromatography, ICEF, 11th International Congress on Engineering and Food, Athens, Greece, 22. - 26.05.2011

Voswinkel, L., Kulozik, U.: Tangential-flow adsorptive membranes for high throughput fractionation of whey proteins, Milchkonferenz, Bern, Switzerland, 12. – 13.09.2011

Voswinkel, L., Kulozik, U.: Whey Protein Ffractionation in One Unit Operation: A Lab Scale Approach, 6th International Whey Conference IWC 2011, Chicago, USA, 18. – 22.09.2011

Voswinkel, L., Kulozik, U.: Chromatographische Proteinfraktionierung aus unfiltrierten Lebensmitteln und Fermentationsbrühen. Technologieseminar Weihenstephan „Konzentrieren und Trocknen von Lebensmitteln", Freising-Weihenstephan, Germany, 27. - 28.10.2011

Voswinkel, L., Kulozik, U.: Separation of Whey Proteins with Fast Flow Ion Exchange Chromatography, 8th International PhD Seminar on Chromatographic Separation Science, Freudenstadt-Lauterbad, Germany, 04. – 06.03.2012

Voswinkel, L., Kulozik, U.: Fractionation of whey proteins with chromatographic membranes at alb and pilot scale, ProcessNet Jahrestreffen der Fachgruppe Lebensmittelverfahrenstechnik, Hohenheim, Germany, 19. – 21.03.2012

Voswinkel, L., Kulozik, U.: High-throughput chromatographic processes for the separation of dairy proteins and peptides, DLG-Symposium Innovative Products and Processes for the Dairy Industry, ANUGA FoodTec, Köln, Germany, 28.03.2012

Voswinkel, L., Kulozik, U.: Cascaded anion and cation exchanger monolithic columns for the fractionation of major and minor whey proteins, 5th Monolith Summer Symposium 2012, Portoroz, Slovenia, 04. – 06.06.2012

Voswinkel, L., Kulozik, U.: Direct Capture Chromatography at Pilot Scale: Fractionation of Whey Proteins with CAscadedIon Exchnagers, 6th International Congress on the Food Factory for the Future, Laval, France, 04. – 06.07.2012

Voswinkel, L., Kulozik, U.: Comparative study of fast flow whey protein fractionation with membrane and monolith based ion exchange chromatography, International Symposium on Preparative and Process Chromatography, PREP, Boston, USA, 16. – 18.07.2012

Voswinkel, L., Kulozik, U.: Gewinnung von Einzelproteinkomponenten aus komplexen Systemen wie Milc, Molke und Ei. Technologieseminar Weihenstephan „Verfahrens- und Strukturoptimierung in der Lebensmittelherstellung", Freising-Weihenstephan, Germany, 25. - 26.10.2012

Voswinkel, L., Kulozik, U.: Direct capture membrane adsorption chromatography with crude whey at pilot scale. ADSA Joint Annual Meeting, Indianapolis, USA, 08. – 12.07.2013

Voswinkel, L., Kulozik, U.: Grundlagen zur chromatographischen Fraktionierung. Technologieseminar Weihenstephan „Neue Ansätze für industrielle Trenntechniken in der Lebensmittel- und Biotechnologie", Freising, Weihenstephan, Germany, 23. - 26.10.2013

Voswinkel, L., Kulozik, U.: Molkenproteinfraktionierung mittels konvektiv unterstütztem Stoffaustausch in chromatographischen Membranadsorbern und Monolithen. Technologieseminar Weihenstephan „Neue Ansätze für industrielle Trenntechniken in der Lebensmittel- und Biotechnologie", Freising, Weihenstephan, Germany, 23. - 26.10.2013

Voswinkel, L., Kulozik, U.: Mass transfer in Sartobind Direct Capture compared to membrane adsorber systems dominated by diffusion or convection. 10th International PhD Seminar on Chromatographic Separation Science, Egmond aan Zee, 23. – 25.02.2014

Voswinkel, L., Kulozik, U.: Removal of β-lactoglobulin as single target protein from whey to reduce the allergenic potential of whey products. Technology Seminar Weihenstephan „Food Bioprocessing: New functionalities through production, concentration and stabilization of biologically active components", Freising-Weihenstephan, Germany, 17. - 19.09.2014

6.3 Poster presentations with first authorship

Voswinkel, L., Kulozik, U.: Adsorptive tangential-flow membranes for highthroughput fractionation of whey proteis from cheese whey. International Congress on Membranes and Membrane Processes, ICOM, Amsterdam, Netherlands, 14. – 15.10.2011

Voswinkel, L., Kulozik, U.: High throughput whey protein separation at pilot scale with CIM® monolithic columns. 26th PREP International Symposium on Preparative and Process Chromatography, Boston, USA, 14. – 17.07.2013

Voswinkel, L., Kulozik, U.: Direct Capture Membrane Adsorption Chromatography for Crude Cheese Whey at Technical Scale. IDF World Dairy Summit, Yokohama, Japan, 28.10. – 01.11.2013

6.4 Curriculum vitae

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Scientist, DSP Development, Sandoz Biopharmaceuticals, Langkampfen, Austria

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Department of Natural Products and Biotechnology, Mediterranean Agronomic Institute of Chania, Greece

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