PEPTIDE AFFINITY EXTRACTION OF β-LACTOGLOBULIN FROM MILK

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Dedicated to my beloved and graceful Master, spiritual guide and Supreme being ~ Baba Gurinder Singh Dhillon.

Abstract

A major challenge faced by dairy industries is the extraction of whey proteins from milk without pre-treatment, because of the presence of colloidal solids and the complex composition of milk. Various methods have been investigated to isolate whey proteins but other than the extraction of lactoferrin, none have been successful because of high processing costs and low product yield. To overcome some of these challenges, this thesis focused on the specific isolation of β-lactoglobulin, in particular without involving pre-treatment of the milk. Previous investigations showed β-lactoglobulin to be allergenic towards some infants and hence its removal from whey and milk was the main objective of this thesis. Isolating this protein from milk using a single-step extraction process has not been demonstrated previously.

A Protein A Mimetic (PAM) ligand, originally designed for isolation of Immunoglobulin G from a variety of sources, was shown in previous unpublished work to have an unexpected affinity for β-lactoglobulin. In the current work, the equilibrium/static binding capacity of this PAM ligand immobilized on a chromatography resin was determined using varying concentrations of pure β-lactoglobulin in neutral aqueous solutions. An affinity chromatography resin was created by amine-coupling the ligand to an activated resin (N-hydroxysuccinimidyl-Sepharose 4 Fast Flow), and its equilibrium binding capacity was found to be 54.11$\pm$0.03 mg β-lactoglobulin/g wet, drained resin.

PAM tetramer ligand showed some cross-reactivity with caseins and immunoglobulins, but the majority of these proteins were recovered in the regeneration step. The majority of the β-lactoglobulin was recovered during elution, with minor traces of α-lactalbumin and immunoglobulins.

A novel, cost effective and scalable method was developed to isolate β-lactoglobulin from milk using an immobilized tetramer peptide ligand, which had previously shown high affinity towards β-lactoglobulin in the presence of other milk proteins. Previous research had shown that the peptide ligand maintained selectivity over a range of buffer pH values, but the optimum selectivity was found to be at pH 7. At buffer pH 7 and a conductivity of around 8 mS/cm, the affinity resin bound almost all β-lactoglobulin present in whey/milk, with only minor leakage in flowthrough fractions. An advantage to this process was the use of low salt during the elution process. Bound β-lactoglobulin was observed to elute at a buffer

conductivity of 22 mS/cm.

The results confirmed that β-lactoglobulin could be selectively removed from milk, thereby producing modified milk suitable for infant intake. In addition, β-lactoglobulin has uses in the confectionary and protein supplement industries and can be used as a gelling and foaming agent in functional foods. Suggestions for further work are expected to support development of this process and to provide a higher recovery of the bound protein, to minimize product loss.

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Abbreviations

α-lac	α-lactalbumin
β-lac	β-lactoglobulin
BSA	Bovine Serum Albumin
BCCA	Branched-chain amino acids
CFM	Cross flow microfiltration
DEAE	Diethylaminoethyl
DMF	Dynamic membrane filtration
EUF	Electro ultrafiltration
ELISA	Enzyme-Linked Immunosorbent Assays
EBA	Expanded bed adsorption
FAO	Food and Agriculture Organization
Fc	Fragment crystallizable
Ig	Immunoglobulin
IPA	Isopropanol
pI	Isoelectric point
LF	Lactoferrin
LP	Lactoperoxidase
MW	Molecular weight
NF	Nanofiltration
NHS	N-hydroxy succinimide
PAM	Protein A Mimetic
RO	Reverse osmosis
SEC	Size exclusion chromatography
UF	Ultrafiltration

CHAPTER 1 - INTRODUCTION

1.1 BACKGROUND AND SIGNIFICANCE

Milk, more commonly known as nature's most complete food, has been used as an excellent food source for many centuries (Park, 2009). Amongst the different sources available, milk from domestic animals such as cows and goats has been preferred, depending on their availability (Agostoni et al., 2000). Milk also serves as a raw material for a diverse family of food products. The primary function is to meet the complete nutritional requirements, mainly of neonates. It is the most adaptable and flexible of all food materials, simplifying its use in producing various products (Fox et al., 2015).

Bovine milk has been the major source of dairy products in developed countries, especially in the Western World (Park, 2009). Although bovine milk is consumed worldwide, there is a clear difference between this and human milk with respect to their bioactive component profiles, especially protein content. A comparison of protein content present in bovine and human milk is shown in Table 1-1. The most critical difference between these, in the context of this thesis, is the absence of β-lactoglobulin (β-lac) in human milk, whereas bovine milk contains nearly 3 g/L of β-lac. Bovine milk also has a total protein content that is three times higher than that of human milk (Addlesperger, 2008).

Table 1-1: Comparison of protein composition in human and bovine milk (Lönnerdal et al., 1976)

	Human milk (g/L)	Bovine milk (g/L)
Casein	2.5	27.3
Lactoferrin	1.7	Trace
Lysozyme	0.5	Trace
Serum albumin	0.5	0.4
Immunoglobulin A	1.0	0.03
Immunoglobulin G	0.03	0.6
Immunoglobulin M	0.02	0.03
α-lactalbumin	2.6	1.1
β-lactoglobulin	---	3.6

Human and bovine milk are considered to be the most important source of natural bioactive components comprising lipids, carbohydrates, peptides and specific proteins (Agostoni et al., 2000). Initially, it was believed that milk contained only one type of protein but many years ago it was shown that proteins in milk could be fractionated into two well-defined groups comprising caseins and whey proteins.

Identification of major caseins and whey proteins has been based on their primary structures. Casein is the best characterized milk protein and constitutes over 70%-80% of total bovine milk protein. Whey represents a source of protein ingredients for many novel food products. Whey proteins are well known for their high nutritional value as well as for their functional properties such as emulsification, stabilization, gelation and foaming in food products. These proteins also improve the texture of various food products including dairy products and confectionaries (Patel et al., 1990).Normal bovine milk contains about 3.5% protein by

weight, of which casein constitutes about 80% and whey proteins constitute 20% (Korhonen and Pihlanto-Leppälä, 2004).

Casein, the principal protein present in bovine milk can be further classified into α-casein, β-casein and κ-casein (Thompson et al., 1965), while the major constituents of bovine whey include immunoglobulins (Igs), β-lac, α-lactalbumin (α-lac) and bovine serum albumin (BSA). Bovine whey proteins are comprised of approximately 50–60% β-lac (Table 1-1). β-lactoglobulin has been demonstrated to be an allergic protein for infants and this limits the use of cow's milk for manufacturing infant formulae (Businco et al., 1993).

To take greater advantage of the nutritional and functional properties, many processes to recover and concentrate whey proteins have been investigated, including demineralization and crystallization. One of the oldest methods for isolating whey proteins involves acid precipitation and denaturation. Using these methods, proteins retain their nutritive value but because they are insoluble in water, the proteins lack other functional properties such as texturizing characteristics and binding properties (Marshall, 2004).

Other methods proposed for fractionation of whey proteins from acid whey include membrane filtration (Zydney, 1998), chromatography (Fee, 2010) and selective precipitation (Casal et al., 2006b, Lozano et al., 2008). These methods have proven good for laboratory purification purpose but have not been widely used for industrial-scale purpose because of their complexity, high processing cost, poor selectivity and product degradation during extreme heat treatment and excess salt usage.

The complex nature of milk has proven to be disadvantageous for extraction of proteins and has remained a challenge for several years. For instance, the presence of fat globules and casein micelles prevent flow through the interparticle void spaces of a packed bed chromatography column, thereby causing an increase in back pressure, leading to low or zero flow rates (Fee and Chand, 2006). More recently, new and improved technologies are increasing the potential to extract milk proteins directly from whole milk.

Previously, Igs have been recovered from complex fluids and the more commonly used technique for recovery of Immunoglobulin G (IgG) includes either Protein A or Protein G affinity chromatography (Linhult et al., 2005, Kochan et al., 1996). Less costly and more robust oligpeptide-based ligands with similar affinity towards Igs (Protein A mimetics or PAMs) were developed, mainly to avoid the use of the original bacteria-derived Protein

A/Protein G ligands. These PAM ligands were proven to show affinity towards IgG, but unexpectedly showed (in unpublished research by a PhD student in the Biomolecular Engineering Research Group at the University of Canterbury) affinity towards β-lac as well. This hence offers a new opportunity to isolate β-lac from milk (Billakanti, 2009).

To purify milk for use in infant formulae, it is necessary to make sure that β-lac is either totally or partially removed from milk products intended for infant intake. There have been a number of techniques used in the past to isolate this protein from whey solutions, as will be described in Chapter 2, but the use of the PAM ligand to achieve this separation has not been attempted previously.

1.2 OBJECTIVES

The objectives of this research were as follows:

- Investigate and characterize the PAM peptide as an affinity ligand for selective isolation of β-lac from whey and milk
- To determine the equilibrium binding capacity of tetramer peptide resin to β-lac
- To calculate the recovery of bound protein by performing elution experiments

1.3 THESIS ORGANIZATION

The remainder of this thesis is organized as follows:

Chapter 2: This chapter outlines the background of the research and reviews the existing literature related to this study. In the first section, a brief description of milk components, including proteins and their uses in the dairy industry is provided. This section mainly focuses on the various available β-lac extraction technologies. In the second section, an elaborate description of our protein of interest is discussed, including its potential allergenic effects on infants. The final section of this chapter describes various relevant protein extraction techniques. This chapter also present potential applications of PAM peptide-based affinity ligands in the isolation of β-lac from milk.

Chapter 3: In this chapter, a description of the equipment used is provided, along with the preparation of buffers and details of experimental protocols are also described.

Chapter 4: This chapter presents the results of experimental work, including the equilibrium

binding capacity of the PAM resin with regard to pure β-lac. Also presented are the results of using the synthetic peptide ligand to isolate β-lac from milk and whey. The advantages and disadvantages of other methods for isolating this protein are compared with those of the PAM-based affinity chromatography method proposed in this research.

Chapter 5: Conclusions and recommendations for future work are summarized in this chapter.

CHAPTER 2 - LITERATURE REVIEW

2.1 INTRODUCTION

Previously, experiments have been performed to isolate various proteins from milk for applications in food industries (Manji and Kakuda, 1986, Goodall et al., 2008). Because milk is complex, it has been difficult to extract and quantify milk proteins (Pampel et al., 2007). Various techniques commonly used to fractionate proteins include membrane filtration, adsorption, chromatography and precipitation. Direct chromatographic capture of proteins from milk has been of great interest and has been used in the recovery of pharmaceutical proteins from transgenic animals. The purification processes are mainly affected by the presence of solids and fat globules in milk, causing clogging of chromatographic column (Shah, 2000). Hence, purification methods have mainly been carried out on defatted milk/whey streams to prevent clogging of membranes and columns. The current processes for protein recovery from milk streams precede chromatographic purification methods with a number of steps designed to remove major milk components, including caseins and lipids (Shah, 2000, Swaisgood, 2003). These pre-treatment steps are deemed necessary to safeguard chromatographic column integrity and performance but overall suffer from serious product loss. Hence, to overcome these losses and obtain a high initial product recovery, direct capture of proteins of interest, without pre-treatment would be ideal.

The following section (Section 2.2) includes a brief presentation of the global milk production practices as a background to the objectives of this research.

2.2 GLOBAL MILK PRODUCTION

According to recent reports released by the FAO (Food and Agriculture Organization, US) in 2014, the total amount of milk production around the world in 2013 was approximately 636 million tonnes. The USA was the largest cow's milk producer in the world in 2013, accounting for 14.4% of world production. Nearly 56.1% of the production was from ten countries, ranking USA as the highest and NZ as the eighth largest producer of cow's milk in the world (Figure 2-1). Approximately 150 million households around the globe are engaged in milk production (Agriculture and Horticulture Development Board, 2015).

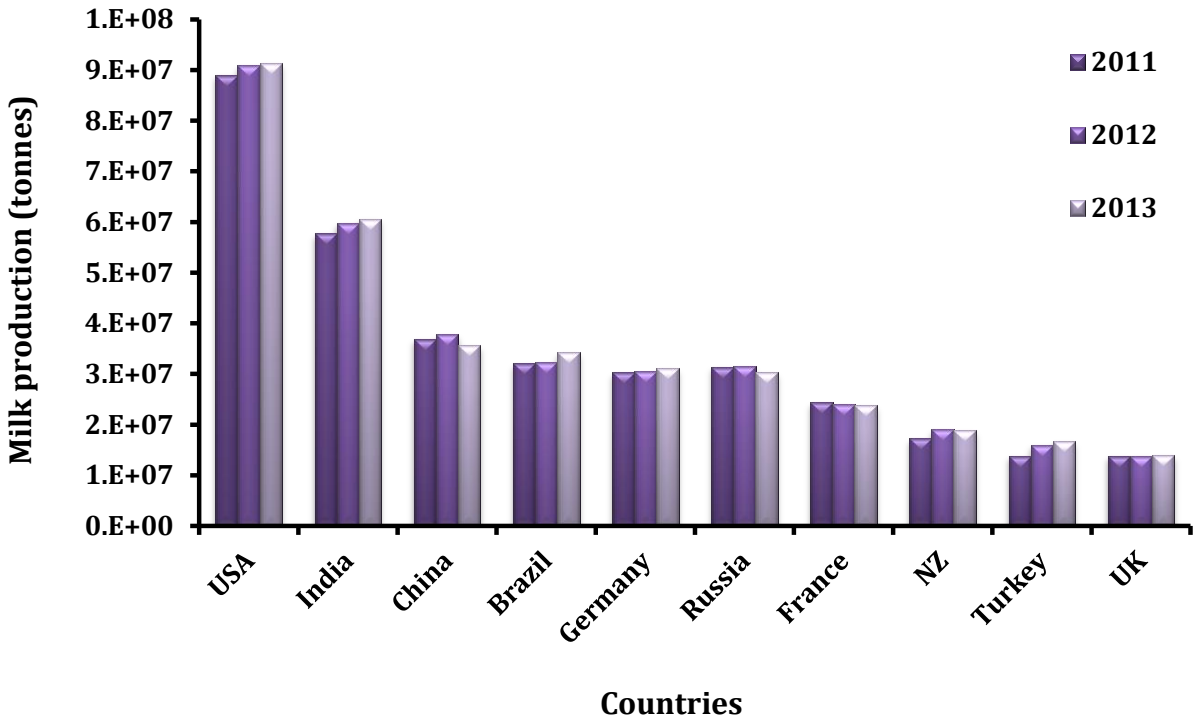

Figure 2-1 Milk produced in the World past 3 years (Agriculture and Horticulture Development Board, 2015)

With annual exports in excess of NZ $13.7 billion, the dairy industry is New Zealand's biggest export earner, accounting for more than 29% by value of the country's merchandise exports. Around 95% of the milk New Zealand produces is exported.

Dairy productivity has risen markedly over the past decade. Milk solids (kg) include the total of milk proteins and fat. A gradual increase in production of milk solids has been observed over the past few years (Figure 2-2). Major exporting destinations for New Zealand dairy commodities include USA, Australia, China, Europe, Malaysia and Japan (Dairy Companies Association of New Zealand, 2015).

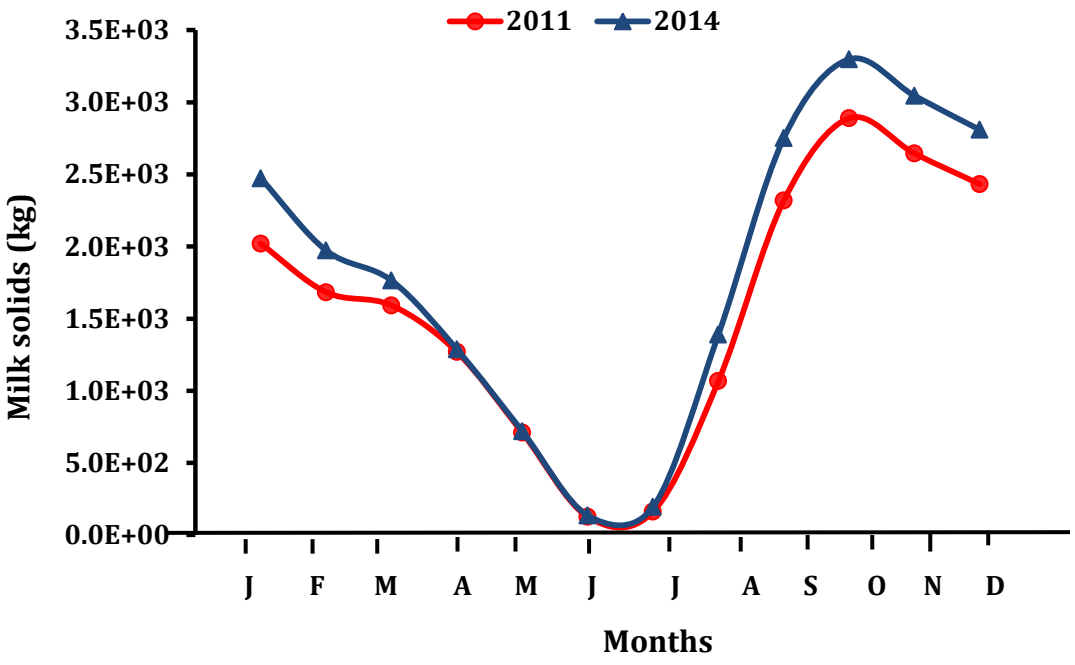

Figure 2-2: NZ milk production for years 2011 and 2014 (Dairy Companies Association of New Zealand, 2015)

According to recent reports (Figure 2-2), a gradual increase in NZ milk production has been observed for the period 2011-2014.

2.3 MILK CONSTITUENTS

The composition of bovine milk is of greatest importance in the dairy industry (Smithers, 2008). Milk of different ruminant species, directly or as dairy products comprise food of outstanding importance for humans (Rose et al., 1970). With some exceptions, milk contains all nutrients required for the growth and development of neonates and is known as the primary source of nutrition for infants. Although studies have been carried out on milk obtained from several species such as cow, goat, and sheep, cow's milk has proven to serve benefits to human health because of its resemblance to human milk (Madureira et al., 2007, Andersson and Mattiasson, 2006). Table 2-1 gives a comparison of nutrients present in milk of different species.

Table 2-1: Comparison of milk nutrients from different species (Bramaud et al., 1997)

Nutrients	Human (g/L)	Cow (g/L)	Buffalo (g/L)	Goat (g/L)
Total solids	129	125	171	130
Proteins	10	34	38	29
Caseins	4	28	32	23
Whey proteins	5.5	6	6	6
Fat	38	31	75	45
Lactose	71	48	49	45
Oligosaccharides	3.8	0.03-0.06	---	41
Riboflavin	0.00043	0.0018	0.0010	0.0044
Ash	2	7	8	8
Calcium	0.34	1014	1.85	1.3
Phosphorus	0.14	0.93	1.25	1.06

Bovine milk is a rich source of bioactive compounds that are increasingly being used as nutraceuticals. It is a complex heterogeneous fluid consisting of essential nutrients such as proteins, fat globules, minerals and other minor components (Gurgel et al., 2001). Overall, milk comprises approximately 87% water and 13% milk solids. These milk solids are comprised of 8.9% non-fat solids, 3.7% triglycerides, esters and amino acids. The non-fat solids are further classified into a mixture of proteins (3.4% whey proteins and caseins), lactose (4.8%) and other minor components (0.7%) (Jackson et al., 2004). An extensive study has been carried out with regard to milk proteins in terms of their structure and functional properties (Rose et al., 1970) (Table 2-2).

Table 2-2: Bovine milk protein properties (Jackson et al., 2004, Gurgel et al., 2001)

Proteins	Concentration (g/kg of solids)	% total protein	Amino acid residues	Molecular weight (kDa)	Isoelectric point (pI)
CASEINS					
α-s_1-casein	12-15	30.6	199	23.6	4.9-5.3
α-s_2-casein	3-4	8	209	25.3	4.9-5.3
β-casein	9-11	30.8	209	24	5.2
κ-casein	2-4	10.1	169	19	5.8
WHEY PROTEINS					
α-lac	0.6-1.7	3.7	123	14	4.4
β-lac	2-4	9.8	162	18.3	5.4
BSA	0.4	1.2	582	67	5.1
Lactoferrin (LF)	0.02-0.1	0.3	690	77	7.9
Lactoperoxidase (LP)	0.03	0.1	612	78	9.6
Igs					
IgG1	0.3-0.6	1.2	---	161	5.5-6.8
IgG2	0.05	0.15	---	150	7.5-8.3
IgM	0.09	0.3	---	1000	5.5-7.4
IgA	0.01	0.03	---	385-417	4.5-6.8

Milk is recognized as a complete food for newborn mammals which contains a number of bioactive components including oligosaccharides, vitamins, minerals, proteins and lipids. Research has proved that milk contains high levels of Igs and other physiologically active compounds for warding off infection in the newborn (Séverin and Wenshui, 2005). Amongst all milk nutrients, proteins have a major impact on human health (Shah, 2000). These proteins are broadly classified into caseins and whey proteins. All proteins have shown differences in their physical and functional properties (Wong, 2012).

As seen from Table 2-1 and 2-2, a clear difference in β-lac content of human and bovine milk is observed. Human milk contains all proteins necessary for infant nutrition and hence has been used for their intake, but the presence of β-lac in bovine milk may limit its use for

infants. Hence a process that would make bovine milk similar to human milk (devoid of β-lac) may be very beneficial for manufacturing infant formulae.

2.3.1.1 Milk proteins – Properties and applications

Milk proteins contain a plethora of components, all of which contribute to the nutritional value of milk and are the best characterized amongst all the food proteins (Swaisgood, 2003). Whey has been produced as a by-product of cheese manufacturing for decades and has been considered as a waste product. Whey disposal has remained a big issue that poses environmental problems in many countries. To recover value, initially, milk proteins were recovered from whey streams by various precipitation methods and selective isolation methods (Smithers, 2008, Hidalgo and Hansen, 1971). In the recent past, research has mainly focussed on isolation of whey proteins from milk because these proteins have numerous applications in food and healthcare industries. Achievements have been reported for extraction of major whey proteins such as α-lac, β-lac from acid whey solutions (Smithers, 2008). A detailed description of the different milk proteins is described in the sections below.

2.3.1.1 Caseins

Caseins are the best characterized milk proteins, constituting nearly 70%-80% of total bovine milk proteins. These proteins are known to be a good source of essential elements (such as calcium, amino acids and inorganic phosphates) and hence provide nutrition to neonates (Rose et al., 1970). All caseins are essentially found in micelles which can be then isolated by centrifugation or by size-exclusion chromatography. Another common method to fractionate caseins is by precipitation at pH 4.6, because acidification causes the casein micelles to aggregate by reducing the electric charge to its isoelectric point (Zhang, 2002).

Caseins are broadly classified into: α-, β-, κ- and γ- caseins. α-casein is the most dominant casein in milk and is known to be calcium sensitive. In bovine milk, 65% of the casein fraction is comprised of the $\alpha\text{-}_{S1}$ and $\alpha\text{-}_{S2}$ subunits of molecular weights 23.6 and 25.2 kDa, respectively (Bhattacharyya and Das, 1999). $\alpha\text{-}_{S1}$ casein has been identified with five genetic variants (A, B, C, D and E) and the structure is known to consist of hydrophobic domains. On the other hand, $\alpha\text{-}_{S2}$ casein has been identified with two genetic variants (A and D) and is more hydrophilic when compared with $\alpha\text{-}_{S1}$ casein. β-casein is also known to be calcium-sensitive, whereas κ-casein is known to be resistant to calcium precipitation. This protein plays a vital role in stabilization of caseins in presence of calcium (Farrell Jr et al., 2004).

2.3.1.2 Whey proteins

Whey proteins are known to be rich in sulphur-containing amino acids such as cysteine and methionine and are highly soluble in aqueous buffers. Whey proteins include macropeptides, major proteins such as β-lac, α-lac, BSA, Igs and minor proteins such as membrane proteins, LP (Lactoperoxidase) and LF (Lactoferrin). The main antibody constituent of bovine milk is IgG, but it also contains low levels of IgM and IgA. These proteins can be denatured by heating them to temperatures above 65-70°C.

Whey proteins are typically known as globular proteins. In comparison with caseins, these proteins are more sensitive to heat treatment but are highly stable to pH variations (Korhonen and Pihlanto-Leppälä, 2004). Whey proteins possess a number of applications to human health and have been observed as a rich source of amino acids (Madureira et al., 2007). Recently, researchers have become interested in producing individual whey proteins during whey protein fractionation, with well-characterized functional and biological properties through processes that will not denature the proteins but retain their nutritional and other properties (Bhattacharyya and Das, 1999).

α-lactalbumin

α-lactalbumin is the most abundant whey protein in human milk whey and the second most abundant protein in bovine milk whey. This protein comprises approximately 3.4% of total milk protein and 20% of total whey protein and is nearly 14.2 kDa in weight and has a theoretical pI of 4.82. α-lac concentration in bovine whey protein ranges between 1.2-1.5 mg/mL (Andersson and Mattiasson, 2006). On the other hand, α-lac is the predominant whey protein in human milk and has a concentration of 2.44$\pm$0.64 mg/mL, after 30 days of lactation (Jackson et al., 2004).

α-lac is the preferred source for infant formula because of its high tryptophan and cysteine content, high digestibility and low potential for causing infant allergies (Gurgel et al., 2001). It also possesses therapeutic uses and is therefore used in nutraceutical industries (Konrad and Kleinschmidt, 2008). Commercial availability of bovine α-lac allows the production of infant formulae that contain amino acid compositions very similar to that of human milk. Human and bovine α-lac shares a 72% amino acid sequence homology. Currently available studies suggest that α-lac digestibility is as high as that of casein and better than β-lac (Heine et al., 1996).

A number of techniques have been developed for the rapid separation of α-lac from other milk proteins. The optimum conditions for selective precipitation of α-lac from bovine whey was investigated previously by Bramaud et al. (1997). Using anion exchange chromatography, Manji and Kakuda (1986) described the rapid separation of both α-lac and β-lac whey proteins. Reviews and articles have been discussed regarding the structure, biological activities (Madureira et al., 2007) and nutritional aspects of α-lac (Indyk et al., 2007, Shah, 2000).

Although previous research has described the use of ion exchange chromatography to isolate α-lac from bovine whey, this process is not highly biospecific and traces of β-lac are expected to elute with α-lac, because both the proteins possess a similar pI. Hence, developing a ligand that behaves selectively towards β-lac would definitely be beneficial because we could use this to isolate β-lac alone, by affinity in comparison with the remaining milk proteins. This study has therefore been carried out to investigate the use of the PAM peptide as an affinity ligand to selectively isolate β-lac from whey and milk.

β-lactoglobulin

β-lactoglobulin, our protein of interest, is known to be the most abundant whey protein in ruminant's milk, comprising nearly 70-80% of whey proteins (Yousefi et al., 2013). It is the only protein without any counterpart in human milk (Sélo et al., 1999). It represents nearly 58% of whey protein or 10% of the total protein in milk (Lozano et al., 2008). In whey, the concentration of β-lac is in the range of 2-4 mg/mL (Andersson and Mattiasson, 2006). Six genetic variants of β-lac have been identified so far; amongst these variants A and B are the most common. Because variant A has an additional negative charge in terms of the amino acid composition, it possesses a slightly lower pI value (~5.1) compared with variant B (~5.2), although the MW of both variants are the same (Yamamoto and Ishihara, 1999). This protein has a tendency to react with several caseins and α-lac to form a complex by hydrophobic interactions (Wang et al., 1997).

The complex formed (β-lac and κ-casein) will lead to clogging of the chromatography column, thereby causing problems in the protein separation process. Hence, as mentioned previously, research has been carried out involving pre-treatment of milk, leading to removal of fat globules. This simplifies separation of milk proteins and has been the most convenient mode of separation. The issue with these separation modes is the involvement of pre-

treatment which is not regarded safe for infant intake.

The primary structure of β-lac contains 162 amino acid residues, with a total MW of 18.4 kDa and binds well to a number of ligands (mainly hydrophobic ligands) (Wu et al., 1999). The secondary structure comprises of nine strands of β structure, a short helix segment and three helical turns. The quaternary structure depends on the medium pH and it occurs mainly as a stable dimer (pH between 5.2-7.5), with a MW of 36.7 kDa (Teixeira et al., 2014). Except in the small pH range around its pI, β-lac is present in the form of dimers at equilibrium. At physiological conditions (pH between 5.2 and 7.5), β-lac forms a dimer and at pH values between 3.5 and 5.2, it exists as an octamer with a MW of 140 kDa (Taulier and Chalikian, 2001, De Wit and Fox, 1989).

The amino-acid sequence and 3-dimensional structure of β-lac show that it is a member of lipocalin family, a widely diverse family, most of which bind small hydrophobic ligands and thus may act as specific transporters (Kontopidis et al., 2004).

β-lactoglobulin possesses several positive functional attributes but some adverse behavior associated with allergenicity, which are explored in the following section.

Advantages and disadvantages of β-lactoglobulin

Hambling et al. (1992) and Liberatori (1976) reviewed the physico-chemical properties of β-lac and its functional applications in food, healthcare and cosmetic industries. β-lac serves as an excellent gelling and foaming agent (Giarrocco et al., 1997). It can also be used in the manufacture of confectionaries (Zayas, 1997). β-lactoglobulin is the ingredient of choice in the formulation of modern food and beverages. It has a high concentration of essential and branched – chain amino acids (BCCAs). Amongst the BCCAs, leucine is known to significantly help in tissue growth and repair due to its solubility in water, Apart from these applications, β-lac also possesses biological applications (Madureira et al., 2007).

On contrary, β-lac has proved to behave as an allergic protein for infants (Businco et al., 1993). The protein was diagnosed to be a major allergen in 1.9 - 2.8% of the general population of infants in Europe. Allergenicity of β-lac has already been shown to be associated with the four peptides derived from cyanogen bromide cleavage of β-lac (Sélo et al., 1999). Hence, the use of this protein for manufacturing infant formulae is limited. However, there are many commercially available infant products that contain large amounts

of β-lac, such as Jarrow 12 formulae (Kunz et al., 2000). It is mainly because of the difficulty in developing an economical process for total removal of β-lac from milk that no process has yet been developed for its isolation.

If a new process could be developed for selective removal of this protein from milk, it could have high potential for preparation of pure infant formulae (Billakanti and Fee, 2009). As a result of this process, the purified milk produced would be devoid of β-lac and the protein isolated could be used as an additive in food products. β-lactoglobulin is expected to elute with low salt strength (nearly 100 mM NaCl) which is compatible with its use in food products; although probably with minor traces of α-lac present. The milk produced would retain all other nutrients intact. On commercialisation, the resultant product (purified milk), will be preferred by those responsible for the care of infants who are allergic to standard milk.

Bovine Serum Albumin

Bovine Serum Albumin, a globular protein, exists in whey at a concentration between 0.3 and 0.6 mg/mL and has a MW of approximately 69 kDa. The pI of this protein ranges between 4.7and 4.9, slightly lower than β-lac and it contains 582 amino acid residues. It comprises nearly 8% of whey proteins in milk (Andersson and Mattiasson, 2006). This protein is reported to exist as a single phenotype with no variants but it does dimerise, mainly through a single unpaired cysteine residue in the primary structure.

The protein is used in food industries because it possesses good gelling properties (Matsudomi et al., 1991) and it is also used widely in therapeutic industries (Zydney, 1998). BSA has also been used widely in biochemical applications (as a blocking agent) including immunoblots and ELISA (Enzyme-Linked Immunosorbent Assays) to prevent non-specific binding of antigens and antibodies. It is known to bind strongly free fatty acids because of its hydrophobic nature. This protein has been extracted along with α-lac and β-lac because the pIs of these proteins are similar to that of BSA (Billakanti, 2009).

Immunoglobulins

Immunoglobulins are gamma globulin proteins that are found in all lactating species. In humans, Igs are classified into five classes: IgA, IgD, IgE, IgG and IgM. Amongst these, IgG is the most abundant immunoglobulin, and it comprises to 75% of total Igs. Each Ig possesses

a characteristic type of heavy chain and the physicochemical properties of the Igs also vary between different classes.. The MW of Igs lie between 144 and 960 kDa with their pI's lying between 5 and 7.2 (Billakanti and Fee, 2009).

Immunoglobulins act as the first defence system against infections and diseases. Enriched bovine colostrum, produced from hyper immunized cows is a very good source of Igs and has been used as a raw material to provide passive immunity (Tomasi Jr, 1972). The concentrations of Igs found in bovine and human serum and mammary secretions are listed in Table 2-3.

Table 2-3: Immunoglobulin concentrations in bovine and human serum and mammary secretions (Hurley, 2003)

Species	Immunoglobulin	Concentration (mg/mL)		
		Blood serum	**Colostrum**	**Milk**
Bovine	Total IgG	25	32-212	0.72
	IgG_1	14	20-200	0.6
	IgG_2	11	12	0.12
	IgA	0.4	3.5	0.13
	IgM	3.1	8.7	0.04
Human	Total IgG	12.1	0.4	0.04
	IgA	2.5	17.4	1
	IgM	0.9	1.6	0.10

Previously, IgG purification has been achieved using PAM peptide ligand because of its selectivity to the F_C portion of IgG (Fassina et al., 1996).

Viral and microbial infections can be reduced and improved immune activity can be provided to consumers by adding Igs to infant formulae and other food products (Gapper et al., 2007).

Lactoperoxidase

Lactoperoxidase is one of the valuable enzymes found in bovine milk and weighs nearly 78 kDa with 612 amino acids, pI is approximately found to be 9.6 (Arnold, 1881). Bovine milk

contains 20 times higher amount of LP than that present in human milk (Gothefors and Marklund, 1975). The concentration of LP in bovine milk is about 30 mg/L constituting about 1% of the whey proteins. The secondary structure of LP consists of 65% β strand and 23% α helix (Fox and Kelly, 2006).

LP catalyzes the oxidation of thiocyanate by hydrogen peroxide and generates intermediate products with antibacterial properties which inhibit the growth of wide range of fungi and bacteria (Seifu et al., 2005, Korhonen and Pihlanto-Leppälä, 2004). One of the major advantages of LP is that it has been previously used to preserve raw milk during transportation from the farm to the dairy plant, where the use of a mechanical fridge is not feasible (Seifu et al., 2005).

Lactoferrin

This protein is an iron-binding glycoprotein of the transferrin family and is expressed in most biological fluids. It is a major component of the mammalian innate immune system (González-Chávez et al., 2009). Bovine LF and human LF have approximately 69% similarly in amino acid residues. The concentration of LF in bovine milk and whey is relatively low and varies throughout the milking season, generally falling within the range 20-400 mg/L (Palmano and Elgar, 2002). LF weighs nearly 80 kDa and has a pI of 8.6 (González-Chávez et al., 2009). It is one of the major whey proteins in human milk with concentrations of 1.4–2.0 mg/mL but only a minor component in bovine milk with only one tenth (0.1–0.2 mg/mL) of concentration found in human milk (Elagamy et al., 1996, Playne et al., 2003).

Isolation of LF has been achieved in both laboratory and lab-scale applications using cation exchange, size exclusion, affinity and hydrophobic chromatography methods (Chand, 2006, Indyk and Filonzi, 2005, Okonogi et al., 1988). In comparison to other milk proteins, LF has a much higher pI but has been easily isolated from milk using cation exchange chromatography (Billakanti, 2009).

Lactoferrin has been used commercially as a natural bioactive ingredient in supplement foods (e.g., infant formulae and dietary supplements) and oral healthcare products (Yamauchi et al., 2006) Because of its distinctive properties, LF has emerged as an important antimicrobial milk protein and recent studies show that this protein possesses applications in human health, by preventing growth of pathogens (González-Chávez et al., 2009, Ward et al., 2002). A highly purified form of LF also finds applications in cosmetics, functional food, beverages

and animal feed (Horton, 1995, Tomita et al., 2002).

Before understanding the separation techniques, it is vital to gain some knowledge regarding the various constituents present in milk.

2.4 PROTEIN PURIFICATION IN DAIRY INDUSTRY

One of the important requirements for advancements made in biotechnology has been the development of various techniques for protein purification. Protein purification is a series of processes that are intended to separate them from one another within a mixture of nutrients.

Generally, protein purification is performed based on protein native properties such as hydrophobicity and size (Matthews, 1984). In certain cases proteins are isolated by affinity ligands (Huse et al., 2002). A few multi-step processes have been developed but typically increase the production cost (Fee and Chand, 2006). Hence, there is a need to develop a chromatographic process which minimizes loss whilst maximizing the product yield. A wide range of techniques are available for protein separation based on end user applications and commercial viability. The most common methods used include salting out and adsorption chromatography (Etzel et al., 2000, Scopes, 2013). Although these methods have been investigated for decades, methods available for industrial-scale extraction of proteins are limited. These industrial-scale extraction methods include precipitation, chromatography, adsorption and membrane filtration. Depending on the end user applications, these processes were used to isolate the protein of interest from complex mixtures (Bhushan and Etzel, 2009, AbdEl-Salam, 2006, Hjorth, 1997, Etzel et al., 2000). For example: centrifugation is one of the commonly used methods to isolate milk fat components, thereby isolating major caseins and whey proteins (Korhonen and Pihlanto-Leppälä, 2004). Expanded bed adsorption has proved to be a potential alternative method for industrial-scale processing of complex protein mixtures (Noel, 2007). On the other hand, membrane filtration techniques are attractive in the dairy industry for bulk processing of bioactive components (Goodall et al., 2008).

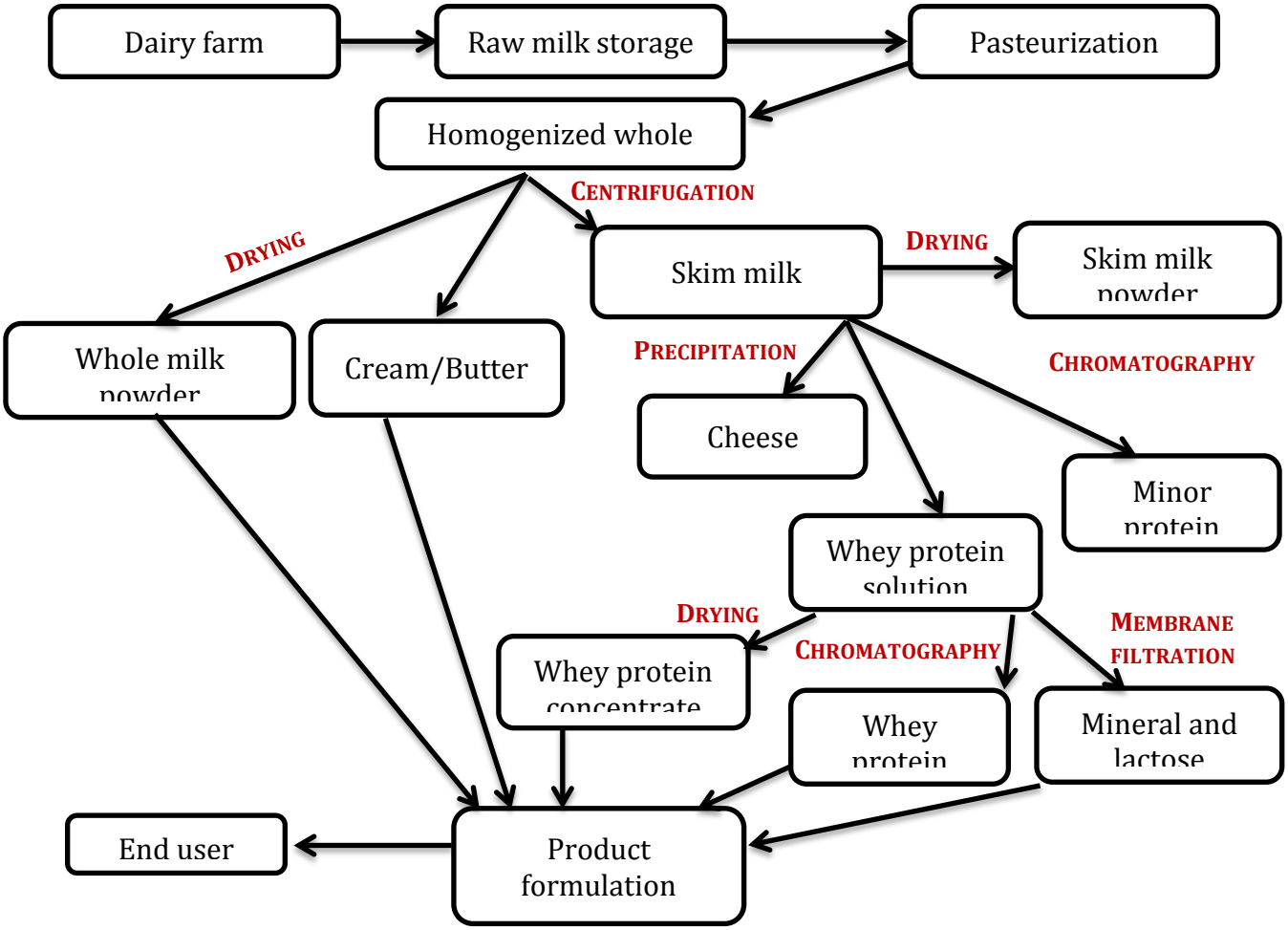

Figure 2-3: Commercial milk product formulation in dairy industry (Billakanti, 2009)

The above-mentioned (Figure 2-4) methods possess benefits and limitations. Some of the milk-derived components were found to be susceptible to harsh treatments, usually employed in isolation processes. Additionally, is has been observed that multiple step processes increase the final product cost but decrease the product yield and activities (Chand, 2006). Because of an increasing demand and applications of dairy proteins in healthcare, food and pharmaceutical industries, cost effective processes need to be developed for bulk productions.

2.4.1 Precipitation

Precipitation is a widely used technique for recovery of proteins and other biological products. The selective precipitation technique involves adjusting the physical properties of components in solutions by addition of solvents, salts and/or heating to promote aggregation. Salting out is one of the most commonly used protein precipitation methods. This method has

been commonly used for extraction of whey proteins from whey, in laboratory scale (Billakanti and Fee, 2009). Using selective precipitation method, aggregation of caseins was observed. This was achieved by adjusting the pH of milk to around 4.6 at 40°C. In addition to this, selective precipitation methods have also been used to separate whey proteins from whey solutions.

Previously, a variety of techniques have been carried out to isolate whey proteins from whey solutions. Bramaud et al. (1997) used selective precipitation for the isolation of α-lac from whey under heat treatment. A study was carried out using citric acid and observing the effect it had on the precipitation of whey proteins. Using phosphate precipitation and chitosan causing precipitation, Casal et al. (2006a) and Al-Mashikhi (1987) isolated β-lac from cheese whey. Several other precipitation methods had been developed to concentrate and isolate whey proteins from whey streams (Stanley et al., 1950, Richter et al., 1974). However, precipitation methods are not suitable for all dairy proteins and bioactive components because of their limitations in food and health care applications. Another limitation is their high cost when applied to industrial scale basis and waste disposal problems (Billakanti and Fee, 2009).

2.4.2 Membrane Filtration

Membrane filtration is commonly used to concentrate milk-derived components in the dairy industry. Separation of fat globules in cream manufacturing, reduction of bacteria and spores in skim milk and concentration of casein micelles as a pre-treatment in cheese manufacturing were achieved using membrane processes (Brans et al., 2004, Brisson et al., 2007). As in Figure 2-5, membrane processes operate over a range of particle sizes from 1 nm to 10 μm. A number of membrane process technologies find application in the dairy industry including cross-flow microfiltration (CFM), ultrafiltration (UF), reverse osmosis (RO), dynamic membrane filtration (DMF), electro ultrafiltration (EU), and nano-filtration (NF). Recent developments in membrane filtration have provided exciting new opportunities for large-scale protein and lactose fractionation including the development of a membrane filtration process for whey processing on a pilot-scale basis.

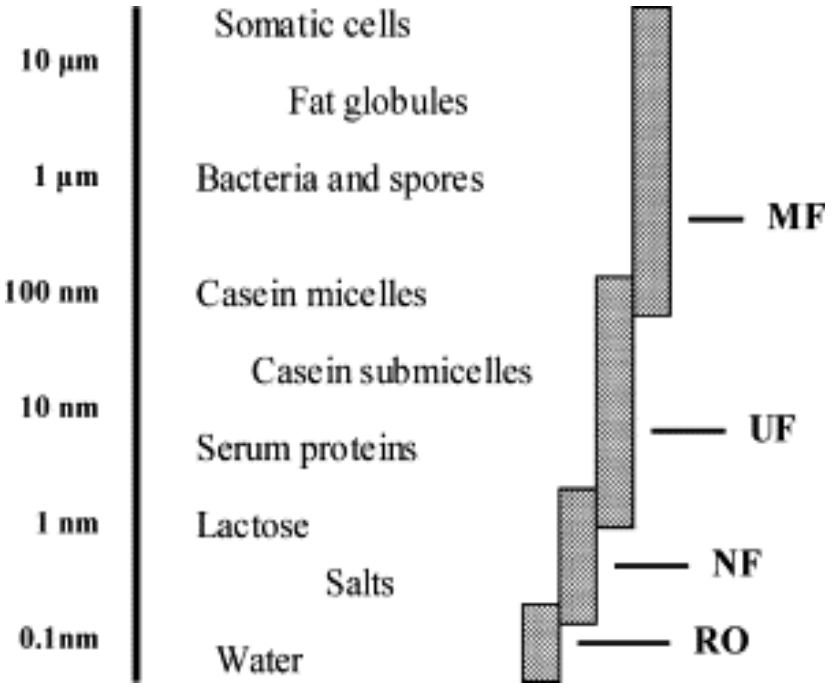

Figure 2-4: Membrane processes for separating desired dairy components (Brans et al., 2004)

The size difference between the components to be separated by membrane processes should be at least a factor of 10. Single whey protein fractionation by conventional membrane filtration (UF particularly) is impossible because whey protein components are of similar size. In the dairy industry, membrane processes have been used in combination with other separation processes for isolation of proteins, however high costs and complicated operational procedures pose a challenge to its application (Chik and Saufi, 2010).

2.4.3 Chromatography

The term "chromatography" refers to a wide variety of separation techniques, based on the partitioning of a sample between a moving phase and a stationary phase. The stationary phase is usually packed into a column and a mobile phase "buffer", containing a mixture of solutes, is pumped into this column. The separation occurs either through differences in the rates of solute movement through the column (caused by differences in interactions of species with the stationary phase) or through differential binding of species onto the stationary phase and subsequent elution. Separation can be modified by changing the packing chemistry or elution buffers. This technique has been used widely for decades for isolation, extraction and

purification of peptides and proteins from complex mixtures. In general, proteins or peptides are separated with the help of chromatography on the basis of their physico-chemical properties such as charge, size and hydrophobicity. Chromatography processes can be operated in either batch or continuous modes in packed bed columns, expanded (or fluidized) bed columns or stirred (suspended) tanks and, more recently, adsorptive membranes.

2.4.3.1 Modes of Chromatography

Packed bed chromatography

Packed bed/column chromatography is described as a technique that consists of a stationary phase placed inside a column through which a liquid mobile phase is passed. Column chromatography has been widely used in dairy industry to isolate whey proteins from milk (Doultani et al., 2004). It is a multi-step process that requires pre-treatment of the feed material, usually centrifugation or filtration through a filter membrane of a particular pore size (usually 0.22 or 0.45 μm) to avoid clogging/blocking of the column by suspended solids. The diameters of chromatography columns are designed to avoid clogging of the column and high back pressure problems. Also, large bead media, for example, Sepharose Big Beads (SP BB) (GE Healthcare, Sweden) have been advantageous to reduce clogging problems because larger beads have larger intraparticle void spaces within the packed beds, allowing passage of some suspended solids. This would also be advantageous for milk and whey processing. Milk is complex in nature and the presence of milk fat globules and casein micelles as suspended solids would otherwise cause clogging of the chromatography column, thereby leading to less complexity. Previously, large-scale fractionation of whey proteins from milk and whey has been achieved using this media (Etzel et al., 2000, Fee, 2010).

Fluidized bed chromatography

An alternative method used previously for isolation of proteins from crude mixtures has been fluidized or expanded bed adsorption (EBA) (Hjorth, 1997). This has been thoroughly investigated and has been confirmed to isolate proteins in a single step, without requiring pre-column filtration to remove suspended solids. Using EBA, Hansson et al. (1994) recovered fusion proteins from a fermentation broth without the need of any pre-treatment. The adsorption method has also been used for large-scale isolation processes and Shiozawa et al. (2001) successfully isolated LF from skim milk using EBA and obtained high product yields.

Conrado et al. (2005) used EBA involving a hydrophobic resin to investigate the concentration of α-lac in cow milk whey. EBA has also been used in minor protein extraction in dairy industries has increased over time. The key attribute of EBA is that bed expansion provides large inter-particle spaces that allow suspended particles to pass through the bed. EBA thus reduces some of the limitations associated with conventional (packed bed) chromatography processes, such as column clogging and high-pressure drops (Billakanti, 2009).

Stirred tank chromatography

Stirred tank/batch adsorption chromatography has offered a few advantages over conventional packed bed chromatography and is relatively new to the dairy industry. This type of chromatography has been used for on-farm fractionation of whey proteins from milk due to the variable fat content in individual cow's milk (Chand, 2006). Batch adsorption has also been involved in isolating minor milk proteins such as LP, LF and Igs from milk (Foley and Bates, 1987). An advantage of this method is that fluids containing suspended solids can be processed easily, including whole milk. As whole milk contains suspended particles, conventional chromatography has not played a significant role in isolating dairy proteins from whole (full-fat) milk.

This method also includes a few limitations, such as, it involves long processing time. An increase in filtration time is expected to be caused by stirring because this can cause breakdown of the resin and the fines that develop in turn lead to an increase in process time because of clogging of resin capture filters (Chand, 2006). Also, large tanks are necessary for this method because the tank must hold large volumes of the feed material and resin (Chand, 2006). Hence, this method is not preferred in the dairy industry to isolate milk proteins.

2.4.3.2 Classification of chromatography on the basis of adsorbent chemistry

Based on the different chromatography resins used, chromatography can be classified into different types. The most commonly used resins in the dairy industries include ion exchange and affinity resins.

Ion exchange chromatography

Ion exchange chromatography is the most widely used separation method, wherein proteins are separated based on their charge. This process depends mainly on the pH and salt

concentrations of buffer solutions. Elution of bound proteins occurs by varying the buffer pH or salt strength (conductivity). For example: an anion exchange adsorption surface with diethylaminoethyl (DEAE) moieties will facilitate binding of negatively charged proteins because DEAE is positively charged.

The higher the concentration of charged salt molecules in the solution, the greater is the competition for binding to the ligands of the matrix, thus the tendency for protein to dissociate from the ion-exchange resin. The protein solution is applied to the column in a solution of low salt concentration. The counterions with which the column has been charged are not permanently bound but are held by electrostatic interaction (Scopes, 2013). According to the charge on the ion-exchange ligands, the ion exchange matrices are divided into two types (Selkirk, 2004):

a. Cation exchange chromatography – The resins are negatively charged and in turn bind to positively charged proteins/particles. At a pH below their isoelectric point, the proteins are positively charged. Cation exchange is used at a pH below the isoelectric point of the protein to be bound.
b. Anion exchange chromatography – The resins are positively charged and facilitate binding of negatively charged proteins/particles. At a pH above their isoelectric point, the proteins are negatively charged. It is used at a pH above the protein's isoelectric point.

Based on the protein of interest, anion/cation exchange matrices are used and this has been the most commonly used separation technique in dairy industries.

Size exclusion chromatography

In this type of chromatography, the protein or peptide is dissolved in an appropriate solvent and is injected into a column packed with particles of defined pore size because size exclusion chromatography separates molecules based on the size. This chromatography is also known as gel permeation or gel filtration chromatography. As the molecular size of the protein/peptide decrease with respect to the average pore size of the packing, molecules penetrate into the pores and as a result elute at a later time. High-molecular-weight material elutes first from an SEC column, followed by low-molecular-weight components (Mori and Barth, 2013).

Whey proteins have been previously isolated from whey filtrate by size exclusion chromatography (Bayram et al., 2008). This type of chromatography plays a role in separating milk proteins which differ widely in their size but poses a limitation because it cannot be used to separate milk proteins that are similar in size. Another disadvantage is the limiting capacity for high-volume industries such as the dairy industry because this is a very slow process. Hence, it has limited use in dairy industry.

Reversed-phase chromatography

Reversed-phase chromatography (RPC) is an important analytical technique that uses a hydrophobic ligand as a stationary phase, and has been used for analysis and purification of wide variety of substances (Vailaya, 2005). This chromatography has been the most widely used method for whey protein quantification. A major limitation of this process is its application in dairy industry for separation of whole or skim (low-fat) milk because the casein and whey protein elution peaks overlap (Billakanti, 2009). If proteins and peptides possess some degree of hydrophobic character, high resolution separation can be achieved by reversed phase chromatography but this is mainly of interest analytically because proteins must be eluted from RPC columns by harsh solvents that can denature the product.

Affinity chromatography

Affinity chromatography is the second most popular type of chromatography used in the dairy industry. It separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix. This is the only known technique that enables the purification of a biomolecule on the basis of its individual chemical structure. Also, affinity chromatography is used to isolate pure substances present at low concentrations in large volumes of crude sample. In affinity chromatography, the immobilised ligand is unique and has very specific binding to its counterpart, called an antigen. Some of the affinity interactions include those between enzyme and inhibitor, antigen and antibody and hormone and receptor (Janson, 2012).

Ortin et al. (1991) extracted β-lac from bovine milk using an affinity counter-current distribution in an aqueous two-phase system. The efficiency of this method for purifying β-lac was because of the strong hydrophobic binding ability of this protein. The method was not preferred for large scale purification of milk due to its high processing cost and large-scale techniques involved. Purification of β-lac from milk was achieved using immunoaffinity

chromatography on an antibovine IgG column, followed by anion exchange chromatography (Q Sepharose Fast Flow column) to remove residual traces of other whey proteins (Sélo et al., 1999). Affinity separation of β-lac in its native form with all-trans-retinal immobilized on calcium bio-silicate was scaled up and applied to isolate it from industrial whey by Vyas et al. (2002). This method also removed α-lac during the isolation process and hence is not suitable for purifying milk.

In general, the most commonly used affinity adsorbents for isolation of Igs from different sources are either Protein-A or Protein-G affinity media (Hober et al., 2007, Kochan et al., 1996). Affinity adsorbents offer a number of advantages over ion exchange adsorbents, including high purity of products and selectivity. Although it possesses advantages, a few limitations also exist, including the high cost of adsorbents, the need for pre-treated feed material and difficult sanitization conditions (Linhult et al., 2005).

Previously, affinity separation of β-lac was achieved using all-trans-retinal immobilized on calcium biosilicate. This process was scaled up and applied to industrial sweet whey solution. The research focussed on developing a scale-up process rather than producing a β-lac free product (Vyas et al., 2002). Investigation on isolation of β-lac from whey was carried out using affinity chromatography without causing any modification to the native structure of this protein. This method was applied to whey streams but not to skim and full milk because the main aim of this thesis was to use cheese whey for experimental trials (MacLeod, 1995). Because of the complex composition of milk, affinity methods have not previously been investigated for isolating β-lac from milk.

2.5 PACKING MATERIAL

2.5.1 Protein A Mimetic peptide ligand

Protein A Mimetic ligand appeared to bind β-lac with high specificity while Fee (2010) was investigating IgG isolation from bovine milk. Thus, PAM ligand has been first described here in relation to its use for affinity binding of Igs.

A peptide mimicking Protein A for its ability to recognize the fragment crystallizable region **(**Fc region) in Igs was identified through screening of a synthetic multimeric peptide library. Compared with Protein A or G, these ligands possess several benefits such as high stability towards chemical and biological reagents, absence of contaminants and low production costs.

Previously, PAM peptide ligands have been screened, synthesized and investigated for their specificity and applications in chromatography process development (Roque et al., 2004, Yang et al., 2005).

Isolation of various classes of Igs from bacterial cell culture broths and human serum were successfully investigated using PAM peptide ligand, known as TG19318 tetramer peptide ligand (Fassina et al., 1996). Another PAM peptide ligand, known as PAM hexamer peptide was identified and characterized for selectivity towards Igs of different classes (Yang et al., 2005). Also, PAM has previously been shown to be an excellent lead compound for downstreaming processing of antibodies in small-scale applications (Dinon et al., 2011). PAM has previously been immobilised on a variety of matrices, including Sepharose, Eupergit C30N, HyperDW, EmphazeW and Protein-Pak (Fassina et al., 1996). However, to date, the applicability of these peptide ligands for isolation of β-lac from dairy streams has not been reported.

PAM is a synthetic peptide which mimics Protein A and has been previously used for the recognition of Igs (Billakanti and Fee, 2009). This peptide was purchased from Genscript (New Jersey, U.S.A) and has a molecular weight of 2141 a.m.u. The main objective of the current research was therefore to investigate the specificity of the tetramer peptide ligand towards bovine β-lac.

PAM peptide ligands are attractive materials because these ligands not only serve as alternative affinity media but are also robust and have low production costs. In single-step process methods, these ligands have been used as an affinity media to isolate Igs from various complex fluids (Fassina et al., 1996, Fassina et al., 2002).

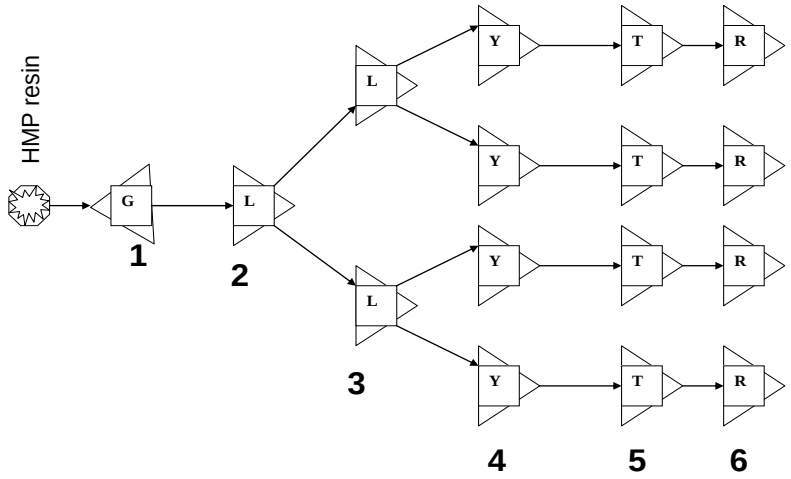

Figure 2- 5: Schematic representation of tetramer peptide (PAM)

2.6 KINETIC MODELS-PROTEIN ADSORPTION

In this study, two different kinetic models-Langmuir and Freundlich were briefly studied. Previously, these models have been used for reversible adsorption. These are described in sections 2.6.1 and 2.6.2.

2.6.1 Langmuir model

The Langmuir model, derived by Langmuir in 1916, is one of the simplest adsorption models used to describe the kinetics of protein adsorption (Brusatori and Van Tassel, 1999). The ability to characterize adsorption equilibria accurately is vital in chemical processes and several two- or three-parameter isotherms are used for this purpose .(LeVan and Vermeulen, 1981). The kinetic equation for the Langmuir model can be written in terms of surface concentration ρ, as:

$$\frac{d\rho}{dt} = k_a C_o(\rho_{max} - \rho_t) - k_d \rho_t \qquad (1)$$

where, ρ_{max} is the surface concentration of protein, ρ_t is the amount of protein adsorbed onto the surface at time *t*, C_o is the protein concentration in the bulk liquid phase , *t* is the time, and k_a and k_d are adsorption and desorption rate constants, respectively. As time approaches infinity, the attainment of equilibrium conditions are observed, a condition corresponding to

an equilibrium amount of protein adsorbed ρ_e. This equilibrium value depends on the specific bulk protein solution concentration of the experiment and can be calculated from the following expression obtained by rearranging in Equation (1).

$$\rho_e = \rho_{max} \frac{C_o}{C_o + k_d} \tag{2}$$

In this study, three linearization plots - Scatchard plot, Eadie-Hofstee plot and Lineweaver-Burk plot were studied. These plots are briefly described in the following sections.

2.6.1.1 Scatchard plot

The Scatchard equation is used to calculate the affinity constant of a ligand in contact with the protein (3). This plot is a ratio of concentrations of bound ligand to unbound ligand against the bound ligand concentration (Debye, 1942).

$$C = Q_0 \frac{C}{Q} - K \tag{3}$$

In the above equation, Q is the adsorption capacity at equilibrium concentration C, Q_o and K are constants determined by adsorption experiment. In the experiments performed, solute concentration is expressed as mg/mL and adsorption capacities are expressed in mg of β-lac per g of tetramer peptide resin.

2.6.1.2 Eadie-Hofstee plot

The Eadie-Hofstee plot was developed by Eadie and Hofstee and is one of the graphical procedures for determining values of the kinetic parameters for an enzyme-catalyzed reaction that conforms to Michaelis-Menten kinetics. The Eadie-Hofstee equation (3) is used to determine Q_o and K.

$$Q = -K \frac{Q}{C} + Q_o \tag{4}$$

This plot has been derived from Michaelis-Menten equation describing the dependence of enzyme-catalyzed velocity on the concentration of the substrate. As larger amounts of substrates are added to a reaction, the available binding sites are filled and hence get saturated, ceasing the reaction rate to increase. This concept has been observed in case of binding reactions as well, where the binding sites of the resin get occupied with the

amount of molecules available in solution, thereby saturating the sites.

2.6.1.3 Lineweaver-Burk plot

Lineweaver–Burk plot is a graphical representation of enzyme kinetics which was developed by Hans Lineweaver and Dean Burk in 1934. Equation (5) has been previously used to determine important parameters in enzyme kinetics such as the Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) (Lineweaver and Burk, 1934).

$$V = V_{max} \frac{[S]}{(K_m + S)} \tag{5}$$

Equation (5) is derived from (4) and is used in turn to obtain Langmuir constants.

$$\frac{1}{Q} = \frac{K}{Q_0} \frac{1}{C} + \frac{1}{Q_0} \tag{6}$$

2.6.2 Freundlich model

The Freundlich adsorption kinetics is mathematically expressed as:

$$\frac{d\rho}{dt} = k_\alpha \rho^{\alpha_1} - k_d \rho^{\alpha_2} \tag{7}$$

At attainment of equilibrium, Equation (7) reduces to:

$$\rho_e = K_D^f C_o^\alpha \tag{8}$$

where, α_1 and α_2 are the adsorption and desorption reactions, respectively.

The Freundlich isotherm is applicable only within certain concentration limits because, given an exponential distribution of binding sites, the number of sites increases indefinitely with a decreasing association constant, implying that there are an infinite number of sites (Umpleby Ii et al., 2001).

SUMMARY

The demand for dairy bioactive components has seen to rise over the past few years, focusing the application of whey proteins in food, healthcare and pharmaceutical industries. To achieve this, there is a need to develop economical and improved methods to reduce production costs and increase product yield. Due to inherent limitations, the processes available at present are not suitable for industrial-scale applications.

Presence of β-lac was identified as an allergen in bovine milk and the isolation of this protein was the main aim of this thesis. This protein is absent in human milk but its presence in bovine milk limits its use for infant intake. Previous research has involved the isolation of this protein from whey streams. Because of the complex composition of milk, identifying a particular ligand showing specificity towards β-lac is a challenge. Another challenge faced more recently is the application of this ligand for large-scale purposes.

The lack of suitable extraction technologies for isolation of β-lac from milk has been a major limitation in the production of human-like infant formulae. Previously, the use of PAM ligand was observed to isolate Igs from milk and unexpectedly seems to have a high specificity towards β-lac; therefore characterization of this ligand was necessary. This ligand has also been used to isolate various classes of Igs from cell culture broth and good yield was obtained. Thus, optimization of the process will also help in isolating β-lac from milk without the need of any pre-treatment.

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CHAPTER 3 - MATERIALS AND METHODOLOGY

This chapter outlines the materials used in this study followed by stepwise protocol of the experiments performed.

3.1 IMMOBILISATION OF TETRAMER PEPTIDE ON A CHROMATOGRAPHY MATRIX

3.1.1 Preparation of solutions

All chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO, U.S.A). The buffer solutions were prepared using ultra-high pure Milli Q water. To produce Milli Q, a unit purchased from Merck Millipore (Billerica, MA) was used. Once the solutions were prepared, these were filtered using a 0.22 μm filter (500 mL disposable sterile units, Global Science and Technology, Auckland, NZ). Filtration was carried out to avoid clogging of the column while performing the chromatography process. The solutions were then sonicated for 10 min to degas the solutions to avoid air bubbles entering the chromatography column.

3.1.1.1 Buffer solutions

a. 10 mM Tris HCl (pH 6.85) – This was prepared by adding 0.6057 g Tris HCl (MW - 121.14 g/mol) to 450 mL Milli Q water. The pH was then adjusted to 6.85 by dropwise addition of 5 M hydrochloric acid and this was made up to 500 mL by adding Milli Q water.

b. 10 mM Tris HCl + 1 M NaCl (pH 6.85) – The buffer was prepared as mentioned in (a) and 29.22 g sodium chloride was added. The pH of the buffer was then adjusted to 6.85 by addition of 1 M sodium hydroxide.

c. Immobilisation buffer/ coupling buffer (pH 8.5) – Coupling buffer was prepared by adding 100 mM sodium bicarbonate ($NaHCO_3$; MW - 84.007 g/mol) and 500 mM NaCl to 800 mL Milli Q water. The pH was then adjusted by dropwise addition of 1 M NaOH. Finally, the buffer prepared was made up to 1 L by addition of Milli Q water.

d. 10 mM sodium phosphate buffer/ binding buffer (pH 7) – 1 L of 10 mM monosodium phosphate (NaH_2PO_4; MW 119.98 g/mol) and 10 mM disodium hydrogen phosphate

(Na_2HPO_4; MW 141.96 g/mol) were prepared separately. The two solutions were then combined to obtain a pH of 7.

e. 10 mM sodium phosphate buffer containing 1 M sodium chloride (pH 7) – 500 mL of 10 mM sodium phosphate buffer was prepared and 29.22 g/mol NaCl was added to this. The buffer pH was then adjusted to 7 by dropwise addition of NaOH.

3.1.1.2 Coupling solutions

a. 1 M Tris HCl/ blocking solution (pH 8.5) – 1 M Tris HCl was prepared by addition of 12.114 g to 100 mL Milli Q water. pH was adjusted to 8.5 by dropwise addition of 5 M HCl.

b. 1 mM hydrochloric acid – 17 μL concentrated HCl was added to 150 mL Milli Q water. The solution was then made to 200 mL by addition of Milli Q water.

c. 1 M sodium chloride – 11.6 g of NaCl (MW – 58.44 g/mol) was dissolved in 200 mL of Milli Q water.

To retain maximum binding capacity of the pre-activated medium prior to the coupling step, solutions were kept in an ice box (0 to 4°C).

3.1.1.3 NHS activated resin

NHS resin (25 mL pack size) was purchased from GE Healthcare Life Sciences, Connecticut, USA. Because NHS resin is supplied in 100% isopropanol suspension (IPA), instructions for removal of isopropanol from the medium and preparation for coupling the ligand prior to use are given in Section 3.1.2.

3.1.1.4 Protein A Mimetic peptide

PAM peptide was purchased from Genscript (New Jersey, U.S.A). The peptide was dissolved in the coupling buffer prior to performing the immobilisation process.

3.1.2 Immobilisation process - protocol

All necessary solutions were prepared prior to coupling the ligand (Section 3.1.1). The steps for coupling include:

- The ligand to be coupled PAM was dissolved in the coupling buffer at a concentration of 10 mg/mL. The ratio of volumes chosen was coupling solution/medium – 1:0.5. NHS-ester groups rapidly hydrolyzed at high pH.

- 5 mL of NHS-activated sepharose resin was washed thoroughly with three medium volumes of Milli Q water.
- Washes of the resin were then performed with three medium volumes of cold 1 mM HCl in order to remove any IPA present.
- The resin was again washed with two medium volumes of Milli Q water following which washes were performed with three medium volumes of coupling buffer.
- To the resulting resin, 2.5 mL of 10 mg/mL of PAM peptide was added and left on the spinning wheel for 24 hr.
- Once coupling was done, non-reacted groups were blocked with the addition of 2 mL of blocking solution (pH 8.5). This was left on the spinning wheel for three hours.
- After coupling, the medium was washed with 1 M NaCl. This was repeated thrice and the resulting supernatant was discarded.
- The coupled medium was stored in 20% ethanol until it was ready to be used.

Once immobilisation was complete, trials were performed to determine the equilibrium capacity of the tetramer peptide resin (Section 3.2).

3.2 AMINO ACID SEQUENCE COMPARISON

Previously, Protein A Mimetic (PAM) peptide has shown specificity towards IgG since the peptide ligand was observed to bind to the F_C portion of IgG. A study was carried out using SIM – Alignment Tool for protein sequences, to compare the similarities between the F_C portion of IgG and Bovine β-lac.

3.3 EQUILIBRIUM/STATIC BINDING CAPACITY DETERMINATION OF RESIN

Initially, protein standards were prepared as described in the following Section (Section 3.2.1).

3.3.1 Protein standards

The whey proteins used in this research (α-lactalbumin, β-lactoglobulin and BSA) were purchased from Sigma Aldrich. Varying concentrations of protein solutions were prepared to obtain a calibration curve. Protein solutions were prepared by dissolving known amounts of proteins in the binding buffer.

3.3.2 Nanodrop 1000 UV Spectrophotometer

Nanodrop 1000 UV Spectrophotometer was used to measure the concentration of samples without the need of dilution.

- Before loading the sample onto the nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, U.S.A), the samples were centrifuged at 12,000 rpm at 4°C for 1 min.
- 2 μl of the supernatant was pipetted onto the end of a fiber optic cable (the receiving fiber).
- A second fiber optic cable (the source fiber) was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends.
- The gap was controlled to 0.2 mm paths.
- A pulsed xenon flash lamp provided the light source and a spectrometer utilizing a linear CCD array was used to analyze the light after passing through the sample.

The instrument was controlled by PC based software, and the data was logged in an archive file on the PC.

3.3.3 Langmuir isotherm

To determine the maximum adsorption capacity of the resin (Q) in contact with a pure β-lac protein solution, an adsorption isotherm is necessary. This was achieved by performing batch adsorption experiments involving trials using different concentrations of pure β-lac protein solution and the adsorption capacity was then compared at varying time intervals. Equilibrium adsorption/desorption of tetramer peptide resin was determined using the Langmuir isotherm (Equation 1).

$$Q = \frac{Q_o\, C}{(K+C)} \tag{9}$$

where Q is the adsorption capacity at a given equilibrium solution concentration C, and Q_o and K are constants determined by the adsorption experiment, with Q_o representing the maximum binding capacity. Solute concentration is expressed in mg/mL and adsorption capacities are expressed in mg of β-lactoglobulin per g of tetramer peptide resin.

The constants in this equation were calculated from a linearization plot (the Scatchard plot, Equation 2) involving the adsorption capacities of the resin at varying concentrations of pure β-lac protein solution.

$$C = Q_0 \frac{C}{Q} - K \quad (10)$$

From Equation 2, the Langmuir constants were obtained and were hence used to plot a Langmuir isotherm.

3.3.3.1 Preparation of resin

To determine the isotherms, resin was used in two forms – wet and dry basis. To obtain fully dried resin, two washes on filter paper were repeatedly performed using 10 mM sodium phosphate buffer (pH 7) at RT and the filtrate was discarded. The remaining resin was left to dry at 26.4°C for 48 h. The weight of the dried resin was checked at regular time intervals to ensure that it was free of moisture. Once the weight remained constant, the weight of resin was obtained for use in dry-basis experiments.

To obtain wet resin, the weight of the resin was measured after discarding the filtrate. This hence gave the weight of resin in wet-basis terms (drained wet resin).

3.3.3.2 Relation between dry resin and wet resin

Dry resin was used to perform experiments using batch 1 peptide (purity~99%) whereas batch 2 peptide (purity~98%) was used to perform experiments with drained wet resin.

To obtain a relationship between dry resin and wet resin quantities, dry resin was initially weighed and repeated washes (3 times) were alternatively performed with 1 mL each of methanol and water at RT. While performing washes, the resin in water/methanol was sonicated for 5 min to ensure all the pores of the resin were thoroughly wetted. The weight of drained wet resin was then checked and a linear plot was drawn to obtain a relation between dry and wet resin.

3.3.3.3 Comparison of adsorption capacities at varying time intervals

Initial experiments were carried out in 5 mL pre-weighed Eppendorf tubes using dry/wet resin to which varying concentrations of pure β-lactoglobulin were added. This was left to react on the spinning wheel (4^oC) and was then centrifuged at 10,000 rpm for 1 minute at 4^oC. The concentration of the protein remaining in the supernatant was then determined using the Nanodrop spectrophotometer. This was repeated at varying time intervals – 10 min, 1, 2, 3, 4 and 24 h and duplicate measurements of the concentration were noted.

Once Q was determined, the slurry was packed into a 5 mL Pharmacia column/empty column (GE Healthcare Life Sciences) and trials were performed with regard to isolating β-lac from whey/milk samples.

3.4 ISOLATION OF B-LAC FROM WHEY/MILK SAMPLES – DYNAMIC CONDITIONS

3.4.1 Preparation of whey solution

Anchor trim milk was purchased from a local supermarket. In order to remove caseins, milk was brought to room temperature (RT) and 5 M HCl was added dropwise with thorough mixing until the pH dropped to 4.6. This was centrifuged using an Eppendorf 5810R refrigerated benchtop centrifuge at 125 x g at 4°C for 1 minute. The clear supernatant was then withdrawn for further use using a pipette and 1 M NaOH was added dropwise to adjust the pH to 7.

3.4.2 AKTA Explorer 10

Protein purification experiments were performed using an AKTA Explorer 10 system (GE Healthcare Life Sciences, Uppsala, Sweden). The purification system was fully automated and controlled by Unicorn software 4.0.

The main system components include an injection valve, sample selection valve, column selection valve, system pumps, outlet valve, pH and wavelength detector, flow restrictor, autosampler and fraction collector (Frac-950). The autosampler was used to increase throughput because it enabled overnight runs (Figure 3-1). The Frac-950 was used to collect samples during the purification process. Based on different stages of purification, varying fraction sizes were collected. A 10 mL Superloop (a sample holder that holds a large volume of sample for repeated injections) was used for larger sample volume injections.

The system possessed monitors as described below:

- Monitor pH/C-900 – Monitor pH/C-900 recorded pH, conductivity and temperature readings. It also consisted of a control unit, an optional flow cell for conductivity and temperature, an optional flow cell with a holder for the pH electrode and the pH electrode. The pH cell was not used during chromatography runs

- Monitor UV-900 – This can record three wavelengths simultaneously and was used here to monitor at 280 and 215 nm wavelengths as a high sensitivity monitor.

Figure 3-1: AKTA Explorer 10 system

Chromatography experiments were conducted for isolation of whey proteins (particularly β-lac). Varying flow rates were used for varying columns as mentioned in Section 3.3.4. The buffer flow rate was controlled by a positive-displacement pump and was normally kept constant while the strength of the buffer varied.

3.4.3 Chromatography column preparation

- The coupled media was thoroughly washed with binding buffer to remove the storage solution (20% ethanol).
- A 5 mL Pharmacia column was dismantled and immersed in a beaker full of water. This was sonicated for 10 min. Alternating with water, 70% isopropanol (IPA) was used and sonication was again performed (3X with each solution individually). It was ensured that the column was clean for use.

- The bottom end of the column was closed with an adapter which allowed dropwise flow of any liquid passing through the column.
- Binding buffer was poured through the top of the column, using a 2 mL Pasteur pipette.
- The slurry was mixed well using a Pasteur pipette and was slowly poured into the column, letting the buffer pass out through the outlet.
- It was ensured that there was no air bubble trapped in the column.
- Once the entire slurry was poured into the column, the slurry was pushed slowly using a packing adapter to help settle all packing uniformly.
- The packing adapter was then carefully removed and a normal adapter was replaced to seal the column.

The column was connected to the lines of AKTA Explorer 10 for use.

3.4.4 Peptide affinity chromatography

- Initially, pump washes were carried out using water.
- Lines A and B were placed in respective buffers and the buffers were flushed through the lines to ensure that lines were free of the storage solution (20% ethanol).
- The column was then equilibrated with the immobilisation buffer/binding buffer, depending on the experiments being performed.
- The column was then equilibrated with the start buffer:
  - Superdex peptide column – 1.5 column volumes (CV) at 0.75 mL/min, backpressure limit – 1.8 MPa
  - 5 mL packed column – 5 CV at 1 mL/min, backpressure limit – 0.3 MPa
- Using different samples loops, varying concentrations of protein samples and whey solutions were separately injected and an existing program was used to control the protein purification conditions.
- Flowthrough fractions and elution fractions were collected in a 96-well microtitre plate.
- For further analysis, these samples were run on a gel electrophoresis set up for comparison with a ladder (MW size marker).
- The columns were stored in 20% ethanol for long-term use.

3.5 FREEZE DRYER

A Labconco freeze dryer/lyophilizer (Total Lab Systems, Auckland, NZ) was used to freeze

dry products under a high vacuum. The temperature and pressure were maintained at -47°C and 0.070 mBar respectively. With lower injection of whey solution/milk into the AKTA 10 Explorer system, the absorbance was observed to be low. The resultant isolated proteins needed identification and this was achieved by passing them through gel electrophoresis. To observe visible bands on the gel, the samples needed to be concentrated and this was achieved using the freeze dryer.

CHAPTER 4 - RESULTS AND DISCUSSION

Initial trials were performed to confirm the immobilisation of the peptide on the resin (Section 4.1). To perform these trials, varying ratios of peptide solution to resin volume were used as described in Sections 4.1.1 and 4.1.2. Once the immobilisation process was confirmed, a relationship between dry resin and wet resin quantities was obtained (Section 4.2). This relationship was used for further experiments to convert dry resin quantities to wet resin quantities or vice-versa. Section 4.3 contains a set of batch experiments that were performed to determine the equilibrium binding capacity of the tetramer peptide resin. Following this, experimental trials were carried involving chromatographic separation of β-lac from whey and milk samples.

4.1 PEPTIDE IMMOBILISATION ON A CHROMATOGRAPHY MATRIX

Initially, to obtain tetramer ligand-resin immobilisation, coupling was performed as described in Section 3.2.2. Two different ratios of ligand-resin were chosen to obtain immobilisation (Sections 4.1.1 and 4.1.2).

4.1.1 Ratio of peptide solution (concentration-10 mg/mL): resin volume~0.5: 1 mL

The first method used for coupling involved a ratio of peptide:resin (P:R) of 0.5:1 mL. Once coupling was complete, samples collected every hour were passed through a Superdex HR Peptide 10/30 column. In Figure 4, the chromatogram depicts the absorbances at wavelengths 280 and 215 nm. The start material was purely PAM peptide solution (10 mg/mL), without contact with NHS-activated Sepharose 4 Fast Flow resin. On comparison, the wavelength at 215 nm showed a higher absorbance value. This is because the absorbance at 280 nm is dependent upon the presence of aromatic amino acids in a peptide sequence, whereas absorbance at 215 nm is caused by the presence of peptide bonds. As shown in Figure 2-6, PAM peptide contains only one aromatic amino acid (tyrosine) which explains why absorbance at 215 nm was higher than 280 nm for the start material.

To further investigate the immobilisation process, the peptide-resin slurry was left to mix by inversion on a slowly rotating wheel (RT) to react and the process was monitored every hour.

It was observed that the sample isolated after 1 hour of reaction showed greater absorbance at 280 nm than that at 215 nm. This showed that the solution at the end of the immobilisation process contained the by-product of the reaction (succinic-acid) because succinic-acid is known to possess a higher absorbance at 280 nm than at 215 nm.

Using a Superdex HR 10/30 peptide column, elution of peptides, proteins and other biomolecules ranging between 100-7000 g/mol is usually observed at around 17-25 mL at a flow rate of 0.75 mL/min (GE Healthcare Life Sciences, 2014). Figure 4-1 represents a chromatogram showing the absorbance (mAU) of samples obtained from the immobilisation solution over time. It can be observed that the product eluted as a result of the chromatographic process was not PAM peptide but succinic acid (MW-118.9 g/mol), which was the by-product of NHS/PAM immobilisation reaction. .As expected, the chromatogram confirms the presence of succinic acid in the eluted product because it has a higher absorbance at 280 nm. Apparently, the 280 nm peaks for 1 and 3 h data overlap which clearly shows that there was no further absorbance after the first hour of the reaction. Similar overlapping of peaks was observed for 215 nm wavelength. This suggested that 1 h was sufficient for maximum immobilisation to occur.

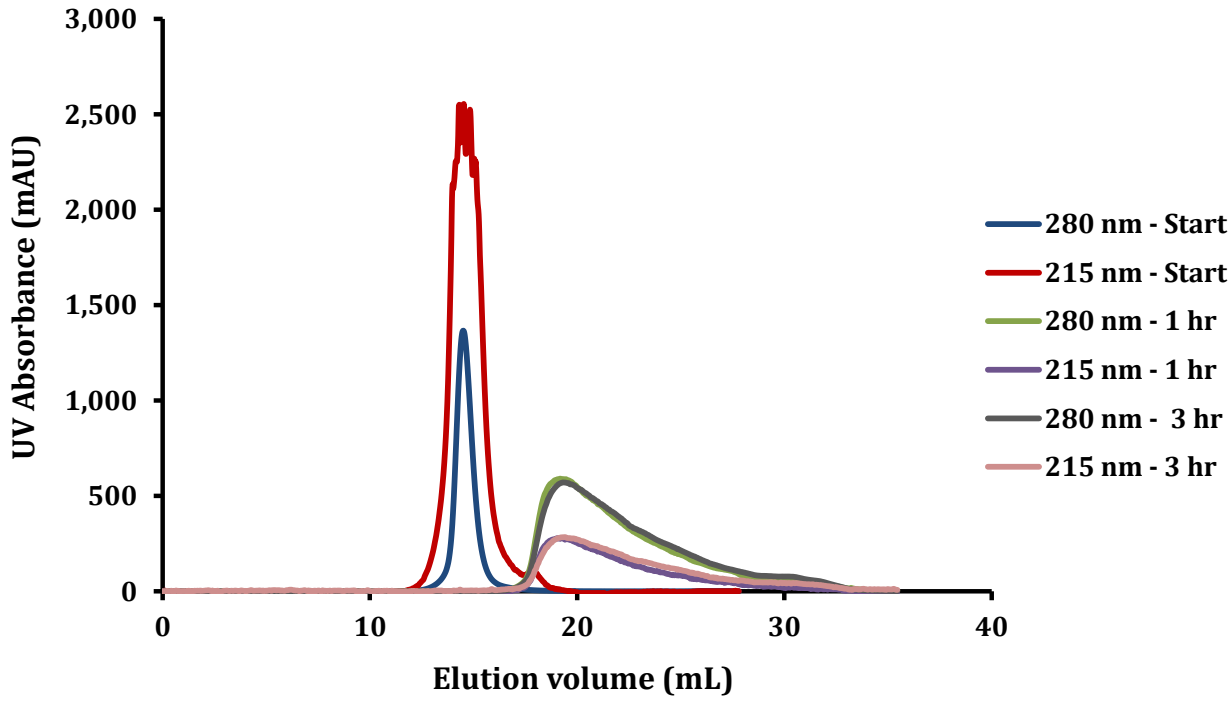

Figure 4-2: Chromatogram showing the absorbance of samples taken from the supernatant of the immobilisation solution over time, showing the immobilisation process, (P:R – 0.5:1 mL)

4.1.2 Ratio of peptide solution (concentration-10 mg/mL): resin volume~1:0.5 mL

The second method followed to achieve coupling involved the use of a P:R ratio of 1:0.5 mL, to check if an increase in the amount of peptide caused an increase in binding capacity of the resin.

Table 4-1 shows a comparison with regard to the area of the individual peaks (mAU•mL) at 280 and 215 nm wavelengths. There is no clear separation of peaks observed which states that there is a mix of PAM peptide and succinic acid (by-product) present. An increase in area of the individual peaks at 280 nm was observed on comparing it with the starting sample which can be justified due to the mixture of products present. The ratio used for the immobilisation process confirmed the saturation of the resin, thereby confirming the absence of binding sites remaining for uptake of more β-lac from the solution.

Table 4-1: Area of the individual peaks (mAU•mL) at 280 and 215 nm wavelengths comparing immobilisation occurring over varying time intervals (Start, 1 and 3 h) to depict the uptake of β-lac by the tetramer peptide resin

Sample	Wavelength (nm)	Area under the peak (mAU•mL)
Start	280	1164.33
	215	3448.70
1 h	280	1976.65
	215	364.24
3 h	280	1445.54
	215	293.27

Figure 4-2 clearly shows that the eluted sample had an absorbance at 215 nm. The successful saturation of the resin was confirmed by comparing the elution volume (mL) to that of the start material. This confirmed the presence of residual peptide in the supernatant. A conclusion was hence drawn that the entire peptide was not immobilized to the resin even after a prolonged time. This is likely to have been because there were no more binding sites present on the resin to allow immobilisation to occur.

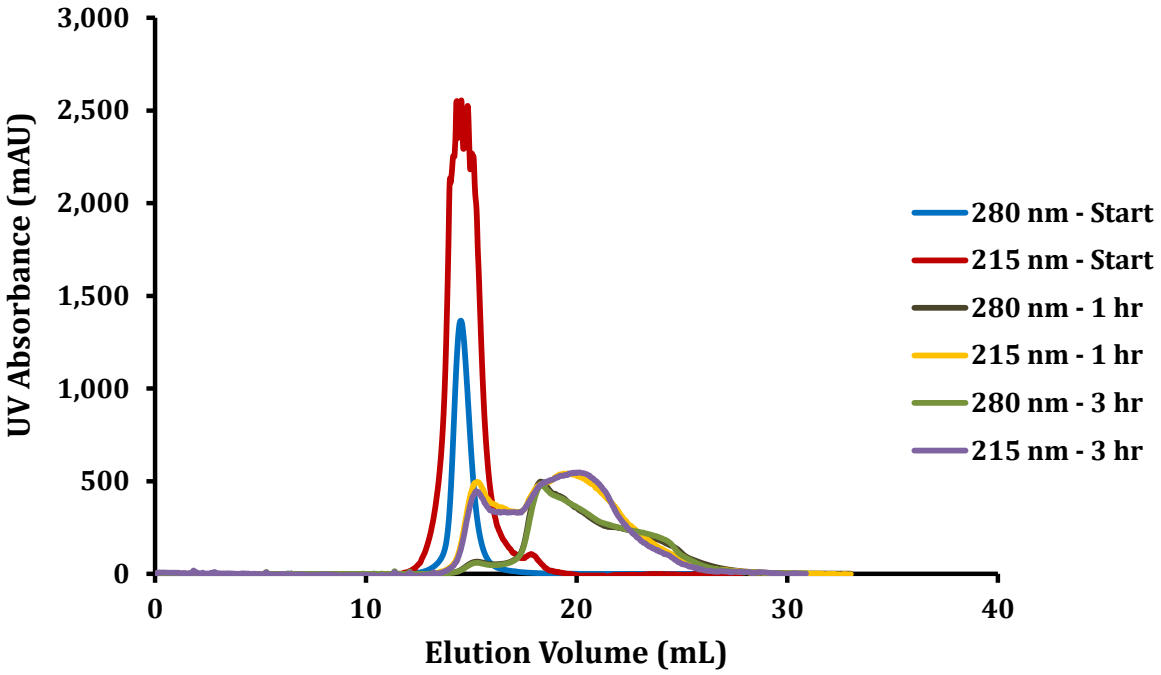

Figure 4- 3: Chromatogram showing the absorbance of samples taken from the supernatant of the immobilisation solution over time, showing the immobilisation process, (P:R–1:0.5 mL)

4.2 RELATION BETWEEN DRY AND WET RESIN

Following the steps as mentioned in Section 3.2.3.1, dry resin and wet resin were separately prepared. A few experimental trials were performed to obtain a relation between dry resin and wet resin quantities. Initial experiments involved the use of dry resin followed by use of drained wet resin for subsequent experiments. This alternative method of resin usage was suggested to check and confirm if there was any variation in the equilibrium binding capacity of the resin (*Q*). Wet resin was preferred for the remaining experiments because this has been the most commonly used form to determine *Q* (Burton et al., 1999, Fee, 2010). With dry resin, it is almost impossible to achieve acceptable packing of a chromatography column with small particles (<50 μm) due to electrostatic interactions, which suggests that wet resin (in the form of a slurry) is preferable (Costa and Cabral, 2012). Another drawback of using dry resin is the time required to ensure that it is completely dry before the experiments. In many cases in the literature, the method for determination of the binding capacity is not stated,

which makes it difficult to compare the binding capacity of the resins. It was expected that *Q* would remain the same for both dry resin and wet resin quantities, when compared on the same basis. The reason to obtain a relationship between dry resin and wet resin was because this could be used to help convert dry resin to wet resin or vice-versa, thereby leading to a consistent basis for comparing *Q* values.

A linear plot was obtained which showed a direct correlation between dry resin and wet resin (R^2 = 1.00) (Figure 4-3).

$$y = 3.79x \quad (1)$$

Equation 1 shows that 3.79 was used as the conversion factor to convert wet resin to dry resin quantities. Hence, this correlation obtained was used for further analysis.

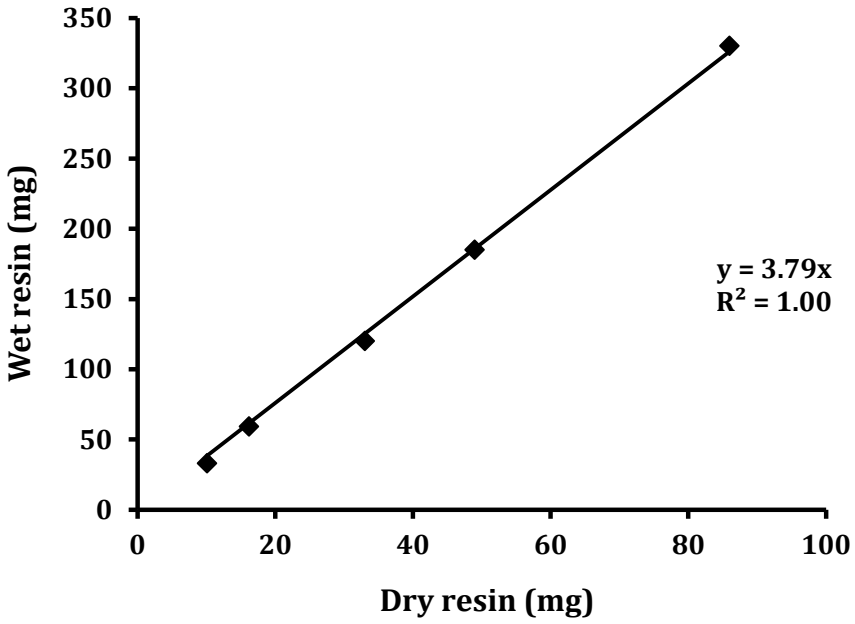

Figure 4-4: Relation between dry and wet resin

4.3 BATCH EXPERIMENTS

4.3.1 Equilibrium/ Static binding capacity of Tetramer Peptide resin

The objective of the work presented here was to obtain the static binding capacity of NHS-activated Sepharose 4 Fast Flow resin. Protein binding capacity (static/equilibrium) is a

critical parameter for chromatography media because it determines how much media is needed to purify a certain amount of protein (GE Healthcare Life Sciences, 2011). To find the maximum adsorption capacity of the tetramer peptide resin in contact with a pure β-lac protein solution, an adsorption isotherm was required.

To determine *Q* (mg/g), linearization plots were first obtained and the plot which best fit the data was chosen to determine the Langmuir constants. Using the two P:R ratios as mentioned in Section 4.1, different plots (Section 4.3.1.1 and 4.3.1.2) were obtained to determine *Q*. For the sake of consistency, the pH of sodium phosphate buffer was set to 7 in all tests. All other conditions were maintained constant in both the cases to allow a direct comparison between different experiments.

4.3.1.1 Ratio of peptide solution (concentration-10 mg/mL): resin volume~0.5:1 mL

Wet resin Basis Measurement of Equilibrium Binding Capacity

Following the first ratio used (Section 4.1.1) and the protocol described in Section 3.2.2, β-lac was allowed to remain in contact with the tetramer peptide resin for 3 h. Figure 4-4 shows the Scatchard plot obtained which was observed to be the best fit for the data points. In Table 4-2, R^2 values are observed to vary with every plot used and Scatchard plot was ultimately seen to be the best fit, although the Lineweaver-Burk plot was almost as good. This was hence used to determine the Langmuir constants (Figure 4-4).

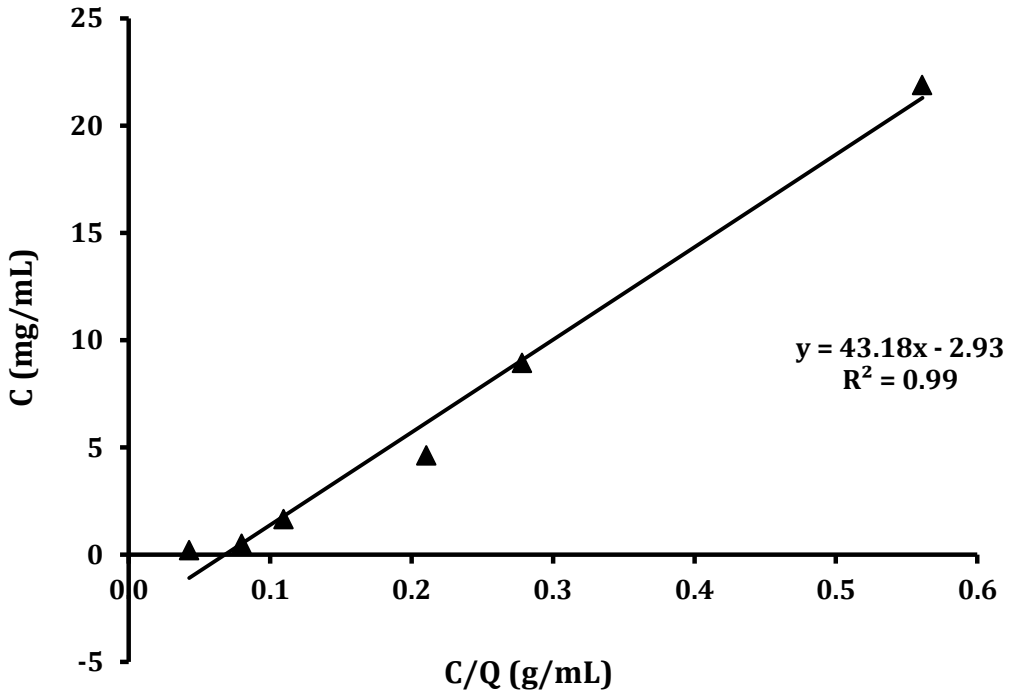

Figure 4- 5: Scatchard plot for wet resin at 0.2, 0.5, 1.65, 4.62, 8.93 and 21.89 mg/mL β-lac, after 1 h binding

Table 4-2: Comparison of varying Linearization plots determining the best fit for wet resin obtained after 1 h biniding

Linearization method	**Equation**	**Constants (mg/mL)**		**R^2 value**
		Q_0	**K**	
Scatchard plot	$C = Q_0 \frac{C}{Q} - K$	43.18	2.93	0.99
Eadie-Hofstee plot	$Q = -K \frac{Q}{C} + Q_0$	34.20	1.32	0.83
Lineweaver-Burk plot	$\frac{1}{Q} = \frac{K}{Q_0} \frac{1}{C} + \frac{1}{Q_0}$	31.04	1.13	0.98

The time interval chosen was to allow maximum binding time, which is clearly depicted in Figure 4-5 which also shows a clear comparison between the varying time intervals (1, 2 and 3 h) with respect to the varying binding capacities corresponding to β-lac concentrations.

Figure 4-5 is a clear illustration in explaining the fact that 1 h was sufficient for allowing all the peptide in the slurry to bind onto the resin thereby saturating the binding sites present.

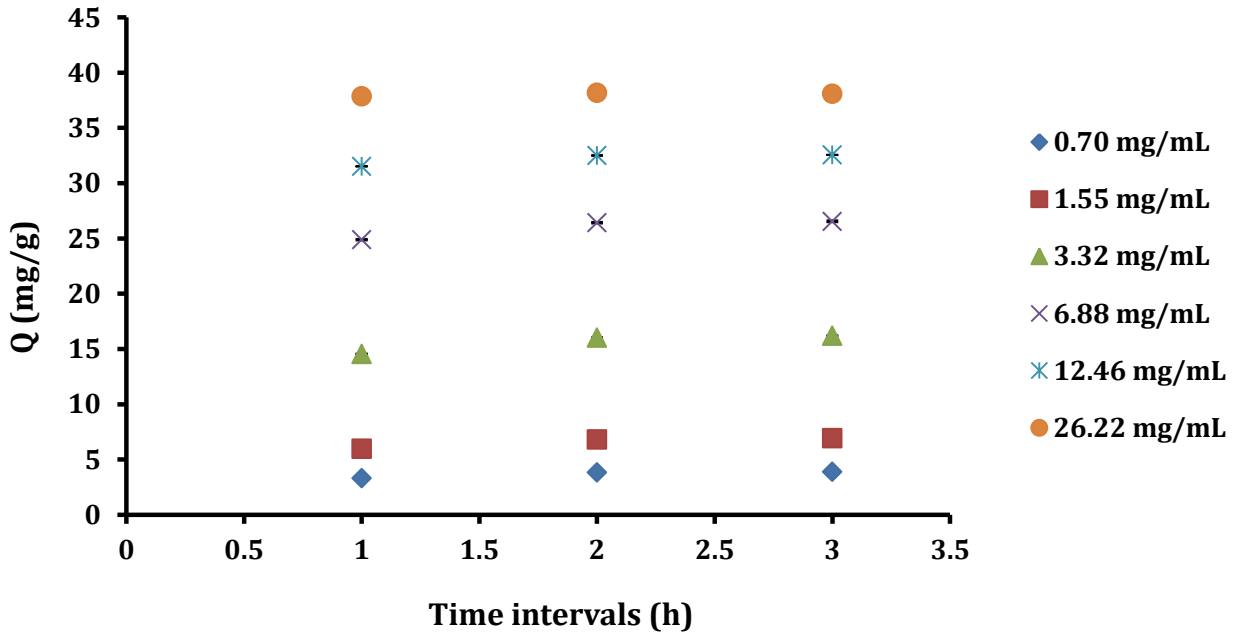

Figure 4-6: Adsorption capacities (mg/g wet resin) at 0.7, 1.55, 3.32, 6.88, 12.46 and 26.22 mg/mL β-lac, after 1, 2 and 3 h binding

Using the Langmuir constants (Q_o = 43.18 mg/mL, K = 2.93 mg/mL) as obtained from Figure 4-4, the Langmuir isotherm was plotted (Figure 4-6), showing a good fit to the data. It was observed that increasing β-lac concentrations led to an increasing trend. Examining this gradual increase, there was a possibility for the data points to fit either a Langmuir/ Freundlich plot. Due to time constraints, Q for higher concentrations of β-lac was not determined. Examining further data would help confirm which isotherm would be the best fit for the data obtained.

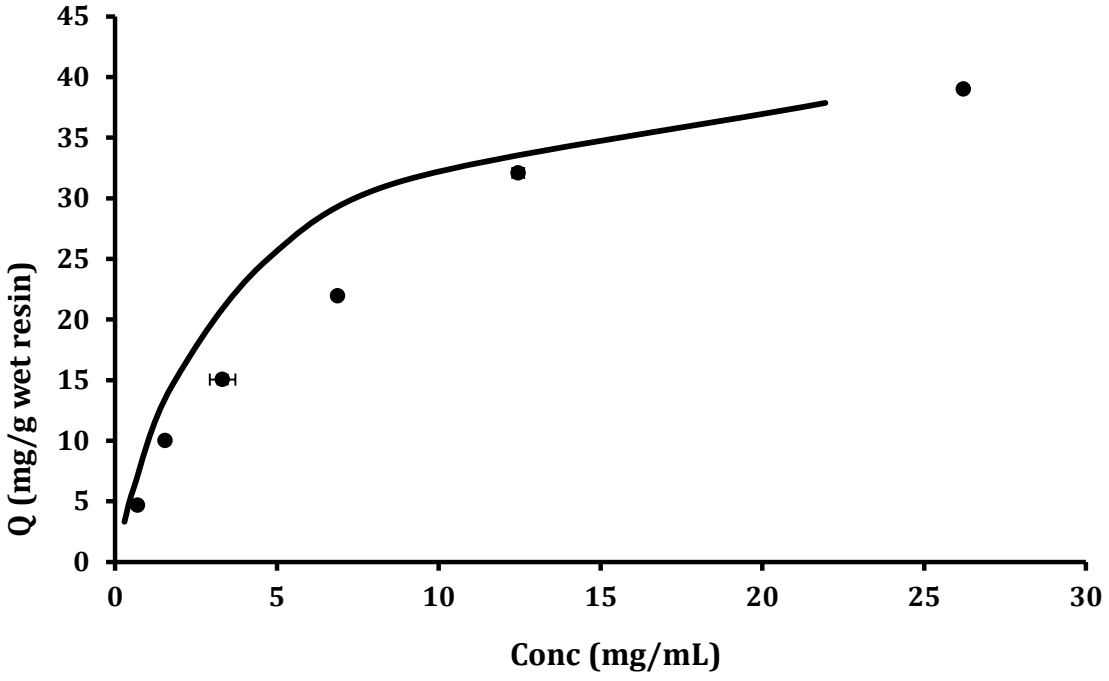

Figure 4-7: Langmuir isotherm for β-lac adsorption to a resin prepared with peptide solution:resin volume~0.5:1 mL, after 1 h binding

Dry resin Basis Measurement of Equilibrium Binding Capacity

Experiments involved using tetramer peptide-resin in the form of dry basis were also performed. The protocol described in Section 3.1.2 was followed with a minor change of using dry resin rather than drained swollen wet resin. In this case, β-lac was allowed to remain in contact with the tetramer peptide resin for 4 h to determine *Q*. Figure 4-7 shows a Scatchard plot which is observed to be the best fit for the data points whereas Table 4-3 compares the R^2 values of varying plots. The values hence obtained were used to determine the Langmuir constants.

Table 4-4 illustrates the varying initial concentrations of β-lac that were determined using the Nanodrop. A random range of initial concentrations was chosen to determine *Q*. Considering lower concentrations of β-lac (0.58 and 1.22 mg/mL), there was no variation observed in the binding capacity of the resin (Table 4-4). This likely was expected due to experimental error because a gradual increase in *Q* with the remaining concentrations of β-lac was noticed and this was consistent. In all cases, the dry resin data was converted to wet resin basis quantities to maintain consistency.

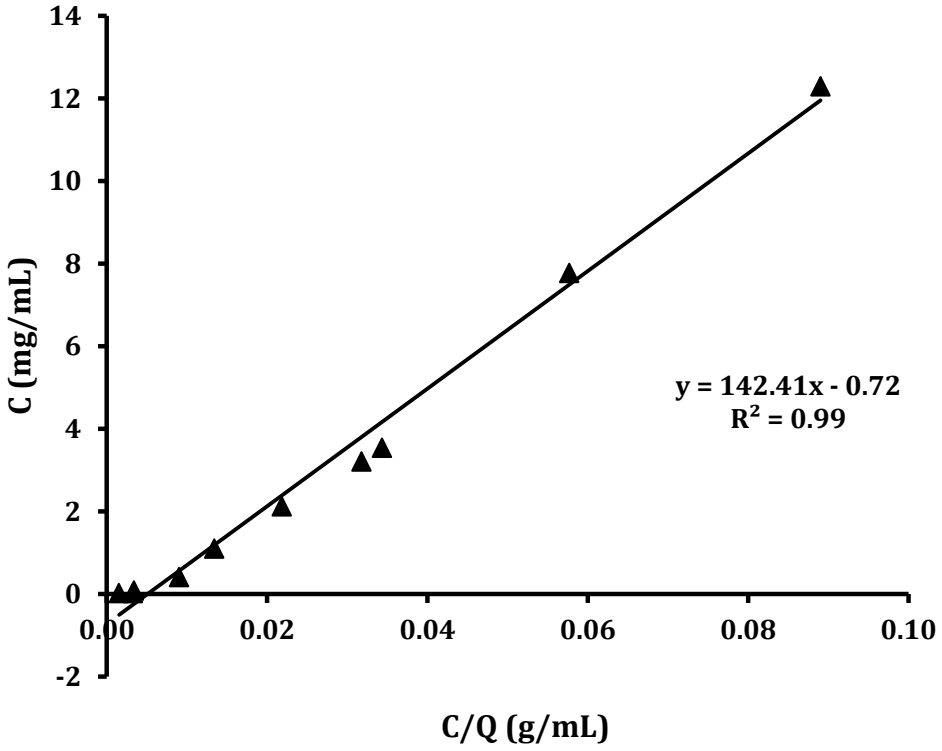

Figure 4-8: Scatchard plot for dry resin experiments (converted to wet resin basis) at 0.02, 0.02, 0.08, 0.41, 1.1, 2.12, 3.2, 3.54, 7.77 and 12.29 mg/mL β-lac after 1 h binding

Table 4-3: Comparison of Linearization plots determining the best fit for dry resin (converted to wet resin quantities), after 1 h binding

Linearization methods	**Constants (mg/mL)**		**R^2 value**
	Q_o	**K**	
Scatchard plot	142.41	0.72	0.99
Eadie-Hofstee plot	105.00	0.2	0.7
Lineweaver-Burk plot	97.69	0.22	0.83

Table 4-4: Equilibrium binding capacities at varying β-lac concentrations, after 1 h binding

Initial conc of β-lac (mg/mL)	**Conc of residual β-lac after 1 h (mg/mL)**	***Q* (mg/g)**
0.58	0.02	3.59
1.22	0.02	3.59
2.43	0.08	14.36
4.83	0.41	51.16
9.17	1.10	85.85
11.70	2.12	106.20
13.14	3.20	116.16
13.70	3.54	118.23
21.04	7.77	130.27
25.90	12.29	134.49

Figure 4-8 shows the change in *Q* with time obtained corresponding to varying β-lac concentrations. It is clearly observed that the maximum adsorption capacity was obtained after the first hour of the reaction. This indicates that the resin was saturated and hence all binding sites were occupied, which prevented further binding. A decrease in binding was observed at 4 h interval and this seemed to be an outlying data point because it was inconsistent considering other concentrations of β-lac solution.

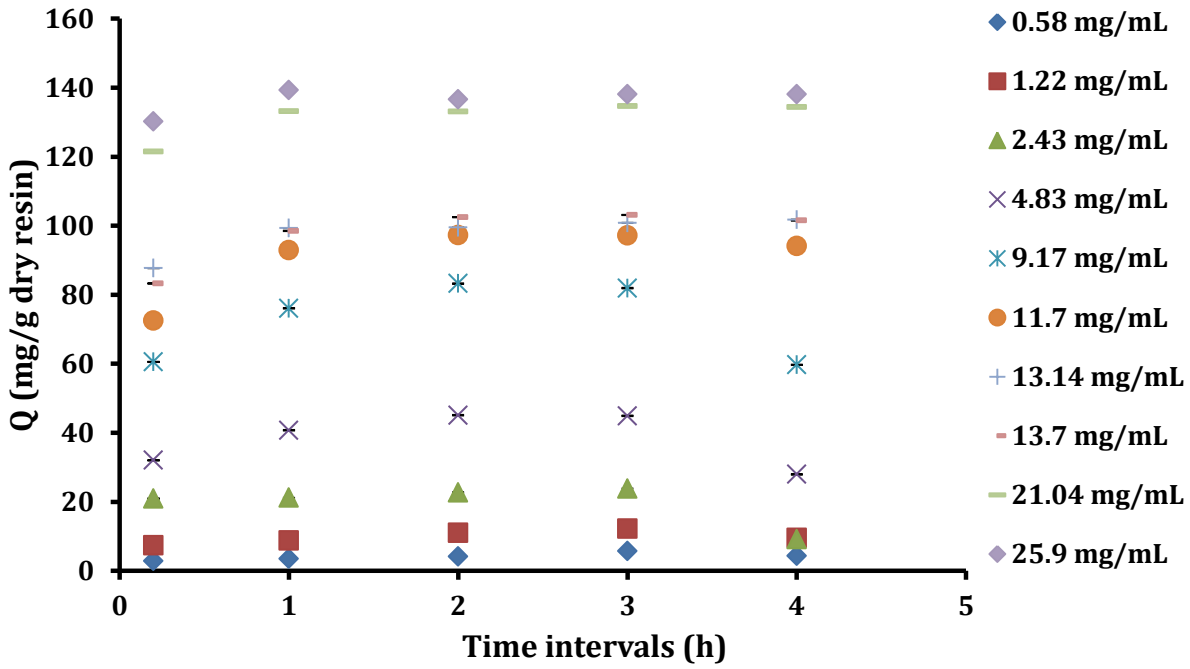

Figure 4-9: Adsorption capacities (mg/g wet resin) at 0.58, 1.22, 2.43, 4.83, 9.17, 11.7, 13.14, 13.7, 21.04 and 25.9 mg/mL β-lac, after 1, 2, 3 and 4 h binding

The Langmuir constants obtained from Scatchard plot (Table 4-2) were used to plot a Langmuir isotherm. Figure 4-9 shows that the tetramer peptide-resin reached its maximum equilibrium adsorption capacity of 134.49 mg/g wet resin at a concentration of 12.29 mg/mL pure β-lac solution.

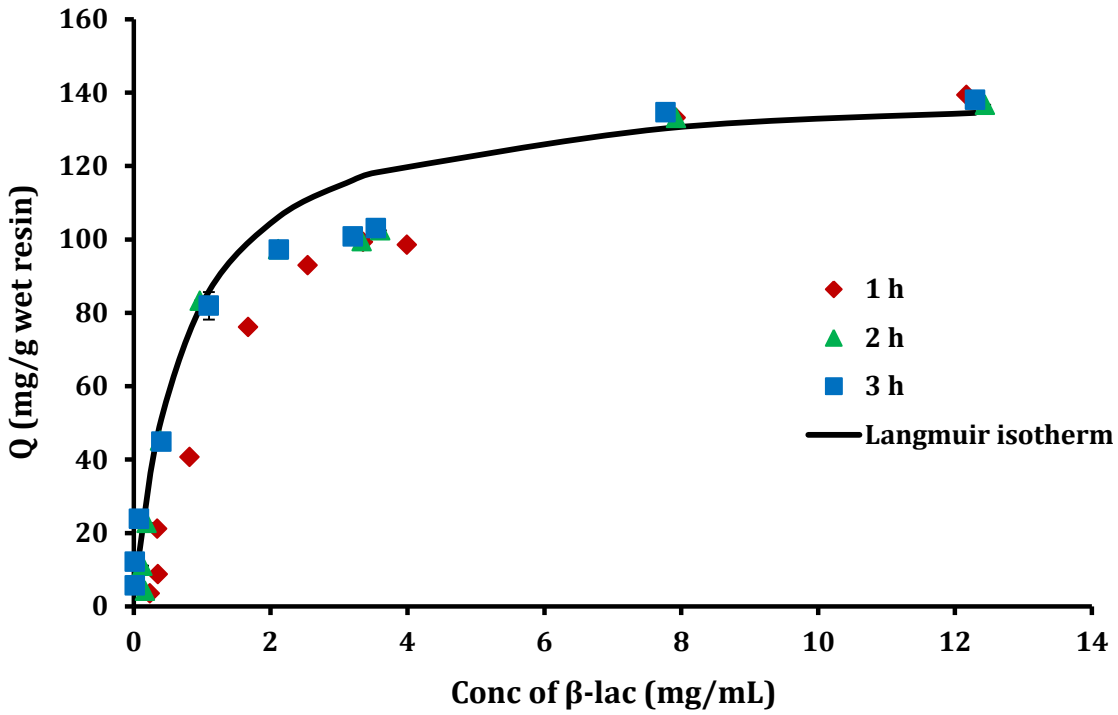

Figure 4-10: Langmuir isotherm for β-lac adsorption to a resin prepared with peptide solution:resin volume~1:0.5 mL, after 1 h binding dry resin

Comparing Figures 4-6 and 4-9, *Q* increased approximately three-fold in the latter case. Although both the Langmuir isotherms were plotted following the same experimental conditions and protocol, the plot shown in Figure 4-9 was for data from a resin that contained triple the amount of peptide as that in Figure 4-6. A calibration curve (Appendix) was used for this plot and hence the variation observed was triple when compared to Figure 4-6. It is obvious that an increase in peptide concentration during immobilisation increased the ligand on the resin surface, which in turn led to a higher binding capacity. However, further work is needed to quantify and confirm this behavior.

4.3.1.2 Ratio of peptide solution (10 mg/mL):resin volume~1: 0.5 mL

Considering the ratio as mentioned in Section 4.1.2, Langmuir constants were determined to plot a Langmuir isotherm. From Table 4-3, the Scatchard plot was observed to be the best fit and it was therefore used to obtain the Langmuir constants (Figure 4-10).

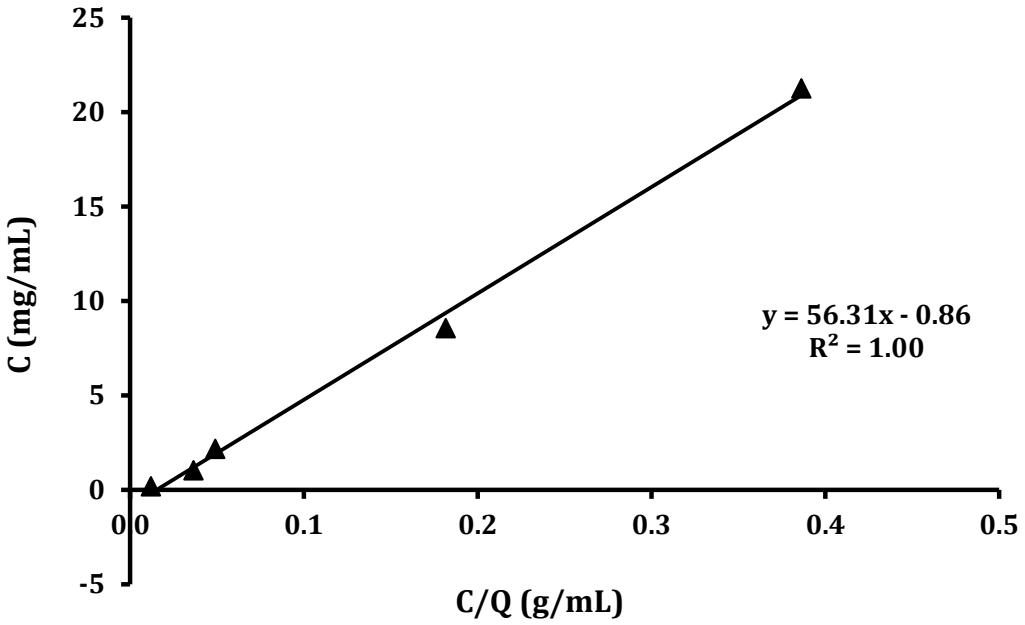

Figure 4-11: Scatchard plot for dry resin (converted to wet resin basis) at 0.18, 1.03, 2.16, 8.55 and 21.25 mg/mL β-lac, after 1 h binding

Table 4-5: Comparison of Linearization plots determining the best fit for dry resin (converted to wet resin quantities), after 1 h binding

Linearization methods	**Constants (mg/mL)**		**R^2 value**
	Q_o	**K**	
Scatchard plot	56.31	0.86	1.00
Eadie-Hofstee plot	50.52	0.45	0.86
Lineweaver-Burk plot	48.04	0.40	0.96

Similar to Figure 4-6, a plot was drawn to determine the maximum binding capacity of the resin. Trials were performed for 2 h duration and equilibrium appeared to to attained after the first hour, which is consistent with the above results. This confirmed that 1 h was sufficient to complete β-lac binding to the tetramer peptide resin.

Figure 4-11 shows a time comparison plot of the amounts of β-lac bound to the resin. The quantities of β-lac bound to the resin remained essentially constant within experimental error. The conclusion drawn from the above results hence remained consistent, proving that 1 h was sufficient to saturate the resin and occupying all binding sites that were available for binding.

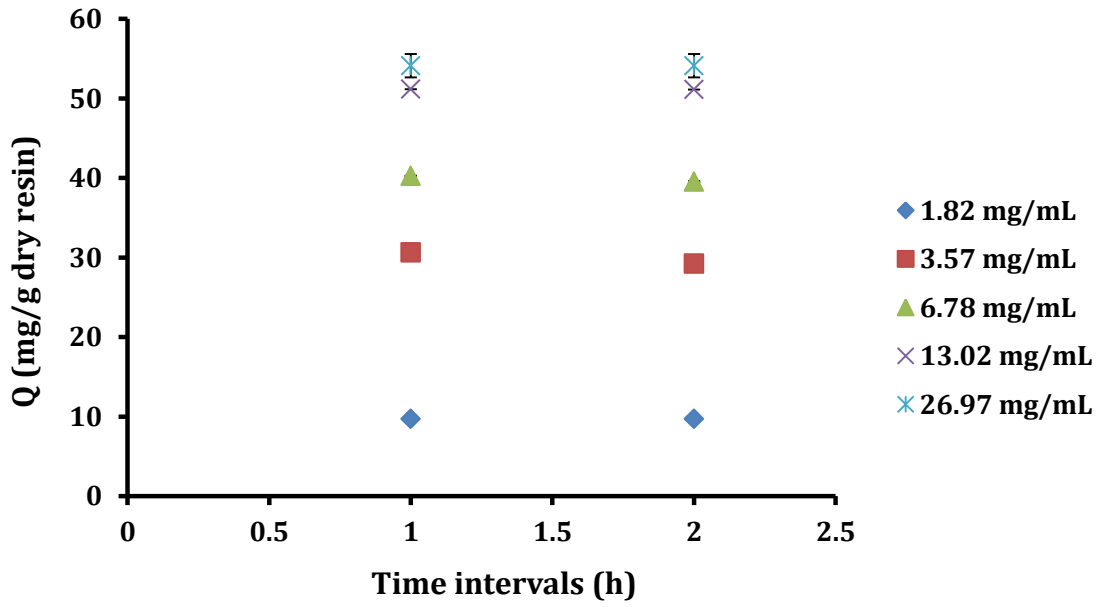

Figure 4-12: Adsorption capacities (mg/g dry resin) at 1.82, 3.57, 6.78, 13.02 and 26.97 mg/mL β-lac, after 1 and 2 h binding

The Langmuir constants obtained from Table 4-5 was used to plot a Langmuir isotherm (Figure 4-12). Similar to Figure 4-6 the trend seemed to increase at higher concentrations of β-lac and there was no stability observed. Time limitations did not allow trials with higher concentrations of β-lac to confirm whether Langmuir isotherm or Freundlich isotherm was the best fit but this should be done in future work.

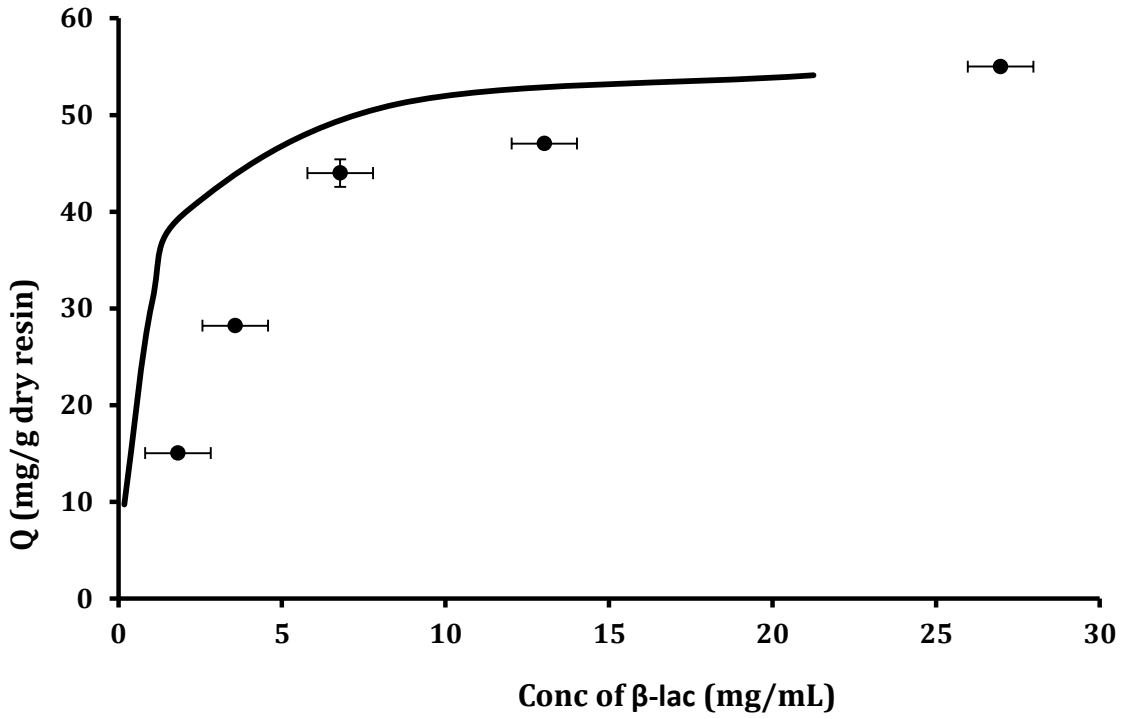

Figure 4-13: Langmuir isotherm for β-lac adsorption to a resin prepared with peptide solution:resin volume~1:0.5 mL, after 1 h binding

From Figure 4-12 it is observed that tetramer peptide resin reached its maximum equilibrium adsorption capacity of 54.11 mg/g resin at a concentration of 21.25 mg/mL pure β-lac solution. Hence as expected, an increase in peptide concentration confirmed an increase in *Q*. As shown in Figure 4-6 and 4-12, *Q i*s observed to be 39.01 and 54.11 mg/g dry resin respectively but Figure 4-9 corresponds to an increased *Q* of 134.49 mg/g wet resin (wet resin and dry resin were interchangeable using the linear relation as shown in Section 4.2). To confirm the concept that with an increase in peptide concentration a higher *Q* is obtained, repetitive trials need to be performed which was not achieved in this research because of time constraints.

4.3.2 Langmuir isotherm with varying salt strengths

Following the determination of *Q*, experimental runs were performed using varying salt strengths to measure the dependence of Langmuir isotherms on salt concentration. Figure 4-13 shows a gradual decrease in *Q* with increasing salt concentrations. Duplicates were performed to ensure consistency and the results seemed to be consistent over the varying

concentrations of β-lac.

The data for the curve denoted “No added NaCl” contained only sodium phosphate buffer with no added NaCl. The remaining isotherms were obtained in presence of varying salt strengths. In the case of ion exchange chromatography, it is always noted that higher salt strengths weaken the strength of protein binding (Yamamoto et al., 1988, Kaltenbrunner et al., 1993) and therefore one would expect a progressively lower maximum binding with increasing salt addition.

Because initial experiments showed that a high salt concentration (1 M NaCl) effectively elutes β-lac from the resin, it was proposed that PAM peptide might behave as an ion exchange material. However, the expected drop in *Q* at higher salt concentrations did not occur (Figure 4-13) but rather the Langmuir isotherm increasingly showed a linear form.

Qualitatively, Langmuir isotherms are known to follow a linear trend where the number of adsorption sites greatly exceed the available adsorbent molecules but saturate beyond a given concentration due to an increase in occupancy sites (Sattler, 2010). This may be one reason for the trend observed in Figure 4-13. However, more work is necessary to draw any firm conclusions regarding the high-salt behavior of the tetramer peptide.

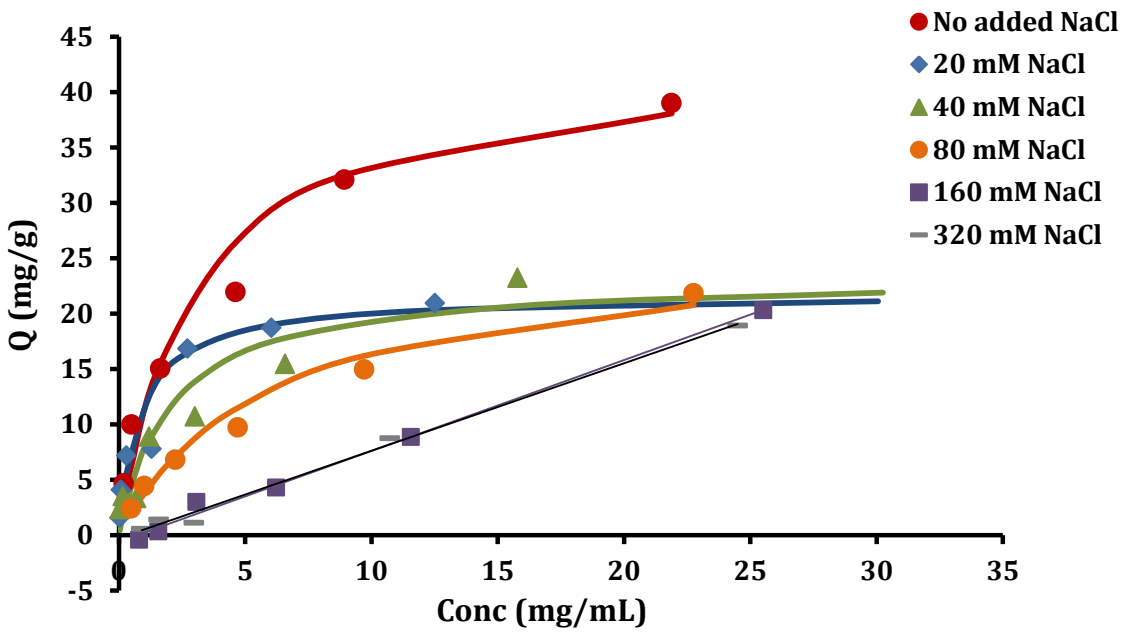

Figure 4-14: Determination of adsorption capacities (mg/g dry resin) in presence of 20, 40, 80, 160 and 320 mM NaCl

4.3.3 Elution of bound β-lactoglobulin

After binding of β-lac by the tetramer peptide resin, it must be eluted without causing damage to the structure/function of the protein. Trials were performed initially involving 0.1 M citric acid and variation in the salt strengths maintaining constant pH and concentration of β-lac solutions. This did not seem to give a high recovery percentage; the use of citric acid and varying salt strengths at varying pH was henceforth chosen to check the effectiveness of β-lac elution by pH change.

Citric acid is commonly used to elute Igs off Protein A and other proteins from affinity chromatography matrices (Hahn et al., 1998, Boardman and Partridge, 1955). Because the ligand was originally designed as a Protein A mimetic, trials were performed involving 0.1 M citric acid. For consistency, the pH of citric acid was maintained constant (~6.02).

Figure 4-14 shows the recovery of bound β-lac. Citric acid eluted β-lac but 100% recovery was not observed. The percentage of β-lac eluted at varying salt strengths remained almost consistent at 60%. Varying salt strengths and pH change are known to elute the bound protein from a chromatography matrix, in case of ion exchange chromatography. As a result of this

trial, a conclusion was drawn that there was no change observed in the percentage recovery, with varying salt strengths.

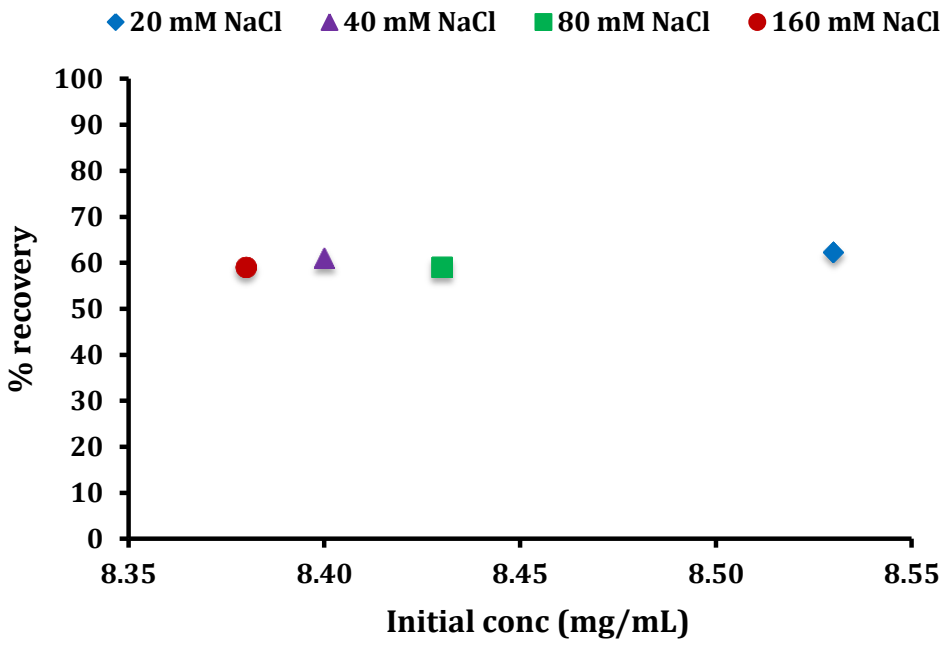

Figure 4-15: Elution of bound β-lac using 0.1 M citric acid in presence of 20, 40, 80 and 160 mM NaCl at constant pH (~6.02)

Trials were then performed using 0.1 M citric acid at varying pH. A particular concentration of β-lac (26.1 mg/mL – 26.6 mg/mL) was chosen and 0.1 M citric acid (varying pH) was added to the pellet to observe if varying the pH value caused any variation in the elution of the bound protein. From Figure 4-15 it is clearly observed that pH did not affect the elution of bound β-lac. The percentage of β-lac eluted varied between 32% and 35% with varying pH values. Hence, pH variation did not affect the elution process.

Thus the presence of salt in citric acid showed a higher recovery percentage compared with a variation of citric acid pH in absence of NaCl.

A conclusion regarding mode of binding of the ligand could not be drawn because the major properties of ion exchangers include elution of bound material involving a variation in the ionic strength and pH of elution buffers (Hahn et al., 1998, Williams and Frasca, 2001). The result in Figure 4-14 shows the matrix tending to behave as an ion exchanger but, on the

contrary, the result in Figure 4-15 does not support the ion exchange property of the resin since a variation in pH of the buffer did not cause any variation in elution and the recovery percentage of the bound protein remained consistent. Due to time constraints, further investigation into the mode of binding was not carried out.

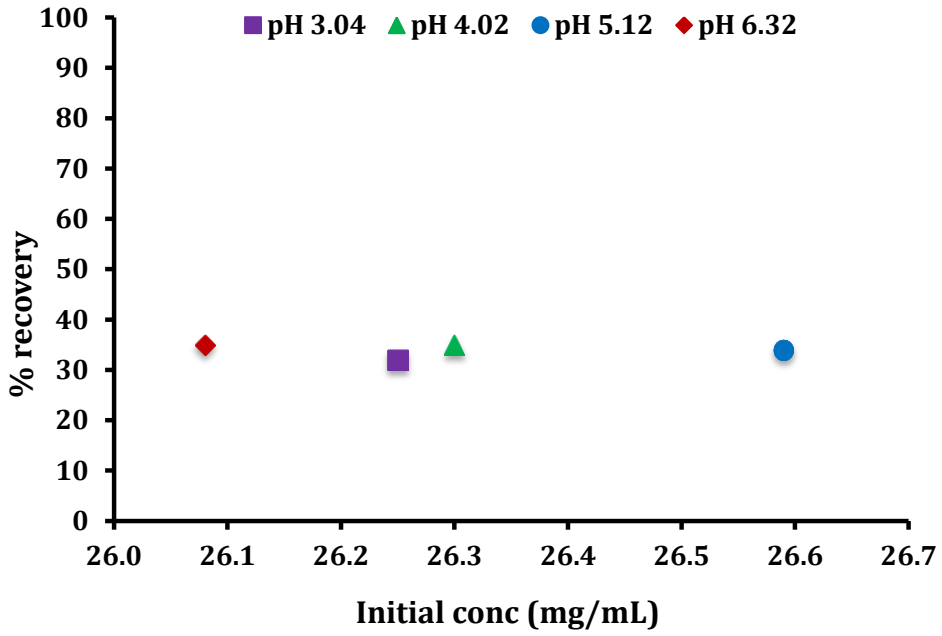

Figure 4-16: Elution of bound β-lac using 0.1 M citric acid at varying pH (3.04 – 6.32)

Once conditions were tested with regard to the elution of bound protein, a study was carried out to isolate β-lac by feeding whey solution and milk through a chromatography column, carefully packed as described in Section 3.3.3.

4.4 DYNAMIC CONDITIONS – SEPARATION OF B-LAC

The main objective of the work presented here was to test the separation of β-lac from

- Whey solution
- Milk

To isolate β-lac, whey solution was initially passed through a 5 mL chromatography column (packed with slurry). These trials were performed using the ratios as described in Section 4.1.1 and 4.1.2. The results obtained are clearly shown in the chromatograms as in the

following sections.

4.4.1 Ratio of peptide solution (10 mg/mL):resin volume~0.5: 1 mL

Initial trials were performed by passing varying volumes of sample through a chromatography column packed with the prepared tetramer peptide resin.

4.4.1.1 Whey solution~varying injection volumes

Injection – 0.25 mL, 0.5 mL and 4 mL whey samples

Figure 4-16 (a) shows a clear comparison between varying injection volumes of acidic whey (pH 6.8). The result seems consistent when comparing the elution volume of each injection. Another factor considered for analysis was the conductivity (mS/cm). β-lactoglobulin was observed to elute at the same conductivity (15-15.8 mS/cm) in every trial. As expected, with higher injection volume there was a higher absorbance observed. To characterise the components of the various peaks in the chromatogram shown in Figure 4-16, gel electrophoresis was performed.

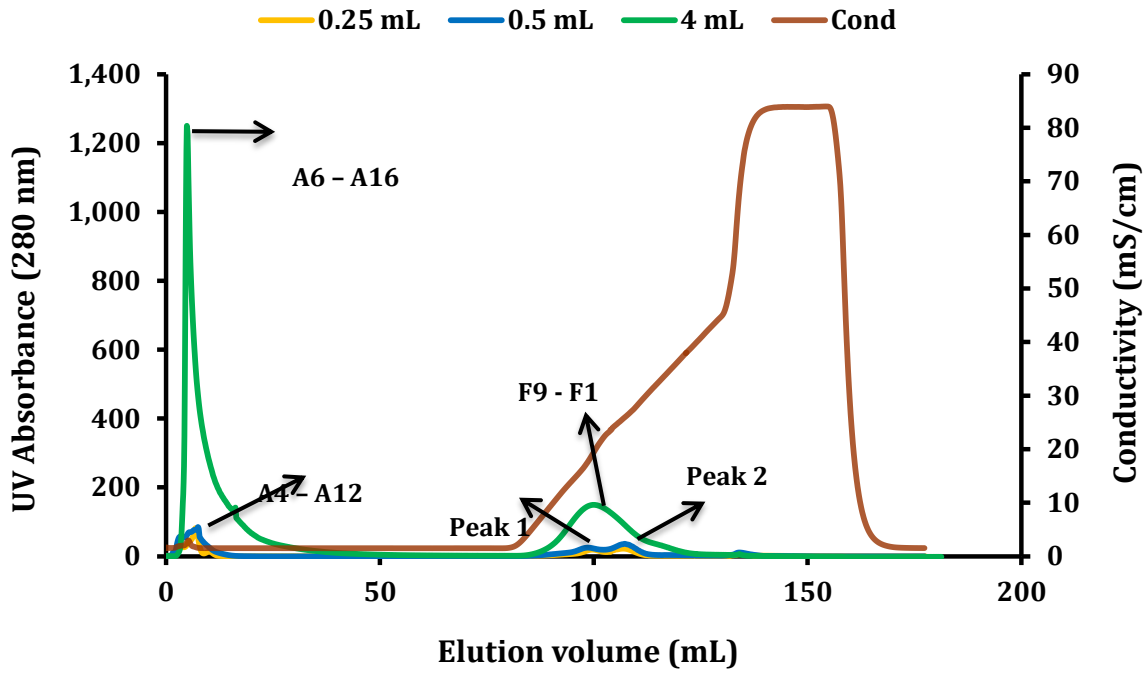

Figure 4-17: Chromatogram for various injection volumes of whey solution (0.25, 0.5, 4 mL whey solution) at 1 mL/min – pH 6.8

The SDS-PAGE gel image shown in Figure 4-17 shows a clear separation of proteins that were obtained as an outcome of the chromatographic separation process. This was compared with a molecular-weight size marker/ ladder (L) which was used as a standard to help identify the approximate size of the protein. The pooled elution fractions were loaded in lane 2, corresponding to flowthrough fractions A4-A12. Figure 4-17 confirms the presence of β-lac as a dark band at the expected molecular size (18.6 kDa). Lane 3 (corresponding to Peak 1 in Figure 4-16) contained both α-lac and β-lac with minor traces of BSA.

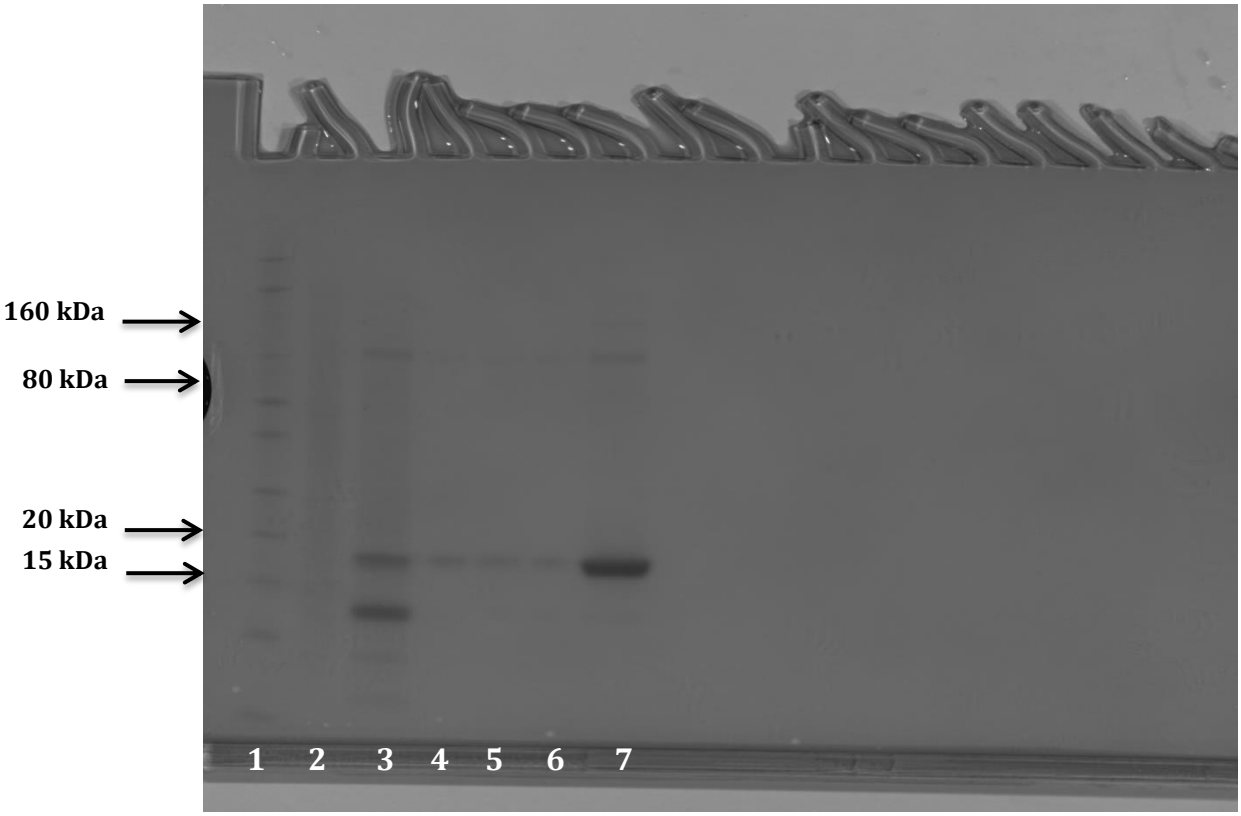

Figure 4-18: SDS-PAGE gel image, showing the protein composition of feed, flowthrough and elution fractions from chromatogram in Figure 4-16 – for 0.5 mL whey solution

In lane 2, various proteins present in whey solution are shown, which is the starting sample (Figure 4-18). Fractions A6 – A16 (lanes 3-6) were flowthrough fractions which consisted of proteins that did not bind to the column matrix. A mix of whey proteins was observed in these

fractions which include α-lac, β-lac, BSA, and Igs. Lanes 7-18 shows the presence of β-lac and minor traces of BSA. To prevent loss of protein (β-lac) in the flow through fractions, it is preferred to load low volumes of samples, as shown in Figure 4-17.

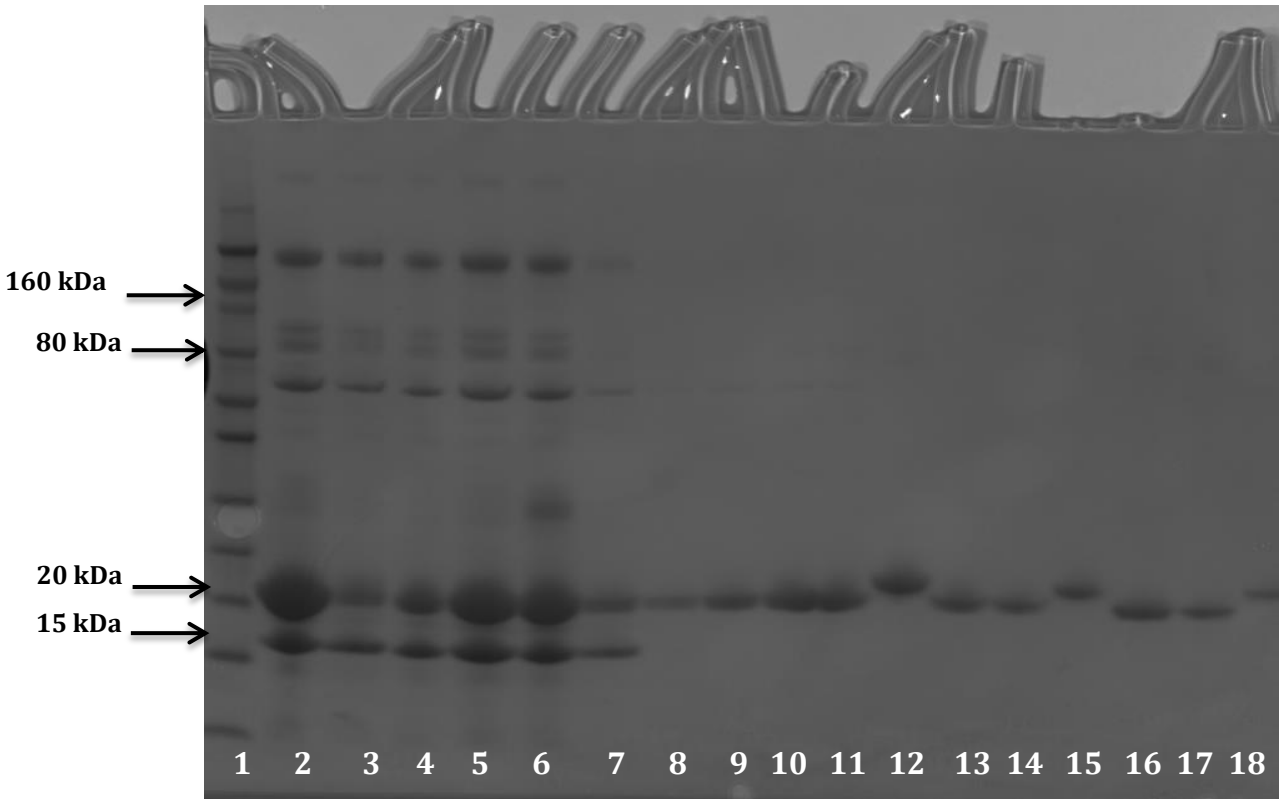

Figure 4-19 : SDS-PAGE gel image showing the protein composition of feed, flowthrough and elution fractions from chromatogram in Figure 4-16 for 4 mL whey solution

Injection – 10 mL whey sample

As expected, an increase in β-lac binding with an increased injection volume was observed as a result of injecting 10 mL of whey. The chromatogram in Figure 4-17 shows the separation of various whey proteins as a result of 10 mL whey injection. Fractions A4–A12, and B12–B7, also known as flow through fractions, consists of unbound whey proteins. The bound and unbound proteins as a result of the chromatographic process were confirmed by running the samples through a gel electrophoresis (Figure 4-20 and 4-21).

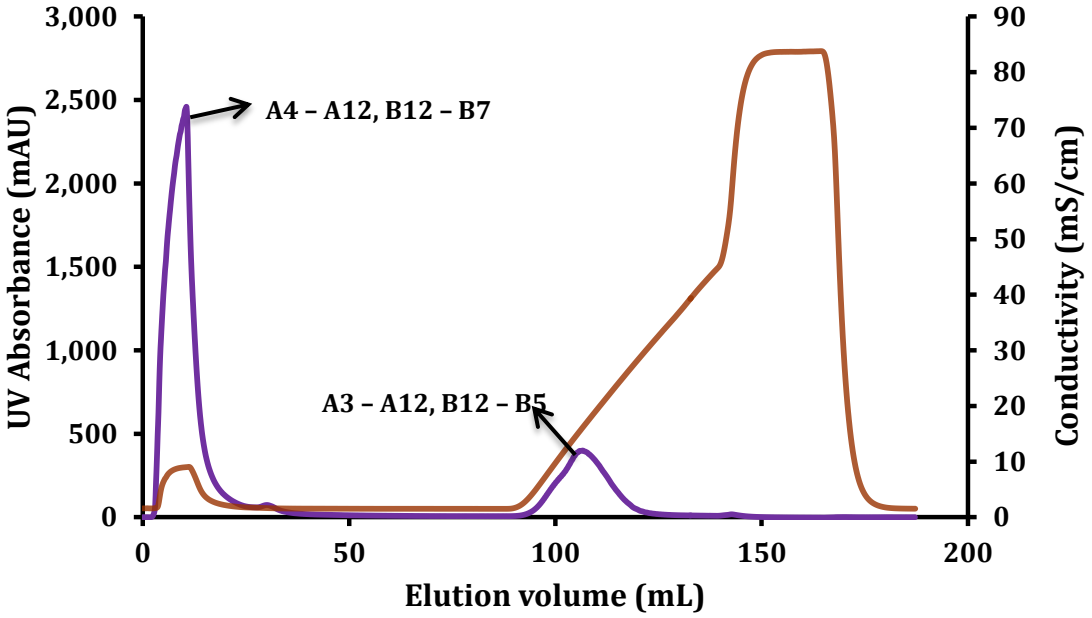

Figure 4-20: Chromatogram for 10 mL whey solution volume injected at 1 mL/min – pH 6.8

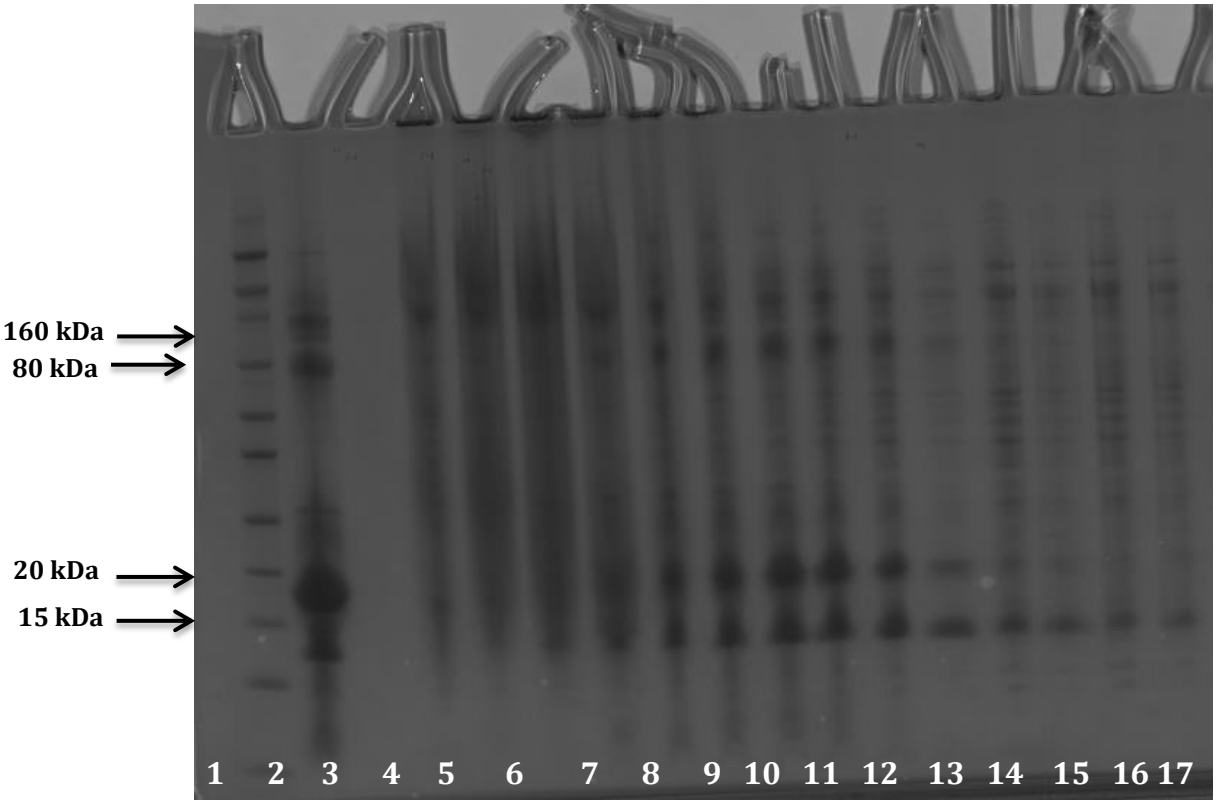

Figure 4- 21: SDS-PAGE gel image, showing the protein composition of flowthrough fractions from chromatogram in Figure 4-19 for 10 mL whey solution

Figure 4-20 shows bands of whey proteins that passed through the column without binding. The presence of bands throughout the lanes was explained by the number of proteins present in milk. Milk used for the experimental trials did not involve any pre-treatment, which meant there was no dilution before entering the chromatography column.

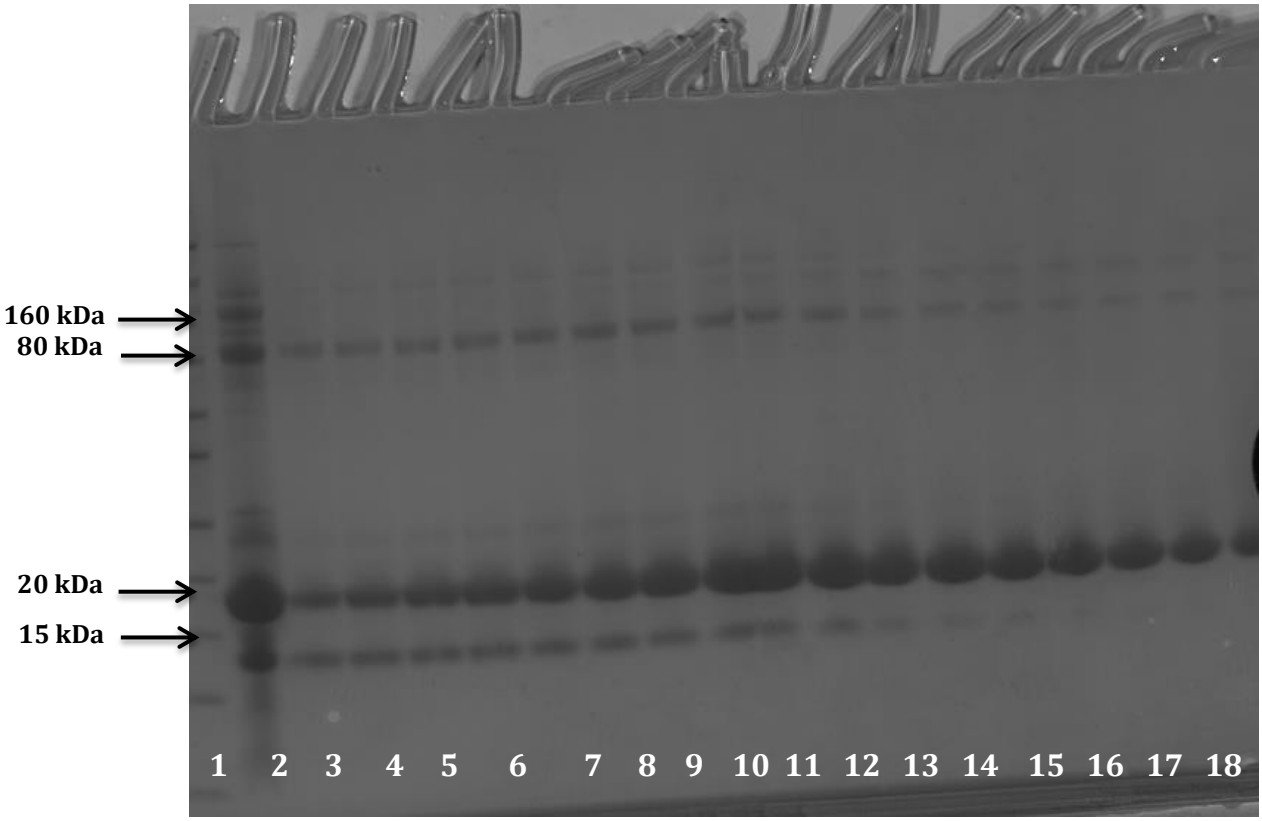

Figure 4-22: SDS-PAGE gel image, showing the protein composition of elution fractions from chromatogram 4-19 for 10 mL whey solution

The elution fractions were seen by gel electrophoresis to contain a mix of BSA, β-lac and α-lac (Figure 4-21). Similar to Figure 4-17, elution fractions possessed a mixture of whey proteins. Because only traces of other proteins were present, it was concluded that the tetramer peptide resin showed a strong affinity for the protein of interest (β-lac).

4.4.1.2 Injection – 1 mL pasteurised milk

To obtain separation of β-lac from milk, the first trial involved an injection of 1 mL pasteurised milk. Figure 4-18 clearly shows the separation of a major portion of milk proteins that were observed in the flow through fractions (A1 – A3). The elution fractions (D11 – D4)

represent the proteins that were eluted at a conductivity of 15.3 mS/cm. The eluted proteins were observed to have a low absorbance and had to be concentrated to enable clarity of the bands depicting different proteins when loaded onto the gel. These eluted proteins were hence lyophilized using a freeze dryer. The concentrated samples were clearly visible on the SDS gel electrophoresis (Figure 4-23).The starting sample contained full-concentration milk without pre-treatment and the flow through fractions seemed to be highly concentrated due to the presence of the remaining milk proteins. To obtain a clear chromatogram it was suggested to load a diluted sample of milk with higher injection volume. This would give an initial confirmation of the clear separation of milk proteins, which could further be carried on for in-depth study.

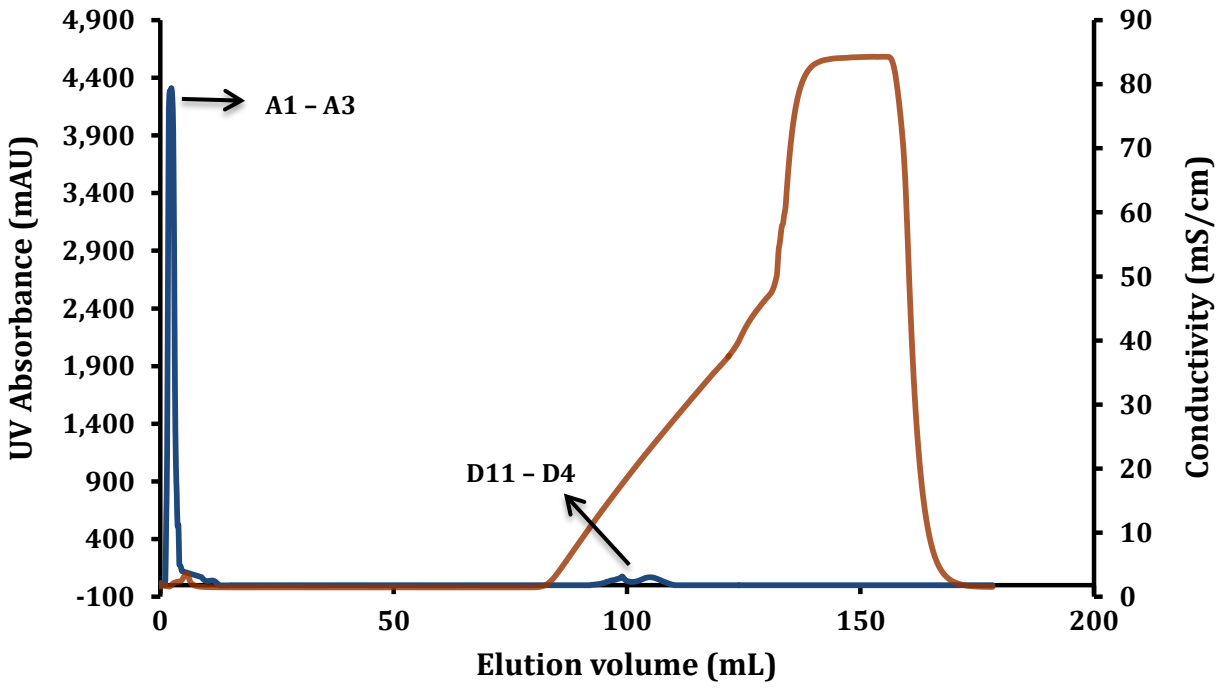

Figure 4-23 : Chromatogram for 1 mL untreated milk as injection volume at 1 mL/min – pH 6.9

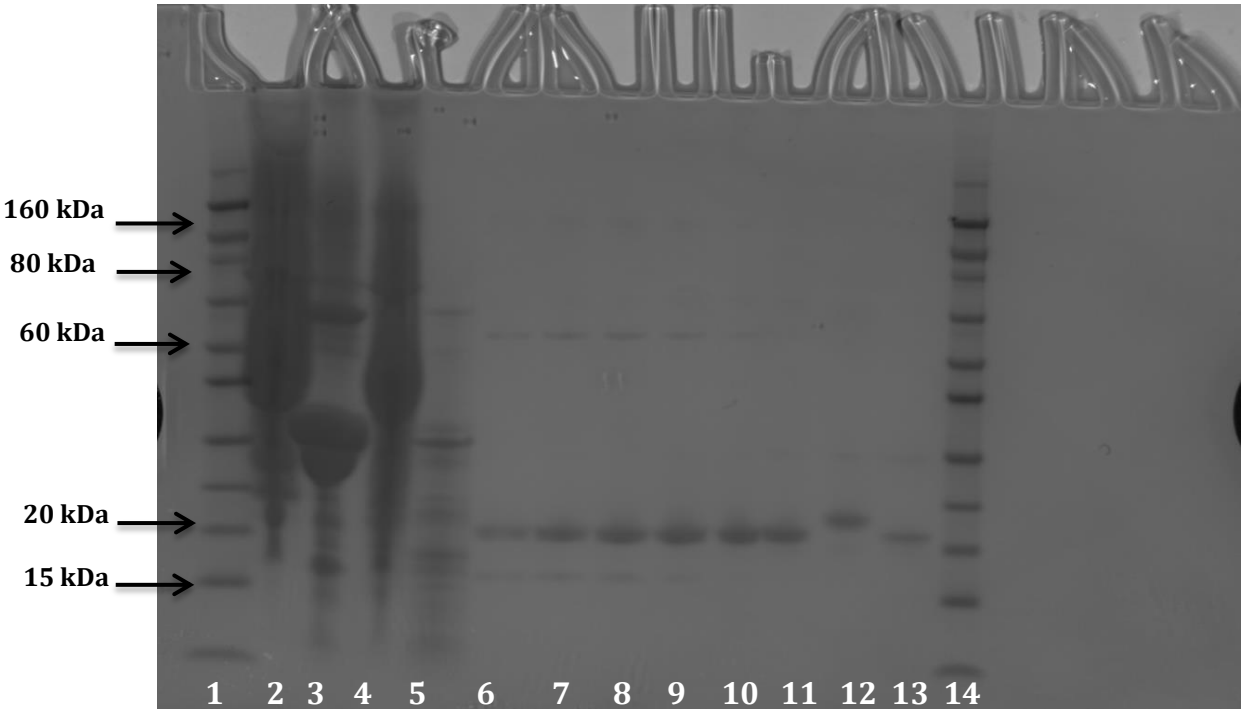

Figure 4-24: SDS-PAGE gel image showing the protein composition of feed, flowthrough and elution fractions from chromatogram as in Figure 4-22 for 1 mL milk

Further experiments were performed based on the varying ratio of tetramer peptide resin which is described in the following sections. The experiments were performed to obtain a comparison between the two different procedures followed for immobilisation of peptide to the resin involving different ratios of peptide and resin.

4.4.2 Ratio of peptide solution (10 mg/mL):resin volume~1:0.5 mL

After the above trials were successfully completed, the column was packed with freshly prepared tetramer peptide resin mixture and varying samples were injected into the column.

4.4.2.1 Whey samples~varying volume injection

On comparing injection volumes of whey solution (1 mL and 4 mL), the elution of the bound protein seemed consistent with both the cases. This was in turn proved by running a gel electrophoresis (Figure 4-25). A clear separation of bound β-lac was observed and traces of BSA were also present in the elution fractions.

A comparison was made between Figure 4-17 and 4-25 to draw a conclusion as to which

method gave a better separation of the bound protein. In Figure 4-17, β-lac eluted at a conductivity of 15.2 mS/cm with an absorbance of 126.65 mAU. Figure 4-25 showed that the bound protein eluted at a conductivity of 15.6 mS/cm and had an absorbance value of 135.65 mAU.

Though there was not much difference seen between the varying ratios used as packing material, Figure 4-25 was seen to possess a slightly higher absorbance value compared to Figure 4-17. With the same injection volume (4 mL whey), the ratio of P:R~1:0.5 mL gave a better separation of β-lac, which was supported by Figure 4-12. As expected, with a higher binding capacity better separation of β-lac was obtained.

Figure 4-25 shows a higher absorbance at 280 nm which suggests that the ratio of P:R~1:0.5 mL is preferred over P:R~0.5:1 mL. Higher binding capacity obtained (mg/g wet resin) strongly suggests that better separation of β-lac can be achieved with the immobilisation ratio of P:R~1:0.5 mL. Hence, this result supports the fact that with a higher peptide concentration during ligand immobilisation, better β-lac isolation from milk is achieved.

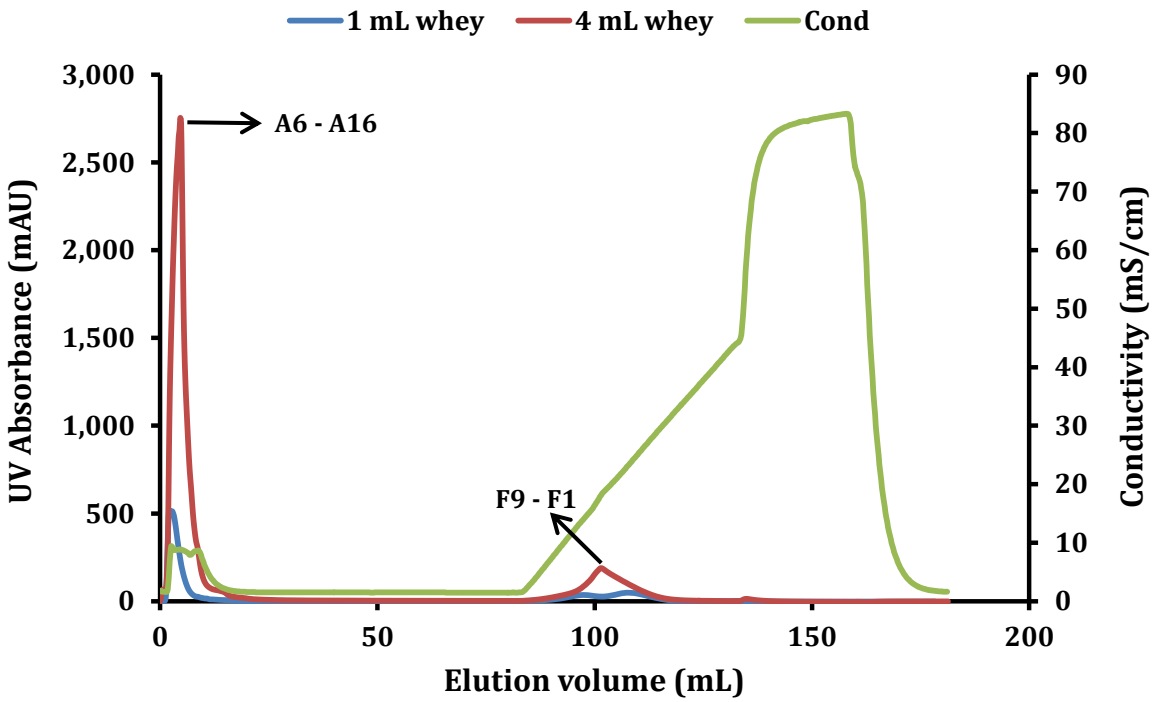

Figure 4-25: Chromatogram showing a comparison for 1 mL and 4 mL whey solutions as injection volume at 1 mL/min – pH 6.8

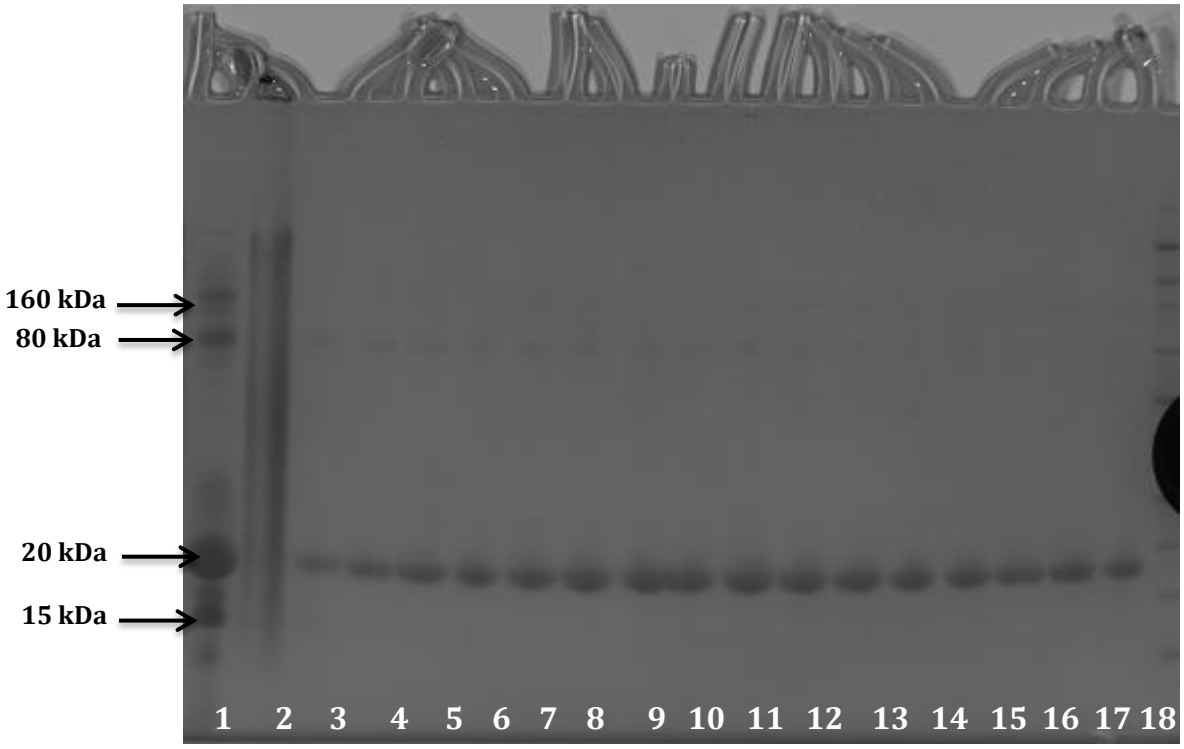

Figure 4-26: SDS-PAGE gel image showing the feed, flowthrough and elution fractions of chromatogram in Figure 4-24 for 4 mL whey solution

4.5 AMINO ACID SEQUENCE COMPARISON – F_C AND β-LAC

As described in Section 3.2, the SIM-Alignment Tool was used to compare the amino acid sequence of F_C portion of IgG and β-lac. As shown in Figure 4-26, 100% identity in five residues - RTPEV (red), 75% identity in four residues – PTPE and PAPE (yellow) and 75% identity in four other residues - PAVF and PSVF (blue) overlap was observed. It was expected that the identified regions showed specificity towards β-lac and was responsible for successful binding of β-lac to the tetramer peptide resin. This needs to be further investigated to confirm if the identified regions were the actual sites causing binding of β-lac to the tetramer peptide resin.

β-lac:

MKCLLLALAL TCGAQALIVT QTMKGLDIQK VAGTWYSLAM AASDISLLDA
QSAPLRVYVE ELKPTPEGDL EILLQKWENG ECAQKKIIAE KTKIPAVFKI
DALNENKVLV LDTDYKKYLL FCMENSAEPE QSLACQCLVR TPEVDDEALE
KFDKALKALP MHIRLSFNPT QLEEQCHI

F_C portion of IgG:

TCPPCPAPEL LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV DVSHEDPEVK
FNWYVDGVEV HNAKRKPREE QYNSTYRVVS VLTVLHQDWL NGLEYKCKVS
NKALPAPIEL TISKAKGQPR EPQVYTLPPS REEMTLNQVS LTCLVKGFYP
SDIAVEWESN GQPENNYKTT PPVLDSDGSF FLYSKLTVDK SRWQQGNVFS
CSVMHEALHN HYTQKSLSLS PGK

Figure 4-27: Amino acid sequences of bovine β-lac sequence and the F_C region of IgG, showing a matching set of amino acids RTPEV (red), PTPE and PAPE (yellow) and PAVF and PSVF (blue) in each

CHAPTER 5 - CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

This thesis describes an attempt to characterize an affinity ligand for the isolation of β-lac from bovine milk. The overall findings of this study are briefly described below.

5.1.1 Tetramer peptide resin immobilisation

PAM was successfully immobilized to NHS-activated Sepharose 4 Fast Flow resin via amine coupling. Immobilisation was confirmed by observing a gradual decrease in peptide concentration over time when incubating a PAM solution with the activated resin. In particular, by the end of immobilisation, there was no absorbance observed at 215 nm, which indicated complete uptake of the peptide by the resin. The sample collected after 1 h of immobilisation process when passed through the chromatography column, was observed to have a higher absorbance at 280 nm than 215 nm. Succinic acid, being the by-product of the immobilisation reaction, was observed on completion of the immobilisation process. This was confirmed by comparing the absorbance values, of which 280 nm was higher, as expected.

Investigations were carried out involving two different P:R ratios and succinic acid was clearly visible in both cases from the chromatograms obtained. As expected, the ratio of P:R containing a higher peptide concentration was observed to release a higher amount of succinic-acid, thereby indicating the saturation of the reaction sites present on the resin.

5.1.2 Adsorption/Batch experiments

On completion of immobilisation, it was necessary to determine the equilibrium/static binding capacity of the resin. To achieve this, adsorption/batch experiments were performed, as a result of which it was observed that the uptake of β-lac by the tetramer peptide resin depended on the concentrations of pure β-lac solution. Varying time intervals were chosen to identify the entire uptake of β-lac present in the solution by the tetramer peptide resin. Trials were performed to determine the consistency and in all cases 1 h was observed to be sufficient after which no further immobilisation occurred.

Results confirmed that increasing the peptide concentration during immobilization led to more uptake of the protein from the solution during subsequent binding and hence led to a higher binding capacity. As expected, the maximum binding capacity, *Q*, of the slurry possessing a higher peptide concentration was observed to be 54.11 mg/g wet resin and the slurry containing a lower peptide concentration had a lower binding capacity of 39.01 mg/g wet resin.

Following the determination of *Q*, an experiment was performed using the concentration of PAM peptide, obtained as a result of the calibration curve. The slurry hence prepared contained peptide concentration three times higher than the previous trials. As expected, there was a three fold increase in *Q* observed. Hence, increasing the peptide concentration resulted in higher *Q* (134.49 mg/g dry resin).

5.1.3 Elution Experiments

Once the binding capacity of the resin was determined, elution of bound β-lac was investigated. Varying the salt strength in presence of citric acid and simultaneous variations in the pH of the solution were tested in an attempt to obtain a high recovery of the bound protein so as to utilize the resultant product in further applications. The recovery of β-lac varied between 59%-62% (for varying pH), but with a change in pH, only 35% of bound protein was seen to elute. Hence, a change in pH did not cause the entire protein to elute off the resin. Because of time constraints, further experiments were not performed to obtain a higher recovery of bound β-lac.

5.1.4 Peptide affinity separation of β-lac from whey solution and milk

Using tetramer peptide slurry, a 5 mL column was packed and whey/milk samples were injected to obtain clear separation of the protein. The results showed a clear separation of β-lac from whey and milk samples, confirmed using gel electrophoresis (Section 4.4).

Investigations carried out were consistent with the tetramer peptide resin behaving as an affinity matrix rather than an ion exchanger. This could be confirmed by performing repetitive runs with varying injection volumes of milk to obtain consistency. The tetramer peptide resin was used in this research only at small scale but should be tested for use in industrial separation of β-lac from milk.

5.1.5 Applications

The tetramer peptide ligand characterized in this work has potential advantages over conventional chromatography methods for selective isolation of β-lac from whey/milk. Although this ligand has been used previously for selective isolation of Igs from human serum and cell culture broths, the use of this peptide for removal of β-lac from dairy streams has not been reported previously.

The buffers used in the isolation process were very mild (neutral pH and with a conductivity of less than 8 mS/cm). Because elution of β-lac can also be achieved using a very mild salt strength (15 mS/cm), the purified milk has applications in the manufacture of infant formulae. β-lactoglobulin can also be concentrated after separation from milk using a lyophilizer and then used as an additive in confectionary and protein supplement industries or as a gelling agent or binder for food.

5.2 RECOMMENDATIONS FOR FUTURE WORK

In this study, a few conclusions were drawn based on the experiments performed but because of time constraints a few experiments must be repeated to improve confidence in the results. Some of the recommendations for future work are as follows:

- Trials were performed by increasing the peptide concentration during ligand immobilisation, which gave a higher binding capacity value but repetitive trials are needed to confirm this. For example, doubling the peptide concentration could be attempted to help compare it with the result obtained.
- A Langmuir isotherm was used in all cases to fit the data points but checking the binding capacity of the resin by trying a few more concentrations of pure β-lac would be helpful to compare whether Langmuir or the Freundlich isotherm was the best fit.
- Having identified that the presence of NaCl (in 0.1 M citric acid) gives 60% recovery of the bound protein, trials could be performed to increase the recovery percentage. Addition of 0.2 M acetic acid as an eluting agent may be worthwhile to try, because this has been previously used for recovering IgG and a high recovery percentage was attained.
- Investigation in this research indicated that the PAM peptide behaves as an affinity matrix, because it showed affinity towards β-lac in particular, with only minor

traces of α-lac present in the eluted product. Increasing the injection volume may possibly help eliminate α-lac binding and this should be investigated.

- The PAM peptide structure is quite complex and would be expensive to produce. Therefore, trials with various PAM peptide structural analogues, in particular simplified structures, preferably with a linear structure, can also be performed to attempt affinity isolation of β-lac from milk.
- Further investigation needs to be carried out to confirm if the identified region on the amino acid sequence of β-lac was the actual cause for binding to occur, due to its similarity to the F_C portion of IgG.

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APPENDICES

APPENDIX A: β-LAC UV ABSORBANCE CALIBRATION CURVE

Initial experiments were performed to obtain the extinction coefficient of β-lac. Trials were performed at varying concentrations of pure β-lac solutions. The absorbance at 280 nm was measured using the Nanodrop Spectrophotometer 1000 and the extinction coefficient was determined.

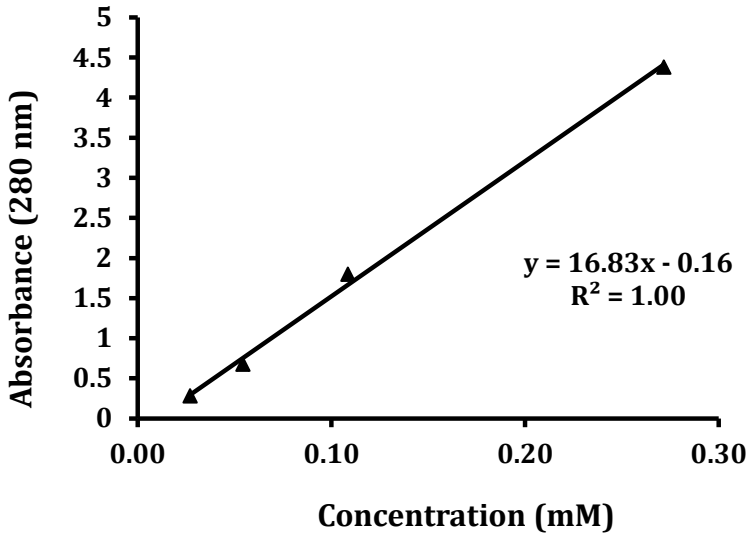

Figure A-1: Determination of extinction coefficient of β-lac

A calibration curve was plotted using absorbance at 280 nm for known concentrations of pure β-lac solutions (Figure A-2). This calibration curve was used to determine unknown concentrations of β-lac in experiments (Section 3.3.1).

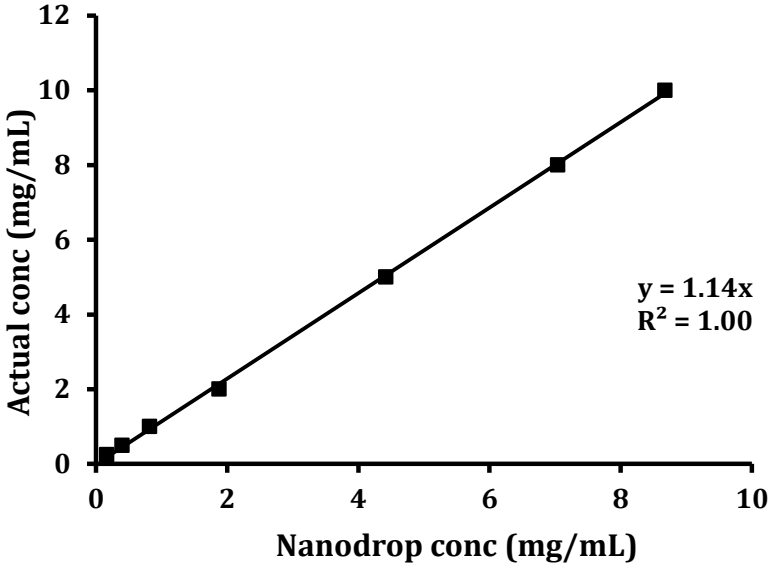

Figure A-2: Calibration curve of β-lac to determine unknown concentrations

APPENDIX B: PAM UV ABSORBANCE CALIBRATION CURVE

To maintain consistency with varying concentrations of PAM peptide, the calibration curve (Figure A-4) was used. Using the calibration curves unknown concentrations of PAM peptide were determined and Langmuir isotherm was hence plot (Figure 4-9).

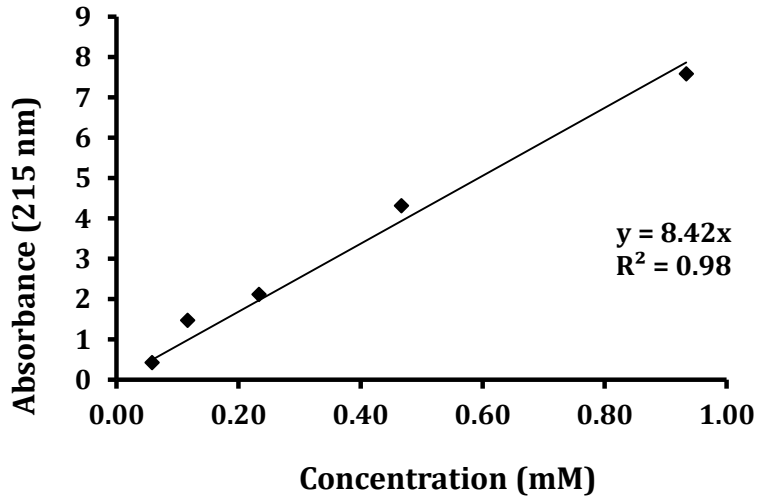

Figure B-1: Determination of extinction coefficient – PAM

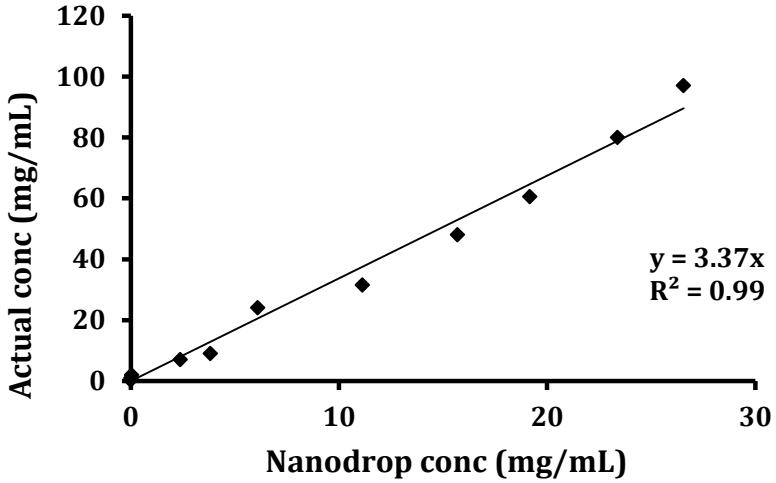

Figure B-2 : Calibration curve of PAM to determine unknown concentrations