MIXED MATRIX MEMBRANE CHROMATOGRAPHY BASED ON LEWATIT MP500 ANION EXCHANGER RESIN AND LEWATIT CNP105 CATION EXCHANGER RESIN FOR WHEY PROTEIN FRACTIONATION

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ABSTRACT

The concept of mixed matrix membrane (MMM) chromatography is an alternative that provides a simple method for the preparation of adsorptive membranes, yet results in high quality membrane chromatography performance. MMMs are prepared by incorporating an ion exchange resin (or any adsorptive resin) into a membrane polymer solution prior to membrane casting. In this study, MMM will be prepared by combining cation and anion simultaneously in EVAL based polymer solution. The membrane will be used for whey protein fractionation to recover both acidic and basic protein. Whey is the liquid byproduct of casein precipitation of milk in the cheese or casein manufacturing industries and of milk concentration prior to milk powder production (Saufi, 2010).

Rather than using the high cost ion exchange resin that purposely design for protein fractionation, this study will using a low cost ion exchanger resin that commonly used at the industry. The 7.5 wt% anion resin (MP500) will be bland with 7.5 wt% cation resin (CNP105) in the 15wt% EVAL based polymer solution. This mixed mode interaction MMM will applied for whey protein fractionation. The feasibility of MMM was tested in whey protein fractionation processes. WPI solutions were diluted to 2mg/mL with different pH binding buffer solution (pH 4, 5, 6, 7, and 8). The whey protein compositions after binding were assayed using UPLC column ACQUITY UPLC PrST using a C4 Jupiter column. A combination of pH and salt elution using different pH elution buffers will be performed to selectively elute the bound protein on the membrane. This membrane had a maximum static binding capacity of 0.260 mg/mL β-Lac, 0.214 mg/mL BSA, and 0.118 mg/mL α-Lac per membrane at pH 5. The membrane was successfully applied to bind acidic and basic proteins simultaneously from a mixture. Based on batch elution experimental results, a productivity of 0.156 mg whey protein mL^{-1} membrane h^{-1} was calculated using this customized mixed mode MMM.

ABSTRAK

Konsep campuran matriks membran (MMM) kromatografi adalah alternatif yang menyediakan kaedah yang mudah bagi penyediaan membran serapan, namun mampu memberi prestasi yang berkualiti tinggi kromatografi membran. MMMs disediakan dengan menggabungkan pertukaran resin ion (atau mana-mana resin serapan) ke dalam larutan polimer membran sebelum membran dihasilkan.Dalam kajian ini, MMM akan disediakan dengan menggabungkan kation dan anion pada masa yang sama dalam larutan polimer berasaskan EVAL. Membran akan digunakan untuk protein whey fraksinasi untuk mendapatkan kedua-dua protein berasid dan asas. Whey adalah hasil sampingan cecair kasein dalam susu dalam industri pembuatan keju atau kasein dan konsentrasi susu sebelum pengeluaran susu serbuk (Saufi, 2010).

Daripada menggunakan kos ion pertukaran resin yang tinggi yang sengaja mereka bentuk untuk protein fraksinasi, kajian ini akan menggunakan ion kos penukar resin rendah yang biasa digunakan di industri. 7.5% berat anion resin (MP500) akan dicampur dengan 7.5% berat kation resin (CNP105) dalam larutan polimer berasaskan 15 wt% EVAL. Mod campuran interaksi MMM ini akan digunakan untuk protein whey fraksinai. Kesesusaian MMM akan diuji dalam proses whey proteinfraksinai. Larutan WPI akan dicairkan kepada 2mg/mL dengan menggunakan larutan berbeza mengikut pH (pH 4, 5, 6, 7, dan 8). Komposisi protein whey selepas proses ikatan telah dicerakin menggunakan kolum UPLC ACQUITY UPLC PrST yang menggunakan C4 Jupiter. Gabungan pH dan garam digunakan dengan larutan pH yang berbeza untuk menceraikan protein yang terikat pada membran.Membran ini mempunyai kapasiti mengikat statik maksimum 0,260 mg / mL β-Lac, 0.214 mg / mL BSA, dan 0.118 mg / mL α-Lac setiap membran pada pH 5. Membran telah berjaya digunakan untuk mengikat protein berasid dan asas pada masa yang sama dari campuran. Berdasarkan keputusan eksperimen kelompok elution, produktiviti sebanyak 0.156 mg whey protein mL-1 membran h-1 yang dikira menggunakan mod campuran ini disesuaikan MMM.

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LIST OF ABBREVIATIONS

Symbols

WR	weight of resin, (g)
WP	weight of polymer, (g)

Abbreviations

BSA	bovine serum albumin
CEW	chicken egg white
DI	deionized
DMSO	dimethysulfoxide
EVAL	ethylene vinyl alcohol
Hb	hemoglobulin
HCl	hydrochloric acid
HIgG	human immunoglobulin
IgG	immunoglobulin G
Igs	immunoglobulins
LF	lactoferrin
LMH	L m^{-2} h^{-1}
LP	lactoperoxidase
LZY	lysozyme
MMM	mixed matrix membrane
MW	molecular weight
NaCl	sodium chloride
NaOH	sodium hydoxide
Na_2SO_4	sodium sulphate
$(NH_4)_2SO_4$	ammonium sulphate
RPC	reverse phase chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscope
SP	sulfopropyl
TFA	trifluoroacetic acid
WPC	whey protein concentrate
WPI	whey protein isolate
α-Lac	α-lactalbumin
β-Lac	β-lactoglobulin

1 INTRODUCTION

1.1 Background

An operation to recover and purify protein products from various biological feed streams is called protein bio-separation. In the pharmaceutical, biotechnological and food industry it is an important unit operation. The cost of protein purification is the main concerns in the production of bio-product as it contributes major cost which is about 50%-90% of total cost production. A bioreactor product mostly contains a lot of impurities that need to be removing to get the final desired bio-product.

The physical and/or chemical properties of the individual protein must be utilized to be able to isolate a specific protein from a crude mixture. There is no single or simple way to purify all kinds of proteins. Procedures and conditions used in the purification process of one protein may impact in the inactivation of another. When choosing purification method, the final goal also has to be considered. The purity required depends on the purpose for which the protein is required.

In the early days of protein chemistry, the only practical way to separate different types of proteins was by taking advantage of their relative solubility. Part of a mixture was caused to precipitate through alteration of some properties of the solvent e.g. addition of salts, organic solvents or polymers, or varying the pH or temperature. Fractional precipitation is still frequently used for separation of gross impurities, membrane proteins and nucleic acids. Under certain conditions, proteins adsorb to a variety of solid phases, preferably in a selective manner. Calcium phosphate gels have frequently been used to specifically adsorb proteins from heterogeneous mixtures. The adsorption principle is further explored in column chromatography. Due to their high resolving power, different chromatography techniques have become dominant for protein purification.

Biological molecules can be separated from one another by exploiting differences in their size, charge, or affinity for a particular ligand or solid support. Chromatography is the laboratory technique that allows separation of molecules based on their differential

migration through a porous medium. Although there are many different types of chromatography, the principle behind the separation of the molecules is the same: a mixture of compounds will have different affinities for the stationary phase (solid support or matrix) on which it is adsorbed and the mobile phase (buffer or solvent) passing through the stationary phase.

Recently, most chromatography systems use packed bed columns to contain the stationary phase or matrix. However few limitations have been identified within this system. The limitations of packed bed column chromatography, such as high pressure drop, long processing times due to slow intraparticle diffusion and complicated scale up procedures (Ghosh 2002; Kawai et al. 2003; Klein 2000; Van Reis and Zydney 2001; Zou et al. 2001), have encouraged the search for new kinds of column material and configuration.

Membrane chromatography, which uses adsorptive membranes, is one of the alternatives that can overcome some of the limitations of packed bed column chromatography. Adsorptive membranes which have a specific functionality or ligand will bind to the target protein with different adsorption mechanisms depending on the type of ligand. Membrane chromatography systems have shown great advantages such as (Ghosh 2002; Kawai et al. 2003; Wickramasinghe et al. 2006; Zou et al. 2001):

a) Low pressure drop across the column
b) Convective mass transfer properties of solutes to the binding sites
c) High flow rate operation with maintain performance
d) Easy column packing with low column clogging tendency
e) Uncomplicated scale up procedures
f) Low cost and the ability to be used as a single disposable device, especially for pharmaceutical or food applications which require very stringent cleaning protocols

Normally, preparation of adsorptive membranes involves complex and harsh chemical modifications. The concept of mixed matrix membrane (MMM) chromatography, developed by Wessling's group (Avramescu et al. 2003), is an alternative that provides a

simple method for the preparation of adsorptive membranes, yet, results in a high quality membrane chromatography performance. Mixed matrix membranes (MMMs) only need the physical incorporation of an ion exchange resin (or any adsorptive resin) into the membrane polymer solution prior to membrane casting. The chemical modifications necessary for incorporating adsorptive ligands are thus decoupled from the membrane preparation step. The availability of various low cost resins suitable for protein adsorption can make this technique cost effective.

In previous study, Saufi and Fee (2011) had prepared a single matrix that incorporated simultaneous cation and anion exchange functionalities. A mixed mode MMM (M5) was synthesized by incorporating SP Sepharose cation resin and Lewatit MP500 anion resin into an EVAL base polymer solution at a ratio based on the relative levels of acidic and basic proteins present in whey. However, the SP Sepharose cation resin used is costly. In the current study, an alternative cation resin which is Lewatit CNP105 was used for whey protein fractionation. The MMM was bind to whey protein solution at different pH and eluted with different elution solution.

1.2 Objectives

The main objective of this research is to produce, characterize and performance evaluation of mixed mode interaction MMM chromatography of Lewatit MP500 anion exchanger resin and Lewatit CNP105 cation exchanger resin for whey protein fractionation.

1) Anion exchange MMM chromatography is developed by incorporating a several potential anion exchanger resins, Lewatit MP500 into ethylene vinyl alcohol (EVAL) base membrane. The anionic MMM is expected to bind acidic whey proteins such as β-Lactoglobulin, α-Lactalbumin, Bovine serum albumin and Immunoglobulins.

2) A cation exchange MMM is developed using CNP105 resin to recover lactoferrin and lactoperoxidase from whey protein. The performance of the membrane is

evaluated and the feasibility of using batch fractionation in the separation process is studied.

3) Using the MMM concept, we are able to capture the positively and negatively whey proteins in one run. However, further elution strategy study is needed to elute the protein of interest that bound on mixed mode interaction MMM. Elution protocol of a combination of pH change and salt elution is also investigated to explore the possibility of eluting bound proteins individually from 1.5mL whey protein injected into the M5 (Mixed Mode Mixed Matrix Membrane).

1.3 Scope of research

In order to fulfill the research objective, the following scopes has been outlined.

i) To study the whey protein binding to mixed mode interaction MMM at different pH from pH 4 to pH 8.

ii) To prepare and determine the static binding capacity of mixed mode interaction MMM.

iii) To perform the elution study using different elution buffers in fractionation of whey protein component using mixed mode interaction MMM chromatography.

2 LITERATURE REVIEW

2.1 Whey protein

Whey is a liquid by-product of casein precipitation of milk in the cheese or casein manufacture in the dairy industry and of milk concentration prior to milk powder production. After the casein curd separates from the milk, following coagulation of the casein proteins through the action of chymosin (rennet) or mineral/organic acid, the remaining watery and thin liquid is called whey (Zadow, 1994) as shown in Figure 2.1. Its colour depends on the quality and type of milk used. It has a yellow/green colour, or sometimes even a bluish tinge. Whey can be made from any type of milk mostly ruminant's milk, with cow's milk being the most popular in western countries, while in some regions of the world, goat's, sheep's, and even camel's milk can be used in the manufacture of dairy products that result in the generation of whey. Whey protein comprises ~50% β-Lactoglobulin, ~20% α-Lactalbumin, ~15% glycomacropeptide (in renneted whey only), and ~15% minor protein/peptide components (e.g., immunoglobulins, lactoferrin, lactoperoxidase, serum albumin, lysozyme, and growth factors).

Table 2.1: Comparison of the proximate analysis of bovine milk and whey (Zadow, 1994; Smithers et al., 1996).

Component	Content (%, w/v)	
	Milk	Whey
Casein protein	2.8	< 0.1
Whey protein	0.7	0.7
Fat	3.7	0.1
Ash	0.7	0.5
Lactose	4.9	4.9
Total solids	12.8	6.3

Whey once considered a waste product. The disposal of whey causes severe environmental pollution problems as it having a biological oxygen demand (BOD) value of about 35- 60 g L^{-1} and a chemical oxygen demand (COD) value of 80-100 g L^{-1} as sewage (Bhattacharjee et al., 2006). Thus in most countries, the government and other regulatory authorities have restricted or banned the disposal of untreated whey (Smithers 2008). This legislative

restriction on whey disposal encouraged a deeper exploration of whey protein-based ingredients. Finally, whey has become a new source of functional ingredients when stricter environmental laws were approved and more attention was given to its benefits (Marshall, K., 2004). Now, whey is generally considered as a functional food, which has measurable effects on health outcomes, and the bioactive properties of whey proteins and whey protein fractions are becoming increasingly recognized (Kruger et al., 2005; Marshall, 2004; Michaelidou & Steijns, 2006; Toba et al., 2000). Enormous quantities of whey are produced annually worldwide. At 130 million tons is estimated for the world production of cheese whey per year, accounting for around 780,000 tons of protein (Monteiro et al., 2008).

Whey proteins consist to about 18–20% of the total milk proteins and its major components are β-Lactoglobulin, α-Lactalbumin, bovine serum albumin and immunoglobulin, representing, 50%, 20%, 10% and 10% of the whey fraction, respectively (Jovanovic et al., 2007). Whey proteins also consist of numerous minor proteins, such as lactoferrin, lactoperoxidase, proteose peptone, osteopontin, and lizozyme (Hahn et al., 1998). The detailed whey protein profile, including general chemical and physiochemical properties, is depicted in Table 2.2. Relying on the quality of the milk, 10 kg of milk will produce only 1-2 kg of cheese, whereas the rest of it will emerge as liquid whey (Bhattacharjee et al., 2006). Figure 2-2 shows the main components of bovine milk, where whey protein only represents about 20% of the total protein in milk.

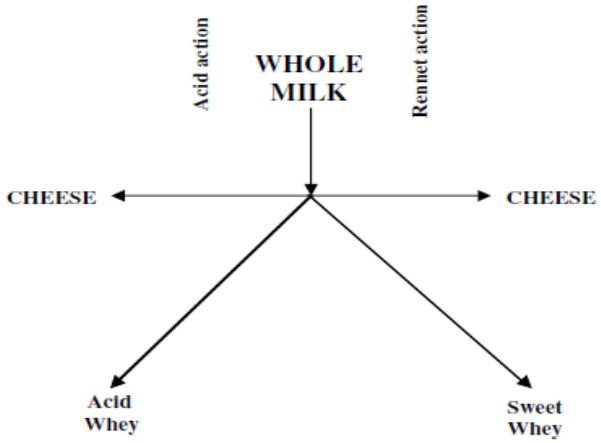

Figure 2.1: Schematic diagram of acid- and rennet-mediated cheese making.

Table 2.2: Protein profiles of whey and primary structure basic properties (de Wit, 1998; Eigel et al., 1984; Brew et al., 1970; Korhonen, 1995; Andersson and Mattiasson, 2006).

Protein	Concentration (g/l)	Molecular weight (Da)	Number of amino acids residues	Isoelectric point (pI)
β-Lactoglobulin	1.3	18, 277	162	5.2
α-Lactalbumin	1.2	14, 175	123	4.5-4.8
Bovine serum albumin	0.4	66, 267	582	4.7-4.9
Immunoglobulins (A, M and C)	0.7	25,000 (light chain) + 50, 000-70,000 (heavy chain)	-	5.5-8.3
Lactoferrin	0.1	80, 000	700	8-9.5
Lactoperoxidase	0.03	70, 000	612	9.5
Glycomacropetide	1.2	6700	64	-

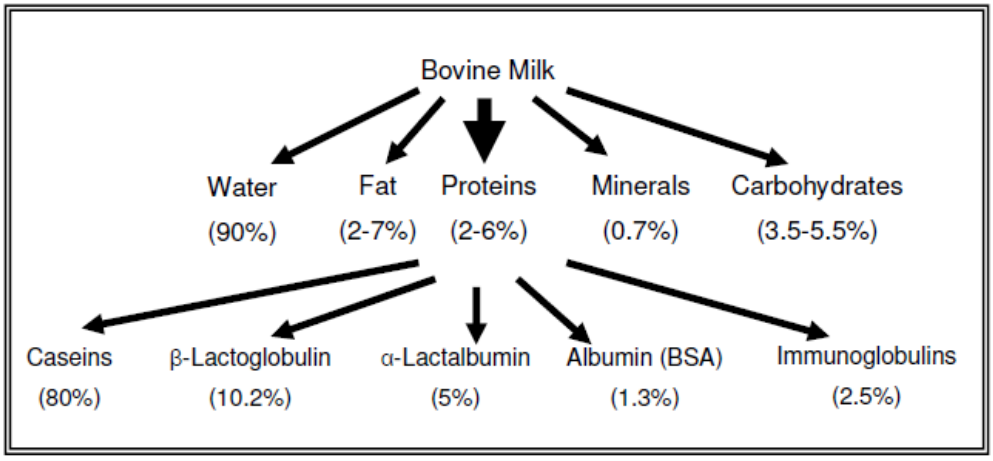

Figure 2.2: Main components of bovine milk and composition of bovine protein (Splitt et al. 1996).

Normally, whey protein products are available in three major forms: concentrates (WPC), isolates (WPI) and hydrolysates (WPH) (Huffman and Harper, 1999). Whey proteins can be converted into whey protein concentrate (WPC) (35-80% protein) or whey protein isolate (WPI) (80-95% protein) for bulk use (Brans et al., 2004). WPC is a mixture of all whey proteins obtained by membrane concentration of whey, retaining significant amounts of the minerals, lipids, and lactose from whey, WPI is a higher quality and higher value mixture of

the whey proteins, manufactured using either membrane filtration or ion-exchange adsorption (Turhan and Etzel, 2004). Particularly due to its high protein content WPI is usually included in sports formulas, infant formulas or medical formulas. WPI also can be used as a source of pure proteins or can be hydrolysed to obtain valuable peptides (Lucena et al., 2007).

In the past two decades in order to earning a significant income from individual proteins from whey, the dairy industry globally has moved from being based solely on commodity food production (Fee and Chand 2006; Horton 1995; Huffman and Harper 1999). The added value of whey has increased dramatically over the past 50 years as illustrated in Figure 2.3, along with the advances in science and technology (adapted from Smithers 2008). Whey components, mainly proteins and peptides, will increasingly be preferred as ingredients for functional foods and nutraceuticals and as active medicinal agents (Smithers 2008).

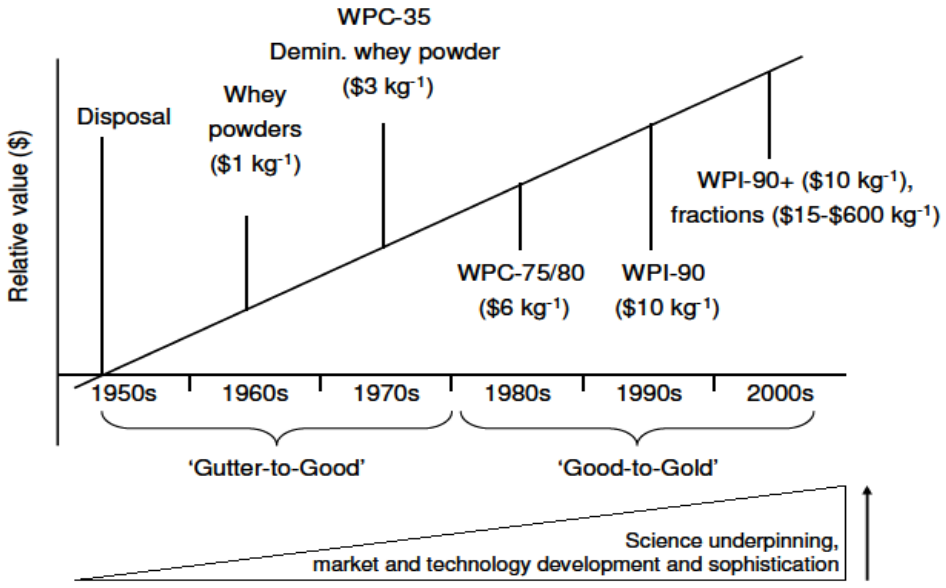

Figure 2.3: Schematic representation of the relative increase in value of whey protein/peptide products with increasing underpinning scientific knowledge of whey solids and advances in technology and marketplace sophistication over the past approximately 50 years (adapted from Smithers 2008).

Each whey protein has unique properties for nutritional, therapeutic, biological and food ingredient applications (Marshall, 2004). Nevertheless, these unique properties are largely unrealized and the acceptance of these products by the food processing industry has limited due to the lack of consistency in the gross composition and functionality, interactions between components, and degradation during processing. Moreover, the presence of lipid and protein impurities can caused WPCs develop a stale off-flavor (Zydney, 1998). Thus, there is a challenge in whey protein fractionation to produce individual whey proteins with well characterized functional and biological properties by a process which will not denature but retain its nutritional and other properties (Bhattacharjee et al., 2006).

2.1.1 Applications and biological activities of whey protein components

The main applications and biological activities of individual major whey proteins are summarized in Table 2.3.

Table 2.3: Main applications and biological activities of individual major whey proteins.

Protein	Biological function	Refrerences
Whole whey proteins	i. Prevention of cancer; • Breast and intestinal cancer • Chemically induced cancer ii. Increment of gluthatione levels; • Increase of tumour cell vulnerability • Treatment of HIV patients iii. Antimicrobial activities iv. Increment of satiety response • Increment in plasma amino acids, cholecystokinin and glucagon-like peptide v. β-Lac free whey would also serve as the primary protein constituent of hypoallergenic infant formulas.	Gill and Cross (2000) Badger et al. (2001) MacIntosh et al. (1995) Hakkak et al. (2000) Rowlands et al. (2001) Parodi (1998) Micke et al. (2001) Micke et al. (2002) Clare et al. (2003) Hall et al. (2003) Casal et al. (2006)
β-Lactoglobulin	i. Transporter • Retinol • Palmitate • Fatty acids • Vitamin D • Cholesterol ii. Enhancement of pregrastic esterase activity	Puyol et al. (1991) Wu et al. (1999) Puyol et al. (1991) Wang et al. (1997) Perez et al. (1992) Warme et al. (1974) Farrell et al. (1987)

	iii. iv. v. vi. vii. viii. ix. x.	Transfer of passive immunity to the newborn Regulation of mammary gland phosphorus metabolism Binding and transport of retinol, vitamin D and palmitic acid Enzymic synthesis of prostaglandins Olfactation, opiodergic, cryptic coloration Anti-hypertensive, anti-cancer, hypocholesterolemic Due to its good solubility, β-Lac has a potential use in power drinks and is a good source of essential amino acids Suitable for confection production due to its good gelling formation and better foam stabilizer.	Flower, D.R. (1996) Horton (1995) Cowan and Ritchie (2007) Zydney (1998) Maduira et al. (2007)
α- Lactalbumin	i. ii. iii.	Due to its high tryptophan content, high digestibility, and lower potential for causing allergies, α-Lac is the preferred protein source for infant formulas, when compared to β-Lac. Applicable as a nutraceutical owing to its high content in tryptophan. Possesses therapeutic uses due to its high	Gurgel et al. (2000) Zydney (1998) (Konrad and Kleinschmidt (2008) de Wit (1998) Markus et al. (2002) Ganjam et al. (1997) Greene, L.H. et al. (1999)

	cytotoxicity. iv. Contraceptive agent because it has strong affinity for glycosylated receptors on the surface of oocytes and spermatozoids. v. Prevention of cancer vi. Lactose synthesis vii. Treatment of chronic stress-induced disease viii. Binding of calcium, absorption ix. Lactose synthesis x. Tumor cells apoptosis	Maduira et al (2007)
Bovine serum albumin	i. Fatty acid binding ii. Anti-mutagenic function iii. Prevention of cancer iv. Immunomodulation • Disease protection through passive immunity v. Transport, metabolism and distribution of ligands vi. Protection from free radicals vii. Contribution to osmotic pressure of blood viii. Widely used in food and therapeutic applications ix. Has a good gelling property x. Nutrient in cell and microbial culture xi. In restriction digests, BSA is used to stabilize	Maduira et al (2007) Walzem, Dillard, and German (2002) Bosselaers, I.M. et al.(1994) Laursen et al. (1990) Ormrod and Miller (1991) Mitra et al. (1995), Tomita et al. (1995), Loimaranta et al. (1999) Farrel, H.M. et al. (2004) Zydney (1998) Matsudomi et al. (1991)

	some enzymes during digestion of DNA and to prevent adhesion of the enzyme to reaction tubes and other vessels. xii. Used to determine the quantity of other proteins, by comparing an unknown quantity of protein to known amounts of BSA.	Geselowitz and Neckers, (1995).
Immunoglobulins	i. Antibacterial activity • HIV ii. Antifungal activity iii. Opioid activity iv. Immunological protection against microbial pathogens and toxins v. Protect mammary gland against infection vi. Antigen binding vii. Capable of preventing the adhesion of microbes to surfaces viii. Inhibiting bacterial metabolism by blocking enzymes Agglutinating bacteria and neutralizing toxins and viruses	Oona et al. (1997) Freedman et al. (1998) Okhuysen et al. (1998) Sharpe et al. (1994) El-Loly (2007)
Lactoferrin	i. Broad spectrum antimicrobial activity ii. Promotion of iron transfer and absorption iii. Cancer prevention	Adlerova et al. (2008) Horton (1995) Lu et al. (2007)

	iv. Cell proliferation and differentiation v. Antiviral, antibacterial and antiparasitic activity vi. Oral administration of LF exerts beneficial effects on the health of humans and animal; • Anti-infective • Anticancer • Anti-inflammatory effects vii. Commercially used as a; • Natural bioactive ingredient in supplement foods (e.g. infant formulae and dietary supplement tablets) • Skin care • Oral health care products	Tomita et al. (2009) Wakabayashi et al. (2006) Wakabayashi et al. (2006) Yamauchi et al. (2006)
Lactoperoxidase	i. Catalyzes the oxidation of thiocynate by hydrogen peroxide. ii. Generates intermediate products with antibacterial properties which kill or inhibit the growth of wide range of bacteria, viruses, fungi, molds and protozoa. iii. Preserve raw milk quality during transportation from the farmer to the dairy plant when not possible to used mechanical refrigeration.	Korhonen (2009) Seifu et al. (2005) Shakeel ur et al. (2002) Seifu et al. (2005)

2.2 Technologies for whey protein fractionation

2.2.1 Column based chromatography

In downstream processing of protein mixture, chromatography is a very well known unit operation. In chromatographic techniques, the different migration of the component of interest between the stationary phase (i.e. matrix phase) and continuous phase (i.e. solvent) in the system is the cause of the principle separation occurred. Depending on the process scale, chromatography media (i.e. stationary phase) is normally packed into a column from several centimeters to several meters diameter. Various types of chromatography mode or interaction are available, such as size exclusion, ion exchange, hydrophobic interaction and reverse phase chromatography. They differ in terms of the separation mechanism and selection of stationary and continuous phase. Table 2.4 gives a brief performance comparison between several types of chromatographic interaction that are normally used in column chromatography (Chaga, 2001; Suen et al., 2003). The advantages and disadvantages between different formats of stationary phase in chromatographic system are given in Table 2.5 (Ghosh, 2003).

Previous studies on whey fractionation using column chromatography are summarized in Table 2.6. Most of them appeared in the literature for the past 10 years.