

**Efficiency of two cation exchange methods for isolating
lactoperoxidase and lactoferrin from Gouda and
Cheddar cheese whey**

BY

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I declare that the thesis herewith submitted for the M.Sc. Food Science degree at the University of Pretoria, had not been previously submitted by me for a degree at any other university or institution of higher education.

ABSTRACT

EFFICIENCY OF TWO CATION EXCHANGE METHODS FOR ISOLATING LACTOPEROXIDASE AND LACTOFERRIN FROM GOUDA AND CHEDDAR CHEESE WHEY

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In South Africa about 2.2 million tonnes of milk are produced annually. Approximately 18% of the milk is used for cheesemaking. The yellow-green liquid that separates from the curd during manufacture of cheese is known as whey. For a long time whey has been considered by the dairy industry to be a waste product. The increased pressure of anti-pollution regulations and cost of disposal to municipal sewages have encouraged the dairy industry to reappraise waste management in general and more specifically whey. Searching for ways to dispose of whey without polluting the environment is becoming costly to the food industries. Thus finding alternative ways for management that focuses on maximizing the value of the available components in whey is becoming more important.

The main objectives of this study were to isolate and quantify lactoperoxidase and lactoferrin in concentrated Gouda cheese whey, non-concentrated Gouda cheese whey and concentrated Cheddar cheese whey by using cation exchange

chromatography and cation exchange membranes and to compare the yield from these two methods.

One-step isolation and stepwise elution (0.3 M NaCl for lactoperoxidase and 0.9 M NaCl for lactoferrin) were used so that the method can be easily transferable to large scale operation.

The results showed that the largest amounts of lactoperoxidase and lactoferrin were present in concentrated Gouda cheese whey followed by concentrated Cheddar cheese whey, and non-concentrated Gouda cheese whey containing the least. The amounts found in the non-concentrated whey corresponded with the amounts reported in the literature. Whey contains about 6% total solids of which lactoperoxidase and lactoferrin form part. It was thus expected that the two proteins would have been present in the concentrated whey (approximately 56% solids) at an approximately 9 times higher level than in the non-concentrated whey, but the increase was only about 4 times. The reason for this might be the heat treatments applied to the milk for cheesemaking and during whey concentration.

By comparing the yields from both methods statistically it was observed that the methods showed no significant differences. However, cation exchange chromatography needed larger amounts of salt solution to elute these two components as compared to the cation exchange membrane method.

The lactoferrin fractions obtained with both methods were rather pure but the lactoperoxidase fractions from both methods appeared to be contaminated with small amounts of minor whey proteins.

OPSOMMING

DIE DOELTREFFENDHEID VAN TWEE KATIOONUITRUILING METODES VIR DIE ISOLASIE VAN LAKTOPEROKSIDASE EN LAKTOFERRIEN UIT GOUDA EN CHEDDARKAASWEI

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Ongeveer 2.2 miljoen ton melk word jaarliks in Suid Afrika geproduseer. Bykans 18% van die melk word gebruik vir die vervaardiging van kaas. Die geel-groen vloeistof wat van die wrongel skei tydens die kaasmaakproses staan bekend as wei. Wei word meestal as 'n afvalprodukt van die proses geklassifiseer.

Die toenemende druk van anti-besoedelingsregulasies en die afvalverwyderingskoste van munisipale rioolstelsels het daartoe gelei dat die suiwelindustrie afvalbestuur (en spesifiek weiverwerking) weer in heroorweging geneem het. Die wegdoening van wei op so 'n wyse dat die omgewing nie besoedel word nie is finansiëel nie lonend nie. Dit word dus toenemend belangrik om 'n wyse te vind waarop waardevolle komponente in wei effektief geïsoleer en finansiëel lonend aangewend kan word.

Die doelwit van hierdie studie was om laktoperoksidase en laktoferriene in gekonsentreerde Gouda-kaaswei, ongekonsentreerde Gouda-kaaswei en on

gekonsentreerde Cheddar-kaaswei te isoleer deur middel van kationuitruilingschromatografie en kationuitruilings-membrane en om die opbrengste van die twee metodes te vergelyk.

Enkelstap isolasie en stapsgewyse eluering (0.3 M NaCl vir laktoperoksidase en 0.9 M NaCl vir laktoferrien) is gebruik sodat die metode maklik op groot skaal toegepas kan word.

Die resultate toon dat gekonsentreerde Gouda-kaaswei die meeste laktoperoksidase en laktoferrien bevat het, gevolg deur gekonsentreerde Cheddar-kaaswei en laastens, ongekonsentreerde Gouda-kaaswei. Die hoeveelheid teenwoordig in die ongekonsentreerde wei het goed vergelyk met waardes wat in die literatuur aangeteken is. Wei bevat ongeveer 6% vaste stowwe, waarvan laktoperoksidase en laktoferrien deel is. Daar is verwag dat die twee proteïene teen ongeveer 9 maal groter vlakke in die gekonsentreerde wei (56% vaste stowwe) teenwoordig sou wees, maar die verskil was slegs viervoudig. Die rede hiervoor is moontlik die hittebehandelings wat toegepas is op die melk tydens kaasbereiding, en gedurende die konsentring van die wei.

Deur die opbrengste van die twee metodes statisties met mekaar te vergelyk, is gevind dat daar geen beduidende verskil tussen die opbrengste van die twee metodes was nie. Die kationuitruilingschromatografie benodig egter groter hoeveelhede soutoplossing om die ionuitruiling te fasiliteer as wat met kationuitruilingsmembrane benodig word. Die laktoferrienfraksies verkry met beide metodes was redelik suiwer maar die laktoperoksidase-fraksies was in beide gevalle klaarblyklik besmet met klein hoeveelhede van die mindere wei proteïene.

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DEDICATION

This work is dedicated to my parents Makweba and Lucy, my brothers Josephat, Boniphace, Pamba, Charles, Benesta and Amos.

Whoever love instruction loves knowledge, but he who hates correction is stupid.

Proverbs 12:1

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GLOSSARY OF TERMS

The following alphabetical list of terms are used in this report

Affinity: the presence of sites on the molecule which can interact with other molecules in a biospecific or "lock and key" binding interaction.

Analyte: the substance to be detected by an assay or the target molecule.

Antibiotics: natural, semi-synthetic or wholly synthetic antimicrobial compounds which are effective at low concentration.

Anti-oxidants: any substances that can delay the onset or slow down the rate of oxidation of autoxidizable materials.

Arthritis: an autoimmune disease of the joints involving an attack by the body's immune system upon the synovial.

Bactericidal: able to kill at least some types of bacteria

Bacteriostatic: able to inhibit the growth and reproduction of at least some types of bacteria.

Biological oxygen demand (BOD): the method used to estimate the amount of oxidizable organic matter in waste water. The method measures the amount of dissolved oxygen consumed in a portion of water that has been aerated and to which a sample of wastewater and an inoculum of microbes have been added.

Chemical oxygen demand (COD): the method used to estimate the amount of organic matter in waste water that is readily oxidized by dichromate ($K_2Cr_2O_7$) in the acid reflux method. It is generally interpreted as organic matter.

Chemical preservatives: commercial compounds (both organic and inorganic substances) that are used to prevent or retard microbial growth.

Crude sample: the sample that has not passed through the column or membrane.

Electrophoresis: a procedure by means of which the members of a heterogeneous population of charged particles can be separated by virtue of their dissimilar migration characteristics in an electric field.

Eluent: a mobile phase used to elute molecules from a column or membrane.

Hepatitis: inflammation of the liver. Hepatitis may be non-microbial in origin, it may be caused by a virus or it may occur as a symptom of certain other infectious diseases.

Hydrophobic: water-hating character of the molecule or functional groups within the molecule.

Hyposideremia: the disease associated with iron deficiency.

Immunodeficiency: the inability to respond with a normal immune response to antigenic stimulation.

Ischaemia: the temporary reduction of blood supply by physical or chemical means to organs.

Isoelectric point: the pH at which the molecule has no net charge and hence zero electrophoretic mobility.

Murine: of or like a mouse.

Myelopoiesis: formation and development of white blood cells or formation of bone marrow.

Neutrophils: the major type of cells which are involved in the early stages of many forms of acute inflammation.

Nutraceutical: food which contain additional components that provide them with specific medical or physiological benefits beyond the traditional nutrients they contain.

Permeate: the stream of mobile phase emerging from the outlet of the column or membrane.

Polymorphonuclear: the white blood cells possessing distinct cytoplasmic granules and a multilobed nucleus.

Singlet oxygen: an energized and reactive, but uncharged, form of oxygen that can be toxic to cells. It may be produced during the formation of hydroxyl radicals by the reaction between superoxide and hydrogen peroxide.

Xenobiotic: any chemical which is present in a natural environment but which does not normally occur in nature.

LIST OF ABBREVIATIONS

- ABTS:** *2,2'-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt*
- BOD:** biological oxygen demand
- BSA:** blood serum albumin
- Cc:** Cheddar concentrate
- CEC:** cation exchange chromatography
- CEM:** cation exchange membrane
- CM:** caboxymethyl
- COD:** chemical oxygen demand
- Cr/Cc:** crude Cheddar concentrate
- Cr/Gc:** crude Gouda concentrate
- Cr/Gnc:** crude Gouda non-concentrate
- DEAE:** diethylaminoethyl
- DNA:** deoxyribonucleic acid
- Gc:** Gouda concentrate
- Gnc:** Gouda non-concentrate
- GO:** glucose oxidase
- L/W:** load-and-wash
- LF:** lactoferrin
- LP:** lactoperoxidase
- LP-s:** lactoperoxidase system
- MA:** membrane adsorber
- RNA:** ribonucleic acid
- SDS-PAGE:** sodium dodecyl sulfate-polyacrylamide gel electrophoresis
- SH:** sulphhydryl
- ST-TP:** sulphopropyl toyopearl
- TEMED:** tetramethylenediamine
- UVD:** ultra violet damage
- WPC:** whey protein concentrate
- WPI:** whey protein isolate

1 INTRODUCTION

In the manufacture of cheese or casein from milk, curds are formed by the action of rennet enzymes and /or acid. Whey is the liquid remaining after the recovery of these curds and has long been considered by the dairy industry to be a waste by-product. As the volume of whey from cheese factories continue to grow world-wide, analysis of the composition of whey revealed components of particular significance to human beings (Renner & El-salam, 1991; Smithers, Ballard, Copeland, de Silva, Dionysius, Francis, Goddard, Grieve, McIntosh, Mitchell, Pearce & Regester, 1996).

World-wide production of whey appears to be in the order of about 133 million tonnes per annum. About 94% of this quantity results from cheese-making, the residual 6% from casein manufacture (Renner & El-salam, 1991).

The increased pressure of anti-pollution regulations and the cost of disposal to municipal sewage plants, along with the consolidation of dairy processing into large units, have made it impractical to continue to dispose of large quantities of whey by the traditional methods. The disposal of whey results in a loss of a potential food source as well as an environmental and economic burden. As researchers look to produce new products from whey as a result of recent innovative processing technologies, people realize that whey is no longer a waste by-product (Renner & El-salam, 1991; Zall, 1992). About 700 000 tonnes per annum of true whey proteins are available world-wide as an ingredient for many food and dairy products (Smithers *et al.*, 1996; de Wit, 1998). Its value as a resource for many medically active components has been recognized in Japan and European countries (Horton, 1995) but not in Africa.

Individual whey components of significance include α -lactalbumin, β -lactoglobulin, blood serum albumin, immunoglobulins, lactoferrin (LF) and lactoperoxidase (LP). Other minor milk components of biological significance

include milk salts, water-soluble vitamins and components related to casein (Renner & El-salam, 1991; Horton, 1995). Many of these components exhibit biological activity that is valuable in nutraceuticals or antimicrobials (Horton, 1995; Smithers *et al.*, 1996; Stine, 1997; Parodi, 1998).

1.1 PROBLEM STATEMENT

In South Africa about 2.2 million tonnes of milk are produced annually. Approximately 18% of the milk is used for making cheese, which produces an estimated 39 600 tonnes of cheese. The major cheese produced is Cheddar and Gouda, which account for 48% and 43% of cheese, respectively. Only 9% exotic cheese are manufactured. Morr (1992) has reported that approximately 8.7 kg of whey is produced per 1 kg of cheese manufactured, this means about 344 520 tonnes of whey are produced annually as waste product in South Africa.

By-products from the manufacture of cheese are extremely potent pollutants containing about 35 000 mg/litre BOD (biological oxygen demand) and 68 000 mg/litre COD (chemical oxygen demand) (Knipschildt, 1986; Renner & El-salam, 1991; Morr, 1992). Thus, management of this waste stream has, mainly, involved implementation of the most economical alternative way for disposal. Such management has included simple discharge into waterways, into the ocean or incorporation into low cost animal feed. The advent of strict, environmental regulations worldwide has encouraged the dairy industry to reappraise waste management in general and whey disposal more specifically. Searching ways for whey disposal without polluting the environment is becoming more costly to the food industries. Thus, finding alternative ways for whey management that focuses on maximizing the value of the available components in whey is becoming more important.

Isolation of lactoperoxidase and lactoferrin from whey : Literature review

1.2 Objectives

1. The primary objective of this study was to compare two ionic exchange techniques (sulphopropyl cation exchange chromatography and cation exchange membrane) for their efficiency to isolate LP and LF from Gouda and Cheddar cheese whey from cheese factories in South Africa.

1.2.1 Specific objectives

1. To compare the yield of lactoperoxidase and lactoferrin from sulphopropyl cation exchange chromatography and cation exchange membrane techniques
2. To quantify the amount of isolated lactoperoxidase and lactoferrin from whey
3. To compare the concentration of lactoperoxidase and lactoferrin in Cheddar and Gouda cheese whey
4. To determine the purity of each fraction using sodium dodecyl sulfate electrophoresis.

2 LITERATURE REVIEW

2.1 Whey

Whey is the yellow-green liquid that separates from the curd during manufacture of cheese and casein. Whey contains more than half of the solids present in the original milk, including about 20% of the protein and most of the lactose, minerals and water soluble vitamins (Marshall & Harper, 1988; McIntosh, Royle, Le Leu, Regester, Johnson, Grinsted, Kenward & Smithers, 1998). It contains about 0.75% whey protein, 0.05% fat, 4.9% lactose and approximately 6% total solids of which 70% or more is lactose and 10% is protein (Mulvihill, 1992; Zall, 1992; Smithers *et al.*, 1996). Essentially 100% of the total milk carbohydrate (lactose) is present in whey. Carbohydrate and protein components are the ones believed to be responsible for the high putrescibility and biological oxygen demand of whey (Smithers *et al.*, 1996).

There are two principal types of whey, i.e. sweet and acid whey. Sweet whey results from the manufacture of products that principally use rennet enzymes to coagulate the milk at minimum pH of about 5.6, while acid whey occurs as the by-product from the manufacture of dairy products where the coagulum is formed by acidification at a maximum pH of about 5.1 (Zall, 1992). Acid whey has a higher mineral/ash content and if acid has been produced by the action of starter bacteria, the lactose concentration is lower than that of sweet whey (Mulvihill, 1992).

Over the past several years, interest has developed in the market place for specialized fractions of whey components that have potential significance in nutritional and disease ameliorating applications (Marshall & Harper, 1988). Lactoferrin and lactoperoxidase are of commercial interest because of medical and nutritional benefits and may be considered to be "nutraceuticals" (any food or part of a food with medical benefits) (Chiu & Etzel, 1997).

2.2 Whey proteins

Whey proteins are a very heterogeneous group of proteins of diverse characteristics that remain in the milk serum or whey after precipitation of the casein at pH 4.6. The major components of whey proteins are β -lactoglobulin (2-4 g/l), α -lactalbumin (0.6-1.7 g/l), blood serum albumin (0.2-0.4 g/l) and the immunoglobulins (0.5-1.8 g/l) (McIntosh *et al.*, 1998; Parodi, 1998). Minor proteins of great importance include lactoferrin, glycomacropeptide, several enzymes (notably lipase, plasmin, lysozyme, xanthine oxidase, lactoperoxidase, acid and alkaline phosphatases), vitamin binding proteins, a variety of growth factors and hormones (Fox & Mulvihill, 1993; Smither *et al.*, 1996; Chiu & Etzel, 1997; Norris, Tsao, Haggarty & Otter, 1998).

Whey proteins are subject to heat-induced denaturation and interaction that result in coagulation or gelation, depending on protein concentration, pH, ionic composition and temperature. These reactions, which involve hydrogen, hydrophobic, ionic, and sulfhydryl-disulfide group interchange mechanisms, are important for providing viscosity, stabilization, or gel structure, as in the manufacture of restructured food products (Morr, 1992).

The growing interest in the functional use of whey proteins has stimulated the production of a wide range of whey protein products (de Wit, 1989; de Wit, 1990). Because of their various functional properties, whey proteins may be used in a wide range of food applications. However, no WPC (whey protein concentrate) or WPI (whey protein isolate) contains all the functional properties in a single ingredient (Huffman, 1996). It is necessary to match the application requirements with the specified function that the whey protein product has been manufactured for. The functional properties and applications of whey proteins in the food industry has been discussed by Fox, (1989); de Wit (1990); Brandenburg, Morr, Weller (1992); Huffamn (1996); Vaghela & Kilara (1996); de Wit; (1998).

Epidemiological and biochemical evidences have shown that whey protein isolates are capable of inhibiting or reducing development of certain diseases such as colon cancer, head and neck cancer (Smithers *et al.*, 1996; Smithers *et al.*, 1998; Chmiel, 1998; Gorewit & Spitsberg, 1998; Tome & Debabbi, 1998; Smithers, McIntosh, Regester, Johnson, Royle, Le Leu & Jelen, 1998; Huffman & Harper, 1999), arthritis, ischaemia (temporary reduction of blood supply by physical or chemical means to organs or limbs), lipid oxidation, UVD damage (Perraudin & Reiter, 1998), gut disease and wound repair (Regester, Belford, Goddard, Howarth, Smithers, Copeland, de Silva, Toneman & Read, 1998), skin illness (Negishi, Otomo, Gotou, Ueda & Kuwata, 1998), diabetic retinopathy, diabetes mellitus, non-diabetic obesity, glaucoma and immuno-enhancing (Bounous, 1998). The studies have shown that animals fed whey proteins displayed a lower incidence of colon cancer as compared to other sources of protein e.g. meat, casein, soybean etc. These results indicate that native whey proteins can have a positive impact on gastrointestinal health when consumed in the diet and thus have great potential as a physiologically functional food ingredient (IFIS Article, 1997; Neff & Holman, 1997; Gauri, 1998; Schanbacher, Talhouk, Murray, German & Willet, 1998). These findings provide clear messages and promotional opportunities for the dairy industry both in the processing and the marketing of whey proteins as valuable ingredients and in promoting the quality of current products containing whey proteins. Perhaps of greatest significance, the work provides a rational basis for the development of functional foods based on whey proteins that have substantiated health benefits (Smithers *et al.*, 1996; Decker, Tak, Verdecchia & Horgan, 1999).

2.2.1 Beta-lactoglobulin

Beta-lactoglobulin is a major protein in bovine milk, representing about 50% of the total whey protein and 12% of the total protein in milk (Fox & McSweeney, 1998). The protein has a molecular weight of ~ 18 kDa and exists in milk as a dimer at room temperature and at pH 5 to 7 (Marshall & Harper, 1988; Wang &

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Hurley, 1998). It is the most allergenic of the bovine milk proteins for the human infant (Fox & Mulvihill, 1993) and is absent in the milk of human and rodents (Hambling, McAlpine & Sawyer, 1992).

Beta-lactoglobulin is rich in sulphur amino acids which give it a high biological value of 110. It contains 2 moles of cystine, 1 mole per monomer. The cystine is especially important since it reacts, following heat denaturation, with the disulphide of κ -casein and significantly affects rennet coagulation and the heat stability properties of milk; it is also responsible for the cooked flavour of heated milk. The isoelectric point of β -lactoglobulin is pH 5.2 (Fox & McSweeney, 1998).

2.2.2 Alpha-lactalbumin

Alpha-lactalbumin is a metalloprotein (Bramaud, Aimar & Daufin, 1997), and is the second most important species of whey protein and also the smallest (~14 kDa) and most heat resistant of the whey proteins. It is relatively rich in tryptophan (four residues per mole) and is also rich in sulphur (1.9 %) which is present in cystine and methionine (Marshall & Harper, 1988; Fox, 1989; Brew & Grobler, 1992; Fox & McSweeney, 1998).

The isoelectric point of α -lactalbumin is ~pH 4.8 and the minimum solubility in 0.1 M NaCl is also at pH 4.8. In spite of its name, it is not a true albumin. Only variant B is found in the milks of western breeds but milks of African and Indian breeds of cattle contain both A and B variants which differ from each other by only amino acid replacement at position 10, i.e. arginine in α -lactalbumin B, glycine in α -lactalbumin A. The milk of Bali cattle contains a third variant C (Fox, 1989).

2.2.3 Blood serum albumin

This protein (~68 kDa), a major component of blood serum, is synthesized in the liver and gains entrance to milk through the secretory cells, but it comprises only

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about 1.2% of the total milk protein (Walstra & Jenness, 1984; Wang & Hurley, 1998).

2.2.4 Lactoferrin

Lactoferrin is an iron-binding glycoprotein with a molecular weight of ~76-87 kDa related to the transferrin family, present in many external mammalian secretions, such as milk, saliva, vaginal and bronchial secretions and in the granules of polymorphonuclear leukocytes (Chen & Wang, 1991; Yoshida & Xiuyun, 1991; Grasselli & Cascone, 1996; Pakkanen & Aalto, 1997; Wang & Hurley, 1998). Crystallographic analysis has shown that LF consists of a single polypeptide chain structured in two globular lobes, corresponding to the N- and C-terminal halves of the molecule. Each lobe contains one iron-binding site in an inter-domain cleft. The atom of iron is bound to lactoferrin through interaction with four ligands: two tyrosines, one aspartic acid and one histidine (Sánchez, Peiró, Castillo, Pérez & Calvo, 1992). LF is synthesized by neutrophils and glandular epithelial cells (Antonini, Catania, Greco, Longhi, Pisciotta, Seganti & Valenti, 1997).

The LF content in milk ranges from 0.03-0.49 mg/ml (Kawakami, Shinmoto, Dosako & Sogo, 1987). Most of the lactoferrin in milk (about 30-150 mg/l) is retained in the whey (accounting for about 70%) and about 30% is bound to the casein and fat (Chen & Wang, 1991). The isoelectric point of lactoferrin is at pH 7.8-8.0. At pH 6.5 lactoferrin is positively charged, while all other whey proteins, except lactoperoxidase, are negatively charged. Its biological properties include regulation of iron and other metals in the gastrointestinal tract, modulation of production of polymorphonuclear leukocytes and of growth of animal cells, and anti-microbial activity against bacteria and yeasts. It has been found to damage the Gram-negative bacterial outer membrane by causing release of lipopolysaccharide molecules from the membrane and so enhancing bacterial susceptibility to hydrophobic antibiotics and human lysozyme. In infant nutrition,

lactoferrin in milk enhances iron absorption (Chen & Wang 1991; Grasselli & Cascone, 1996; Smithers *et al.*, 1996; Chiu & Etzel, 1997).

Other ascribed functions of lactoferrin are regulation of myelopoiesis, the control of hyposideremia during systemic inflammation, inhibition of antibody synthesis, enhancement of hydroxyl radical production by neutrophils, regulation of leukocyte, cytotoxic activity, and lymphocyte proliferation (Chen & Wang, 1991; Wang & Hurley, 1998). Lactoferrin also acts as an antioxidant; that is, protecting against the highly reactive and damaging oxygen-derived species such as superoxide, hydroxyl, hydrogen peroxide and singlet oxygen (Perraudin & Reiter, 1998). These important functions make lactoferrin a potential source of dietetic food and pharmaceuticals (Kawakami *et al.*, 1986).

The specific role of lactoferrin, both with respect to the defense against gastrointestinal infections and as a promoter of iron transport in the intestinal tract, depends on the fact that it is not digested in the gastrointestinal tract which also means that it is not nutritionally available as a source of amino acids (Hambræus, 1992).

2.2.4.1 Stability of lactoferrin

The stability of LF to heat treatment is increasing greatly with increasing iron saturation, suggesting that the two lobes of LF present a different thermoresistance. The widely used pasteurization treatment at 72 to 74°C for 15 seconds had practically no effect on lactoferrin structure (Sánchez *et al.*, 1992). Furthermore denaturation of LF with or without bound iron is slower when heated in phosphate buffer than in milk. This difference may be due to the decrease in pH of milk with increasing temperature and also the interaction of LF with caseins and some whey proteins.

2.2.4.2 Antimicrobial action of lactoferrin

The antibacterial effects and mode of action of LF have been studied in many bacterial species of clinical importance. LF is capable of retarding the growth of many bacterial species, an effect attributed to its ability to chelate iron, which is essential for microbial growth (Chen & Wang, 1991; Dionysius, Grieve & Milne, 1993; Wang & Hurley, 1998). Although the mechanism by which LF inhibits bacterial growth has not been fully elucidated, more recent findings have implicated cell interactions as the proposed mode of action. LF interacts directly with bacterial surfaces, which leads to damaging of the outer membrane of Gram-negative enteric bacteria, causing the release of lipopolysaccharides from the bacterial cell, thereby sensitizing the cell to antibiotic action. The effect is strain and dose dependent and also depends on bacterial load. A bactericidal effect of LF against *Escherichia coli* was demonstrated at high concentrations and a bacteriostatic effect at low concentrations (Dionysius *et al.*, 1993; Dionysius & Milne, 1997; Sanchez & Watts, 1999). Other microorganisms that have been shown to be sensitive to LF are *Salmonella typhi*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Micrococcus flavus* (Batish, Chender, Zumdegani, Bhatia & Singh, 1988). *Listeria monocytogenes* is a Gram-positive bacterium reported to be inhibited by LF. This organism is able to invade intestinal cells and causes severe infections in humans and is associated with the ingestion of various contaminated foods including refrigerated ones (Antonini *et al.*, 1997).

The discovery of the bactericidal peptide (lactoferricin) hydrolyzed from LF by pepsin lacking an iron-binding site, provides new insight into the anti-microbial mechanism of the LF molecule. The 25-residue peptide is highly basic and has shown an effectiveness similar to that of many clinically useful antibiotics, causing complete inhibition of four strains of *Listeria monocytogenes* at concentrations less than 10 µg/ml (Wakabayashi, Bellamy, Takase & Tomita, 1992; Sanchez & Watts, 1999). The peptide is capable of binding

lipopolysaccharide molecules and releasing them from the outer membrane of bacteria in a similar manner to that of the native LF molecule (Wakabayashi *et al.*, 1992; Sanchez & Watts, 1999). The inhibitory action of LF depends upon its iron saturation level and the presence of citrate ions, which compete with LF for iron (Chen & Wang, 1991).

2.2.5 Lactoperoxidase

Lactoperoxidase is a glycoprotein with a molecular weight of ~78-89 kDa and it contains one haem group per molecule. The iron content is 0.0680-0.0709% and the carbohydrate content is 9.9-10.2% with different compositions of amino acids (Yoshida & Xiuyun, 1991; Björck, 1992). Its amino acid sequence is known and the peptide chain contains 612 amino acid residues, including 15 half cysteins. Lactoperoxidase is a basic protein having a high isoelectric point of pH 8.63 to 9.8 (Al-mashikhi, Li-chan & Nakai, 1988; de Wit & van Hooydonk, 1996).

According to de Wit & van Hooydonk (1996), the carbohydrate content of lactoperoxidase is structured into four or five potential N-glycosylation sites. During the isolation some of the glycosidic fractions may be lost by hydrolysis and this is partly responsible for the electrophoretic and chromatographic heterogeneity of LP. Another cause of heterogeneity is partial deamination which occurs at pH > 9. Despite of all these changes, there appears to be no significant difference in activity between various LP fractions.

The presence of lactoperoxidase has been demonstrated in milk from many species, e.g. bovine, porcine, guinea pig, murine and human. Bovine milk is rich in lactoperoxidase (estimated to be 30 mg/litre) but guinea pig milk, the richest source of lactoperoxidase, contains 10 to 15 times as much (Björck, 1992).

Several different forms of lactoperoxidase have been demonstrated in bovine milk. These are grouped into two fractions, A and B, the former being the more

acidic. A total of 10 different forms of lactoperoxidase, designated A1, A2/I, A2/II, A3, B1, B2/I, B2/II, B3, B4 and B5, were identified (Björck, 1992).

Lactoperoxidase has attracted much interest owing to its antibacterial activity. It catalyses the oxidation of thiocyanate (SCN^-) by hydrogen peroxide (H_2O_2) to hypothiocyanate (OSCN^-), an antimicrobial compound. Thiocyanate occurs naturally in many foods, such as milk. Adding thiocyanate and hydrogen peroxide to milk can lengthen its shelf life by inhibiting bacterial growth. Based on clinical evidence, it was found that brushing teeth with lactoperoxidase-supplemented toothpaste reduced dental caries (Fox & Mulvihill, 1993; Chiu & Etzel, 1997).

Commercial interest in lactoperoxidase is focused on (1) activation of the indigenous enzyme for cold sterilization of milk or as protection of the mammary gland against mastitis, and (2) addition of isolated lactoperoxidase to calf or piglet milk replacers to protect them against enteritis, especially when the use of antibiotics in animal feed is not permitted (Fox & Mulvihill, 1993), and (3) in functional food formulations for its antioxidant properties (Perraudin & Reiter, 1998).

2.2.5.1 Stability of lactoperoxidase

Bovine lactoperoxidase is relatively heat resistant, with the enzyme being only partially inactivated by short-time pasteurization at 74°C , leaving sufficient activity to catalyze the reactions between thiocyanate and hydrogen peroxide (Wolfson & Sumner, 1993). Thermal analysis of native LP by differential scanning calorimetry revealed its denaturation temperature to be between 73°C and 83°C at a heating rate of between $0^\circ\text{C}/\text{min}$ and $20^\circ\text{C}/\text{min}$ respectively. In a kinetic study, a marked effect of calcium on thermal inactivation of LP in the temperature range between 63°C and 70°C has been reported. Furthermore, LP is less stable during heating at acidic pH, a feature which may also be explained by a release of calcium from LP. Normal pasteurization for 15 s at 72°C has been reported to cause about

34% inactivation of LP (50 mg/ml) in 0.1 M acetic acid buffer, whereas nearly 100% inactivation occurred after heating for 2 min at 72°C (Herández, van Markwijk & Vreeman, 1990).

2.2.5.2 Antimicrobial effects of lactoperoxidase

The antimicrobial activity of the lactoperoxidase system is well documented. It is a naturally occurring system that has been proven to be both bacteriostatic and bactericidal to a variety of Gram-positive and Gram-negative microorganisms such as *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Streptococcus mutans*, and *Staphylococcus aureus* (Pakkanen & Aalto, 1997). The lactoperoxidase system can alter many cellular systems, including the outer membrane, cell wall, cytoplasmic membrane, transport system, glycolytic enzymes and nucleic acids. Non-pathogenic bacteria as well as pathogenic bacteria have been shown to be inhibited or killed by the lactoperoxidase system (Wolfson & Sumner, 1993; Pakkanen & Aalto, 1997).

LP-catalysed reactions yield short-lived intermediary oxidation products of SCN^- , which may be further oxidized to end products such as sulphate, CO_2 and ammonia or may be reduced back to SCN^- . The major intermediary oxidation product is hypothiocyanate (OSCN^-) which may be produced in amounts of about 1 mol per mol H_2O_2 (Reiter & Härnolv, 1984; Wolfson & Sumner, 1993; de Wit & van Hooydonk, 1996).

The oxidation of sulphhydryl (SH) groups of enzymes and other proteins have been considered to be the key to the antimicrobial action of the lactoperoxidase system. The LP system (LP-s) can be inhibited by substances containing SH-groups, either by direct binding to the haem group of the enzyme or by scavenging thiocyanate ions. In the latter case, the inhibitor is competing with the target substance (Reiter & Härnolv, 1984; de Wit & van Hooydonk, 1996).

It appears that the bacterial cytoplasmic membrane is structurally damaged or changed because organisms exposed to the lactoperoxidase system immediately leak potassium, amino acids and polypeptides into the medium. Uptake of glucose, purines, pyrimidines and amino acids as well as synthesis of protein, DNA and RNA is also inhibited (Reiter & Härnolv, 1984; Wolfson & Sumner, 1993).

2.2.5.3 Applications of the lactoperoxidase system and lactoferrin

The use of chemical preservatives in foods has been a controversial consumer concern. In an era of nutrition awareness, it has become fashionable for consumers to question the use of preservatives (Jager, 1994). A number of preservatives have been questioned regarding their safety over the past few years, some of them have been banned or their use limited (Branen, 1993). This perception has forced food scientists to look for alternatives to synthetic chemical preservatives. Emphasis has been on naturally occurring compounds and natural antimicrobial systems (Roller, 1995). The naturally occurring systems include those derived from microorganisms, animals and plants.

LP and LF are naturally occurring compounds which have been proven to be both bacteriostatic and bacteriocidal to a variety of microorganisms while being harmless to proteins and enzymes of the host organism (Batish *et al.*, 1988; International Dairy Federation, 1988; Antonini *et al.*, 1992; Wakabayashi *et al.*, 1992; de Wit & Hooydonk, 1996; Sanchez & Watts, 1999).

The activated LP-s (supplemented with thiocyanate and hydrogen peroxide) may be useful in milk preservation and improving performance of calves fed on raw milk and reconstituted milk replacers (Ekstrand, Mulla & Waterhouse, 1985). The lack of milk cooling facilities in many areas of the world continues to have a severe negative impact on the dairy industry with its heavy dependence on refrigeration for production, transportation, and processing. The lack of means for

inhibiting milk spoilage, even for a few hours in many cases, has to some extent stifled attempts to increase milk production and availability to the increasing populations of many developing countries. The LP-s has been proposed as a means of treating milk either on farms or at collection centres in areas where cooling is not possible (Zajac, Gladys, Skarzynska, Härnolv & Björck, 1983; Ridley & Shalo, 1990).

2.2.6 Immunoglobulins

Immunoglobulins are antibodies synthesized in response to stimulation by macromolecular antigens foreign to the animal (Walstra & Jenness, 1984). There are five isotypes of immunoglobulins. All have a similar basic structure, but there are differences in the organization structure as well as the amino acid sequences and carbohydrate groups present (Larson, 1992).

The dominant species of immunoglobulins in bovine milk, IgG (~150 kDa), is identical to blood serum IgG in virtually all mammalian species. IgG is the carrier of passive immunity to the newborn. The classical "euglobulin" fraction contains secretory IgA and IgM, of which IgM is related to the well-known cold creaming phenomenon of raw milk (de Wit, 1998). From a strictly nutritional point of view, the immunoglobulins are of less interest since their role is not primarily as nutrients. It is however of interest that they have an essential role in the defense mechanism against gastrointestinal infections, even though they are not nutritionally available as a source of amino acids (Hambræus, 1992).

2.2.7 Lactose

Lactose is the major carbohydrate in the milk of most mammals, being synthesized in the mammary gland from galactose and glucose. The lactose content of the milk from the given species can vary widely in concentration. For cow's milk with average lactose content between 4.8 and 4.9%, reported values

for large scale studies have ranged from 3.23 to 5.77% (Visser, Van den Bos & Ferguson, 1988; Harper, 1992). Almost 100% of the total milk lactose is present in whey (Smither *et al.*, 1996).

2.2.8 Minerals

Liquid whey typically contains 0.7-0.8 g/liter of total minerals, chiefly calcium, sodium, potassium, phosphate, citrate and chloride (Mangino, 1992; Morr, 1992). These minerals are partly bound to proteins and partly in solution. During ultrafiltration, the protein bound portion is retained by the membrane and concentrated together with the proteins, and the soluble minerals may pass through the membrane so that a close to constant concentration is maintained in the water phase of the retentate (Renner, 1992).

2.3 Fractionation of lactoperoxidase and lactoferrin

Several techniques have been developed to separate these components from whey or skim milk on a laboratory or industrial scale. Laboratory scale analytical techniques include ion exchangers such as:

- ❖ Diethylaminoethyl (DEAE) Sephadex (Yoshida & Xiuyun, 1991)
- ❖ CG-50-NH₄ (Hernández *et al.*, 1990)
- ❖ ion-exchange membrane (Chiu & Etzel, 1997)
- ❖ ion-exchange chromatography (Shimazaki, Tanaka, Kon, Oota, Kawagushi, Maki, Sato, Ueda, Tomimura & Shimamura, 1993)
- ❖ Carboxymethyl (CM) Sephadex (Kawakami *et al.*, 1987; Yoshida & Xiuyun, 1991)
- ❖ affinity chromatography (Al-mashikhi *et al.*, 1987; Chen & Wang, 1991; Grasselli & Cascone, 1993).

Other techniques include:

- ❖ isoelectric focusing (Ng-kwai-hang, 1992)

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- ❖ ultrafiltration (Turgeon & Gauthier, 1990)
- ❖ ion complex precipitation (Yoshida, 1989)
- ❖ gel filtration chromatography (Shimazaki *et al.*, 1993)
- ❖ chelating chromatography (Al-mashikhi *et al.*, 1987; Thomas, Cordle, Westfall & Barefoot, 1992)
- ❖ hydrophobic interaction chromatography (Yoshida, 1989)
- ❖ Immobilized monoclonal antibodies (Kawakami *et al.*, 1987).

Some of these methods such as gel filtration and DEAE require harsh conditions including exposure to high salt concentrations or strong acids. They also require a long process of gel filtration or time consuming dialysis using a large quantity of buffer for the DEAE chromatography (Xiuyun, Yoshida & Ng, 2000). While sulphopropyl-toyopearl and ion exchange membrane requires no further steps such as gel filtration and dialysis and therefore they are rapid (Chiu & Etzel, 1997; Yoshida & Xiuyuni, 1991).

Buffers of different ionic strength and pH can be used to elute LF and LP from the chromatographic resin. Usually it takes almost 16.7 h when cation exchange resin is used. To overcome this problem, ion exchange membranes can be used (Björck, 1992; Chiu & Etzel, 1998).

There are two major modes of ion exchange separation: isocratic and gradient elution. In isocratic elution only a single eluent (concentration) is employed. The eluent ion competes with the sample ions for the exchange sites of the resin. This causes the sample ions to move down the column or membrane at different rates and leave the exchanger as separate peaks. The concentration of the eluent should be high enough to elute sample ions rapidly, but if the concentration of the eluent is too high, the early eluting peaks may not be resolved (Gjerde & Fritz, 1987). The separation is based on the fact that each component has a different affinity to the column. The length of time (retention time) that a component stays in the column depends on the void volume. For

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example there are always a few voids between the sulfonated polystyrenedivinylbenzene in a cation exchanger (Smith, 1988). The structure of a polymer-type sulfonic-acid is shown in Figure 1.

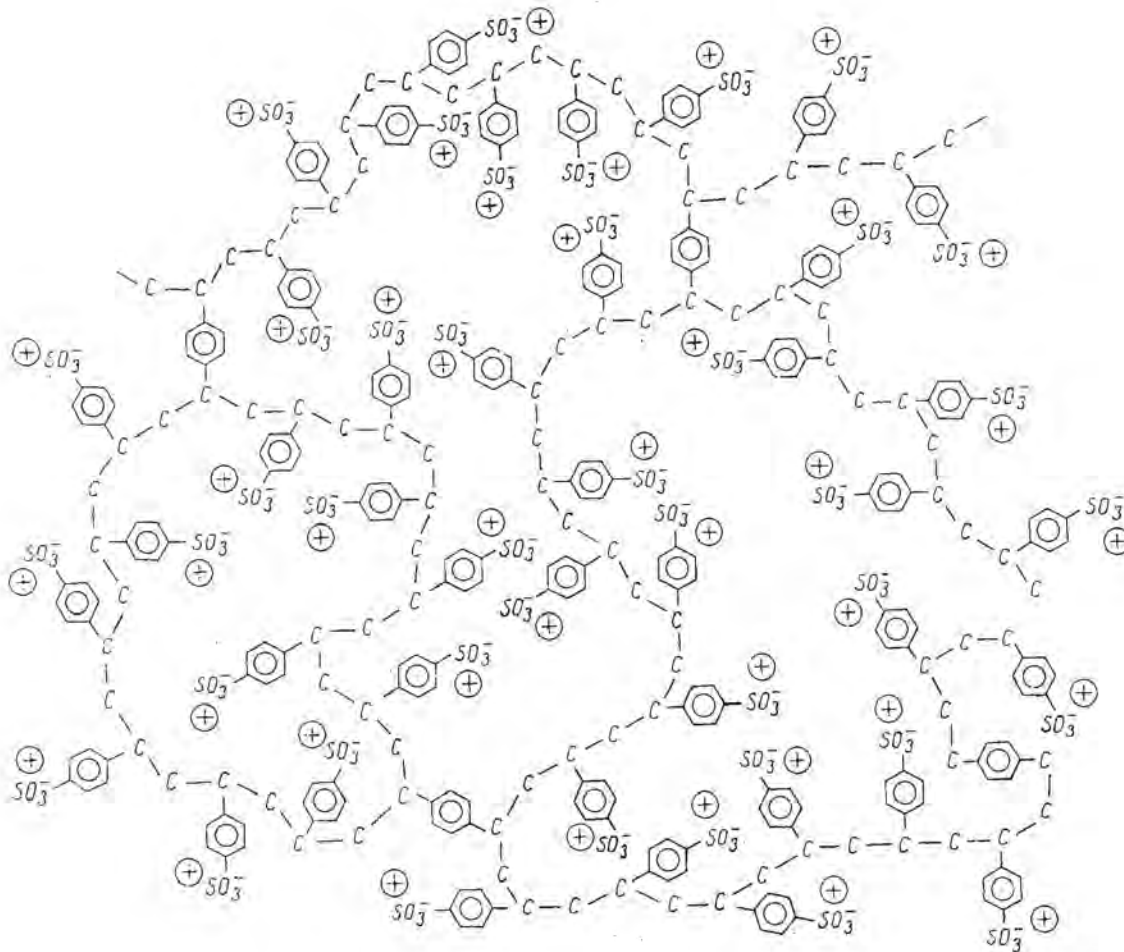


Figure 1: Representation of a swollen sulfonic-acid cation exchanger with a styrene-divinylbenzene matrix containing univalent ions: \oplus -counter-ions; solvent not indicated (Minczewski, Chwastowska & Dybczynski, 1982).

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Stepwise elution has been found to be useful in ion exchange. The conditions are adjusted such that an all or nothing situation prevails. In this mode, the eluent condition is adjusted so that one ion type passes quickly through the ion exchanger. The eluent condition is then changed so that the second ion is rapidly eluted (Gjerde & Fritz, 1987).

Stepwise elutions are easy to achieve, for example if cation A is converted to a neutral or anionic complex, it will pass quickly through a cation exchange chromatograph or membrane. A second cation B that is not complexed will be strongly taken up by the ion exchanger, B can then be eluted by changing to an appropriate eluting salt solution (Chiu & Etzel, 1987; Gjerde & Fritz, 1987). Figure 2 shows chromatographic separation from a sample containing two components.

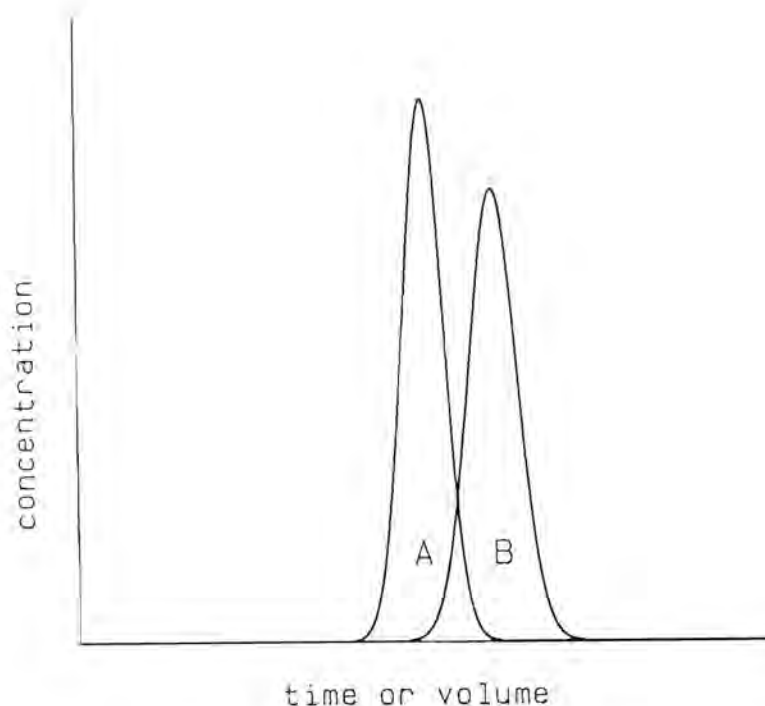


Figure 2: Partial chromatographic separation of two species (Small, 1989).

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Elution by gradient is, in principle, a rather simple matter of adjusting the starting and ending eluent concentrations and gradient duration in column volumes (which sets the slope) for optimal resolution of all the molecules of interest in the mixture. The concentration of the eluent increases as a linear function of time. The problem associated with gradient elution is the difficulty in obtaining a steady detector signal (and therefore a good baseline) as the eluent concentration is continually changing (Gjerde & Fritz, 1987). One would think that results from a quick run with a steep gradient covering a wide range in eluent concentration could be used directly by setting concentrations at the elution point of the first and last peak of interest, and the duration by the number of peaks in between. Unfortunately, due to the behaviour of molecules on a column, such a simplistic approach rarely, if ever, actually works (Gjerde & Fritz, 1987; PerSeptive Biosystems, 1996).

When eluting in a gradient, molecules are always moving down the column. The rate at which a molecule moves at any time is determined by its isocratic retention at the instantaneous eluent concentration. At very low concentration, it may move very slowly, often imperceptibly. As the eluent concentration increases during the gradient, the molecules begin to move down the column at an ever-increasing rate until it shows no retention and moves at the eluent flow rate, at which point it certainly will elute from the column. However, elution may occur well before that point, if the gradient slope is low enough, so that the molecule reaches the bottom of the column during the "slow movement" stage (Smith, 1988; PerSeptive Biosystems, 1996).

The degree to which the elution point shifts with changing gradient slope depends upon the number of sites of interaction between the bound molecule and the surface, as well as the mode of chromatography. For smaller molecules, the isocratic retention often changes rather slowly with changes in eluent

concentration, whereas for large proteins the changes may be extremely sharp (PerSeptive Biosystems, 1996).

The primary objective in an ion exchanger, though, is not just to elute a component, but to separate it from other components. The efficiency can be measured in terms of its stability to resolve the individual components (selectivity i.e. column volume needed to elute peak 2 versus peak 1). The column selectivity is directly related to its efficiency (Smith, 1988).

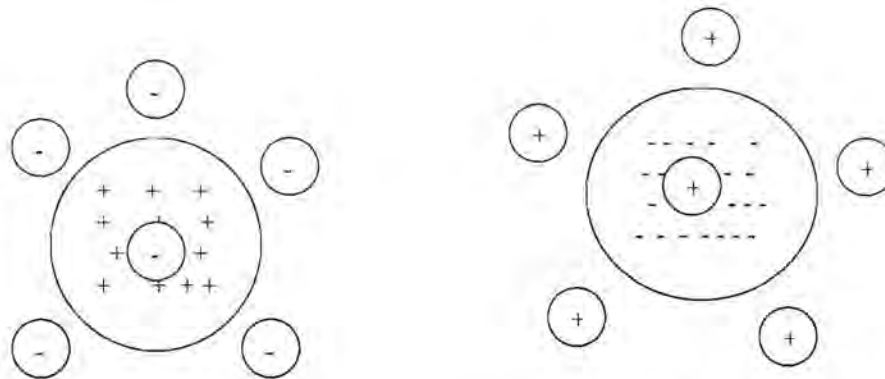
Isolation of 57-91 mg of LF from 1 litre of skim milk using carboxymethyl (CM) cation exchange chromatography, 15.4 mg of LP and 23.1 mg of LF from 1 litre of pasteurized sweet whey using S-Sepharose, have been reported (Yoshida & Xiuyun, 1991). Also, isolation of 41 mg of LP and 87.6 mg of LF from 1 litre dialysed whey by passing it through a column of CM resin at pH 7.7 has been achieved (Yoshida & Xiuyun, 1991). All of these methods require more time.

2.3.1 Ion exchange chromatography

Traditional chromatography uses porous particles packed into columns. As liquid flows through the column around the beads, bio-molecules in the liquid diffuse into the pores of the particles to binding sites on the inner surface of the pores. The rate-limiting factor in the low-pressure column chromatography is the time required for the molecules to diffuse into and out of the pores where the binding sites are. The various steps of equilibrating, loading, washing, elution and regeneration can take hours (Sartorius AG, 2000).

An ion exchanger consists of an insoluble matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter-ions. These counter-ions can be reversibly exchanged with other ions of the same charge without altering the matrix (Khym, 1974; Gjerde & Fritz, 1987; Pharmacia Fine Chemicals A.B., 1984). Figure 3 shows ion exchanger with respective counter-ions.

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Anion exchanger with exchangeable counter ions.

Cation exchanger with exchangeable counter ions.

Figure 3: Ion exchanger with exchangeable counter-ions (Pharmacia Fine Chemicals A.B., 1984).

The matrix may be based on inorganic compounds, synthetic resins, polysaccharides etc. The nature of the matrix determines its physical properties such as its mechanical strength and flow characteristics, its behaviour towards biological substances and to certain extent, its capacity (Pharmacia Fine Chemicals A.B., 1984; Small, 1989).

The presence of charged groups is a basic property of an ion exchanger. The type of group determines the type and strength of the ion exchanger, their total number and availability determines the capacity. There is a variety of groups which have been chosen for use in ion exchangers (Pharmacia Fine Chemicals A.B., 1984; Johnson, 1987). Some of these are shown in Table 1.

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stage is sample application and adsorption. Unbound substances can be washed out from the exchanger bed using a running buffer solution. In the second stage, substances are eluted from the column, separated from each other.

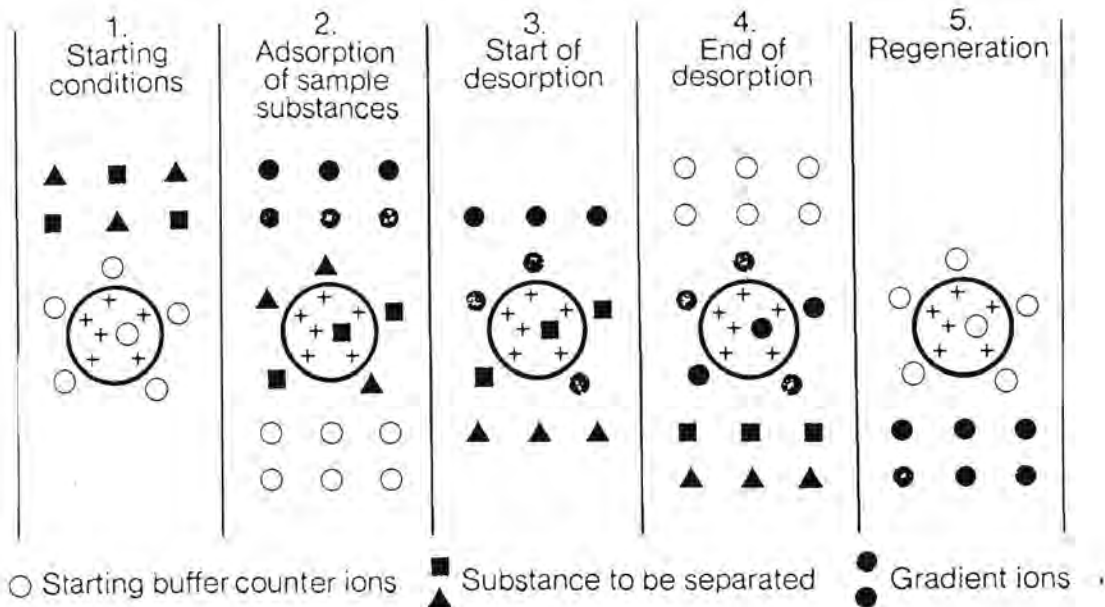


Figure 4: Separation in ion exchange chromatography (Pharmacia Fine Chemicals A.B., 1984).

The separation is obtained because different substances have different affinities for the ion exchanger due to differences in their charges. These affinities can be controlled by varying conditions such as ionic strength and pH. The differences in charge properties of biological compounds are often considerable, and since ion exchange chromatography is capable of separating species with very minor differences in properties such as two proteins differing by only one amino acid, it is a very powerful separation technique indeed. This is particularly so when ion exchange is coupled with a technique such as gel filtration, which separates by a different parameter, that of size. In ion exchange chromatography one can

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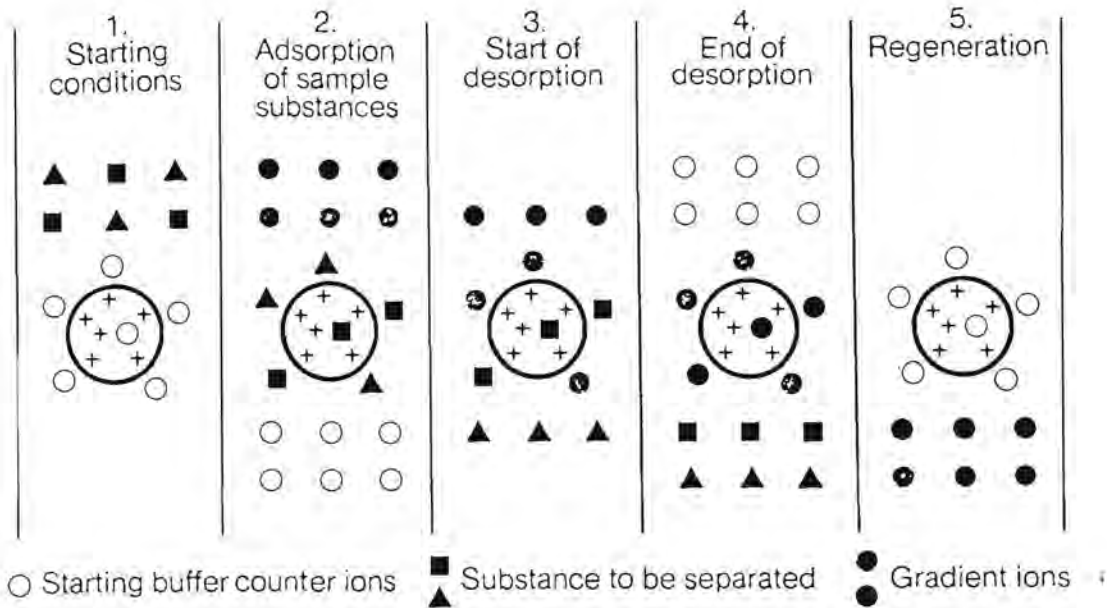


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choose whether to bind the substances of interest, or adsorb out contaminants and allow the substance of interest to pass through the column. Generally it is more useful to adsorb the substance of interest, since this allows a greater degree of fractionation (Pharmacia Fine Chemicals A.B., 1984).

2.3.2 Sulphopropyl cation exchange chromatography

Sulphopropyl cation exchange chromatography has been reported as a rapid method for isolation of LF and LP. The results indicated that rennet whey is most suitable for the isolation of LP while acid whey is good for isolating LF. The SP-TP cation exchange chromatography is advantageous because it can adsorb these functional proteins from rennet and acid whey without dialysis; while other techniques e.g. CM-TP, require dialysis. The yield of LP and LF by using the SP-TP method has been reported as 20.1 mg and 39.5 mg respectively from 350 ml of acid whey; while the yield from rennet whey was 11.8 mg LP and 15.9 mg LF (Yoshida & Xiuyun, 1991).

2.3.3 Fractionation of lactoperoxidase and lactoferrin by using cation exchange membranes

Ion exchange membranes are one of the most advanced separation membranes and have been used in various industry fields; electrodialytic concentration or desalination of electrolyte solutions, separations of ions from non-ionic substances, separator for electrolysis, diffusion dialysis to recover acids and alkalis from waste acids and alkalis, separation of acidic gases such as carbon dioxide by carrier transport, separation of amino acids etc (Sata, Nojima & Matsusaki, 1999; James, McMaster, Newton & Miles, 2000; Shahi, Thampy & Rangarajan, 2000; Takata, Yamamoto & Sata, 2000; Sata, Kawamura & Matsusaki, 2001; Sata, Kawamura, Matsusaki & Higa, 2001).

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Though the performance of ion exchange membranes has been improved, these membranes are ineffective in the separation of ions with the same charge (Sata, Kawamura & Matsusaki, 2000), therefore further improvement to produce membranes with varied degrees of permselectivity, high chemical and thermal stability, which meet the growing demands in different industrial processes, is important. Their performance also depends on the properties and the associated operating conditions. The distribution of functional groups in a membrane is directly related to the spatial variation of the fixed charge (Hsu & Jiang, 1999).

Little work has been done to isolate whey proteins, more specifically LP and LF, by using ion exchange membranes, probably because there was no membrane developed for the isolation of bio-molecules. The rapid growth in the field of biotechnology has led to an increase in the demand for efficient, large-scale protein purification processes (Ghosh, Cui, 2000). Sartorius (AG, 2000) developed membranes which are capable of isolating biological molecules from a biological mixture in solutions. These membranes contain different functional groups such as sulfonic acid, quaternary aminoethyl, carboxyl and diethylamine.

Membrane adsorbers (built-in ion exchanger groups) offer exciting new possibilities for rapid isolation and purification. The use of these membrane adsorbers (MA) in the pressure filtration mode forces the protein solution through the sub-micron pores of the membrane bringing protein direct into contact with the binding site. This direct contact with the binding sites eliminates the rate dependence on diffusion into beads, which characterizes chromatography, without sacrificing capacity or the precision of the separation and reduces various steps and time which are found in traditional chromatography (Chiu & Etzel, 1997; Sartorius AG, 2000). As a result, membrane adsorbers provide high dynamic capacity at high flow rates. Incorporated into complete disposable or reusable units they supplement the benefit of very short process times with easy handling, flexibility and compatibility with syringe, peristaltic pumps or existing work stations, so that no time is lost in changing from a traditional

chromatography column to the new membrane ion exchangers. A membrane adsorber with very low non-specific adsorption and high binding capacity (up to 2 mg/cm²) can be used at high flow rates for rapid separation or concentration (Sartorius AG, 2000).

The reusable membrane adsorber units with adsorption areas of 15 cm² and 100 cm² are recommended for larger volumes (up to 20 L) and higher protein quantities. High dynamic binding capacity, high flow rates (up to >200 ml/min/unit) and ease of handling allow the use of syringes or peristaltic pumps for rapid binding (adsorption) and elution (desorption) in concentration and purification steps. These advantages can be utilized for many applications such as rapid protein concentration (Table 2), protein purification, removal of contaminants and screening tests (Sartorius AG, 2000).

Table 2: Protein concentration with MA 15 (Sartorius AG, 2000)

MA unit with syringe	S15	Q15	
Protein (50 ug/ml)	Cytochrome C	BSA	
Volume (ml)	200	200	
Loading buffer (A)=	10 mM	10 mM	
Sodium phosphate	pH 7.0	pH 7.0	
Washing buffer	10 ml (A)	10 ml (A)	
Elution (1 M KCl)	3 ml	3 ml	
Result for each protein	Before MA	After MA	Evaluation
Volume (ml)	200	3	98.5% (reduction)
Concentration (mg/ml)	0.05	6.75	66 fold reduction
Recovery of total protein			95-100 %
Total time needed			5-7 min

2.4 Protein quantification

Several techniques have been developed for the estimation of protein concentration in samples. The appropriate choice of method depends on five major criteria: the amount of protein available to assay, the concentration of the protein, the specificity of the assay, the presence of chemicals which may interfere with the assay and the ease and reliability of performing the assay (Stoscheck, 1990). Among these are spectrophotometric methods, some with protein assay dye reagents (e.g. Bio-Rad and Coomassie blue G 250), chemical methods and enzyme-linked immunosorbent assays (Kawakami *et al.*, 1987; Yoshida & Xiuyun, 1991; Kruger, 1996; Wang & Hurley, 1998). Proteins actively absorb light in the ultraviolet region with two maxima, 280 and 200 nm (Stoscheck, 1990).

Advantages of ultraviolet light absorption methods are: (1) they can be performed directly on the sample without the addition of any reagents, (2) they can be performed very rapidly since no incubations are required, and (3) the relationship between protein concentration and absorbance is linear (Stoscheck, 1990). Since all proteins absorb light at 280 nm, any contamination by other proteins can skew the results.

2.4.1 Measurement of enzyme activity

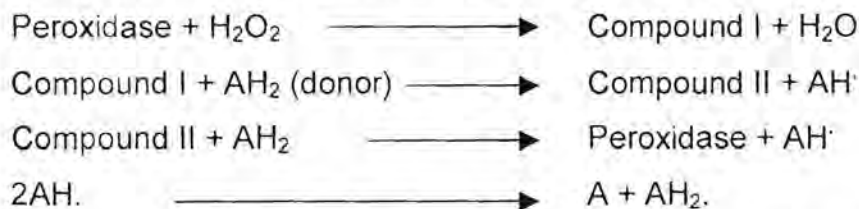
Methods of measuring enzyme activity have traditionally been divided into three classes: continuous, coupled, and discontinuous. The three methods differ with regard to the matter of separation. Thus, whereas the first two do not require the product to be separated from the substrate, the last does. In addition, the first do not require a termination step, whereas the last usually does (Rossomando, 1990).

Several assays for measurements of peroxidase activity have been used. It is however, difficult to compare reported results since there are no standardized peroxidase units upon which such comparisons may be used. Most assays are based on the principle that the electron donors in oxidized form absorb visible light and can be determined by a spectrophotometric assay. Examples of such substances that have been utilized in peroxidase assays are pyrogallol, guaiacol, and o-dianisidine. All these substances have the drawbacks that they are not stable in solution and they are classified as being carcinogenic or otherwise harmful (Björck & Mullan, 1993).

A new electron donor, without the disadvantage mentioned above, has come into use, that is, *2,2'-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt* (ABTS). This electron donor is today most frequently used for assaying various types of peroxidase (Björck & Mullan, 1993; Pütter & Becker, 1993).

2.4.1.1 Principle of the method

The kinetics and mechanisms of peroxidase reaction systems generally catalyses the following reactions and represented on Figure 5:



When halides or thiocyanate ions are present, compound I reacts directly with the donor in one step with transfer of two electrons from the donor to compound I to produce the oxidized donor and regenerate the native enzyme (Björck & Mullan, 1993).

Isolation of lactoperoxidase and lactoferrin from whey : Literature review

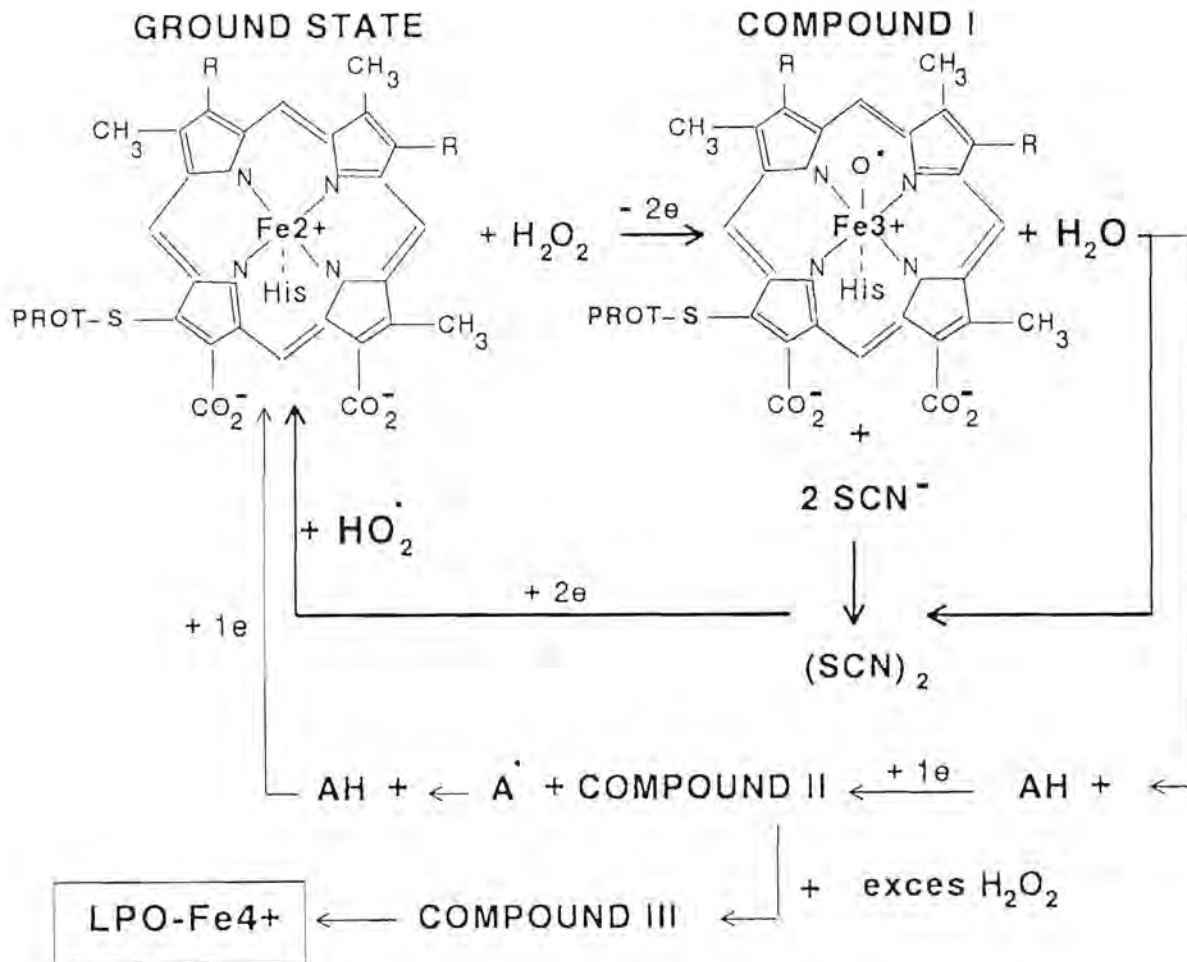


Figure 5: Pathways in the lactoperoxidase catalysed reaction mechanism. The normal peroxidatic cycle includes compound I. Insufficient 2-electron donors lead to compound II, and access of H₂O₂ results in the formation of compound III (de Wit & van Hooydonk, 1996).

2.4.2 Protein quality

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis is the most widely used method for qualitatively analyzing protein mixtures. It is particularly useful for monitoring protein purification, and because the method is based on the

Isolation of lactoperoxidase and lactoferrin from whey : Literature review

separation of proteins according to size, the method can also be used to determine the relative molecular mass of proteins (Walker, 1996). The method is rapid, sensitive and capable of a high degree of resolution (Stryer, 1995).

The electrophoretic process involves the movement of charged species under the influence of an external electric field. Sodium dodecyl sulphate (anionic detergent) is used to solubilise and to give the proteins a uniform charge distribution. The proteins are then loaded onto a polyacrylamide gel which act as a support and the electric field applied. The protein samples are concentrated into a sharp band in the stacking gel before it enters the main separating gel where the protein gets a sharper band. The proteins are separated based on their relative molecular sizes since they have a uniform charge distribution (Walker, 1996; Hawcroft, 1997).

3 MATERIALS AND METHODS

3.1 Preliminary experiments

In order to establish the quantity of lactoperoxidase and lactoferrin from whey samples, preliminary trials were done following the methods described below. From these trials it was observed that when loading more than 5 ml and 10 ml of whey concentrate and non-concentrate, respectively, some of the analyte passed through and some was bound in both methods. Therefore 5 ml and 10 ml were the volumes of the crude sample taken before diluting to 100 ml with the respective load-and-wash (L/W) buffer. The pH of the original samples was found to be 5.60, 5.76, and 5.90 for the Gouda whey concentrate and non-concentrate and Cheddar cheese whey concentrate, respectively.

To avoid variation of samples, all whey samples were taken from the same batch of the respective cheese whey, i.e. Gouda cheese whey from a Clover factory and Cheddar cheese whey from a Dairy Belle factory.

3.2 Main experiment

After establishment of experimental conditions the work was divided into three steps after whey clarification: isolation, quantification and purity determination. Ten replicates from each sample were done by using a cation exchange membrane method and cation exchange chromatography. Figure 6 shows the flow protocol of protein isolation.

Isolation of lactoperoxidase and lactoferrin from whey: Materials and Methods

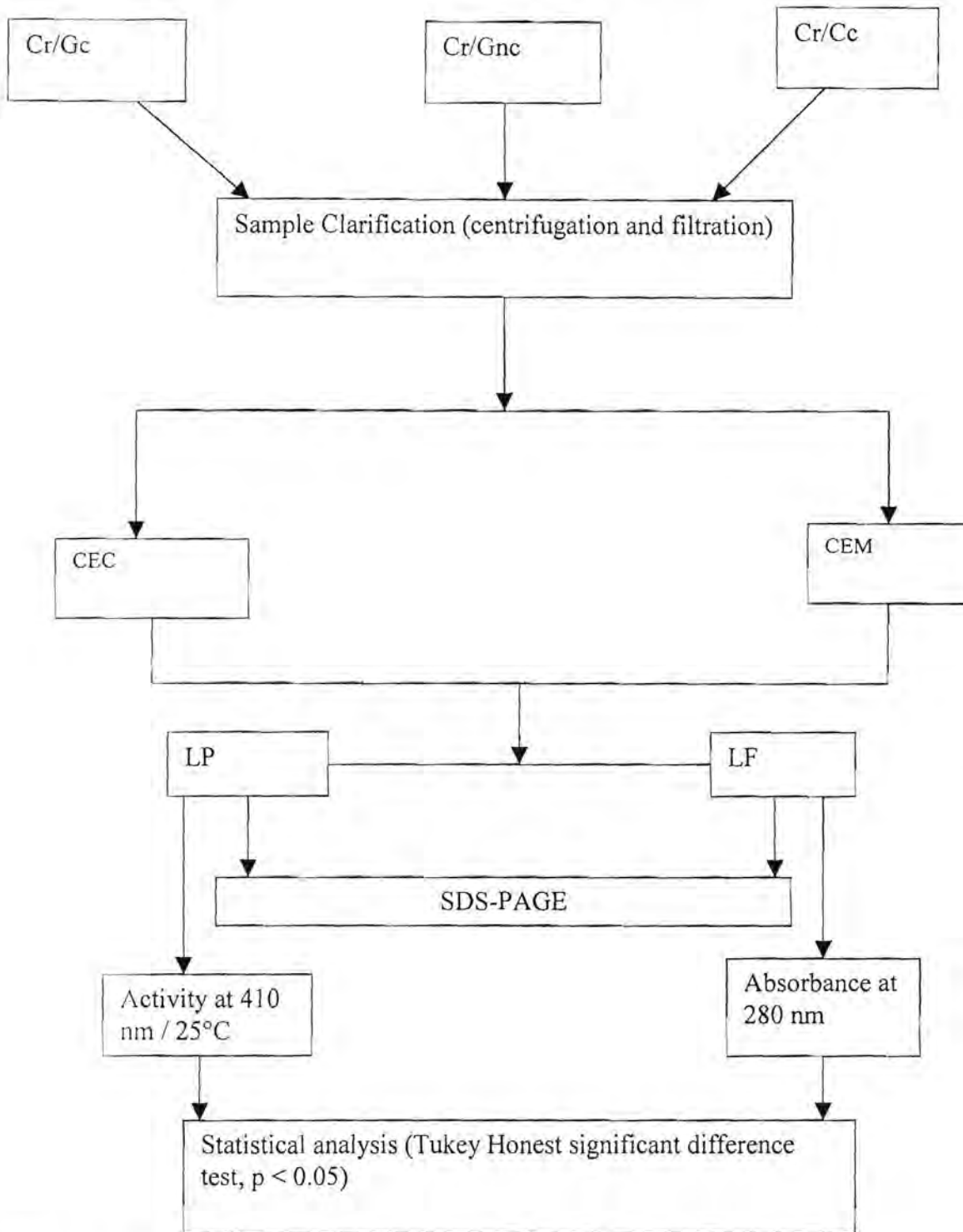


Figure 6: Flow diagram for the isolation of Lactoperoxidase and lactoferrin

3.3 Materials

3.3.1 Cheese whey

The crude (raw) samples of Gouda cheese whey concentrates (56% solids) and non-concentrates were obtained from the Clover factory at Wesselsbron. The Cheddar cheese whey concentrate (52% solids) was supplied by the Dairy Belle factory at Bloemhof.

3.3.2 Centrifuge

A Beckman model J2-21 (USA) was used for whey centrifugation while micro-centrifuge model no. C-1200, 220V/50Hz, serial no. 5761 (Woodbridge, New Jersey) was used to concentrate the samples for purity determination.

3.3.3 Filter paper

A rundfilter (MN GF-1, German) paper, 0.7 μm diameter supplied by Separations SA (Randburg) was used

3.3.4 Vacuum pump

A Watson-Marlow, HR flow inducer (England) pump was used during vacuum filtration.

3.3.5 Chromatography resins

Sulphopropyl Toyopearl (SP-650M) supplied by Separations SA (Randburg) was packed in the column used for separation.

3.3.6 Membrane

A reusable membrane (S-100 module) with an absorption area of 100 cm² in a polysulfone housing material was obtained from Sartonet SA (Midrand).

3.3.7 Peristaltic pump

A minipuls 2 no: 128451 (Gilson, France) was used to pump solutions and samples into the membrane and into the chromatography column.

3.3.8 Spectrophotometer

A computerized spectrophotometer (Beckman 7000, USA) was used to read absorbances of the sample.

3.3.9 Substrate

A one-step ABTS product no: 37615 was used in lactoperoxidase activity determination and was supplied by Separations SA (Pierce, Randburg).

3.3.10 Lactoperoxidase and lactoferrin

Lactoperoxidase and lactoferrin were obtained from Separations SA (ICN, Randburg).

3.3.11 SDS-PAGE Reagents

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis reagents used were supplied by Separations SA (Randburg). The reagents are: acrylamide, bis acrylamide, tris-base, sodium dodecyl sulfate {SDS (C₁₂H₂₅O₄SNa)}, ammonium per-sulfate and tetramethylenediamine (TEMED).

3.3.12 Sample buffer

The sample buffer was made up of tris-HCl, glycine, SDS, 2 β -mercaptoethanol and bromophenol blue supplied by Separations SA (Randburg).

3.3.13 Running buffer

The running buffer consisted of tris-base, SDS and glycine supplied by Sigma-Aldrich SA (Atlasville).

3.3.14 Power supply

A Bio Rad (Richmond, USA), serial no. 215 BR 003455, model 1000/500 was used to supply electric current for gel electrophoresis.

3.3.15 Heating block

A dry block heater, Hägar design was used for denaturation of the samples.

3.3.16 Vortex mixer

A vortex mixer type Reax 2000 (Heidolph, German) serial no. 541.19000.000 was used to mix the samples.

3.3.17. Staining reagents

Gelcode blue reagent supplied by Separations SA (Pierce, Randburg) was used for overnight staining.

3.3.18 Electrophoresis unit

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done in a vertical slab unit (Bio Rad, Mini Protean II, Richmond, USA).

3.3.19 Electric shaker

A Innova™ 400 (New Jersey, USA) shaker was used during staining and destaining of a gel.

3.3.20 Analytical grade reagents

All other chemicals used were of analytical reagent (AR) grade.

3.4 Methods

The precipitated casein and other fines, were removed from the whey by centrifugation at 10 000 x g for 30 min at 10°C, followed by vacuum filtration through a 0.7 µm filter paper. The filtrated whey samples were measured in 350 ml quantities into separate containers and frozen (-20°C) to avoid microbial spoilage during the period of the experiments.

3.4.1 Sample preparation for loading onto the ion exchanger

The same amount of whey sample (depending on the whey type) was used in both methods i.e. cation exchange chromatography and cation exchange membrane. For Gouda and Cheddar cheese whey concentrate 5 ml was taken and adjusted to pH 6.5 by using 0.2 M NaOH before diluting with respective load-and-wash (LW) buffer. For Gouda cheese whey non-concentrate 10 ml was used, the pH adjusted to 6.5 by using 0.2 M NaOH and then diluted with the respective L/W buffer. All samples were diluted to 100 ml before loading.

3.4.2 Sulphopropyl (SP) cation exchange chromatography

Exchange chromatography was carried out on a 2.6 x 12 cm column of Sulphopropyl Toyopearl (SP-650M). In this method 0.05 M phosphate buffer, pH 6.5 was used as L/W solution. The diluted sample (100 ml) was pumped through the column with a peristaltic pump. After passing, 0.05 M phosphate buffer (100 ml), pH 6.5 was used for washing. Lactoperoxidase and lactoferrin were eluted by using, respectively, 0.3 M NaCl (100 ml) and 0.9 M NaCl (100 ml) in a discontinuous step gradient in L/W. The SP resin was regenerated by elution with 0.2 M NaOH (100 ml) and of 0.2 M HCl (100 ml). Before loading the next sample, L/W (100 ml) phosphate buffer was passed through the column.

3.4.3 Cation exchange membrane

A S-100 membrane module containing charged surface moieties ($R-CH_2-SO_3^-$) covalently bonded to internal pore surfaces was used. The dilution of samples in this method was done by using 0.01 M phosphate buffer (L/W), pH 6.5. The diluted sample was pumped through the membrane by using a peristaltic pump. After loading, L/W buffer (50 ml) was used for washing. Lactoperoxidase and lactoferrin were eluted with, respectively, 0.3 M NaCl (60 ml) and 0.9 M NaCl (60 ml) in L/W buffer. The membrane was regenerated by washing with 0.2 M NaOH (50 ml), followed by 50 ml of L/W before loading a next sample.

3.4.4 Determination of lactoperoxidase activity

Lactoperoxidase activity was determined by using UV spectroscopy. One step ABTS was used as substrate. The assay mixture consisted of 0.1 M phosphate buffer of pH 6.4 (2 ml), sample (40 μ l) and ABTS (100 μ l). The absorbance was measured at 410 nm as a function of time (15 s interval) at 25°C by using a computerized spectrophotometer (Beckman 7000, USA). Three replicates from

the same sample fraction were done. The following formula and the factor derived by Björck & Mullan (1993) was used for activity determination.

$$\text{Units/ml} = \frac{\Delta A_{410}/\text{min} \times \text{assay volume (ml)}}{32.4 \times \text{sample volume (ml)}}$$

where ΔA_{410} = change in absorbance at 410 nm

Enzyme activity is defined as: 1 unit (U) of lactoperoxidase activity is that amount of enzyme catalysing the oxidation of 1 μ mole of substrate per min at 25°C (Hernández *et al.*, 1990; Björck & Mullan, 1993).

For the crude sample, 5 ml was diluted to 20 ml with load-and-wash 0.05 M phosphate buffer, pH 6.5. For the assay, diluted sample (40 μ l) and substrate (100 μ l) were used.

3.4.5 Determination of lactoperoxidase and lactoferrin concentration

Standard graphs of known concentrations of LP and LF were prepared. For LP, UV absorbances of known concentrations and of the isolates were measured in duplicate and triplicate respectively, from which a graph of absorbance against time was drawn for each concentration and the sample isolates. The slopes of the graphs of known concentrations were used to construct a single standard graph of slope against known concentrations (Appendix A). The slopes of the graphs of individual isolates were then used to determine the equivalent concentration from the standard graph prepared. All of the absorbances were obtained using a Beckman 7000 spectrophotometer.

Similarly known concentrations of LF standards were prepared and their absorbance read at 280 nm on a computerized Beckman 7000 spectrophotometer in a 2 ml cuvette. A graph of absorbance against concentrations was drawn. The absorbances of the isolates were also measured

at 280 nm and their equivalent concentrations' established from the prepared standard graph (Appendix B).

3.4.5.1 Preparation of lactoperoxidase standard

- ◆ The stock solution of lactoperoxidase was prepared by dissolving 1 mg of lactoperoxidase into 5 ml of phosphate buffer.
- ◆ Six different volumes (LP concentration) were taken from the stock i.e. 5, 10, 15, 20, 25 and 30 μ l. Separately these concentrations were added to the 2 ml phosphate buffer pH 6.4 in a cuvette followed by ABTS. The readings of the absorbances were taken for 3 min at 15 s interval in duplicate. The means of the absorbances were used to construct the graph of absorbance versus time from where the best line was drawn and the slope determined (Appendix C).
- ◆ From the six different lactoperoxidase concentrations 6 slopes were obtained and these slopes were used to construct the graph of slope versus concentration (Appendix A).

The following formula was established to determine LP concentration (mg/ml) of whey (see example in Appendix D):

$$\text{Concentration of LP (mg/ml)} = \frac{X * df_1 * df_2}{V_o}$$

Where, X = Concentration read from the standard lactoperoxidase graph

df₁ = dilution factor of the sample before passing through the membrane or chromatograph

df₂ = dilution factor in the reaction mixture (cuvette).

V_o = volume of the whey before dilution

3.4.6 Determination of LP and LF purity.

LP and LF purity were established by electrophoresis on SDS-page gel in a vertical slab unit according to Laemmli (1970). The separating gel consisted of 40% acrylamide (4.453 ml), bis-acrylamide (0.089 g), distilled water (13.91 ml), 1.5 M tris-HCl buffer of pH 8.8 (6.25 ml), 10% SDS (0.25 ml), 10% ammonium persulfate (0.125 ml), and TEMED (12.5 μ l). The stacking gel consisted of distilled water (6.1 ml), bis-acrylamide (0.018 g), 40% acrylamide (0.926 ml), 0.5 M tris-HCl buffer pH of 6.8 (2.5 ml), 10% SDS (0.1 ml), 10% ammonium persulfate (50 μ l), and of TEMED (10 μ l).

The sample for gel electrophoresis analysis was concentrated by precipitation. The sample (150 μ l) and methanol (600 μ l) were combined and mixed by three to four inversions of the capped tube and centrifuged in a benchtop microfuge for 10 s at 50 Hz. After centrifugation chloroform (150 μ l) was added to the contents of the tube, briefly blended on a vortex mixer and again centrifuged for 10 s at 50 Hz. Distilled water (450 μ l) was added to the contents of the tube, blended thoroughly on a vortex mixer, and centrifuged for 10 s at 50 Hz to separate the two liquid phases that formed on addition of water. The upper phase that formed was carefully removed. About 1-3 mm of the upper phase was left because most of the protein is at the interface. Again methanol (450 μ l) was added, the contents vortex-mixed and centrifuged in a benchtop microfuge for 4 min at 50 Hz. After centrifugation a white protein pellet was obtained, and the bulk of supernatant was aspirated off.

The concentrated samples were dissolved in sample buffer (25 μ l) containing 0.05% (w/v) bromophenol blue and 2 β -mercaptoethanol, heated on a dry heat block for 10 min at 90°C and cooled in crushed ice for 1 min before loading. The electrophoresis was performed at 200 V for 10 min and then reduced to 120 V for 1 h. Gelcode blue stain reagent was used for overnight staining. Distilled water was used for destaining.

4 RESULTS

The following results answer the objective of this study which was to isolate and quantify lactoperoxidase and lactoferrin, using two different techniques, from two types of cheese whey which were obtained from different cheese factories. No lactoperoxidase was found in the Cheddar cheese whey initially used in the experiments. It was then established that the cheese factory concerned preserved their Cheddar cheese whey with H₂O₂. Cheddar cheese whey then had to be obtained from another factory. Because of time constraints, it was decided to include, in the case of the Cheddar cheese whey, only the concentrated form in the studies and to apply only cation exchange membrane for the isolation of lactoperoxidase and lactoferrin from the Cheddar cheese whey concentrate.

4.1 Lactoperoxidase and lactoferrin concentrations in the samples

Table 3 shows the mean values of LP and Table 4 that for LF isolated by cation exchange membrane and cation exchange chromatography, while Figures 7 and 8 show visual interpretation of these results.

At a probability level of $p < 0.05$ there were no significant differences of LP concentration in Gouda cheese whey concentrate (Gc) as isolated by the two methods (CEM and CEC). Similarly there was no significant difference of LP concentration in fractions isolated by the two methods in Gouda whey non-concentrate (Gnc).

The concentration of LP in Gc and Cheddar cheese whey concentrate (Cc) isolated by CEM differed significantly ($p < 0.05$). The amount of lactoperoxidase in crude Gouda whey concentrate (Cr/Gc), non-concentrate (Cr/Gnc) and crude Cheddar cheese whey concentrate (Cr/Cc) did not differ significantly.

Isolation of lactoperoxidase and lactoferrin from whey: Results

The mean values of LF concentration isolated from cheese whey samples differed significantly ($p > 0.05$) between concentrated and non-concentrated cheese whey. There were also no significant differences between the methods.

Table 3: The concentration of lactoperoxidase (LP) in crude samples of concentrated (Gc) and non-concentrated (Gnc) Gouda cheese whey and concentrated (Cc) Cheddar cheese whey and in samples isolated by cation exchange membrane (CEM) and cation exchange chromatography (CEC) methods

Samples	Concentration of LP ¹ (mg/ml)		
	Crude	CEM	CEC
Gc	0.0306 ^c (±0.0078) ²	0.1903 ^a (±0.0501)	0.1897 ^a (±0.0296)
Gnc	0.0330 ^c (±0.0028)	0.0418 ^c (±0.0072)	0.0390 ^c (±0.0053)
Cc	0.0237 ^c (±0.0038)	0.1070 ^b (±0.0005)	N.D

¹Means with different letters as superscripts in the same column and same line are significantly different from each other ($p < 0.05$).

²Numbers in parentheses are standard deviations.

N.D: Not done due to time constrains

Isolation of lactoperoxidase and lactoferrin from whey : Results

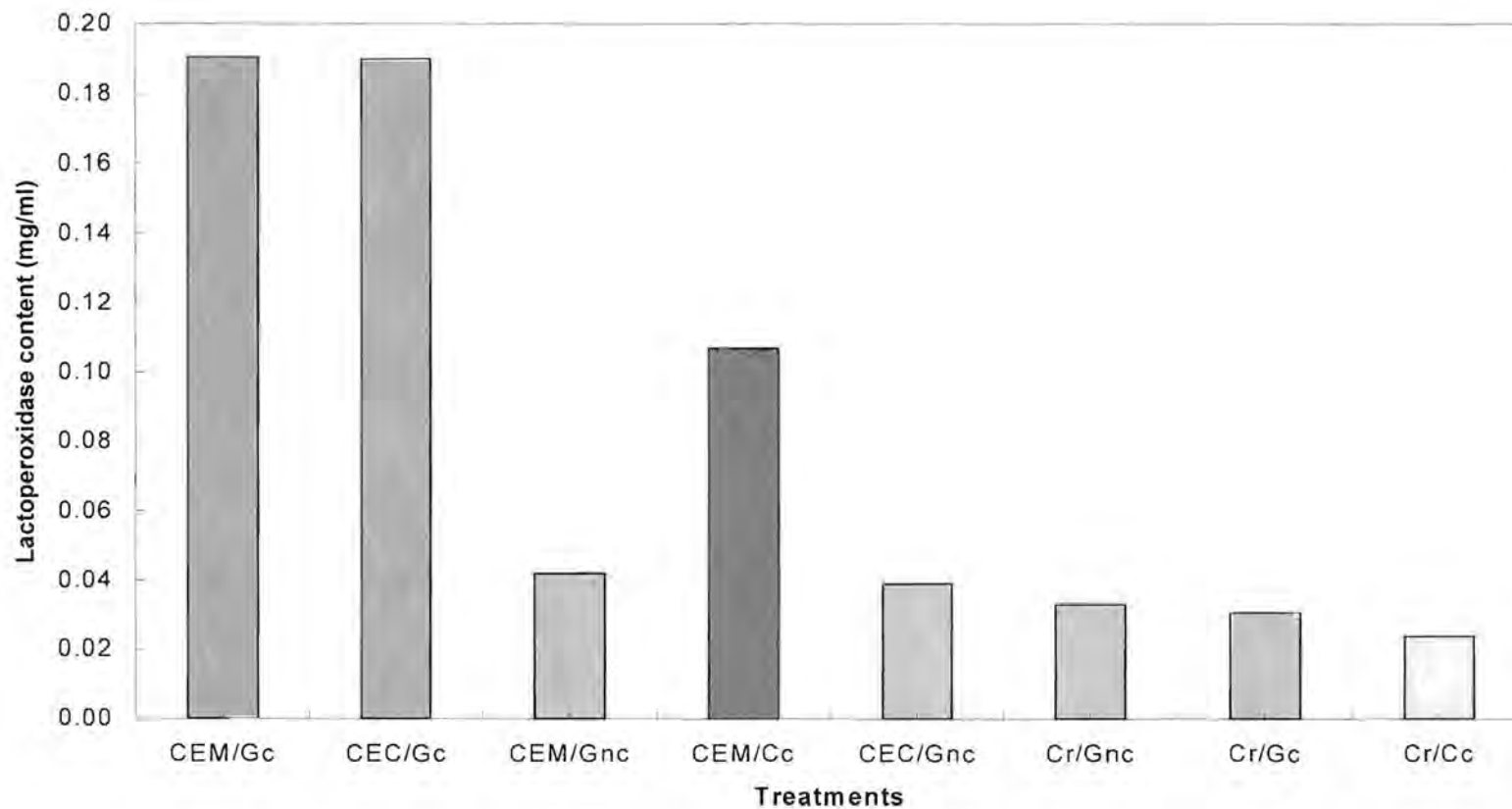


Figure 7: Lactoperoxidase content in the crude samples (Cr) of concentrated (Gc) and non-concentrated (Gnc) Gouda cheese whey and concentrated Cheddar cheese whey (Cc) and in the samples isolated by cation exchange chromatography (CEC) and cation exchange membrane (CEM) methods

Table 4: The concentration of lactoferrin (LF) in samples isolated from Gouda cheese whey concentrate (Gc) and non-concentrate (Gnc) and Cheddar whey concentrate (Cc) by using cation exchange membrane (CEM) and cation exchange chromatography (CEC) methods

Sample	Concentration LF ¹ (mg/ml)	
	CEM	CEC
Gc	0.4464 ^a (±0.0773) ²	0.3949 ^a (±0.0439)
Gnc	0.1186 ^b (±0.0447)	0.0962 ^b (±0.0294)
Cc	0.3893 ^a (±0.0653)	N.D

¹Means with different letters in the same column and line are significantly different from each other ($p < 0.05$).

²Numbers in parentheses are standard deviations.

N.D: Not done due to time constrains

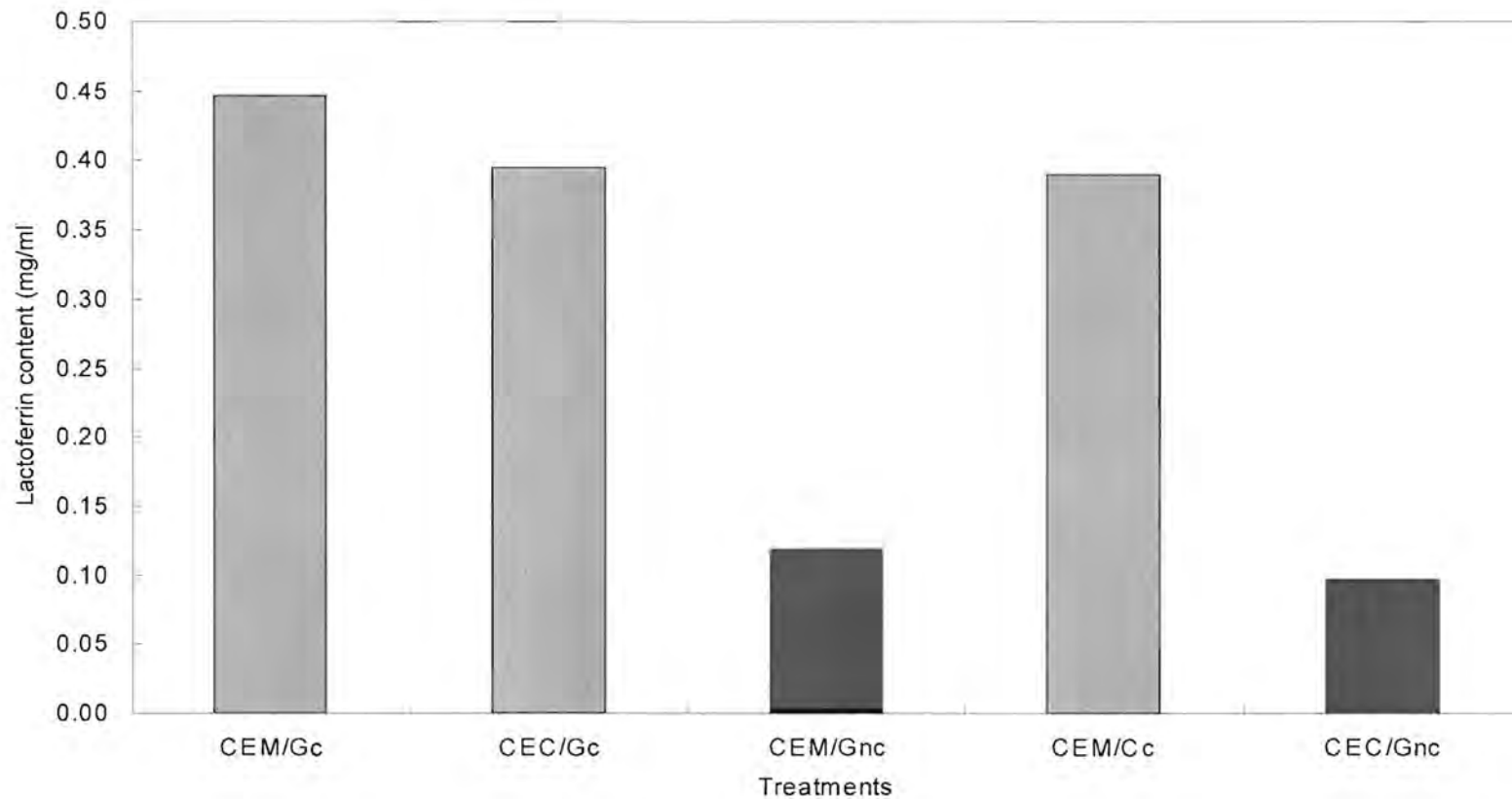


Figure 8: The concentration of lactoferrin in the sample isolated from concentrated (Gc) and non-concentrated (Gnc) Gouda cheese whey and in concentrated Cheddar cheese whey (Cc) by using cation exchange membrane (CEM) and cation exchange chromatography (CEC) methods

4.2 Lactoperoxidase activity

After subjecting ABTS (substrate) to the isolated lactoperoxidase fractions the absorbance reading at time zero started above zero, showing that under normal conditions lactoperoxidase reacts almost instantaneously with ABTS. The reaction was not linear above 3 min, therefore three minutes were taken as maximum time for measuring lactoperoxidase activity from the isolates. The lactoperoxidase activity in the isolates and crude samples are shown in Table 5 and 6 respectively. Visual interpretation of these results is shown in Figure 9.

Generally the rate of ABTS oxidation by lactoperoxidase in the crude samples was low as compared to lactoperoxidase in the isolates. This is due to the fact that the concentration of lactoperoxidase in the samples was determined as the function of ABTS oxidation with time.

Table 5: The activity of lactoperoxidase in samples isolated from Gouda cheese whey concentrate (Gc) and non-concentrate (Gnc), and Cheddar cheese whey concentrate (Cc) by using cation exchange membrane (CEM) and cation exchange chromatography (CEC) methods

Sample	Activity ¹ (U/ml)	
	CEM	CEC
Gc	2.91970 ^a (±0.56158) ²	2.9618 ^a (±0.41681)
Gnc	1.3812 ^{bc} (±0.26978)	1.22898 ^c (±0.15076)
Cc	1.77100 ^b (±0.2351)	N.D

¹Means with different letters as superscripts in the same column and line are significantly from each other ($p < 0.05$)

²Numbers in parentheses are standard deviations.

N.D: Not done due to time constrains

Isolation of lactoperoxidase and lactoferrin from whey: Results

Table 6: The activity of lactoperoxidase in crude Gouda cheese whey concentrate (Gc) and non-concentrate (Gnc) and Cheddar cheese whey concentrate (Cc) samples

Sample	Activity ¹ U/ml
	Crude
Gc	1.62078 ^a (±0.38163) ²
Gnc	1.33320 ^b (±0.13584)
Cc	0.98800 ^c (±0.35557)

¹Means with different superscripts differ significantly from each other at (p<0.05)

²Numbers in parentheses are standard deviations

Isolation of lactoperoxidase and lactoferrin from whey - Results

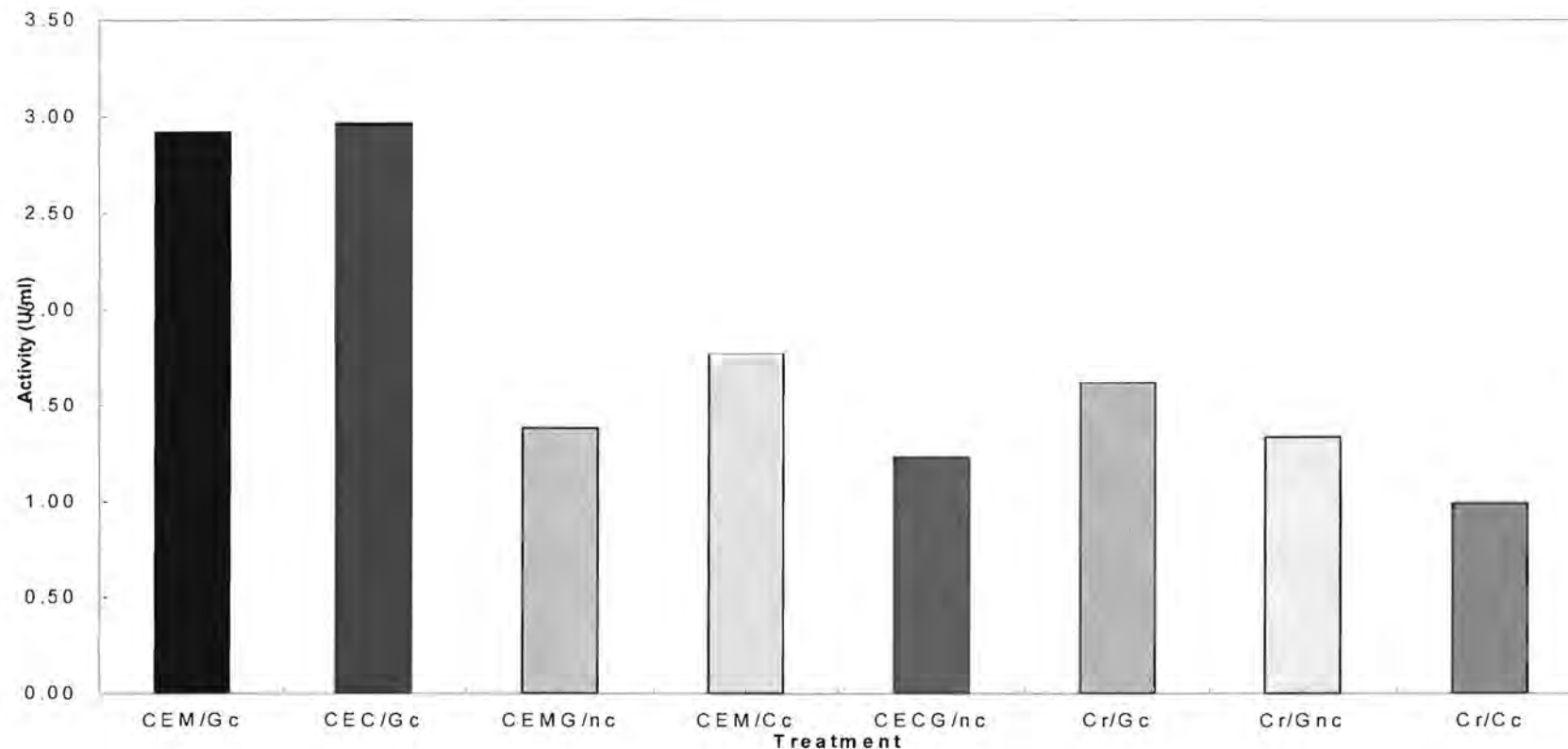


Figure 9: The activity of lactoperoxidase in the samples isolated from concentrated (Gc) and non-concentrated (Gnc) Gouda cheese whey and Cheddar cheese whey concentrate (Cc) by using cation exchange membrane (CEM) and cation exchange chromatography (CEC) and in crude Gouda concentrated (Cr/Gc), non-concentrated (Cr/Gnc) and crude concentrated Cheddar (Cr/Cc) cheese whey samples

Isolation of lactoperoxidase and lactoferrin from whey: Results

The activities obtained for the crude samples were less than that for the isolates. Gouda cheese whey concentrate showed a different trend whereby the rate of reaction started slowly with negative values and later increased. Table 7 shows the means of the 10 readings of the absorbances of each of the three replicates obtained from the same fraction of an individual sample (30 readings in total) and Figure 10 shows its visual interpretation.

Isolation of lactoperoxidase and lactoferrin from whey: Results

Table 7: The mean* absorbances of lactoperoxidase in concentrated (Gc) and non-concentrated (Gnc) Gouda cheese whey and in concentrated (Cc) Cheddar cheese whey isolated by cation exchange membrane (CEM) and cation exchange chromatography (CEC), and in the crude (Cr) Gouda and Cheddar cheese whey samples

Time (s)	Absorbances at 410 nm							
	CEMGc	CEMCc	CEMGnc	CECGc	CECGnc	Cr/Gc	Cr/Gnc	Cr/Cc
0	0.0686	0.0421	0.0664	0.0544	0.0589	-0.0392	0.0658	0.0554
15	0.1332	0.0613	0.1031	0.1084	0.0980	-0.0390	0.1185	0.0780
30	0.1775	0.0712	0.1384	0.1479	0.1306	-0.0391	0.1647	0.1051
45	0.2226	0.1063	0.1905	0.2029	0.1818	-0.0382	0.2119	0.1362
60	0.2901	0.1455	0.2488	0.2593	0.2452	-0.0347	0.2622	0.1709
75	0.3371	0.1870	0.3024	0.3180	0.2928	-0.0265	0.3138	0.2053
90	0.4028	0.2206	0.3532	0.3874	0.3406	-0.0064	0.3653	0.2431
105	0.4814	0.2483	0.3938	0.4430	0.3694	0.0342	0.4169	0.2820
120	0.5338	0.2738	0.4358	0.4976	0.4139	0.1537	0.4633	0.3201
135	0.5766	0.3002	0.4819	0.5581	0.4630	0.1858	0.5104	0.3571
150	0.6260	0.3326	0.5380	0.6097	0.4992	0.3081	0.5625	0.3940
165	0.6775	0.3743	0.6060	0.6616	0.5628	0.4174	0.6162	0.4347
180	0.7322	0.4137	0.6670	0.7077	0.6173	0.5255	0.6659	0.4702

* means of 10 readings of each of three replicates, i.e. a total of 30 measurements

Isolation of lactoperoxidase and lactoferrin from whey: Results

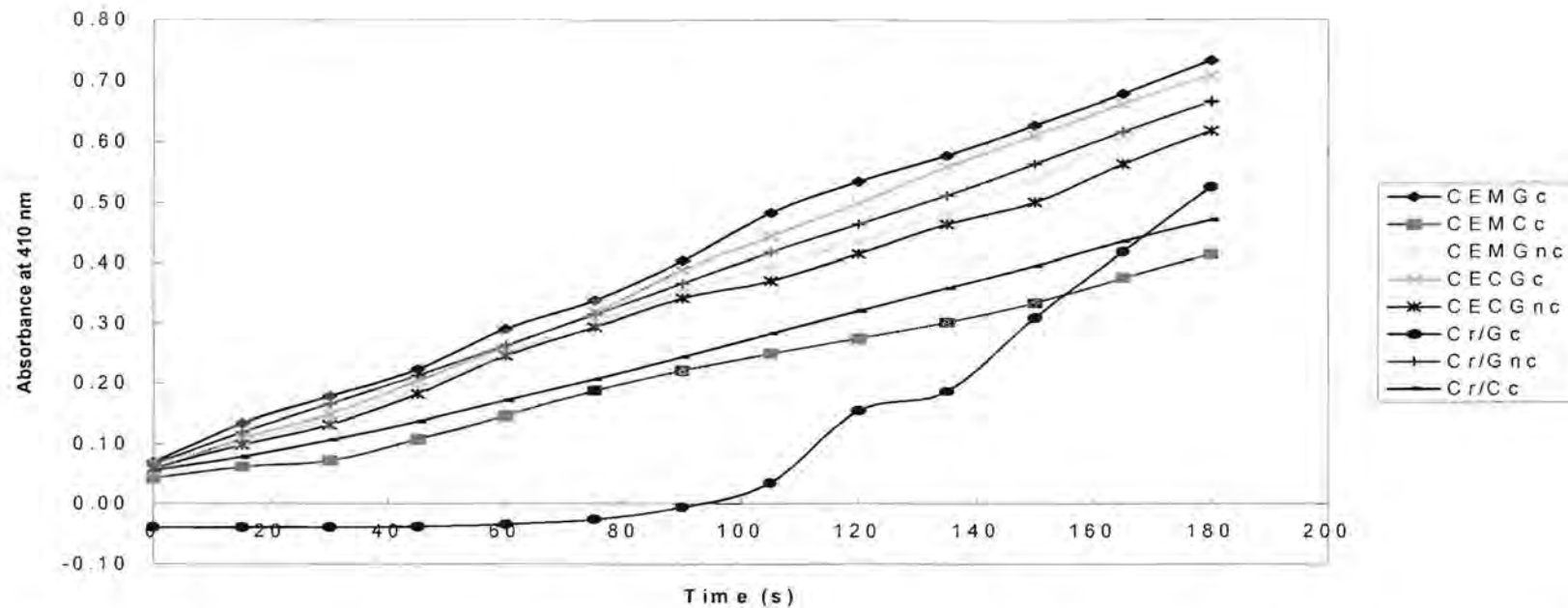


Figure 10: The absorbances of ABTS during oxidation by lactoperoxidase in crude samples of concentrated Gouda (Cr/Gc) and non-concentrated Gouda (Cr/Gnc) and Cheddar (Cr/Cc) cheese whey, and in samples isolated by cation exchange membrane (CEM) and cation exchange chromatography (CEC) methods

Isolation of lactoperoxidase and lactoferrin from whey: Results

At a probability level of $p < 0.05$ there were no significant differences between the methods or the samples. Both methods gave a high LP activity in Gc samples as compared to the rest of the samples. Even though the mean of LP in Cc was higher than in Gnc obtained with both methods, the difference was statistically not significant.

The mean activity of LP in the crude samples differed significantly. Gouda cheese whey concentrate had the highest activity, followed by Gouda cheese whey non-concentrate and Cheddar cheese whey concentrate.

4.3 Purity of lactoperoxidase and lactoferrin in the isolates

The SDS-PAGE patterns of the LP and LF isolates and the LP and LF standards, molecular weight marker and crude sample are shown in Figure 11. The purity of LP and LF obtained by cation exchange membrane and cation exchange chromatography did not differ. As compared to the LP and LF standards, no great difference was observed. The observations revealed that other proteins passed through the column and membrane during loading and the loosely bound proteins were released during washing.

Isolation of lactoperoxidase and lactoferrin from whey: Results

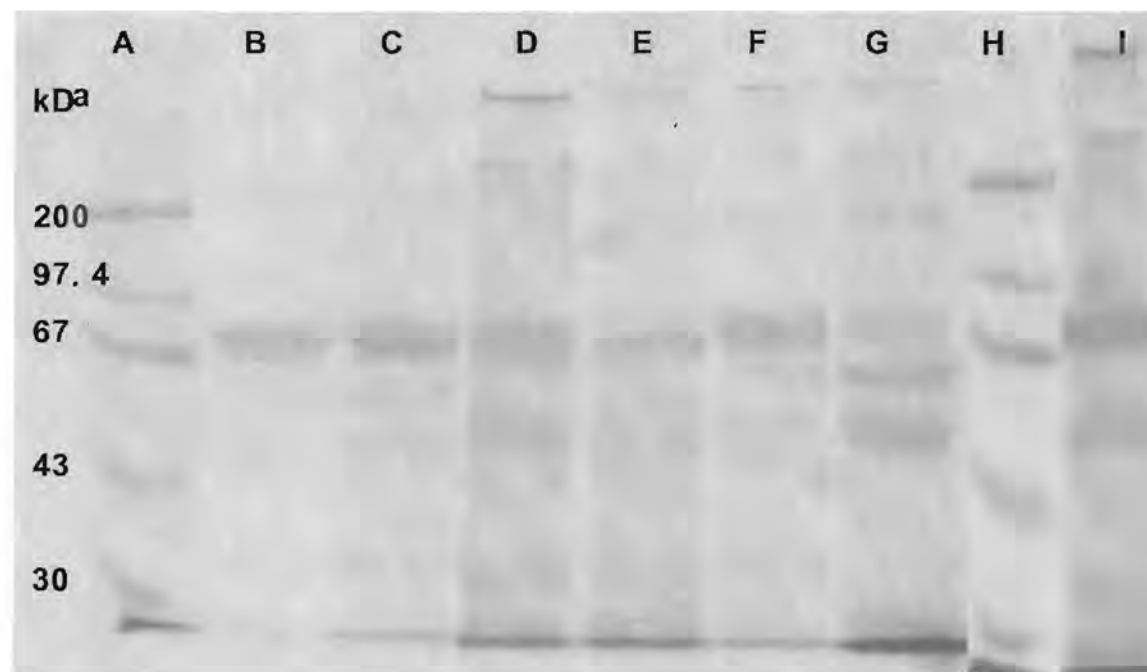


Figure 11: SDS-PAGE profile: A = molecular weight marker, B = lactoperoxidase standard; C = lactoferrin standard; D = lactoperoxidase isolated by cation exchange membrane; E = lactoferrin isolated by cation exchange membrane; F = lactoferrin isolated by cation exchange chromatography; G = crude sample; H= molecular weight marker and I = lactoperoxidase isolated by cation exchange chromatography

5 DISCUSSION

The main objective of this work was to isolate and to compare the lactoperoxidase and lactoferrin content from different whey sources using two cation exchange techniques.

The chemistry of the mobile and stationary phases along with other operating conditions was manipulated to achieve the separation of lactoperoxidase and lactoferrin from the other whey proteins. Such conditions include pH of the sample, pH of the running buffer, concentration of the eluting salt solutions and the flow rate. Proteins are amphoteric polyelectrolytes and the net charge that they carry is dependent of pH. The isoelectric point of lactoperoxidase and lactoferrin is at pH 8.63-9.8 and 7.8-8.0 respectively (Chen & Wang, 1991; Grasselli & Cascone, 1996; Al-mashiki *et al.*, 1996) while other whey proteins have an isoelectric point at pH 4.8-5.2 (Fox, 1989; Fox & Macsweeney, 1998). Below its isoelectric point a protein has a net positive charge and therefore can be adsorbed to the cation exchanger and *vice versa*. The pH of 6.5 to which the samples were adjusted, caused lactoperoxidase and lactoferrin to have a net positive charge while other whey proteins were negatively charged and therefore passed through the cation exchanger unbound (Cheng & Wang, 1991; Chiu & Etzel, 1997).

During binding and elution, molecules from the sample move through the spaces between the packing particles at the same rate as the mobile phase. Some of the molecules bind to the surface with greater or lesser strength. While inside the particles, or bound to the surface, the molecules do not move down the column. Thus the molecules of interest that bind most tightly do not move at all unless the mobile phase conditions are changed i.e. eluting salt solution, and the ones that bind weakly (component of non-interest) move more quickly down the column. Molecules which do not bind at all, elute in the solvent front in a volume which is

equal to the void volume of the ion exchanger (Gjerde & Fritz, 1987; Smith, 1988; PerSeptive Biosystems, 1996).

Due to differences in charge density and affinities for the ion exchanger, lactoperoxidase and lactoferrin were further separated from each other. In addition, minor differences in molecular structure or configuration also contributed in separating these two proteins. There is no preferential adsorption of lactoperoxidase and lactoferrin onto the membrane or chromatography resins (Hernández *et al.*, 1990) and elution depends on the affinity of the protein for the binding site and the strength of eluting salt solutions. Lactoferrin bind more strongly than lactoperoxidase (Chiu & Etzel, 1997) therefore lactoperoxidase eluted before lactoferrin since the elution started with the lower salt concentration.

The flow rate of the samples in both methods was 4 ml/min and the volume of isolates collected was 40 ml for LP and 32 ml for LF. Preliminary experiments (data not shown) showed that cation exchange chromatography and cation exchange membranes had a maximum capacity for binding lactoperoxidase and lactoferrin from samples and above this point some of the analyte passed unbound. This means that functional groups in the ion exchanger were already saturated and other analytes had no space to bind. The number of electrical charges on the resin matrix per unit weight of material expresses quantitatively the capacity of an ion exchanger (Khym, 1974; Gjerde & Fritz, 1987). Stepwise elution of lactoperoxidase and lactoferrin by using eluting salt solution concentrations (0.3 M NaCl for lactoperoxidase and 0.9 M NaCl for lactoferrin) established by Chiu & Etzel (1997) was employed, so that the method could be easily transferable to large scale operation. No lactoperoxidase activity was found in fractions eluted by 0.9 M sodium chloride solution.

Although additional steps increase the purity of the proteins, these steps reduce the yield, jeopardizes reutilization of other valuable whey components if needed

and are time consuming (Kawakami *et al.*, 1987). To avoid these problems one-step separation was employed in this study. This method is simple and thus can scale up easily.

The sample in chromatography passes through a screen before it enters the chromatography matrix, the chances of solid particles to enter into the column matrix is thus reduced. The absence of a screen in the cation exchange membrane allows the sample to enter directly into the membrane. Any solid particles present can enter the membrane and block it resulting in build-up of the pressure inside the membrane housing and thus forcing the sample backwards or spilling it off. These observations were experienced during preliminary trials and therefore a thorough clarification of the samples was done (i.e. centrifugation and filtration).

After passing 100 ml of the diluted Gouda cheese whey concentrate and non-concentrate through the chromatography column, no lactoperoxidase activity was detected in the permeate. This indicated that the entire enzyme was adsorbed onto the column matrix. The bound lactoperoxidase and lactoferrin were released from the column by passing 0.3 M NaCl and 0.9 M NaCl through, respectively. Before releasing lactoperoxidase from the column, about 60 ml of eluting salt solution passed without lactoperoxidase activity being detected in the permeate. After running SDS-PAGE lactoferrin was not found in the last 68 ml of the eluting solution. Likewise when 100 ml of the diluted samples were loaded onto the cation exchange membrane, there was no lactoperoxidase activity detected in the permeate. After washing, the bound lactoperoxidase and lactoferrin were released immediately after changing the strength of the respective eluting sodium chloride solutions, i.e. from low to high concentration. Therefore small amounts of eluting salt solutions were used as compared to the chromatography method. The reason for using more of the eluting salt solution in chromatography than in the membrane method, is that in chromatography the liquid flows through the column and around the beads, bio-molecules in the liquid diffuse into the pores of

the particles to binding sites on the inner surface of the pores where the functional groups are found. The rate-limiting factor in the low-pressure column chromatography is the time required for the molecules to diffuse into and out of the pores where the binding sites are (Sartorius AG, 2000). Another factor that might have contributed to requiring more eluting salt solution in chromatography is the chemical structure and length of the side chain (spacer arm) of the ion exchanger. It has been established that sulfonated polystyrenedivinylbenzene has few voids and therefore long retention time (Gjerde & Fritz, 1987; Smith, 1988). However, in the membrane method pressure forces the liquid through the sub-micropores of the membrane bringing the proteins into direct contact with the binding sites; thus when the eluting solution enters the membrane (Sartorius AG, 2000) the bound bio-molecules (in this case lactoperoxidase and lactoferrin) elute immediately. Similarly there was no lactoferrin in the last 28 ml of 0.9 M of sodium chloride as determined by SDS-PAGE.

The results have shown that Cheddar cheese whey concentrate (Cc), Gouda cheese whey concentrate (Gc) and Gouda cheese whey non-concentrate (Gnc) from cheesemaking factories contained appreciable amounts of lactoperoxidase and lactoferrin. The concentration of lactoperoxidase was found to be 0.033-0.0418 mg/ml in non-concentrated cheese whey and 0.1070-0.1903 mg/ml in the concentrated whey. The amounts of lactoperoxidase in non-concentrated whey were similar to the amounts which have been reported in the literature. Yoshida & Xiuyun (1991) reported a presence of 0.057 mg/ml of lactoperoxidase in acid whey prepared from skim milk by acidifying at 20°C, 0.034 mg/ml in sweet whey made from whole milk heated to 37°C, while Hernández *et al.* (1990) reported an amount between 0.04 mg/ml and 0.05 mg/ml in raw milk and sweet whey. Burling (1989) according to Yoshida & Xiuyun (1991) found 0.0154 mg/ml of lactoperoxidase from pasteurized sweet whey.

Whey contains about 6% total solids (Mulvihill, 1992; Zall, 1992) of which lactoperoxidase and lactoferrin form part. It was thus expected that the two

Isolation of lactoperoxidase and lactoferrin from Gouda and Cheddar cheese whey: Discussion

proteins isolated would have been present in the concentrated whey sample (approximately 56% solids Steyn, FW, Clover SA, 2000 -personal communication) at an approximately 9 times higher level than in non-concentrated whey but the increase was only about 4 times. The reason for this observation might be the heat treatments applied to the milk for cheesemaking and the whey during concentration. Studies have shown that lactoperoxidase is relatively heat stable and is partially inactivated at temperatures between 63°C and 70°C, normal pasteurization at 72°C/15 s causes about 34% inactivation and heating at 72°C/2 min causes 100% inactivation (Hernández *et al.*, 1990). In addition to the heat treatment applied to the milk during cheese manufacturing in South African cheese factories, the whey is further subjected to different temperature treatments. Thus, the Gouda cheese whey concentration involved heating of the whey to ~70-74°C, followed by filtration and separation, heating again to ~93 to 110°C, condensing to 26%, cooling to 8°C, heating to 85°C, and then condensing to ~ 56% solids in a triple effect evaporator (Steyn, FW, Clover SA, 2000 -personal communication). Almost all these heat treatments will reduce the amount of lactoperoxidase in the whey.

The temperatures at which whey was prepared in some of the studies reported in the literature (e.g. Yoshida and Xiuyun, 1991; Hernández *et al.*, 1990) were so low that proteins or enzymes would probably not be inactivated or denatured.

The concentration of lactoperoxidase in raw milk has been reported to be 0.03 mg/ml and this level varies depending on the sexual cycle of the cow and varying also according to summer or winter season, feeding regime and breed (Reiter & Hårnolv, 1984; Björck, 1992; Muir, 1996). It also has been established that heat treatments such as pasteurization affect the structure and properties of whey proteins either reversibly or irreversibly. Reversible changes in the proteins occur mainly at temperatures up to 60°C. These changes are often attributed to pre-denaturation transitions caused by partial loss of the three dimensional protein structure and by changes in protein hydration. Irreversible changes in protein

structure may occur above the denaturation temperature of a protein and are influenced further by environmental conditions such as pH, ionic strength and protein concentration. Protein denaturation may be considered as a two-step process; an unfolding step that may be either reversible or irreversible and an aggregation step that generally follows irreversible unfolding (de Wit, 1998). Denatured protein, in this case lactoperoxidase, can not show activity when subjected to the substrate and can not bind onto the membrane or onto the chromatography resin.

The literature has revealed that lactoperoxidase activity in commercially processed milks is reduced more than 30%, due to either exposure to light after processing or to a more severe heat treatment than 12 s at 75°C, or to a combination of both (Hernández *et al.*, 1990). One or more of the aforesaid factors might also have contributed to the low level of lactoperoxidase found in the concentrated cheese whey samples examined in the present experiments.

The ability of counter-ions (salts) to displace proteins bound to fixed charges is a function of the difference in affinities between the fixed charges and the non-fixed charges of both the protein and the salt (Rossomando, 1990). Whey components such as minerals, lactose and other whey proteins (immunoglobulins, β -lactoglobulin, α -lactalbumin and bovine serum albumin) were removed. These components were either removed immediately during the processes of loading or during washing, and remained with the component of interest, in this case lactoperoxidase, which was bound to the functional groups of the membrane and chromatography resins. It was also observed that lactoperoxidase in Gc and Cc was present in significantly different amounts, being higher in Gc. This might be attributed to the differences in processing conditions. De Wit (1998) has shown that the susceptibility of the whey proteins to heat denaturation appears to decrease as the total solids content of the solution increases, for example thermal denaturation of β -lactoglobulin and α -lactalbumin was retarded by increased lactose content, an effect which is explained by the effect of sugar on

the structure of water. The hydrophobic interaction between pairs of hydrophobic groups appears to be stronger in sugar solutions than in pure water and that mechanism may stabilize proteins against heat denaturation. This phenomena might be applicable in the case of the lactoperoxidase content in Gc and Cc in respect to the solute concentrations. In addition, salts and low pH, have been reported to influence the stability of lactoperoxidase, it being less stable during heating at low pH (Zadow, 1986; Hernández, *et al.*, 1990; Wolfson & Sumner, 1993).

The concentration of lactoperoxidase in the crude (raw) samples was lower than in the isolates, but the differences of lactoperoxidase between the crude samples were not statistically significant. The implication of this observation was that, since the concentration of lactoperoxidase was determined as the function of ABTS oxidation, low oxidation of ABTS in the crude sample indicates a presence of temporary lactoperoxidase inhibitors. An inhibitor is any substance that alters enzyme activity thus reducing the rate of oxidation. One of the lactoperoxidase inhibitors which have been reported is L-cystein (Beumer *et al.*, 1985; Björck & Mullan, 1993). However, in our study, the growth of microorganisms capable of growing at low temperatures was most probably responsible for inhibiting lactoperoxidase activity in the crude samples (Morrison & Hultquist, 1963). These inhibitors (microorganisms) did not bind to the membrane or chromatography resins and were thus removed during the isolation processes i.e. during loading or washing. Gouda cheese whey concentrate showed a different trend where the rate of reaction started slowly with negative values and later increased. This sample probably contained relatively large numbers of microorganisms as compared to the other samples. It is believed that the degree of lactoperoxidase inactivation is parallel with the growth of microorganisms in the samples (Morrison & Hultquist, 1963). Another possible reason for the low rate of reaction which has been suggested by Hernández *et al.* (1990) is the destruction of the enzyme by oxidized ABTS directly after the conversion of ABTS to the oxidized form, when it has not left the enzyme-substrate/product-complex. Also it is most

probably that other whey components might have been interfering the reaction either by forming ABTS-whey component complex and therefore unavailable for lactoperoxidase activity.

The lactoperoxidase activity in sample isolates was found to be the highest in Gouda and Cheddar cheese whey concentrate and least in Gouda cheese whey non-concentrate. This observation reflects the concentration of the enzyme in the respective sample fractions. After isolation, lactoperoxidase activity increased markedly in Gouda and Cheddar cheese whey concentrate respectively and there was no significant change in lactoperoxidase activity between Gouda cheese whey non-concentrate isolates and the respective crude sample. These observations suggested that there probably were no enzyme inhibitors in the crude sample and therefore the enzyme oxidized the substrate without the competition that was present in Gouda cheese whey concentrate.

According to Voet, Voet & Pratt (1999), many substances alter the activity of an enzyme by reversibly combining with it in a way that influences the binding of substrate and/or its turnover number. Inhibitors act through a variety of mechanisms. Some enzyme inhibitors are substances that structurally resemble their enzyme's substrate but either do not react or react very slowly. These substances are commonly used to probe the chemical and conformational nature of an enzyme's active site. Other inhibitors affect catalytic activity without interfering with substrate binding sites. Many do both. In this study, it was observed that after isolation enzyme activity increased dramatically meaning that inhibitors were not isolated along with the analyte.

It is difficult to compare the results obtained from this study with that reported in the literature, since neither a standardized peroxidase unit at international level nor the existence of internationally recommended standard materials is known up to now on which comparison may be based (Björck & Mullan, 1993; Pütter & Becker, 1993).

Isolation of lactoperoxidase and lactoferrin from Gouda and Cheddar cheese whey: Discussion

Isolation of 0.023 mg/ml of lactoferrin from pasteurized sweet whey using S-sepharose and 0.088 mg/ml from dialysed whey by passing it through a column of carboxymethyl resin has been achieved by Yoshida & Xiuyun (1991). The results from our study showed a lower concentration of lactoferrin in unconcentrated whey and higher amounts in the concentrated whey. An amount of 0.09 mg/ml (same as the amount which has been reported) was present in the unconcentrated whey, that means 9 times concentration of whey should have resulted in 9 times the amount of lactoferrin in the concentrated whey i.e. about 0.8 mg/ml. The fact that only about 0.4 mg/ml was found in the concentrated whey means that concentration caused losses of some of the lactoferrin. Statistically there were no significant differences in lactoferrin content between Gouda and Cheddar cheese whey concentrate isolated by using the cation exchange membrane. The reported lactoferrin content in milk ranges from 0.03-0.49 mg/ml (Kawakami *et al.*, 1987) of which about 0.03-0.150 mg/ml is retained in whey. By comparing with the literature, it appeared that heat treatment and other industrial processing parameters during cheese manufacturing caused no significant reduction (denaturation) of lactoferrin. Sánchez *et al.* (1992) reported that the widely used pasteurization temperatures of 72-74°C for 15 s had practically no effect on lactoferrin structure, the phenomena being explained by the stability of lactoferrin to heat treatment when saturated with iron, suggesting that the two lobes of lactoferrin present different thermoresistance. The suggested mechanism by which lactoferrin exert its heat resistance is that the binding of iron to lactoferrin causes a conformational change, the molecule becoming more compact. The iron enters into the open interdomain cleft in each lobe and then the domains close over the iron atom. These changes in lactoferrin structure explain why iron-saturated lactoferrin is more resistant to denaturation and proteolysis than the apo form (especially the C-lobe which is more compact than the N-lobe) (Sánchez *et al.*, 1992). The thermal stability of lactoferrin also depends on both ionic strength and the pH of the medium. The iron-binding ability of lactoferrin has been said to be retained more than 85 % after heating at an ionic strength of 0.01 and temperature of 65-90 °C. At an ionic strength of 0.1,

the iron binding ability decreased markedly with increasing temperature (Kawakami, Tatsumi & Dosako, 1992).

The lactoferrin fractions obtained with both methods were rather pure but the lactoperoxidase fractions from both methods appeared to be contaminated with small amounts of minor whey proteins, presumably light chains of immunoglobulins (Wang & Hurley, 1998) as shown by the SDS-PAGE profile. There are two types of hydrophobic ions interaction that are not suitable in ion exchangers. The first type is the one which has low hydration energy and they form minimal hydration spheres that results in their tendency to be retained within the ion exchangers via both Coulombic and van der Waals adsorption interaction (Wetzel, Pohl & Riviello, 1985; Kaliszan, 1987). The interaction is distorted by introducing a charged solution (eluting salt solution). It is likely that the contaminants found in lactoperoxidase fractions were due to these forces (Coulombic and van der Waals) and therefore eluted together with lactoperoxidase fractions after introduction of eluting salt solution. Another reason of contamination of lactoperoxidase fractions might be the localisation of charges (positively and negatively charged domains/regions) on the proteins which thus interacted with the ion exchanger (Cachia & Hodges, 1991). The loosely or unbound whey proteins, possibly blood serum albumin (~68 kDa), β -lactoglobulin (~18 kDa) and/or α -lactalbumin (~14 kDa), passed the membrane or chromatography column during loading or during washing (Fox & MacSweeney, 1998; Wang & Hurley, 1998) as they were not shown by SDS-PAGE profile.

6 CONCLUSIONS AND RECOMMENDATIONS

Although whey contains valuable nutrients, people have been faced for centuries with the necessity of getting rid of it without question of recompense for handling and other costs. This was because no suitable and profitable processes were available for the recovery of its constituents. Only in recent years has the situation begun to change whereby new technologies have been developed to isolate high quality whey proteins. New uses have been found for whey protein isolates which means that whey can not be considered a waste by-product any more.

The use of available technologies, the recognition of valuable nutrients in whey and nutritional awareness of consumers, create an opportunity for dairy industries to transform the regarded waste by-product into worthwhile products.

This study has shown that all samples of Gouda cheese whey concentrate and Cheddar cheese whey concentrate contained large amounts of lactoperoxidase and lactoferrin, whereas non-concentrated Gouda cheese whey contained smaller amounts of lactoperoxidase and lactoferrin. This study revealed that whey from cheese factories is a potential source of lactoperoxidase and lactoferrin for pharmaceutical and industrial use.

Concentrated whey contained large amounts of lactoperoxidase and lactoferrin as compared to non-concentrated whey. For the purpose of handling, storage and transport it would be economical to encourage whey concentration if whey would be used as a source of lactoperoxidase and lactoferrin. This would reduce bulkiness of whey if the whey has to be transported from one point to another although some of the lactoperoxidase and lactoferrin is lost during concentration. On the other hand the technique to be employed must be thoroughly investigated in terms of technical aspects and operation costs. The study has shown that to elute the bound protein from the chromatography matrix large amounts of eluent

is to be used as compared to the cation exchange membrane method. Since large amounts of eluent is used in the chromatographic method, the time required to get the component of interest, in this case lactoperoxidase and lactoferrin, is also relatively longer as compared to the cation exchange membrane method. Another advantage offered by the cation exchange membrane method is its simplicity and compatibility with simple syringes, peristaltic pumps or existing work-stations, so that no time is lost in changing from a traditional chromatography column to the new membrane ion exchangers.

Even though the yield of lactoperoxidase and lactoferrin from both methods did not differ, the above mentioned reasons makes the cation exchange membrane method to be superior for isolating these components. This will be possible if the initial raw materials to be loaded are clearly clarified because in the cation exchange membrane method the sample enters directly into the membrane which means that if the sample is not properly clarified, the membrane is likely to block, thus causing the building-up of the pressure inside the membrane housing and thus forcing the sample or raw materials backwards. Unlike in chromatography, the presence of the screen reduces the chances of solid particles entering into the column matrix.

It would be interesting to establish the quantity of lactoperoxidase and lactoferrin in whey powder from dairy factories. This will be aimed at trying to reduce bulkiness as well as trying to eliminate low temperature storage of the raw material (whey) and this will have an impact on production costs.

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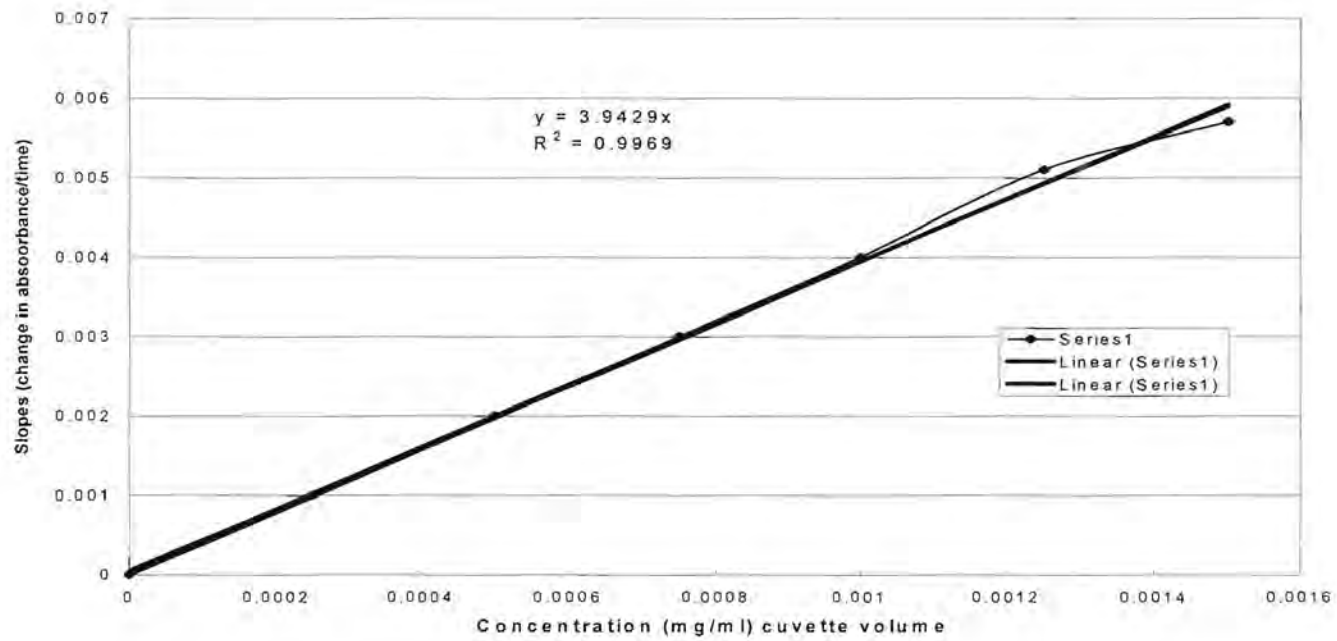
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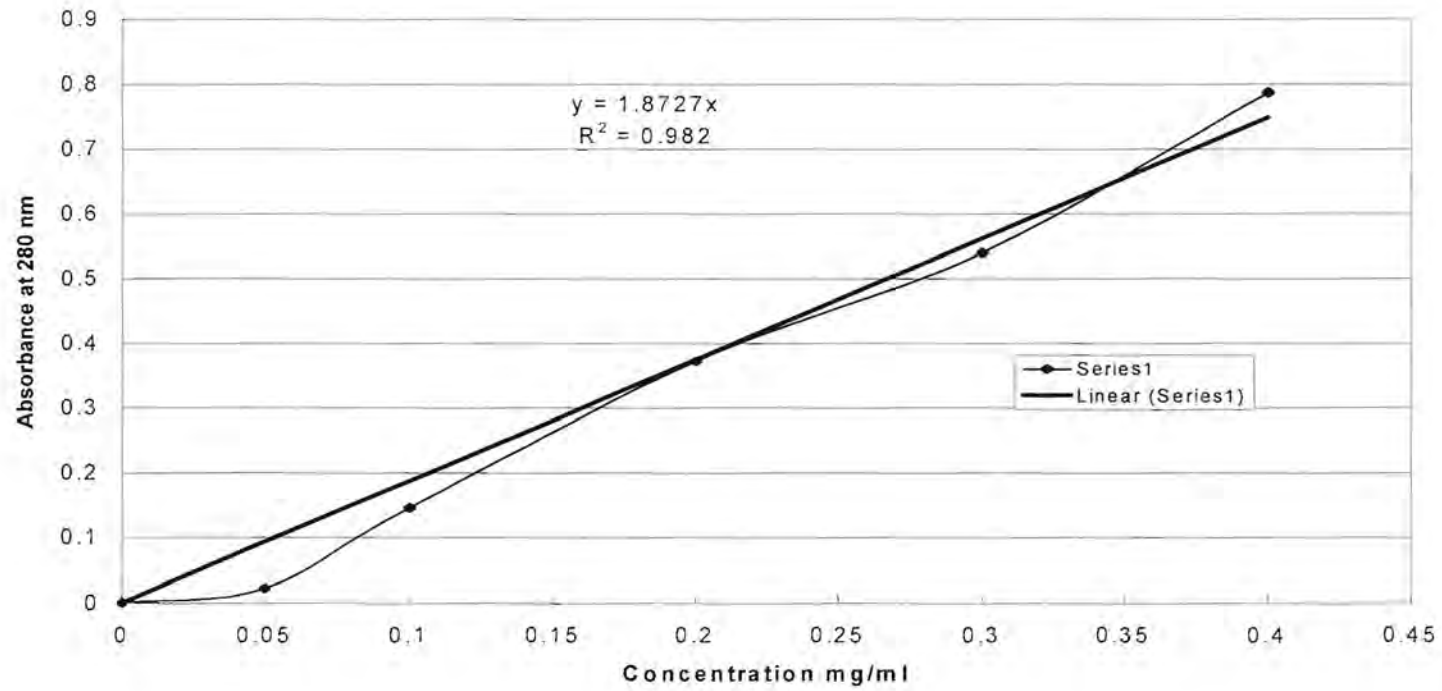
Isolation of lactoperoxidase and lactoferrin from whey: Appendix

Appendix A: Lactoperoxidase standard curve



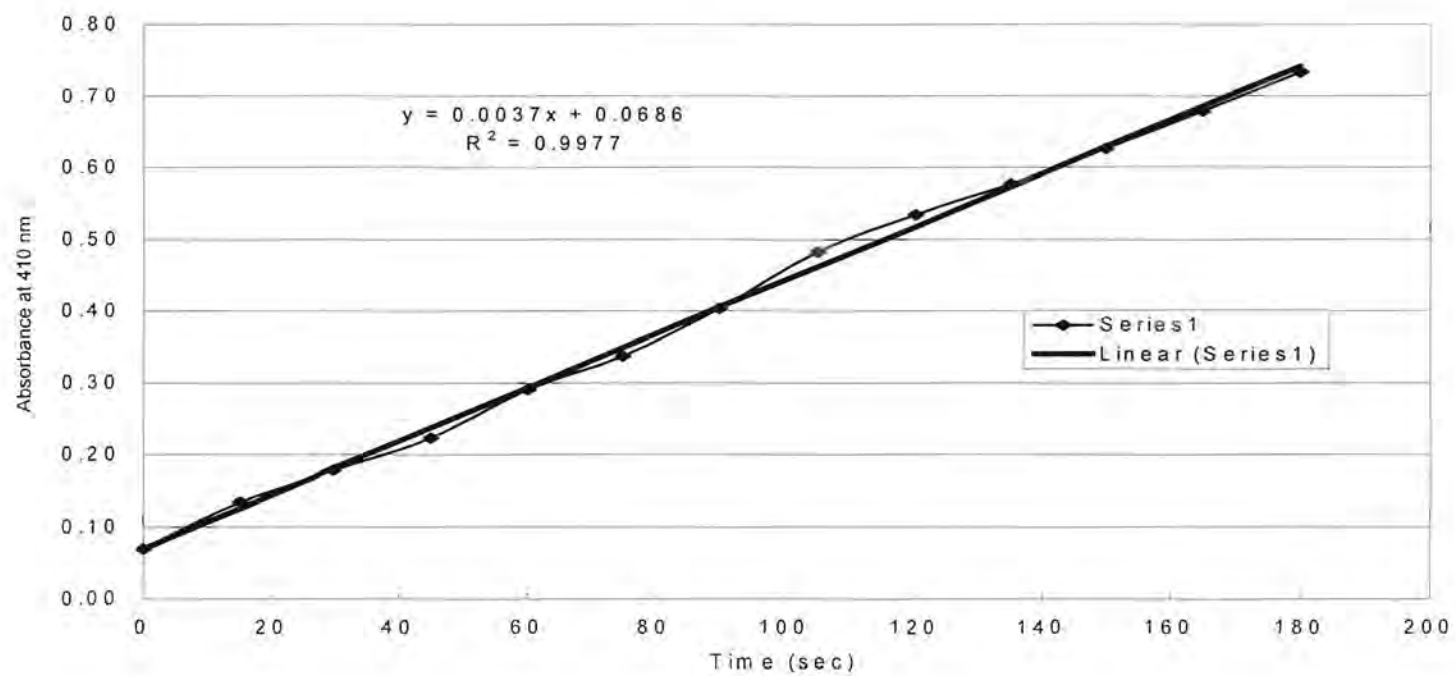
Isolation of lactoperoxidase and lactoferrin from whey: Appendix

Appendix B: Lactoferrin standard curve



Isolation of lactoperoxidase and lactoferrin from whey. Appendix

Appendix C: Oxidation of ABTS by lactoperoxidase



Appendix D: Example of lactoperoxidase calculation

$$\text{Concentration of LP (mg/ml) of whey} = \frac{X * df_1 * df_2}{V_o}$$

(1) slope from Appendix C = 0.0037

(2) $X = 0.00094$ mg/ml (from the standard graph)

(3) $V_o = 5$ ml

(4) $df_1 = 20$; since V_o was diluted with phosphate buffer to 100 ml

(5) $df_2 = 50$; since 40 μ l of sample was added to 2 ml phosphate buffer in a reaction cuvette

Therefore LP concentration (mg/ml) of whey = 0.1877