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Solvent Free Extraction of Bioactives - Process development

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Abstract

The aim of this project was to develop a laboratory scale, cost-effective process to produce:

a) Bovine serum albumin (BSA) at >80% purity from bovine plasma. The research identified an ion exchange chromatographic resin with selectivity for BSA and capable of producing BSA to a purity of >95%. The technology also produced an additional IgG enhanced fraction with an IgG composition of >45%.

b) Chondroitin sulphate (CS) at > 80% purity using selected connective tissues. The research resulted in identifying an enzyme capable of hydrolysing, solubilising and extracting CS from the collagen matrix in minced trachea within a 4 hour period. An ion exchange resin with selectivity for chondroitin sulphate and a membrane capable of purifying and concentrating CS to >80% was also identified.

Implementation of the technology will provide industry with opportunities to significantly recover value from co-product streams such as bovine plasma by producing a high purity BSA product and an IgG enhanced fraction, which could provide the industry with income greater than the current income raised by selling blood for rendering.

Implementation of the same technology will also provide the industry with additional opportunities to recover value from trachea by producing a high purity CS product, which will also provide the industry with income greater than the current income through selling trachea for rendering

Executive summary

In recent years there has been an expansion in the bioactive (neutraceutical, pharmaceutical and functional food) industries due to the increased awareness among the general public about illnesses such as diabetes, Alzheimer's disease, cancer and osteoarthritis. Waste streams from food and agricultural industries are now being utilised more in downstream processing to recover the bioactive components which are reported to be beneficial to human and animal health. These bioactives add value to the products and thereby are beneficial for the economic growth of the industries. In the meat and livestock industry, one of the major waste products is animal cartilage. This waste contains chondroitin sulphate (CS), a bioactive component found naturally in animal cartilage. It is reported to be used in the treatment of arthritis and to have natural anti-inflammatory properties. As a result, large-scale recovery of bovine trachea from bovine offal streams has been implemented in meat industries.

Meat and Livestock Australia (MLA) has estimated that the production of major meat derived bioactives could add a net benefit of ~ $30 million per annum to the meat industry. However, the capital and operating costs to develop a bioactive industry is too expensive due to the cost of current manufacturing processes. Methods used now for industrially fractionating plasma into its components have involved complex series of steps such as the Cohn fractionation method and its many variations. These methods involve the use of solvents such as ethanol and include heating steps to precipitate undesirable protein components. Current processes are labour intensive and costly and the recovery of total proteins as functional products is low. In the case of Chondroitin Sulphate, the majority of the CS in the global market is manufactured by expensive and hazardous solvent precipitation methods. Hence, the development and use of safe and cost-effective technology for the production of CS is of economic importance to the industry.

The aim of this project was to develop a laboratory scale, cost-effective process to produce BSA at > 80% purity from bovine plasma and CS at > 80% purity using selected connective tissues. The technology developed at laboratory scale will be translated and demonstrated as a proof of concept at pilot scale at a later stage.

Literature and patent review indicated that membrane technology could be utilised to fractionate bovine serum albumin (BSA) from plasma, but most of the work carried out by researchers used model systems with low protein concentrations or specialised membranes. The literature and patent review also indicated that alkaline hydrolysis was not a suitable method to solubilise cartilage due to the generation of large quantities of salt and potential deterioration of the extracted CS. Similarly, the use of ethanol to

precipitate CS is also not recommended due to the additional capital and operating costs associated with the flammable and explosive nature of the solvents.

This project was aimed to use Chromatographic separation membranes/resins that are commercially available for future scale up. Four polymeric ultrafiltration membranes, 100 kDa polyvinyl difluoride (PVDF), 100 kDa regenerated cellulose (RC), 100 kDa polyether sulfone (PES) and 300 kDa PES, and three ceramic microfiltration membranes of 0.1 µm, 0.2 µm, 0.5 µm molecular weight cut-off (MWCO) were evaluated for selective separation of BSA. The effect of BSA concentration in the feed between 1.5 and 26.3 mg/ml, plasma pH between 7.8 and 4.6, and conductivity between 0.21 and 13.6 mS/cm on the separation efficiency of the membranes was investigated. Under all tested conditions selective separation of BSA from bovine plasma was not achieved and all the membranes demonstrated very poor flow characteristics due to excessive protein fouling of the membranes because of the higher concentration of proteins in the plasma compared to the model systems used by researchers.

The experimental program identified a cost effective chromatographic resin based on binding capacity for BSA. The laboratory trials produced a BSA product of 97% purity and this purity was comparable to a Sigma standard analysed by ultra performance liquid chromatography (UPLC) and SDS-PAGE profile. The second stream from the chromatographic column produced an IgG enhanced fraction with 47% IgG content.

A further advantage of the chromatographic approach is that the process to separate CS from cartilage has also identified the same resin as the optimum adsorbent. This provides opportunities to produce 3 different products (BSA, IgG enhanced fraction and CS) using the same capital investment, hence reducing commercial risks.

The cost of production (COP) for a commercial plant projected at producing 26 tonnes/year of BSA and 88 tonnes/year of IgG enhanced fraction as a dried powder using chromatography was $26.8/kg of BSA and $2.16/kg of IgG enhanced fraction. The equivalent COP of BSA using the heat shock method was estimated at $51.50/kg of BSA. The COP based on producing 114 tonnes/year of total protein (BSA and IgG) using chromatography was estimated at $7.80/kg of protein. A disadvantage of the heat shock method is the denaturation of IgG and other plasma proteins, limiting the process to the production of BSA as a functional ingredient. The COP included the current income of selling blood at $0.15/kg as an opportunity costs. The two main contributors to the total cost associated with the COP using chromatography were opportunity and labour costs.

The cost of production (COP) of CS (as a dry powder) for a commercial plant projected at producing 49 tonnes/year was $20/kg using chromatography and $13/kg using membranes compared to ethanol

precipitation which was estimated at $70/kg. The COP included the current income of selling cartilage at $0.2/kg as an opportunity costs. The two main costs associated with the COP of the chromatographic and the membrane technology were the opportunity and labour cost. In the ethanol process, 80% of the costs were estimated to be associated with loss of ethanol during processing and in the recovery of ethanol.

This project has demonstrated, at laboratory scale, the technical feasibility and cost effectiveness of chromatographic technology to produce high purity BSA (97% purity) and an IgG enhanced fraction (47% IgG) from bovine plasma and use of membrane technology to produce CS at purity >80% from bovine cartilage.

Therefore, considering the favourable laboratory experimental results, the analysis of COP of producing BSA, IgG enhanced fractions and CS and the potential gain to the industry, further development of the technology aimed at achieving future commercialisation of the technology is recommended. The recommendations include:

• The technology should be further developed to pilot scale which will provide information to make decisions regarding investments into the future:

- Establish scaled up design and operating parameters for the process.
- Validate consistency of the process and of product specification.
- Produce prototype samples for customer evaluation.
- Refine the preliminary COP using data generated at pilot scale.
- Projected estimate of the capital cost of a commercial scale process using data generated at pilot scale.
- Evaluate the economic feasibility of a commercial process using data generated at pilot scale.

• Conduct functionality tests on the BSA fraction to compare the efficacy of current products in the market with the products produced using the proposed technology.

• Conduct bioactivity studies (*in vitro* and *in vivo*) of the IgG enhanced fraction to compare the efficacy of current products in the market with the products produced using the proposed technology.

• Develop technology to further purify IgG as an active ingredient and broaden the product portfolio.

• The use of ethanol to precipitate proteins was not pursued and is not recommended due to the additional capital and operating costs associated with the flammable and explosive nature of the solvents, in addition to the loss of the precipitated proteins.

• Based on the laboratory data, the COP of producing CS using membranes is lower compared to chromatography, but this should be considered in the overall context after including and comparing the capital costs of the two approaches, the products produced and income generated by the technologies.

• The patent by Zheng and Lu (2008), a Chinese patent (CN101280027, 2008) should be scrutinised further if commercialisation of separating CS using a membrane approach is pursued.

• Conduct further research to investigate and compare the efficacy of products in the market with the products produced using the proposed technology, e.g., biochemical characterisation of the products, assessment of anti-inflammatory effects using in vitro assays and in vivo studies to determine the efficacy of orally delivered CS to relieve symptoms of osteoarthritis.

• Characterise and quantify the hydrolysed collagen stream, which is a co-product stream in both the chromatographic and membrane approach.

• Develop technology to recover the hydrolysed collagen, as a novel bioactive product that could provide an additional income stream.

PART A: Condroitin sulphate

Contents

Background and Introduction

Background

The sustainability and development of the meat processing industries is constrained by a focus on meat cuts. Insufficient value is gained from the remaining majority of the animal which is usually processed as render (normally around $0.10/kg). The overall project is designed to add value to meat processing co-product streams by developing innovative cost-effective separation technologies to produce chondroitin sulphate (CS) and bovine serum albumin (BSA) as bioactive molecules for use as dietary supplements, nutraceuticals, cosmeceuticals or functional food ingredients. MLA has estimated that the production of 5 major meat derived bioactives could add a net benefit of ~$30m pa to the meat industry. The major impediments to elicit meat industry investment to develop a bioactive industry have been the capital costs of the technology and its related operating costs.

The majority of CS in the global market is manufactured by solvent precipitation methods which are expensive and hazardous. Similarly, BSA in the global market is manufactured by solvent extraction and heat precipitating methods. In the pharmaceutical industry, high purity BSA is manufactured using a combination of many different chromatographic methods. The solvent extraction and precipitation methods are expensive due to the cost of solvent recovery for re–use. The chromatographic resins used in the pharmaceutical industry are also very costly.

The development and use of cost-effective technology for the production of CS and BSA will make it viable for the meat industry to invest in developing a bioactives industry and help improve the sustainability of the meat industry and provide the impetus to grow additional income.

This project will build upon the knowledge and expertise developed and implemented in the dairy industry for protein separation using ion exchange chromatography, membrane separation and, thermal and acid/base reaction systems. In addition, the knowledge and experience developed in enzyme technology to digest proteins and carbohydrates and the application of the technology to connective tissues to extract marine CS will also be utilised.

This report details the activities and experimental results from the programme undertaken to develop a process to separate CS from bovine plasma and details of the program undertaken to separate BSA will be provided in a separate reported.

Introduction

Cartilage is a flexible connective tissue in humans and animals. Collagen, a protein in the cartilage tissues is associated with proteoglycans which consist of a core protein covalently linked to glycosaminoglycans (GAGs). CS is a member of the glycosaminoglycan family of long chained unbranched polysaccharides consisting of repeating disaccharide units. CS is a sulphated GAG consisting of alternating sugars of N-acetylgalactosamine and glucuronic acid and is attached to proteins through the hydroxyl groups on the serine residues, as proteoglycans.

Proteins or polypeptides are composed of amino acids, which are joined together by peptide bonds to form three dimensional protein structures. Proteins can be degraded to form smaller peptide chains or amino acids through hydrolysis that cleaves the peptide bonds. The hydrolysis of protein can be achieved either with acid or with proteolytic enzymes that cleave specific peptide bonds. Therefore, extracting CS from connective tissues in cartilage using hydrolysis requires the initial solubilisation of the tissue matrix into a liquid form and cleaving of CS from the core-protein chains.

Enzymes are high molecular weight proteins, which play a vital role in physiological processes. Certain proteases are very specific in the peptide bonds that they cleave, while others are non-specific and could completely hydrolyse proteins to amino acids. Generally there are two classes of protease enzymes, namely exopeptidases and endopeptidases. The exopeptidases cleave peptide bonds from the ends of the protein molecule; therefore they are likely to produce free amino acids while the endopeptidase cleaves peptide bonds within the protein molecule, thus producing smaller peptides rather than free amino acids. Exopeptidases are further classified as carboxypeptidases as they hydrolyse amino acids on the carboxy end of the protein and aminopeptidases hydrolyse amino acids at the amino end of the protein.

The activity of proteases is dependent on pH, reaction temperature, substrate and the pre-treatment the substrate receives before hydrolysis. Similarly, the stability of the enzyme is dependent on temperature and pH. The operating conditions of the enzyme reaction are generally based on a compromise between stability and activity of the enzyme in the substrate.

In addition, each enzyme has its own inactivation conditions of temperature, heat hold time and pH.

Ultrasonication is a new technology utilised in recent years in extraction processes, to extract intracellular compounds. Ultrasonic waves generating cavitations disrupt tissues and facilitate the release of matrix components. Ultrasound can also help to intensify the effects of enzyme treatment, and by this reduce the amount of enzyme needed or increase the yield of extractable compounds.

Project objectives

The overall aim of the project is to develop a cost effective laboratory scale process to produce CS at > 80% purity using selected connective tissues, and scaling up the process to produce a gram quantity sample at preparative laboratory scale.

Achieving the objective requires the design and conducting experiments to develop methods to cleave the proteins so that the cartilage matrix is destroyed to assist the solubilisation of the trachea and releasing CS. The separation of CS from the other breakdown products in the solution requires the design and conducting experiments to identify techniques with potential to be scaled up to commercial scale in a safe and economic manner.

The objective of this part of the project was to investigate and select promising extraction and separation technologies and develop laboratory based process to produce CS at >80% from connective tissue. A preliminary analysis of the cost of production of CS was also to be assessed.

Literature and patent review

The methodology used for the literature and patent search is detailed in Appendix 1.

Scientific literature

Cartilage is a tissue formed by a matrix of collagen associated with proteoglycans. The most commonly found proteoglycan in cartilaginous tissues is Aggrecan. This consist of a core protein with approximately 230 kDa molecular mass. GAG chains, which includes approximately 100 CS chains with a molecular weight of 10-25 kDa, 30-60 keratan sulphate chains of 3-15 kDa and *N*- and *O*- linked oligosaccharides are covalently attached to this core protein (Watanabe *et al.,* 1998; Kiani, *et al.,* 2002). GAGs are sulphated polysaccharides and, are alternating copolymers of uronic acids and amino sugars (Volpi and Bolognani, 1993).

Cartilagenous tissues are major sources of CS which is a polymer consisting entirely of D-glucuronosyl-N-acetylgalactosamine repeating disaccharide units, in which the galactosamine is sulphated at C4 or C6 and hence negatively charged. In contrast, most galactosaminoglycans in non-cartilaginous connective tissues (e.g. skin and tendon) are CS/DS (Dermatan Sulphate) copolymers comprised of varying proportions of D-glucuronosyl-N-acetylgalactosamine and L-iduronosyl-N-acetylgalactosamine (Nakano, *et al.,* 2010).

Cartilaginous tissues are considered valuable by–products in the meat and fishery industries. CS is extracted from various natural sources, including shark, chicken and bovine cartilage. CS in the cartilaginous tissues has been reported to have a wide range of health associated properties. Osteoarthritis is the most common form of joint disease, resulting from degradation of cartilage tissues and loss of proteoglycans. Tissue degeneration process (eg. osteoarthritis) is improved, in humans and in animals such as horses and dogs, by supplementing with CS (Deal and Moskowitz, 1999; Jordan, 2003; Volpi, N. 2004; Zang, 2007). CS is also reported to preserve and maintain articular cartilage (Bali *et al.* 2001) and is also regarded as an alimentary preservative with emulsifying properties (Hamano *et al.* 1989).

1.1.1 Extraction of Chondroitin Sulphate

Several researchers have investigated the methods of CS extraction and purification. In general, the method includes the following steps: cartilage hydrolysis (with strong alkalis or proteases)

which releases CS, ethanolic precipitation of the hydrolysates and treatment of the redissolved precipitate (with ion-exchange resins or by dialysis) in order to eliminate remaining peptides and salts (Murado *et al.,* (2010)). Chondroitin sulphate was first isolated from cartilage in 1884, and its constituents and structure were described by Levene in 1925.

When extracting CS from connective tissues in cartilage, the hydrolysis process initially solubilizes the tissue matrix into a liquid form and then cleaves the CS from the core-protein chains. Guanidium hydrochloride (4M) is a commonly used chemical in the laboratory for the complete extraction of proteoglycans from tissues without causing degradation of the compounds. However, a cost effective and safe process is needed and in addition, food safety is important when the end product is used for human consumption.

Several methods have been reported in the literature on extraction of chondroitin sulphate from different sources of tissues. CS can be liberated from the core protein by either enzymic or alkali treatment. Murado *et al.,* (2010) have reported that the treatment of the Skate (*Raja flavirostris)* cartilage or the hydrolysate with strong alkalis tends to deteriorate CS. The extraction process involving alkali treatment dissociates tissues and core proteins to release GAGs which are then subjected to further downstream processing for recovery. The draw-back of alkali treatment is that the down-stream processing needs large volumes of water and therefore raises environmental concerns as well as processing costs (Ashie and Pederson, WO2005037871–A1, 2005). This can be avoided by using proteolytic enzymes.

Digesting with exogenous proteolytic enzymes is the most frequently used procedure to release GAGs from tissues. Jo *et al.* (2004) screened eleven different commercial proteases for the preparation of CS from Skate cartilage. Their results showed that Maxazyme NNP (a neutral protease) was more efficient with respect to CS production (CS yield per enzyme cost). Douck-Choun *et al.* (2001) used sea cucumber as an alternative source of CS for use in the manufacture of nutraceutical products. They investigated the extraction of CS from the body wall of sea cucumber using ten different enzymes. Based on the degree of hydrolysis and the yield of CS, Alcalase was selected as the most suitable enzyme

Some authors have investigated the effectiveness of endogenous enzymes on GAG release in order to reduce the cost of using expensive exogenous enzymes. Nakano *et al.* (1998) extracted

GAG from bovine cartilaginous tissues including nasal cartilage, occipital articular cartilage and temporomandibular joint disc, without using the expensive exogenous proteinase commonly used to extract GAG from tissues. The GAG was extracted with highest yield (~80% of total tissue uronic acid) by incubating nasal cartilage in 0.1 M sodium acetate, pH 4.5, at 37°C. The GAG-peptide purified by diethylaminoethyl (DEAE) ion-exchange chromatography contained approximately 7% protein, 89% chondroitin sulphate and 4% keratane sulphate. Tissue autolysis was suggested to occur under the incubation conditions to release GAG-peptide.

1.1.2 Isolation/Purification of Chondroitin Sulphate

Ethanol precipitation

The CS needs to be recovered from the pool of tissue digest after its extraction from the connective tissues. The most common method to recover GAGs is by ethanol precipitation, although the presence of salt is necessary for complete precipitation. If the GAG concentration is high and sufficient salt is present, 4–5 volumes of ethanol is needed to completely precipitate the GAGs (Silva, 2006). Sequential precipitation with organic solvents (methanol, ethanol or propanol) is employed to isolate CS from other GAGs present in the tissue digest (Volpi, 1996). Increasing volumes of solvent fractionates the GAGs. Heparin is the first GAG to precipitate, followed by dermatan sulphate and lastly chondroitin sulphate.

Murada *et al.,* (2010) observed a high recovery of CS (99%) with increasing volumes of ethanol, but with limited purity (67-70%). This was not considered a practically feasible process due to the high consumption of alcohol by the process.

Lignot *et al.* (2003) have suggested a membrane purification step prior to ethanol precipitation, to produce CS from Skate cartilage. The authors have mentioned that the combined process of ultrafiltration followed by ethanol precipitation allows reducing solvent consumption and related costs.

Ion- exchange chromatography

GAGs are polyanionic due to the presence of sulphated groups. This property of GAGs enables the molecules to bind to anion-exchange resins. Several investigators have employed anion-exchange chromatography to isolate GAGs from tissue digests. The most common resin employed is a DEAE resin. Since GAGs differ in their average negative charge density, an elution step with a continuous salt gradient is often used to elute the bound GAGs. Residual glycoproteins elute in low salt and GAGs elute in high salt concentrations, thus better purity and separation of different classes of GAGs is achieved by this method (Silva, 2006).

Davies *et al.* (2008) purified the GAG extract (mainly consisting of Chondroitin sulphate) from bovine trachea on the Q-Sepharose column. The recovery of GAGs was 101.3 ± 5.7% with negligible protein.

A low cost method of preparation of CS for commercial purposes was developed by Nakano *et al.* (2000), to extract CS from bovine nasal cartilage without using any chemicals except acetic acid used for pH adjustment to 4.5. More than 70% CS is released as crude CS-peptide fraction by incubation at 37°C. The authors suggested that the purity can be improved by 1.4 fold by anion-exchange chromatography. The authors suggested that the preparation may be used as an ingredient for food, cosmetic or pharmaceutical products.

Membrane purification methods

Membrane purification methods are employed to remove salts and contaminating low molecular weight compounds to purify organic compounds. Membranes with pore size smaller than the molecular weight of CS (10-25 KDa) will retain the compound allowing the smaller molecules to go through the membrane.

Murado *et al.* (2010) successfully applied a combination of enzymatic and chemical hydrolysis as well as selective precipitation and ultrafiltration-diafiltration processes, to purify CS from Skate cartilage wastes. The process developed was rapid and highly efficient, with low consumption of reagents and high purity for the chondroitin sulphate (99%). In this process, in order to reduce the aggressive treatments with strong alkalis, a combined step of hydrolysis–precipitation in hydroalcoholic solution was employed.

Lignot *et al.* (2003) developed a low-cost two-step process to produce CS from Skate cartilage. The process consisted of an enzymatic extraction followed by tangential filtration to concentrate and purify CS. The performances of UF and MF membranes were compared in terms of flux and selectivity. The 0.1 μm-pore size membrane appeared to be more efficient to separate CS from other compounds.

Patents

Isolation and purification of chondroitin sulphate based on alcohol precipitation, ion-exchange chromatography and membrane separation, have been employed in novel processes.

Zhang *et al.* (CN 101904491–A, 2010) produced CS, from nose bone, throat bone, trachea, jawbone and foreign cartilages of cow or sheep, by ultrasonicating the cartilage powder in alkali. The process was followed by enzyme treatment with papain followed by neutral protease, alcohol precipitation and drying. The purity of CS produced was not less than 88%.

Huang *et al*. (CN 101358220–A, 2009) prepared CS from nasal bone of cow through synergetic reaction of natural enzyme (homogeneous pancreas of pig) and a protease (2709 alkaline protease), which was followed by alkali extraction and final precipitation with alcohol. The purity and yield of CS obtained was > 95% and > 25%, respectively.

Josse and Josset (WO2004046199–A3, 2004) have described a method to produce CS from bovine or porcine cartilage by enzyme hydrolysis (with *Bacillus licheniformis* alkaline protease) followed by ultrafiltration, dialysis and ethanol precipitation. CS with purity 97–100% and a molecular weight of 15–30 KDa was produced. The by-products from this process are peptones, and amino acids which can be used to produce pharmaceutical, cosmetic or dietetic products.

Raw material of nasal cartilages of fish, especially of salmon heads contains chondroitin sulphate. Takashi *et al.* (JP2000273102–A, 2000) have invented a method to isolate and purify CS from fish nasal cartilage, by alkali treatment and proteolytic treatment followed by ultrafiltration /diafiltration treatment. The concentrated liquid (in the retentate) was either dried as is, or the CS in the retentate was precipitated by ethanol.

A method to extract CS from shark cartilage has been patented by Wang *et al.* (2009). The method comprises the processes in the following sequence., Curing, alkalization, enzymatic hydrolysis, de–proteinization, crystallization, resin pretreatment, resin exchange, ultrafiltration dehydration, calcification of filtrate and crystallization dehydration dying. In the secondary crystallisation procedure, the covalent binding of protein and shark CS is broken by hydrogen peroxide. The authors claimed that this method improved the purity and calcium content of CS.

Song and Yan (CN1903889–A, 2007) have disclosed a method for CS extraction by alkali treatment and enzyme hydrolysis of animal soft bone to obtain the filtrate. Purification was carried out by passing the filtrate through DEAE ion exchange resin and eluting with NaCl and HCl. The eluate was dialysed and freeze-dried. There was an increase in output of CS by 150-170 mg/kg of soft bone compared to the previously known methods of extracting chondroitin sulphate, which has an output of 47-56mg/kg.

Ashie and Pedersen (WO2005037871–A1, 2005) have described a method to extract GAGs such as CS, hyaluronate and keratin sulphate from animal tissue with endoprotease (serine endopeptidase or metalloproteinase) and exoprotease (aminopeptidase or carboxypeptidase) followed by ion-exchange purification. The authors have mentioned that the yield and purity is increased by this method compared to the conventional method with endoprotease alone.

Lee and Park (KR2002096272–A, 2002) obtained CS from the organ of oxen, with improved yield. The method comprised hydrolysing the organ of oxen with pepsin enzyme followed by purification by DEAE anion exchange chromatography.

Tadashi *et al.* (JP1051509–A, 1976) described a process to prepare highly pure chondroitin sulphate from animal cartilage by alkaline or enzymic extraction and purification by adsorbing the extract onto a strongly basic anion exchange resin. The raw materials used were the cartilages of sharks, whales, cattles or pigs. The strongly basic anion exchange resin of large pore size included Amberlite HPA 10 (RTM) and Amberlite IRA 938 (RTM). Salt (NaCl) solution with concentrations 0.5 M and 1.8 M were used for elution. Sodium chondroitin sulphate containing no antigenic substance was eluted at the latter concentration.

Zheng and Lu (CN101280027–A, 2008) reported the production of CS with yield > 37% and purity > 95%, by adopting single enzymolysis technology to replace the traditional alkaline hydrolysis process, and employing ultrafiltration process to substitute for repeated alcohol precipitation.

Khare *et al.* (US20040146993–A1, 2004) invented a process of obtaining chondroitin sulphate from a feed stock that contains connective tissues (can be from bovine, ovine, equine, swine, bird and fish), with high purity (≥ 95%) and yield (> 90%). The process involved digesting the feedstock with a protease, treating the liquid digest with a reagent comprising a divalent hydroxide of an alkaline earth metal to precipitate protein impurities, separating the precipitate and processing the treated liquefied digest using a membrane to form a permeate and a retentate, wherein the retentate comprises chondroitin sulphate.

A novel method of producing and purifying Chondroitin Sulphuric acid from tissues of animal origin, mainly from porcine or bovine origin has been reported by Gérard (2004). The method consists in subjecting the cartilaginous tissues to grinding, enzymatic hydrolysis, filtration, dialysis and precipitation with an alcohol, in order to obtain purified CS in the form of a sodium salt.

Tang *et al.* (CN1486993–A, 2004) claimed that compared with available CS products, their invention produces CS with high purity, easy absorption, high bioavailability and high bioactivity. CS with molecular weight of 10-30 kDa was prepared through conventional alkali treatment, enzyme hydrolysis, superfiltering and nanofiltering membrane separation, purification and concentration.

De Paula and De Alencar (BR200105320–A, 2001) reported the production of CS from bovine trachea. The process comprised hydrolysing the cartilage by trypsin, pepsin and chymotrypsin, followed by precipitation with chitosan. The yield was very low (0.3-0.7%).

Conclusions and recommendations

Several procedures have been described in the scientific literature to extract CS from various cartilage sources and include alkaline hydrolysis, exogenous and endogenous proteolytic enzymes and use of mild acids such as acetic acid. The extraction of CS using these methods has been variable and most likely due to the different sources of cartilage and the quality of the cartilage. The use of endogenous enzymes is not covered widely in the literature, hence its effectiveness is not well established, but the use of different exogenous enzymes is widely described in the literature which suggests that the technique is efficient at extracting CS.

The use of alkaline hydrolysis has the disadvantage of requiring adjustment of pH back to around neutrality, creating a very large salt stream which needs to be disposed. In addition, it is reported that alkali tends to deteriorate CS, hence its effect on structure and efficacy is not known.

There were no studies reported in the scientific literature with the use of ultrasonication to weaken or break the cartilage matrix to assist in solubilising the cartilage, but Zhang *et al.* (CN 101904491–A, 2010) reported the use of ultrasonication with base hydrolysis in their patent.

Ethanol precipitation is widely reported in the literature to separate CS from the solubilised cartilage solution. In the ethanol precipitating technique, 4-5 volumes of ethanol are required to completely precipitate the GAGs. The use of this large volume of ethanol, requiring technology to recover and re-use the ethanol will significantly add to the cost of production. In addition, all the equipment used in the plant will need to be flame proof, in addition to the need for special flame proof electrical wiring of the plant. The safety aspects of using ethanol in the production environment will require strict operating procedures, further increasing the cost of production.

The other laboratory techniques employed by researchers to separate CS from the cartilage hydrolysate have been chromatography, membrane separation or a combination of these two techniques with ethanol precipitation. Chromatography and membrane separation are mild and safe techniques that could easily be scaled up to commercial scale.

Most of the relevant patents on producing CS from cartilage involve the use of alkali treatment and precipitation of CS using ethanol. The methods employed in the patent literature using

exogenous enzymes followed by ion exchange chromatography include the patents by Song and Yan (CN1903889–A, 2007), Ashie and Pedersen (WO2005037871–A1, 2005), Lee and Park (KR2002096272–A, 2002) and Tadashi *et al.* (JP1051509–A, 1976). Since the patent by Tadashi *et al.* (JP1051509–A, 1976) pre-dates the other patents by ~35 years, the validity of these patents are questionable and needs further analysis by a patent attorney.

The patent by Zheng and Lu (CN101280027–A, 2008), a Chinese patent, is based on enzyme hydrolysis to replace the traditional alkaline hydrolysis and use of ultrafiltration to substitute for repeated alcohol precipitation. The patent abstract does not mention any methods for pre-filtering the hydrolysate before ultrafiltration such as use of coarse filtration followed by microfiltration. This patent needs further scrutiny if commercialisation of CS separating using a membrane approach is to be considered.

Methodology – Solubilising bovine trachea by hydrolysis

Materials and equipment

The materials and equipment used for the experimental program included:

Minced trachea (Wagstaff Cranbourne Pty. Ltd., Cranbourne, Victoria), Branson ultrasonicator probe (400 W power and 20 KHz frequency, Emerson Industrial Automation, Conneticut, USA), Blackstone NEY ultrasonicator (Blackstone-NEY Ultrasonics,Jamestown, NY), portable pH meter (TPS Pty Ltd, Australia), temperature controlled shaking water bath (Grant Instruments, Cambridgshire, UK), HR73 Halogen Moisture Analyser (Mettler Toledo, Columbus, OH, USA), microplate reader (Varioskan, Thermoscientific, Flash, USA), 96 well plates (Nunc, Roskilde, Denmark), eppendorf centrifuge 5417R (Hamburg, Germany).

Fluorescamine (4-phenylspirol[furan-2(3H), 1'-phthalan]- 3, 3' –dione;), glacial acetic acid glacial, Sodium tetraborate decahydrate-Borax, Gly-Gly-Gly, and Chondroitin Sulphate A sodium salt from bovine trachea were all purchased from (Sigma-Aldrich, St Louis, Missouri, USA), Blyscan kit (Biocolor Life Science, Northern Ireland, UK), Acetone (Merck, Germany).

Methods

1.1.3 Cleaning and processing bovine trachea

Fresh bovine trachea (4.5 kg) kindly donated by Wagstaff Cranbourne Pty. Ltd., Cranbourne, Victoria, was received at CSIRO Food and Nutritional Sciences (CFNS), Werribee, Victoria, from the Abattoir and was stored overnight at 4°C. The next day the trachea was washed thoroughly in hot water. The membranes and fat attached to the trachea were removed. The cleaned trachea was chopped into small pieces (~ 2cm^2) and minced with a meat mincer. The minced trachea was aliquoted into 200 g lots and stored frozen at -20°C until further use. The trachea used for the experimental procedures is shown in Figure 4.1.

Figure 4.1 Fresh trachea on arrival at CFNS (left), cleaned trachea (centre) and minced trachea (right).

1.1.4 Extraction of chondroitin sulphate from bovine trachea by acid hydrolysis without ultrasonication

The frozen minced bovine trachea were thawed overnight at 4°C. Water was prepared at pH 4.5 by adding acetic acid and pre-heated to 37°C. To produce minced trachea slurry, pre-heated water (pH 4.5) was added at a ratio 1:4 (w/w), mixed well, and pH was re-adjusted to 4.5 with acetic acid. The mixture was incubated at 37°C in a shaking water bath for 24 hours (the pH was checked periodically and re-adjusted to 4.5 with acetic acid). A 'control' sample was prepared in the same manner, except that the pH of the water was not adjusted to 4.5. During incubation, 2 mL aliquots were removed at the start (as soon as the water was added to the minced trachea) and every 30 minutes. The 2 mL aliquots were transferred into two eppendorf tubes and centrifuged for 15 minutes at 10,000g and a temperature of 4°C. The clear mid-supernatants were collected carefully, without disturbing the 'top' fat layer, filtered through a 0.2µM filter and analysed for Glycosaminoglycans (GAGs) by the Blyscan assay method.

1.1.5 Extraction of chondroitin sulphate from bovine trachea by acid hydrolysis with ultrasonication

Pulsed ultrasonication during acid hydrolysis

The frozen minced bovine trachea were thawed and minced trachea slurry prepared as described above. The schott bottles containing the mixtures were placed on the ultrasonication equipment (Blackstone NEY, Jamestown, NY) and incubated at 37°C in a shaking water bath for 22 hours (the pH was checked periodically and re-adjusted to 4.5 with acetic acid). A 'control' sample was prepared in the same manner, except that the pH of the water was not adjusted to 4.5.

During the incubation at 37°C, ultrasonication was applied for 2 minutes every hour (one hour off and 2 minutes on) for 5 hours, at 40 KHz frequency and maximum power of the ultrasonication unit. Incubation at 37°C continued overnight (without the pulse ultrasonication) for 17 hours. In total, the incubation time was 22 hours. Four millilitre aliquots were removed, at the start (as soon as the water was added to the minced trachea), immediately after the '2 minute' ultrasonication and 30 minutes after the ultrasonication, for 5 hours. Aliquots were also removed at the end of incubation period (i.e., after 22 hours). The aliquots were transferred into eppendorf tubes and centrifuged immediately for 15 minutes at 10,000g at a temperature of 4°C. The clear mid-supernatant collected carefully, without disturbing the 'top' fat layer, filtered through 0.2μM filter and analysed for Glycosaminoglycans (GAGs) by the Blyscan assay method.

Ultrasonic pre-treatment of minced trachea

The frozen minced bovine trachea were thawed overnight at 4°C and three different treatments were carried out, which included two controls. Ultrasonication was performed with the Bronson ultrasonicator probe (400W power and 20KHz frequency). During ultrasonication the trachea slurry was kept in an ice bath to avoid a temperature rise. The temperature of the slurry was measured before and after ultrasonication. In the first treatment, the minced trachea and water were mixed at a ratio 1:4 (w/w), ultrasonicated for 90 seconds and the pH was adjusted to 4.5 with acetic acid. In the second treatment, the minced trachea and water were mixed at a ratio 1:4 (w/w), pH was adjusted to 4.5 with acetic acid, ultrasonicated for 90 seconds and the pH was re-adjusted to 4.5. In the 'control' sample, the minced trachea and water were mixed at a ratio 1:4 (w/w) and ultrasonicated for 90 seconds but the pH was not adjusted to 4.5.

Soon after ultrasonication, 4 mL aliquots were removed and the mixtures were incubated at 37°C in a shaking water bath for 22 hours. The pH was checked periodically and re-adjusted to 4.5 with acetic acid. Four millilitre aliquots were removed into eppendorf tubes soon after the temperature of the mix reached 37°C and thereafter every 30 minutes. The aliquots were centrifuged immediately for 15 minutes at 10, 000g at a temperature of 4°C and clear mid-supernatant was carefully removed, filtered through a 0.2μM filter and analysed for Glycosaminoglycans (GAGs) by the Blyscan assay.

1.1.6 Extraction of chondroitin sulphate from bovine trachea by enzyme hydrolysis without ultrasonication

The enzymes used for this experiment were Promod 648L (Biocatalysts), Multifect Neutral (Enzyme solution), Protamex (Novozyme) and Alcalase 2.4L (Novozyme), are listed in Table 4.1 with detailed information on each of the enzymes. These enzymes are approved as food-grade enzymes.

Table 4.1 Properties of enzymes used for extraction of chondroitin sulphate from minced bovine trachea.

Enzyme Name	Origin	Activity	Optimum pH/temp	Description	Supplier	Price
Promod648L	Mixed strains mainly *Bacillus*	235 Casein protease units/gram	Working pH 5-8 Temp range 55°C-65°C Enzyme inactivation, at 90°C for 10 minutes or 85°C for 30 minutes **Trial conditions**: 60°C, pH 7.0, 0.5%(w/w)	• Proteinase blend from plant and bacterial sources used in the extraction of Chondroitin Sulphate from collagenous materials. • A dose of 0.5% w/w on the cartilage is recommended. Incubate in pH 5.5 sodium acetate buffer or water alone, at 65°C for 16 hours	Biocatalysts (Parc Nantgarw, Wales)	$189/Kg (5 Kg pack)
Multifect neutral	*Bacillus amyloliquefaciens*	>1600 AU (azo units)/g (based on azo-casein substrate)	Most effective at operating pH 6-8 (optimum of pH 7.0) Temperature range of 40°C – 60°C Enzyme inactivation, at 80°C for 3-7 minutes or adjusting the pH below 3.0 and holding at > 50°C for 30 minutes	• Metallo neutral endopeptidase. • Hydrolyses a broad range of substrates at neutral pH. • Typical dosage levels range	Enzyme Solutions (Victoria, Australia) / Genencor International (Rochester, NY 14623 USA)	$45/Kg for 28Kg pail. $65/Kg for 5Kg pail

Enzyme Name	Origin	Activity	Optimum pH/temp	Description	Supplier	Price
				from 0.1% - 1.0%		
			Trial conditions: 55°C, pH 7.0, 0.5% (w/w)			
Protamex®	*Bacillus licheniformis* and *Bacillus amyloliquefaciens*	1.5 AU-N/g	pH optimum is 6-8 Temperature optimum is 50°C-65°C. Hydrolysis temp. should be 65°C for good quality Protease inactivation occurs at 85°C for 15 minutes natural pH or instantly at pH 3.0 **Trial conditions:** 60°C, pH 7.0, 0.5% (w/w)	• Used for making animal and protein hydrolysates in the pet food industry. • Recommended dosage 1.0-2.0 g/kg of raw material • Protamex is designed to give optimum hydrolysis and flavour at neutral pH	Novozyme (Bagsvaerd Denmark)	Protamax 1.5 $61/Kg

Enzyme Name	Origin	Activity	Optimum pH/temp	Description	Supplier	Price
ALCALASE® 2.4L FG	*Bacillus licheniformis*	2.4 AU-A/g	pH optimum is 6.5 – 8.5 Temperature optimum Is 55°C-70°C (60°C) Inactivation in 10 minutes at 85°C or higher when pH is 8.0. Also by heating for 30 minutes at 50°C or higher when pH is 4.0 **Trial conditions:** 55°C, pH 8.0, 0.5% (w/w). Inactivated at 90°C for 10 minutes	• Serine endopeptidase. • Enzyme dosage on whey or soy protein is 0.51%	Novozyme (Bagsvaerd Denmark)	$39/Kg

AU-N/g and AU-A/g – activity units per g

The frozen minced bovine trachea was thawed overnight at 4°C and three different treatments were carried out, which included two controls. To the minced trachea, water was added at a ratio 1:4 (w/w), mixed well, and pH was adjusted (to the optimum pH of the enzyme, recommended by the supplier). The schott bottles containing the mixtures were placed in the shaking water bath until the temperature reached the optimum temperature when the enzyme (0.5% w/w) was added. A 'control' sample was prepared in the same manner, except that enzyme was not added. A second 'control' sample was prepared which contained enzyme only in water and adjusted to the desirable pH.

The solutions were incubated at the appropriate temperatures for 24 hours (pH was checked periodically and re-adjusted). Aliquots of 4 ml were removed at the start as soon as the mixtures reached the temperature before enzyme addition (0 hour). After enzyme addition, aliquots were collected into eppendorf tubes after 1, 4 and 24 hours. The aliquots from the controls and the samples were placed in a water bath at recommended enzyme inactivation temperature for the specified time (see Table 4.1) and immediately kept on ice. It was then centrifuged for 15 minutes at 10,000g at 4°C and the clear mid-supernatant collected, filtered through 0.2μM filter and analysed for Glycosaminoglycans (GAGs) and Free Amino Nitrogen by the Blyscan assay and FAN assay, respectively. Dry Contents were determined for the unfiltered supernatants with the Halogen Moisture analyser.

1.1.7 Extraction of chondroitin sulphate from bovine trachea by enzyme hydrolysis, with Alcalase and ultrasonication

The frozen minced bovine trachea were thawed overnight at 4°C and three different treatments were carried out, which included a control. To the minced trachea, water was added at a ratio 1:4 (w/w), mixed well, and pH was adjusted to 8.0. In the first treatment, the mixture was ultrasonicated with the Bronson probe (Power 450 W and Frequency 20 KHz) for 90 seconds (on ice), then transferred to 55°C water bath and Alcalase enzyme added (0.5% w/w) after 10 minutes. In the second treatment, Alcalase enzyme was added (0.5% w/w) to the mixture prior to ultrasonication with the Bronson probe (Power 450 W and Frequency 20 KHz) for 90 seconds (on ice). The mixture was then transferred to the water bath at 55°C. The 'control' sample was

ultrasonicated in and transferred to the water bath at 55°C as above, but Alcalase enzyme was not added to the minced trachea slurry.

The mixtures were incubated at pH 8.0 and 55°C for 24 hours (pH was checked periodically and re-adjusted). Aliquots of 4 ml were removed before and after ultrasonication and after 1, 2, 3, 4 and 24 hours of incubation at 55°C. The aliquots from the controls and the samples subjected to treatment were placed in a 90°C water bath for 10 minutes to inactivate the enzyme and then immediately kept on ice. The cooled samples were then centrifuged for 15 minutes at 10,000g at a temperature of 4°C and the clear mid-supernatant collected, filtered through 0.2μM filter and analysed for Glycosaminoglycans (GAGs).

Results and discussion – Solubilising bovine trachea

Extraction of chondroitin sulphate from bovine trachea by acid hydrolysis, without ultrasonication

Acids hydrolyse proteins by non-specific cleaving of the peptide bonds. There are not many reports in the literature on the use of acid to extract CS from bovine cartilage. The suitability of acid hydrolysis without adding exogenous enzymes to extract CS from bovine trachea was investigated due to the potential cheaper processing cost. Nakano *et al.* (2000) reported that pH is an important factor affecting the yield of soluble CS. The authors mentioned that the majority (73.2% $\pm$ 3.0) of CS (measured as uronic acid) was liberated from bovine nasal cartilage by incubation in water at 37°C for 7 hours at pH 4.5, without introducing any exogenous materials other than acetic acid to maintain pH.

In the experiment investigating the use of acetic acid, the minced bovine trachea was incubated at 37°C for different time periods over a 22 hour period, at pH 4.5 (adjusted with acetic acid) and at pH 6.7 (without acid, control) (see Section 4.2.2). The results in Figure 5.1 show that there is no difference in the concentration of solubilised CS between the two treatments (with and without acid) until 5 hours of reaction time. However, at 22 hours the concentration of CS in the acid treated samples was less than the samples at near neutral. Nakano *et al.* (2000) have reported an eight–fold higher CS yield at pH 4.5 compared to the CS yield at near neutral pH during 7 hours of incubation. The authors recommended bovine nasal cartilage as an appropriate by-product to prepare CS-peptide with high yield. Articular cartilage with a low content of extractable CS (Nakano *et al.* 1998) was not recommended for this purpose.

The low yield of CS with acid hydrolysis in this experiment, based on the Blyscan assay (compared to yield from the enzyme hydrolysis) may indicate that the hard tissues of bovine trachea may not be suitable for acid extraction of CS. At 22 hours, the decreased CS levels compared to that at near neutral conditions may suggest that prolonged incubation in acid could have affected the CS structure. There is no information in the literature on CS extraction at acidic pH at 37°C for 22 hours.

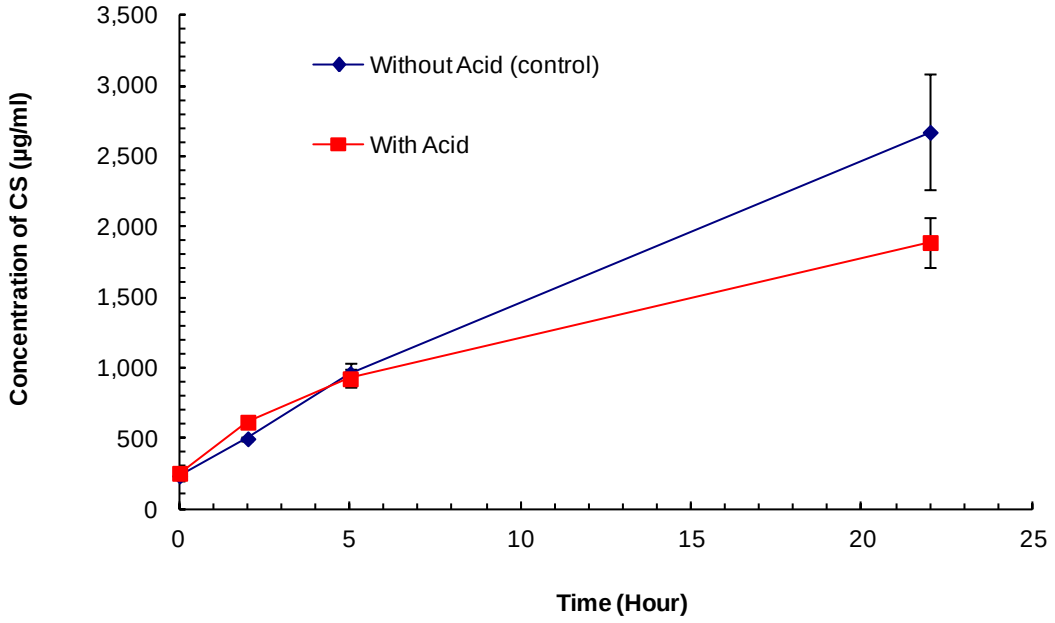

Figure 5.1 Concentrations of Chondroitin Sulphate in the bovine trachea slurry, with and without acid treatment, incubated at 37°C for different time periods. The data represent mean ± standard deviations of 6 determinations.

Extraction of chondroitin sulphate from bovine trachea by acid hydrolysis, with ultrasonication

Ultrasonication is employed to extract intracellular materials. When ultrasonication is used in combination with enzymes, acids or alkalis, it could have an enhanced effect on the yield of the extractable compounds. In the following two experiments, the effect of ultrasonication in combination with acid was examined. Two sonication types were tested, (see Section 4.2.3 for details) (i) Pulsed sonication, with short pulsed intervals for two minutes every hour, for 5 hours (ii) Pre-treatment of the trachea slurry with ultrasonication, prior to incubation at 37°C.

A gradual increase in the concentration of CS in the slurry is observed during the 22 hour incubation at 37°C, in all the treatments, i.e., with and without acid / with pulsed US and with and without acid / without US as shown in Figure 5.2. After the 5 hour incubation period, there was no significant difference in CS concentration, between the treatments, in the slurries. This indicates that neither ultrasonication (at the specified conditions), nor the pH, had any effect on CS extraction at 5 hours.

After 22 hours of incubation, the CS concentration was consistently higher in the slurries without acid compared to the acidified slurries, in both ultrasonicated and none ultrasonicated samples (Figure 5.2). This may indicate that prolonged exposure of CS to acidic conditions is having some effect on CS structure and its stability, thereby resulting in a decreased concentration.

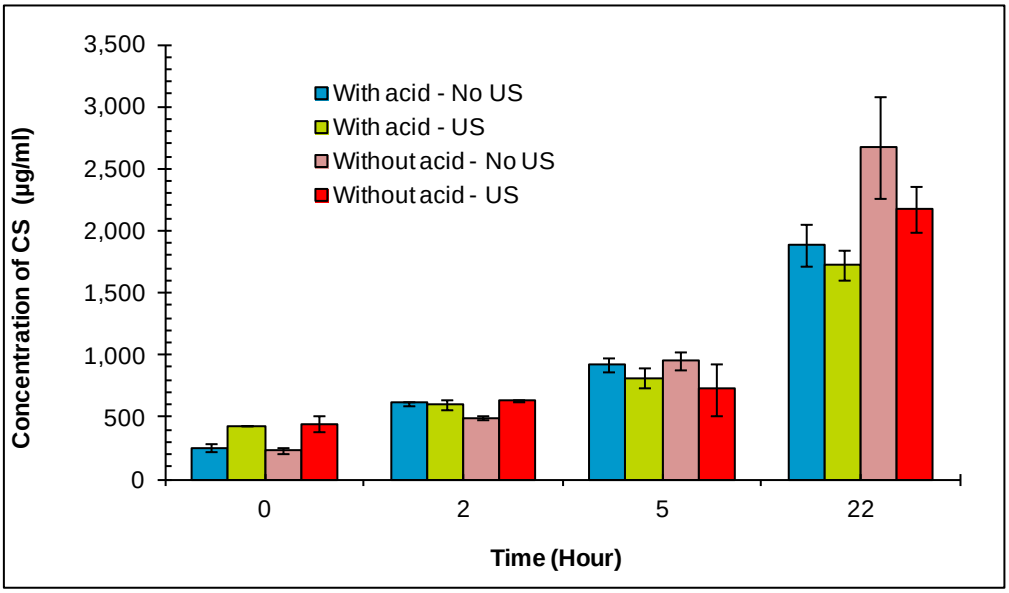

Figure 5.2 Concentrations of Chondroitin Sulphate in the bovine trachea slurry treated with pulsed ultrasonication (US) with and without acid, incubated at 37°C for different time periods. Pulsed ultrasonics was applied for two minutes every hour. The data represent mean ± standard deviations of 6 determinations (controls without ultrasonication also included).

The results (Figure 5.2) show that at 22 hours of incubation, ultrasonication had no effect on CS concentration in the acidified and non-acidified slurries compared to the corresponding 'none ultrasonicated' slurries.

The results of the ultrasonic pre-treatment trials shows that at 5 hour incubation there was no significant difference in CS concentration, between the ultrasonicated slurry without acid and ultrasonicated slurry with acid addition (before US) as shown in Figure 5.3. However, the CS concentration is significantly lower in the ultrasonicated slurry with acid addition (after US). The increased yield in CS when acid was added prior to ultrasonication indicates better mixing of the acid with the trachea slurry.

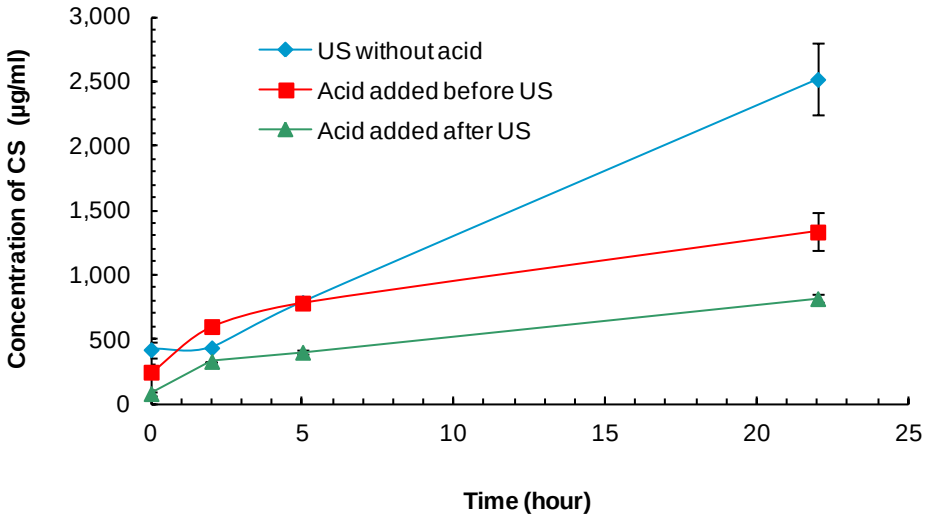

Figure 5.3 Concentrations of Chondroitin Sulphate in the bovine trachea slurry, pre-treated with ultrasonication (US) where acid was added before or after US and without acid addition, incubated at 37°C for different time periods. The data represent mean ± standard deviations of 6 determinations.

At 22 hours of incubation, the concentration of CS in the slurries with the different treatments was; slurry pre-treated with ultrasonication without acid > slurry with acid addition prior to ultrasonication > slurry with acid addition after ultrasonication (Figure 5.3).

This result again indicates that prolonged exposure in acid medium is affecting CS concentration in the trachea slurry.

Extraction of chondroitin sulphate from bovine trachea by enzyme hydrolysis, without ultrasonication

Extraction of CS from cartilaginous tissues by enzyme hydrolysis has been well documented in the literature. Four food-grade enzymes were evaluated for their ability to maximally extract CS from bovine trachea. The enzymes, Promod 648L (Biocatalysts), Multifect Neutral (Enzyme solution), Protamex (Novozyme) and Alcalase 2.4L (Novozyme), are listed in Table 4.1 with detailed information on each of the enzymes. These enzymes were selected based on the information on their ability to degrade collagen and to extract CS. Promod 648L has been recommended by the company as a high performance enzyme which is ideal for extraction of chondroitin sulphate. Multifect Neutral and Protamex (recommended by the companies) are suitable for collagen degradation. Alacalase has been reported in the literature as the most suitable enzyme based on the degree of hydrolysis and CS yield, when extracting CS from sea cucumber (Douck-Choun *et al.* 2001).

Maxazyme NNP (a neutral protease) has been reported in the literature, to be the most efficient with respect to CS extraction among the eleven commercial proteases evaluated (Jo *et al.* 2004). However, suppliers were unable to provide Maxazyme NNP due to the restrictions imposed by the manufacturer. Therefore, Maxazyme was not included in the experimental program.

Figure 5.4 compares the amount of CS extracted by the four enzymes, Protamex, Mulitifect Neutral, Alcalase and Promod. The results show that high levels of Chondroitin sulphate ($\geq$ 6000 μg/mL) can be extracted from bovine trachea by enzyme treatment than with acid treatment ($\leq$ 1000 μg/mL), in four hours. Among the enzymes evaluated Alcalase and Promod performed better than the other two enzymes in extracting CS-A (Figure 5.4) and Alcalase performed the best in hydrolysing the proteins in the trachea (Figure 5.5), resulting in the highest free amino nitrogen content (FAN) in the hydrolysate. Theoretically, 40 g of 'wet' minced trachea (with a dry content 27.3%) when completely solubilised in a total of 200 ml, should produce a slurry containing 5.4% dry content. After 24 hour hydrolysis, the dry contents showed the highest level of ~ 4% compared to the theoretical amount of 5.4% in the slurries treated with Alcalase, Promod and Protamex enzymes (Figure 5.6). However, the higher levels of Free Amino Nitrogen levels exhibited by Alcalase

indicated that this enzyme hydrolysed the proteins into smaller peptides than the other two enzymes (Promod and Protamex). Hence, Alcalase was selected as the preferred enzyme to extract CS free of proteins from bovine trachea. Further, since the cost of Alcalase enzyme ($39/Kg) is much lower than the price of Promod ($189/Kg) and Protamex ($61/kg), Alcalase was selected as the preferred enzyme for the continued development of the process.

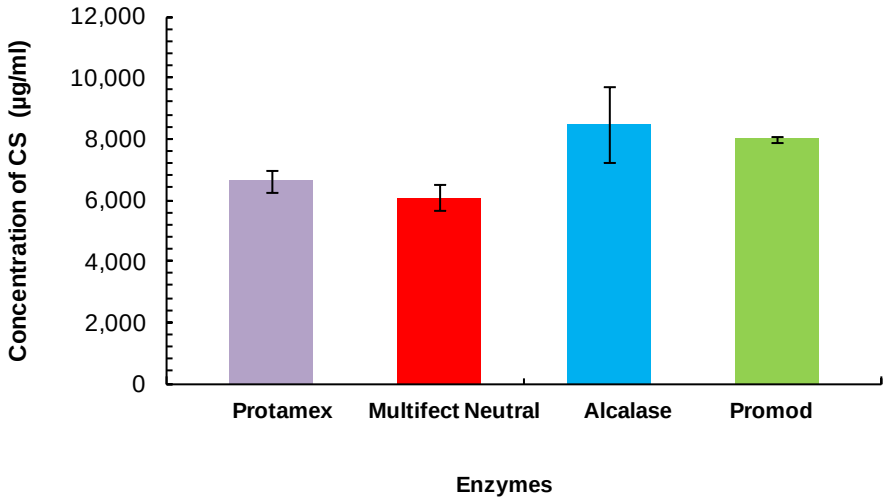

Figure 5.4 Concentrations of Chondroitin sulphate (CS) in the enzyme hydrolysates, after treating the minced trachea for 4 hours with the following enzymes, Protamex, Mulitifect Neutral, Alcalase and Promod. Enzyme reaction conditions used for the trials are detailed in Table 4.1. The data represent mean ± standard deviations of 6 determinations.

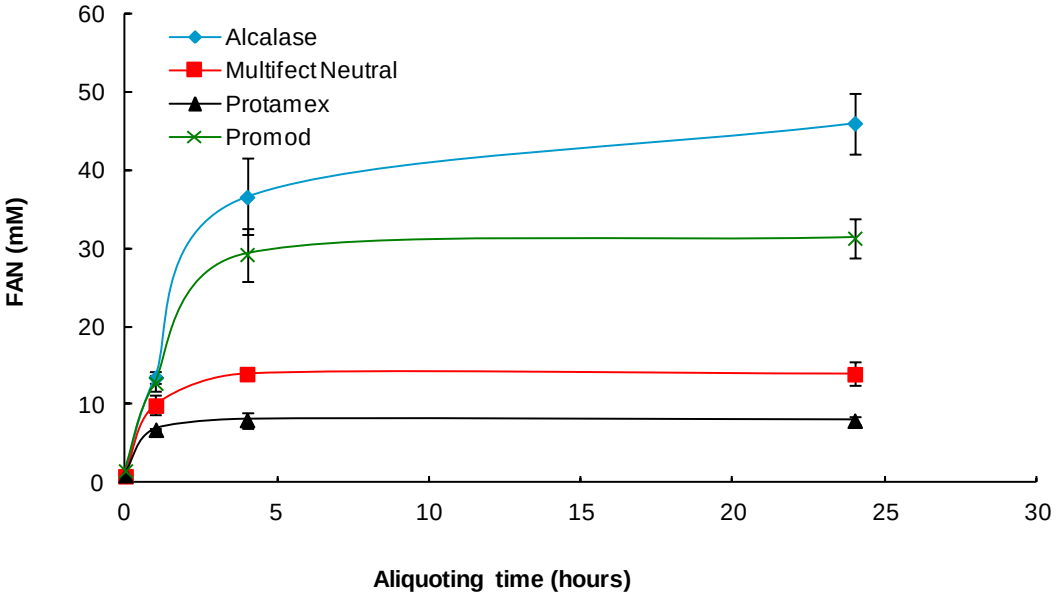

Figure 5.5 Extent of protein hydrolysis in the slurry, by the Free Amino Acid (FAN) assay, after hydrolysing trachea for 1, 4 and 24 hours with the four enzymes, Protamex, Mulitifect Neutral, Alcalase and Promod. Enzyme reaction conditions used for the trials are detailed in Table 4.1. The data represent mean ± standard deviations of 6 determinations.

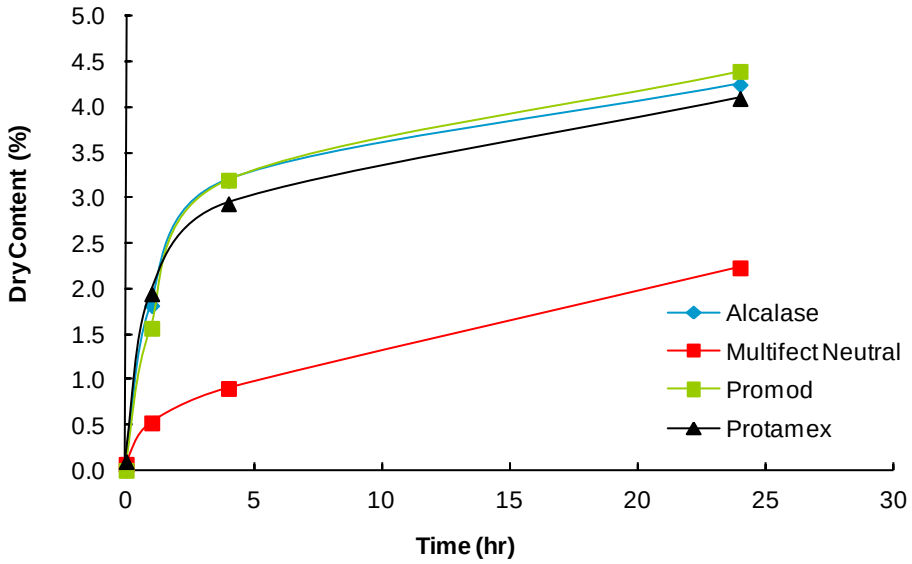

Figure 5.6 Comparing the amount of dry contents in the slurry after hydrolysing the trachea for 1, 4 and 24 hours with the four enzymes, Protamex, Mulitifect Neutral, Alcalase and Promod. Enzyme reaction conditions used for the trials are detailed in Table 4.1.

Extraction of chondroitin sulphate from bovine trachea by enzyme hydrolysis, with Alcalase and ultrasonication

Figure 5.7 shows the results of the effect of ultrasonication on chondroitin sulphate yield before and after addition of Alcalase enzyme. The results demonstrate that better yield of chondroitin sulphate is obtained when Alcalase enzyme is added to the minced trachea before ultrasonication. This may be due to better mixing of the enzyme with the minced trachea. However, there is no significant difference in the yield of chondroitin sulphate extracted by Alcalase enzyme with and without ultrasonication (at the specified conditions) at 4 hours (Figure 5.8).

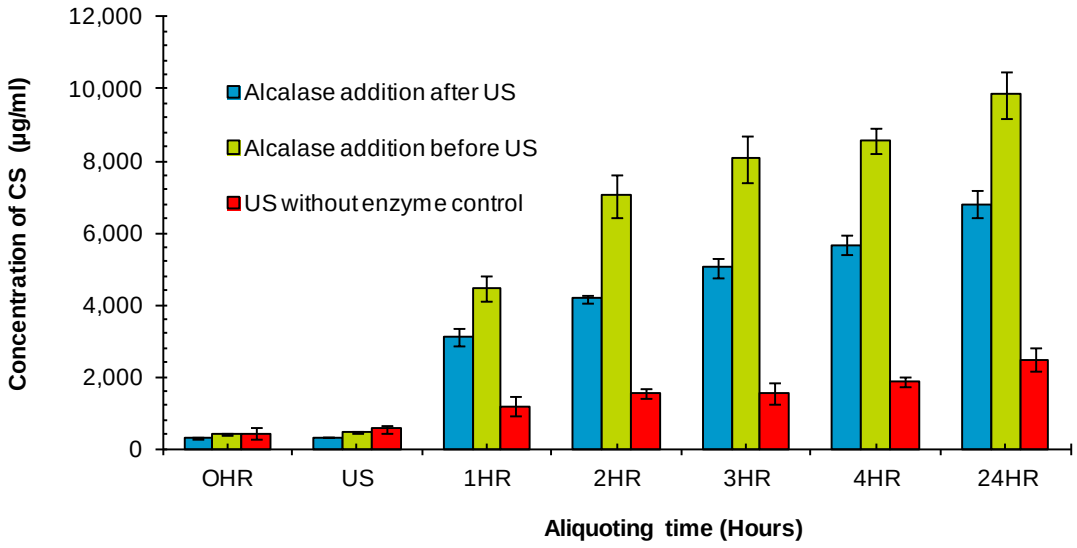

Figure 5.7 Concentrations of Chondroitin sulphate in the ultrasonicated (US) trachea slurry before and after adding Alcalase, at different time periods. Alcalase enzyme reaction conditions used for the trials are detailed in Table 4.1. The data represent mean ± standard deviations of 6 determinations.

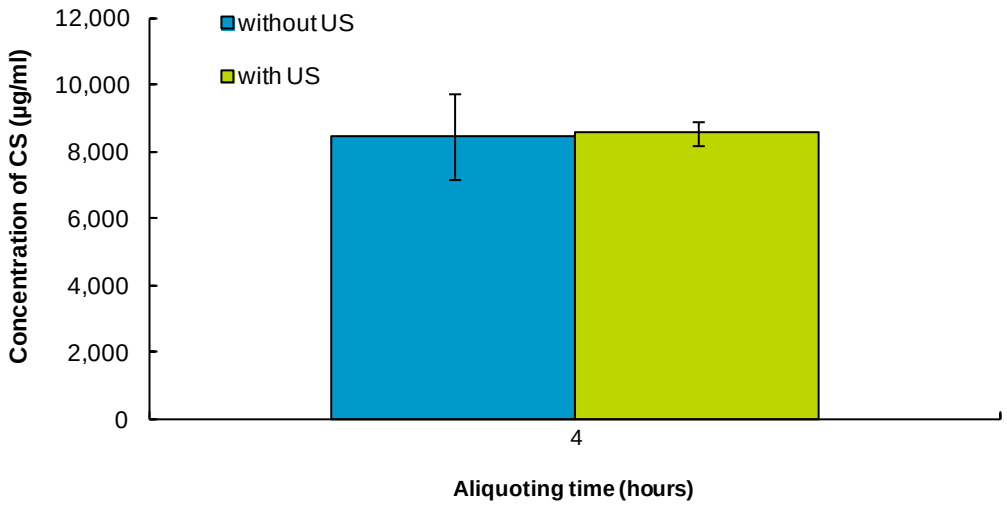

Figure 5.8 Concentrations of Chondroitin sulphate (CS) in the trachea slurry hydrolysed for 4 hours with Alcalase enzyme, with and without ultrasonication (US). The data represent mean ± standard deviations of 6 determinations.

Conclusions and recommendations

The laboratory trials indicated that acidification of minced trachea slurry is unable to significantly extract and solubilise CS as suggested by some literature. This could be due to the nature of the cartilage tissue (bovine trachea). The experimental results also suggested that treating minced trachea with ultrasonics in different combinations of acid or protease enzyme does not improve extraction and solubilisation of CS.

Treating minced trachea with protease enzyme while stirring the reaction mixture results in the highest level of extraction and solubilisation of CS. The protease enzyme Alcalase, a serine endopeptidase was the best performing and the most economical enzyme of the tested enzymes to extract and solubilise CS from the minced cartilage matrix.

It is recommended that the protease Alcalase be used to extract and solubilise CS from minced trachea without any application of ultrasonics. It is also recommended that the Alcalase enzyme reaction be further optimised prior to scale-up of the technology.

Methodology – Separation and purification of chondroitin sulphate

Introduction

Chromatographic separation processes are widely used to isolate and purify value added products in the food industry. A typical chromatographic production process should be robust, easy to be scaled up, readily automated, easy to adapt to new products and should not require any organic solvents. Chromatography is a gentle and reliable technique used for purifying proteins at commercial scale. Ion exchange chromatography is one of the chromatographic techniques that allows the separation of molecules based on their charge.

Ion exchange media are composed of fixed functional charged groups attached to an insoluble matrix with counter ions of opposite charge attached to the functional groups. Ion exchange in biological media is achieved by exchanging the charged molecules with the counter ions attached to the charged functional groups.

There are four main types of ion exchange media (Table 6.1). Cation exchangers attract and exchange positively charged molecules while anion exchangers attract and exchange negatively charged molecules. The dissociation of the functional group and the counter ion is dependent on the pH of the solution and the type of exchanger. In strong exchangers, ionization or dissociation is complete over a wide pH range, whereas in the case of weak exchangers, ionization is dependent on a narrower range of pH:

$R^+ Cl^- \leftrightarrow R^+ + Cl^-$

Where; R^+ is the charged functional group and Cl^- is the counter ion.

CS being a negatively charged molecule due to its sulphation pattern, is an ideal candidate to be separated using anion exchange chromatography.

Table 6.1 Ion exchange media and their functional groups.

Exchanger	Type of exchanger	Functional group
Diethylaminoethyl (DEAE)	Weak anion	-O-CH_2CH_2-$N^+H(CH_2CH_3)_2$
Quaternary aminoethyl (QAE)	Strong anion	-O-CH_2-CH_2-$N^+(C_2H_5)_2$-CH_2-CHOH-CH_3
Quartenary ammonium (Q)	Strong anion	-O-$CH_2N^+(CH_3)_3$
Carboxymethyl (CM)	Weak cation	-O-CH_2-COO^-
Sulphopropyl (SP)	Strong cation	-O-C_3H_6-$SO3^-$

Materials and equipment – chromatographic separation

Resins with Generally Regarded As Safe (GRAS) status were chosen for the initial resin evaluation study. Three ion exchange chromatographic resins were screened to determine their binding capacity and specificity. Characteristics of selected resins as provided by the manufacturers (GE Healthcare Life Sciences and Gibco Invitrogen Corporation) are detailed in Table 6.2. The resins were activated following the manufacturer's instructions.

Table 6.2 Characteristics of ion-exchange resins.

Resin name	Supplier	Type	Functional group	Matrix	Bead Size (microns)	pH range
Q Sepharose Big Beads (BB)	GE	Strong anion	Quarternary amine	Highly cross-linked agarose	100 – 300	2 - 12
QAE GibcoCel HB2 High Substitution	Gibco	Strong anion	Quarternary amine	Reg.Cellulose-hydroxypropyl/ cross link	300 – 600	2 - 12
QAE GibcoCel HB3	Gibco	Strong anion	Quarternary amine	Reg.Cellulose-hydroxypropyl/ cross link	300 – 600	2 - 12

GE - GE Healthcare Life Sciences (Chalfont St Giles

BUCKS, United Kingdom)

GibcoCel - Gibco Invitrogen Corp (Aukland, New Zealand)

Materials and equipment – membrane filtration

Three flat sheet ultrafiltration membranes manufactured by Koch Membrane Systems of different molecular weight cut-off were selected to compare their ability to retain CS from the hydrolysed trachea. All membranes are US Food and Drug Administration (FDA) listed. Properties of selected membranes as provided by the manufacturer are summarised in Table 6.3.

Table 6.3 Specifications of flat sheet membranes tested at laboratory scale.

Nominal molecular weight cut–off (MWCO) (Daltons)	Membrane Material	pH range during filtration	Maximum operating temperature (^{o}C)
5,000	Polyethersulfone	8.0	22
10,000	Polyethersulfone	8.0	22
30,000	Polyvinylidene Fluoride (PVDF)	8.0	22

Ultrafiltration experiments were conducted using Amicon stirred cell 8050 (Millipore) with holding volume of 50 ml and nominal membrane diameter of 43 mm. The flat sheet ultrafiltration assembly is shown in Figure 6.1. The ultrafiltration membrane was placed at the bottom of the cell. The device was pressurised with compressed air to force liquid through the membrane. Gentle stirring was applied to control the concentration and accumulation of proteins on the membrane surface.

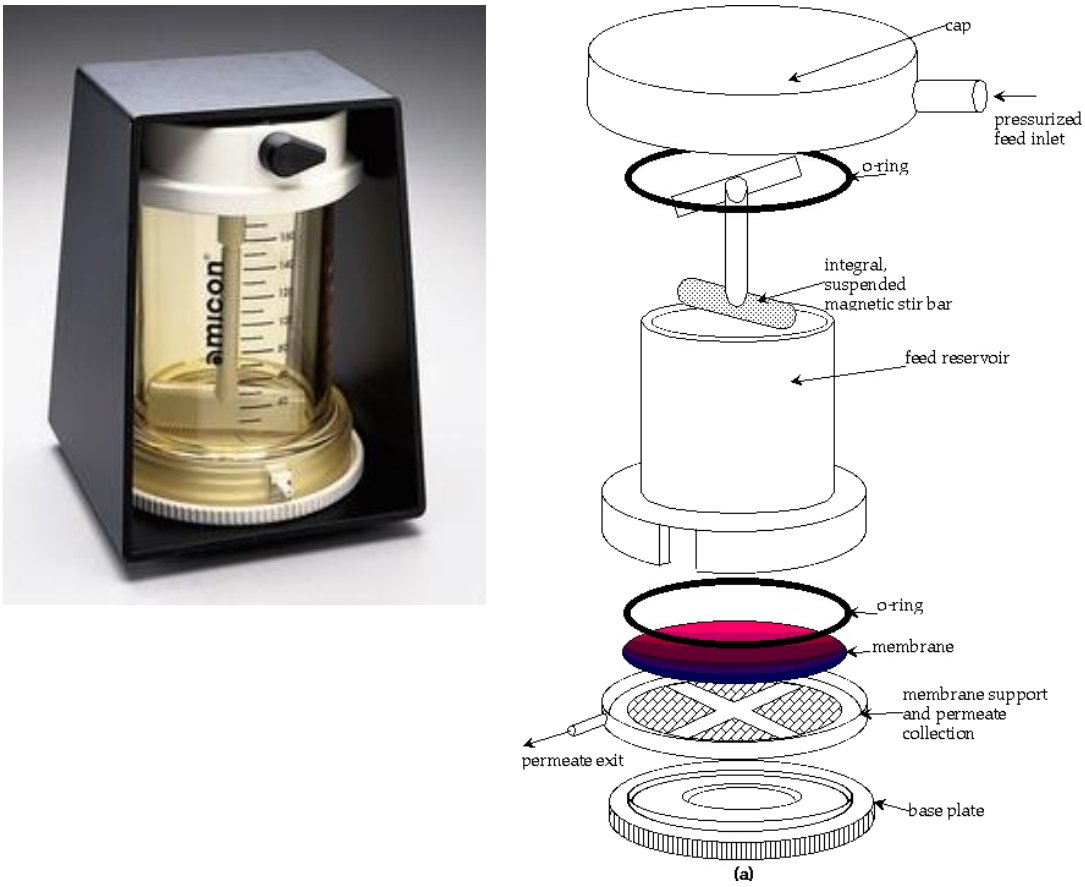

Figure 6.1 Amicon Stirred Cell 8050 components and assembly used for evaluating membrane separation.

Methods

1.1.8 Hydrolysis of bovine trachea for ion exchange trials

Fresh trachea was donated by Wagstaff Cranbourne Pty. Ltd., Cranbourne, Victoria. The trachea on arrival at Werribee laboratories were cleaned, washed and minced using a kitchen mincer. The minced trachea were stored frozen at –20^{0}C until further use.

Minced trachea at a concentration of 20% (w/w, based on wet weight) was brought to 55^0C using a water bath. When the temperature reached 55^0C, the pH of the slurry was adjusted to 8.0 using 5M NaOH. After achieving pH 8.0 and a temperature of 55^0C, Alcalase 2.4L at the rate of 5.0 µl/g of minced trachea was added and the hydrolysis reaction continued for 24 hours at a temperature of 55^0C. After a 24 hour reaction period, the hydrolysate was heated to 90^0C for 10 minutes to inactivate the enzyme. The hydrolysate was centrifuged at 10,000g for 15 minutes at a temperature of 4^0C and the supernatant decanted and stored frozen until further use.

1.1.9 Ion Exchange resin trials

Resin selection by evaluating static adsorption

Static binding experiments were conducted to evaluate adsorption capacity and specificity of three selected resins to adsorb CS. Prior to use, the resins were washed and activated according to the manufacturer's instructions. Excess liquid was removed by suction filtration using a Buchner funnel and the moist resin was stored refrigerated until it was used for the experiments.

In each resin experiment, approximately 5g of activated resins was accurately weighed into 50mL syringes. In each experiment, 30g of hydrolysed cartilage (diluted if required) was weighed into the syringes containing the resin and kept mixing in an orbital shaker for 90 minutes to reach equilibrium. After 90 minutes, the resin was allowed to settle for 10 minutes and the supernatant (flow through, F/T) was decanted. The resin was washed 3 times with MilliQ water and the wash water was decanted each time. After washing the resin, the adsorbed CS was eluted by adding a salt solution into the syringes containing the resin. The syringes with the resin and salt solution were mixed in the orbital shaker for 35 minutes and the liquid (eluate) was then decanted. The eluate was dialysed using MilliQ water to remove salt with 500Da dialysis tubing (Spectrum Laboratories Inc.). The samples collected during the trials were analysed for CS using the Blyscan assay kit.

For the trials to investigate the adsorption capacity of the 3 resins, the elution was carried out with 30g of 1M salt solution.

Equilibrium isotherms of QAE GibcoCel HB3 resin

In the experiments to determine the equilibrium isotherms with QAE Gibcocel HB3 resin, the hydrolysed cartilage was diluted to achieve different CS concentrations in the feed. The cartilage hydrolysate solution was diluted with MilliQ water to achieve solutions of 95, 85, 75, 50, 25, 12.5 and 6.25% feed concentrations and used as the feed for the experiments.

Elution characteristics of QAE GibcoCel HB3 resin

During the trials to investigate the elution characteristics of QAE GibcoCel HB3 resin, the adsorbed CS was eluted from the resin using different salt concentrations. In this protocol, the elution of CS was initially conducted by loading the syringe containing the CS adsorbed resin with 30g of 0.1M salt and mixing in an orbital shaker for 35 minutes. The eluate was decanted and 30g of reverse osmosis (RO) water was added and mixed with the resin for 5 minutes. This water was also decanted and mixed with the 0.1M eluate. Following the 0.1M elution, 0.2M, 0.3M, 0.5M, 0.75M, 1.0M and 2.0M salt solutions were added to the same resin separately and the procedure repeated with each different salt solution with a water rinse between each elution.

Determination of recovery and purity of CS extract

The conditions used to determine the purity and recovery of the CS, and acquire laboratory scale data to investigate the cost of production of separating CS, were based on using trachea hydrolysate that was diluted to achieve a 18% hydrolysate concentration (55g of hydrolysate made up to 300g with MilliQ water). The 300g of diluted hydrolysate was mixed with 200g QAE Gibco HB3 resin and stirred for 20 minutes. The resin/hydrolysate mixture was allowed to settle for 5 minutes and the supernatant decanted. The resin was washed twice with 300g of water where in each case the resin was allowed to settle and the wash water was decanted. The bound CS was eluted by adding 300g of a 2M NaCl solution and mixing the

resin/NaCl mixture for 10 minutes and then allowing the resin to settle. The eluate (supernatant) was decanted and the resin washed with 300g MilliQ water twice. After washing the resin, fresh diluted hydrolysate solution was mixed with the resin and the whole procedure repeated. A total of 3 lots of diluted trachea hydrolysate solutions were used to produce CS using the same batch of resin.

The 3 batches of CS eluates were dialysed using dialysis tubing of 12-14kDa molecular weight cut-off over 24 hours. The dialysed CS eluates were freeze dried and stored until analysed.

Results and discussion – Purification of chondroitin sulphate

Selectivity and static adsorption capacity of strong anion exchange resins for CS

Due to its sulphation pattern CS is a negatively charged molecule and based on this property, three anion exchange resins were selected to evaluate static adsorption capacity and specificity to isolate CS from the trachea hydrolysate. The selected resins were strong anion exchangers - Q Sepharose Big Beads (BB), QAE GibcoCel HB2 High Substitution (HBHS) and QAE GibcoCel HB3 (HB3).

These resins are composed of fixed charged functional groups (quarternary amine groups) attached to an insoluble matrix such as agarose in Sepharose and cellulose GibcoCel resins. Counter ions of opposite charge are adsorbed to these functional groups. In biological media, ion exchange is achieved by exchanging the counter ions adsorbed to the charged functional groups with charged molecules (e.g. proteins, CS). Anion exchangers attract and exchange negatively charged molecules. The dissociation of the counter ion is dependent on the pH of the solution and the type of exchanger. For strong exchangers, ionisation or dissociation is achieved over a wide pH range (2 – 12). The interaction of target charged molecule with the chromatographic stationary phase depends on many factors such as pH, ionic strength of the feed material, additives, accompanying biomolecules and the chemical structure of the resin.

Static binding experiments were conducted to evaluate adsorption capacity and specificity of three selected resins to bind CS as described in the methodology section. The hydrolysate feed, F/T (flow through) fractions (the hydrolysate solution depleted in the target molecule due to the adsorption of the target molecule by the resin) and eluates were analysed for CS by the Blyscan assay. The efficiency of CS adsorption was calculated as the amount of CS adsorbed and eluted from 1mL of resin.

The results of the adsorption trials are detailed in Table 7.1 and graphically shown in Figure 7.1. The CS concentration in the F/T in the experiment with BB was similar to that of the feed suggesting that CS was not adsorbed by BB and this is further confirmed by the very low amount of CS in the eluate, both before and after dialysis. The results suggest that the capacity of BB to adsorb CS was very low. The low CS in the F/T from both HBHS and HB3 experiments indicated that CS was adsorbed by the resin and the lower concentration in HB3 resin indicated that HB3 had a higher capacity for CS. This was further confirmed by the concentration of CS in the eluates. The F/T and eluate results indicated that HB3 had the highest capacity for CS among the 3 tested resins. The price of the GibcoCel resins are lower by a factor of >4 and taking into consideration the higher capacity of QAE GibcoCel HB3 for CS, this resin was selected for further investigation.

Table 7.1 Concentration of CS during adsorption from minced cartilage hydrolysate and elution from 3 different anion exchange resins: Q Sepharose Big Beads (BB), QAE GibcoCel HB2 High Substitution (HBHS) and QAE Gibcocel HB3 (HB3). The data represent means of 3 experiments analysed in duplicate (n=6).

Resin	Feed	F/T	Eluate	Dialysed eluate
			mg/mL	
Q Sepharose Big Beads	9.8	9.8	0.1	0.0
QAE Gibcocel HB2 High Substitution	9.8	2.3	5.8	6.4
QAE GibcoCel HB3	9.8	1.3	7.8	8.0

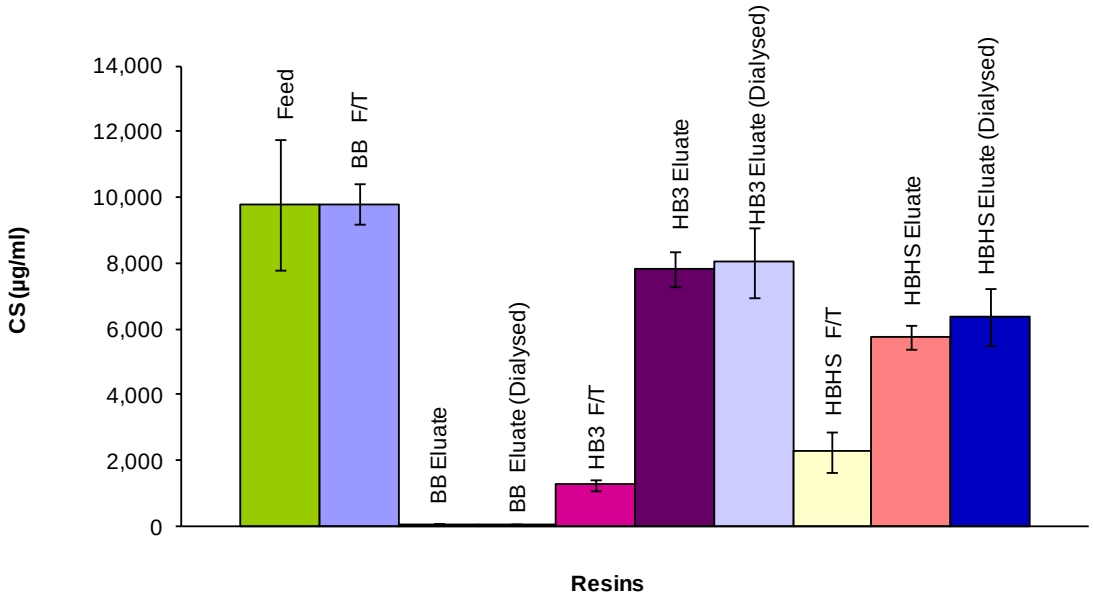

Figure 7.1 Adsorption of CS from cartilage hydrolysate on Q Sepharose Big Beads (BB), QAE GibcoCel HB2 High Substitution (HBHS) and QAE Gibcocel HB3 (HB3) resins. CS adsorbed on the resin was eluted using 1M salt (NaCl) solution. The data represent mean ± standard deviations of 6 determinations.

Equilibrium isotherms

A useful parameter in chromatography is the distribution coefficient (K_d) which is a measure of the distribution and concentration of the molecule of interest in the stationary phase compared to that in the liquid phase under equilibrium conditions, and is described by the following equation:

K_d = Concentration in stationary Phase (q*) / concentration in liquid phase (C*)

(* denotes under equilibrium conditions).

The value of K_d impacts process scale separation through the quantity of resin required to achieve the separation with an increase in K_d resulting in an increased volume of resin required to achieve the separation.

When a charged molecule such as a protein or CS in solution is in contact with an ion exchange resin, the charged molecules from the bulk of the liquid will transfer from the liquid phase and adsorb onto the solid phase by exchanging the counter ion on the resin. This transfer of the charged molecules will take place until equilibrium is reached where there will be no net transfer of charged molecules between the liquid and the solid phase. The distribution of the molecules of interest between the solid and liquid phases under equilibrium conditions is generally described by adsorption isotherms. The shape of the isotherms provides information on the efficiency of the adsorption process, a concave shape indicating a favourable isotherm where the concentration in the solid phase is greater than that in the liquid phase (i.e., high K_d). This type of isotherm is described by the Langmuir model. An unfavourable isotherm, where the concentration of the molecules in the liquid phase is greater than in the solid phase is described by a convex shaped isotherm.

Many of the equilibrium isotherms are described by the Langmuir model which is based on the following assumptions (Doran, 1995):

- Adsorption on the surface is limited to a single layer of molecules;
- Adsorbed molecules are not free to move on the surface of the solid phase; and
- Enthalpy of adsorption is the same for all molecules.

These assumptions for CS adsorption are likely to be valid because when the CS molecules are bound onto the resin no other CS molecules will have access to the sites. In addition, CS molecules will be bound at specific sites and if the CS molecules are to move on the surface of the solid phase, then bonds in specific sites will need to be broken simultaneously.

The Langmuir equation for adsorption is described by:

$$q^* = k_d Q_m C^* / (1 + k_d C^*)$$

Where:

q^* = solid phase concentration of CS in equilibrium;

C^* = liquid phase concentration of CS in equilibrium; and

Q_m = maximum adsorption.

k_d = dissociation constant dependent on the adsorption (k_1) and desorption (k_2) rate constants and determines the curvature of the model. k_1 (adsorption) is a function of liquid phase concentration and the (Q_m - q*) on the solid surface whereas k_2 is a function of adsorbed protein concentration q*.

Figure 7.2 shows the equilibrium curve for adsorption of CS by QAE Gibcocel HB3 resin. The equilibrium curve follows the concave Langmuir adsorption isotherm. Using the solver add-in in Microsoft Excel the Langmuir parameters for the static adsorption of CS by QAE Gibcocel HB3 was determined by minimizing the sum of error squared between the experimental data and the Langmuir model. K_d was determined as 29.15 mg/g wet resin and Q_m as 2.39 mg/g wet resin.

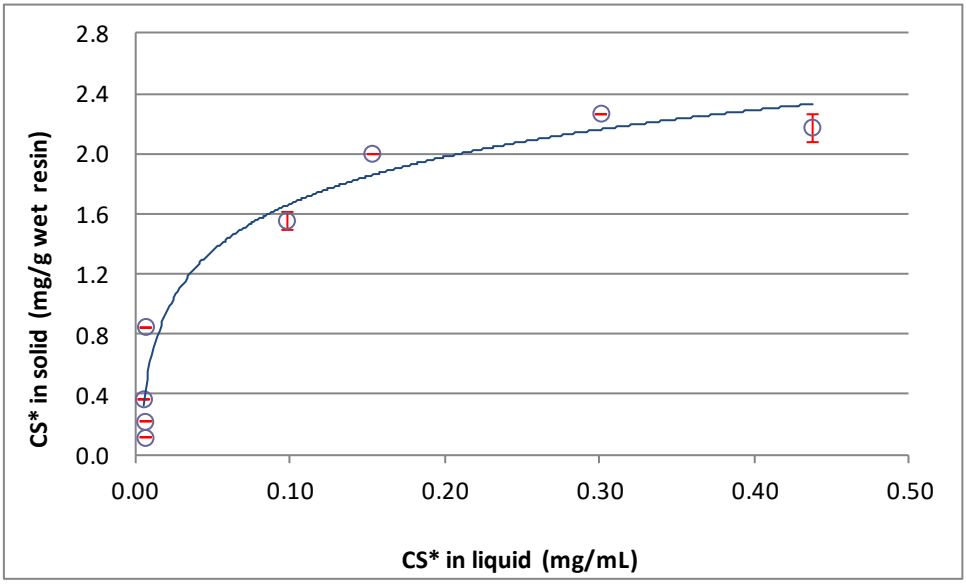

Figure 7.2 Equilibrium isotherm described by the Langmuir model where CS in the liquid is in equilibrium with the CS adsorbed on QAE Gibcocel HB3 resin. The data represent mean ± SEM of 5 determinations.

Elution of CS absorbed on QAE Gibco HB3 resin

The desorption (elution) of the CS adsorbed by the QAE GibcoCel HB3 resin can be achieved by utilising a salt solution. The effect of different salt solutions on the elution of CS from the QAE GibcoCel HB3 resin is shown in Figure 7.3. Approximately 5% of the CS was eluted using a 0.2 M salt solution and a 0.5M salt solution eluted approximately 50% of the CS bound to the resin. A 1M salt solution eluted approximately 80% of the adsorbed CS and complete elution was achieved with 2M salt solution.

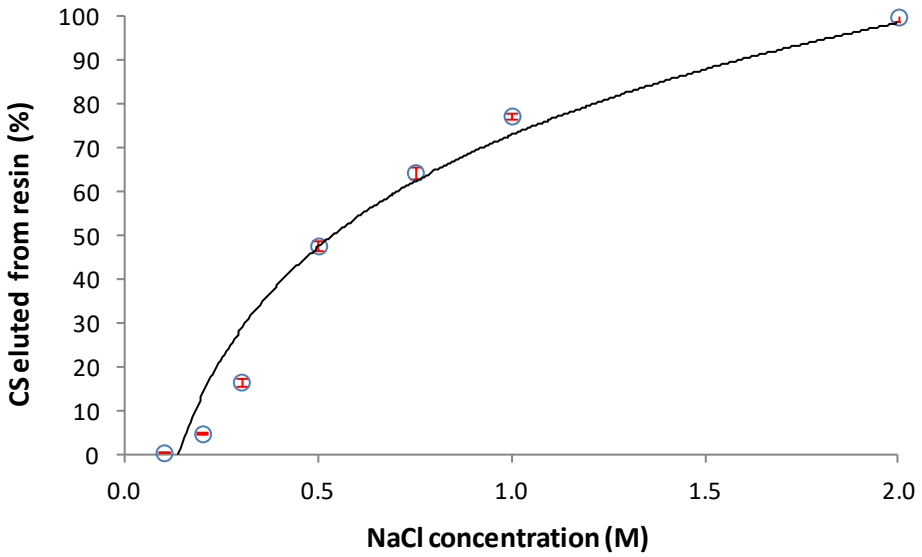

Figure 7.3 Elution of adsorbed CS from QAE GibcoCel HB3 resin using different salt concentrations. The data represent mean ± SEM of 6 determinations.

The elution trial results suggest that a 2M NaCl solution will be required to completely elute the adsorbed CS from the resin.

Retention of CS from trachea hydrolysate using ultrafiltration

Laboratory scale ultrafiltration trials using flat sheet membranes were conducted to determine the molecular weight cut-off (MWCO) membrane size required to separate CS from the cartilage hydrolysate solution. With the aim of scaling up the ultrafiltration process to commercial scale, the membranes available in scale for pilot-

plant and commercial processing were selected. Three membranes 5, 15 and 30 kDa made from polyethersulfone (PES) were used in the membrane screening experiments. The effective filtration area of all selected disk membranes was 13.4 cm^2.

After rinsing the membrane with water, approximately 30mL of the cartilage hydrolysate feed was added to the cell. The gentle stirring was applied to the cell using a magnetic stirrer and the system was pressurised with compressed air (30 psi).

In using membranes to separate CS in desalting the eluate from ion exchange chromatography, CS needs to be separated from the salt and any other small molecular weight substances. Therefore, a suitable membrane should retain CS and permeate all other non-CS molecules. Similarly, in the trachea hydrolysate, the use of protease enzymes will hydrolyse proteins into smaller peptides which will be smaller than CS, therefore, a suitable membrane should retain CS and permeate non-CS substances. The permeation of CS across the three membranes that was investigated resulted in low permeation of CS across the membranes. The permeation through the 30kDa membrane was the worst with 4.1% of the CS in the feed permeating across the membrane. The permeation of CS through the 5 and 10kDa membranes were low at 1.4 and 2.1% of the CS in the feed respectively. The results suggest that all three membranes are able to retain CS with relatively low losses of CS in the permeate stream.

The average permeate rate (flux) achieved with membrane sheets will be inferior to the performance of spiral wound membranes used with pumps, where high cross flow rates can be achieved, resulting in high permeate flow rates, but membrane sheets give a good comparison of the performance of different membranes. The membrane sheets are taken as a guide on the performance of spiral ultrafiltration membranes. The average flow rate of permeate over the trials resulted in 0.83±0.2, 0.57±0.08 and 0.25±0.07 mL/min for the 30, 10 and 5kDa membranes respectively. The average permeation flow rates indicated that 10 or 30kDa membranes are suitable to be considered when scaling up to preparatory scale in the next milestone.

The concentration of CS in the retentate with the membranes that were investigated is shown graphically in Figure 7.4. While the feed was being concentrated in the retentate there was a very low level of CS permeating across all the membranes.

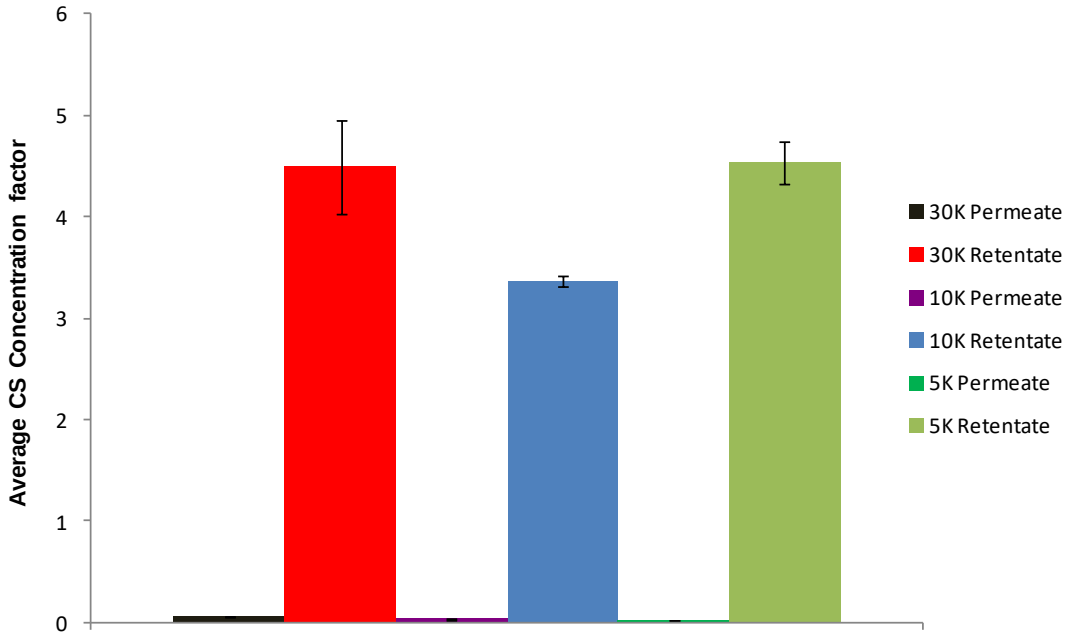

Figure 7.4 Membrane filtration of cartilage hydrolysate using membrane sheets of 5, 10 and 30kDa ultrafiltration membranes in an Amicon stirred cell. The data represent mean ± standard deviations of 6 determinations.

Conclusions and recommendations

Of the resins investigated, QAE GibcoCel HB3 resin was the most suitable resin to separate CS from the trachea hydrolysate solution, based on adsorption capacity and cost of resin. The static equilibrium isotherm indicated that CS adsorption followed the Langmuir model with Q_m at 2.39 mg/g wet resin and k_d at 29.15 mg/g wet resin. The elution trials indicated that a salt strength >1M and ≤2M is required to elute the adsorbed CS. Furthermore, QAE GibcoCel HB3 resin has been selected as a suitable resin to separate BSA from bovine plasma, hence the capability of this resin to be used to produce 2 different bioactives from meat industry co-product streams is an added advantage.

In the application of membranes to purify the CS in the eluate or to separate the CS from the hydrolysate solution containing CS, salts and peptides, the experimental data suggest a membrane of MWCO ranging between 10 and 30 kDa is suitable to separate and purify CS.

Laboratory scale ion exchange trials to determine chondroitin sulphate recovery and purity

Three consecutive ion exchange loading trials were conducted to investigate (a) if the adsorption of CS from hydrolysed trachea is significantly reduced over 3 loadings, (b) recovery of CS from the hydrolysate and (c) purity of the CS produced by ion exchange chromatography. The adsorption and elution conditions used for the trials were not optimised.

The eluate from the ion exchange trials were dialysed using 12-14kDa molecular weight cut-off dialysis membranes. Due to the low volumes of eluate produced during each trial it was practical to use dialysis to desalt the eluate rather than an ultrafiltration module, which requires larger volumes.

The concentration of CS in the hydrolysate feed was 2.5mg/L. The loading conditions, recovery and purity of the CS from the 3 trials are shown in Table 8.1. As the trials progressed, the weight of resin used in each trial was decreasing due to the loss of some resin associated with the washing of resin before re-use. Although the weight of the resin used for each trial was different, the weight of hydrolysate used for each trial was kept constant.

The CS in the F/T was <1% when the hydrolysate: resin ratio was between 1.49 and 1.51, but at a ratio of 1.59, the CS in the F/T was 5.23% of the CS in the original hydrolysate feed, i.e., a potential loss of ~5%. The average amount of CS recovered after 3 trials of ion exchange and dialysis was 68.7 ± 1.2% of the CS in the feed. The consistent recovery figure over the three trials suggests that multiple loadings have no detrimental impact on the adsorption of CS by the resin and elution with 2M NaCl is adequate to elute and regenerate the resin after each chromatographic cycle 9Table 8.1). During dialysis, sample from trial A and B were lost due to damage to the dialysis tubes and this was estimated at ~ 5% from each trial and these losses decreased the recovery of CS from these two experiments.

The purity of the CS produced using ion exchange chromatography and dialysis without any optimisation of the process was 81.6 ± 2.6% on a solids basis. The high purity suggests that the 12-14kDa dialysis membrane was capable of removing (permeating) the salt and any other small molecular weight, non-CS components such as peptides from the eluate, therefore the use of membranes of 10kDa (or a 30kDA) is suitable at larger scale.

Table 8.1 Average CS recovery and purity from three anion exchange resin trials. CS was separated from trachea hydrolysate using QAE GibcoCel HB3 anion exchange resin.

Trial	Resin weight	Hydrolysate weight	Hydrolysate : resin ratio	CS in F/T	Recovery of CS from feed	Purity of CS (on a solids basis)
	g	g		%	%	%
A	200.6	299.5	1.5	0.5	67.2	83.3
B	198.2	299.4	1.5	0.5	70.2	77.9
C	189.5	300.8	1.6	5.2	68.8	83.5

Preliminary estimate of cost of production of CS

Based on the laboratory scale trials, the production of CS at a purity >80% is possible using either a chromatographic or a membrane approach. In the chromatographic approach, the separation is based on adsorbing the negatively charged CS onto an anion exchange resin and the membrane based approach is based on retaining the larger molecular weight (20-30kDa) CS and letting the peptide material permeate through the membrane.

Based on the laboratory scale data, preliminary estimates for the cost of production (COP) for both technologies were carried out. These cost estimates would need to be further refined as preparatory scale samples are produced and more importantly when the laboratory based process is scaled up to pilot scale.

The preliminary estimate of cost of production of 49 tonnes/year of CS was based on several assumptions. The details of the assumptions are in Appendix 4 for ion exchange chromatography and Appendix 5 for membrane separation. The main assumptions used in the COP model were:

- The process is assumed to operate continuously 5 days per week with 2 operators per shift over 48 weeks of the year.
- Based on experience in the applications of ion exchange chromatography in the dairy industry, the life of the resin and membranes was assumed to be 2 years. It is expected that ion exchange resins would last longer than the assumed period.
- The weight of a cleaned trachea was taken as 250g.
- The effluent streams from the process (hydrolysate with CS removed) could be sent for rendering and the salt permeate from the chromatographic system is handled by the existing effluent treatment systems
- Labour cost was taken as $35,000 per year.
- The current income from rendering trachea was taken as $0.20/kg. This was taken as an opportunity cost.

CS production using ion exchange chromatography

The envisaged processing strategy to extract CS from bovine trachea using proteolytic enzymes and separating the CS from the hydrolysate employing ion exchange chromatography is shown in Figure 9.1

It is anticipated that hygienically collected trachea would be cleaned of any fat, membrane and other non-cartilage material and minced. Extraction of CS from the minced trachea will be achieved by using Alcalase proteolytic enzyme to cleave the proteins and break down the cartilage matrix to smaller peptides and release CS into the hydrolysate solution. The enzyme Alacalase is to be added at a dosage of 0.5% (w/w) based on the minced trachea to 20% (w/w) slurry of the minced cartilage, heated to a temperature of 55^0C in a tank with good agitation. The hydrolysis reaction should be continued for 4 hours after which the enzyme is inactivated by heating the hydrolysate solution to 90^0C for 10 minutes. This heating step would also be microbiology control step. The hydrolysate should then be filtered to separate any particulate material using a vibrating fine screen mesh or a bag filter. The filtrate should then be loaded onto chromatographic columns using either traditional batch systems or preferably a more efficient simulated moving bed chromatographic system. The eluate from the chromatographic separation is then subjected to ultrafiltration using a 10-30kDa molecular weight cut-off membrane to remove any salts and peptides and concentrate CS in the solution to ~15%, the maximum concentration due to high viscosity of CS at higher concentrations. The concentrated CS is then dried using a spray drier and packed.

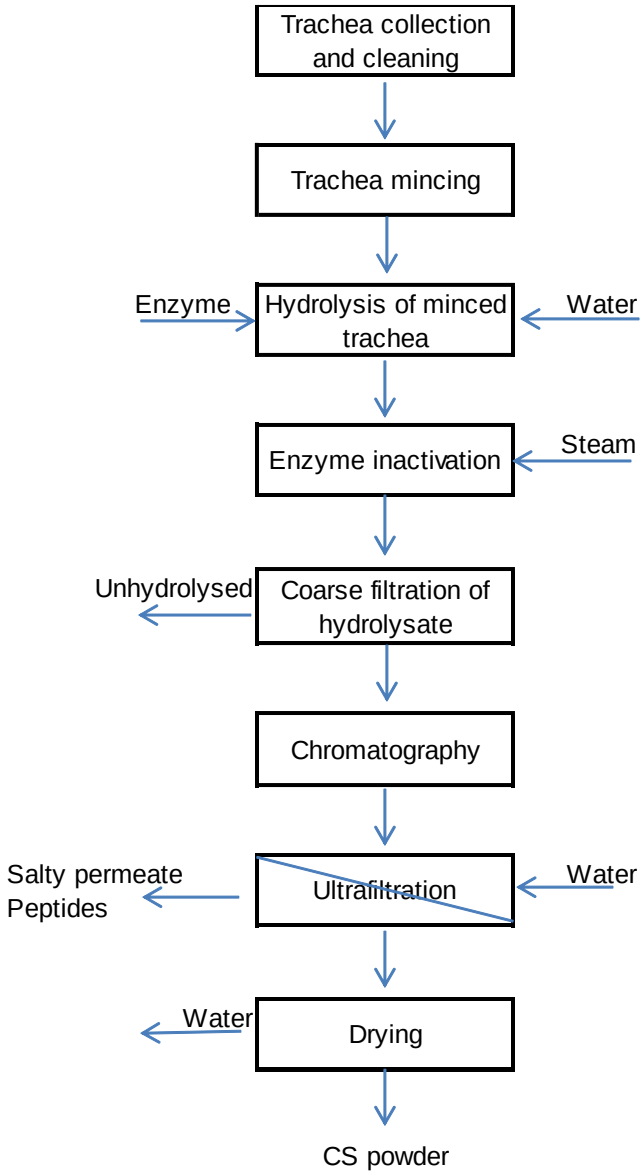

Figure 9.1 Schematic of the conceptual enzymatic and ion exchange chromatographic process to extract and separate CS from bovine trachea.

1.1.10 Estimation of cost of production of CS using ion exchange chromatography

The details of the estimate of the COP to produce 49 tonnes/year of CS are detailed in Table 9.1 (trachea from 20,500 animals/day). The COP includes the current income received by an abattoir through rendering at $0.2/kg as an opportunity cost charged as an expense. The COP with the opportunity costs charged as an expense is estimated at $19.9/kg, but the COP without charging the opportunity costs is

estimated significantly lower at $14.9/kg. The COP was also estimated assuming a contingency based on adding 50% of direct operating cost. The COP with the added contingency expenditure was estimated at $29.80/kg and without the opportunity cost was estimated at $22.30/kg of CS produced.

The COP of production for a smaller process plant producing 25 tonnes/year equivalent to trachea from 10,500 animals per day was also analysed. The COP was estimated at $24.20/kg and without adding opportunity costs was reduced to $19.20. Adding a contingency of 50% to the direct COP resulted in the increase of COP to $36.30/kg and without adding opportunity costs was $28.80/kg of CS produced.

Table 9.1 Preliminary estimate for the COP to produce 49 tonnes per year of CS from bovine trachea using anion exchange chromatography. The cost is estimated on the basis of hydrolysis batch/day and total cost incurred per year. The percentage contribution of each cost item based on the total direct COP was also estimated.

Cost of production	$/batch	$/year	% of direct COP
Trachea opportunity cost	1,025	246,000	25
Enzyme	270	64,760	7
Labour	875	210,000	21
Salt	185	44,407	5
Packaging	103	24,600	3
Water	319	76,515	8
Power	59	14,065	1
Steam	70	16,913	2
CIP	50	12,000	1
Spray drying	66	15,838	2
Resin replacement	1,031	247,464	25
Membrane replacement	27	6,476	1
Direct COP	**4,079**	**979,037**	
COP/kg	**19.9**		
Contingency on direct costs (%)	50		
Direct COP	6,119		
COP/kg	29.8		
CS production (kg/batch)	205		
CS production (tons/year)	49		

Trachea opportunity costs–the income derived by disposing trachea as a waste stream and used for rendering.

Membrane filtration

The envisaged processing strategy to extract and separate CS from bovine trachea using proteolytic enzymes and separating the CS from the hydrolysate employing membranes is shown in Figure 9.2 The CS extraction procedure for the membrane filtration process will be similar to that envisaged for the chromatographic process up to the enzyme inactivation step. The separation of particulate material will need to be very efficient as any particulate material will impact on the efficient performance of the subsequent microfiltration process. The separation of particulate material will require an efficient solids separation system such as a filter press.

The filtrate from the solid separation system is introduced as the feed stream to the microfiltration plant using a membrane of 0.8 -1.2 µm pore size. The microfiltration plant could be either a ceramic or a polymeric membrane system. Permeate from the microfiltration system will consists of all soluble material such as peptides, salts, CS and carbohydrates. Any material >0.8 µm including any precipitated or agglomerated proteins will be retained in the retentate stream. Permeate from the microfiltration plant will be the feed to the ultrafiltration plant.

Permeate from the microfiltration plant is processed through the ultrafiltration system with a membrane of 10-30kDa molecular weight cut-off. The peptides, carbohydrates and salts will permeate across the membrane and the CS will be retained by the membrane. During ultrafiltration, initially the feed will be concentrated to reduce the volume until a CS concentration of ~15% is achieved and at this stage water is introduced to the feed/retentate to diafilter (wash) the stream to further permeate and reduce the amount of peptides, carbohydrates and salts in the retentate. Diafiltration increases the purity of the CS in the retentate stream. The diafiltered retentate is then spray dried to produce a CS powder of >80% purity.

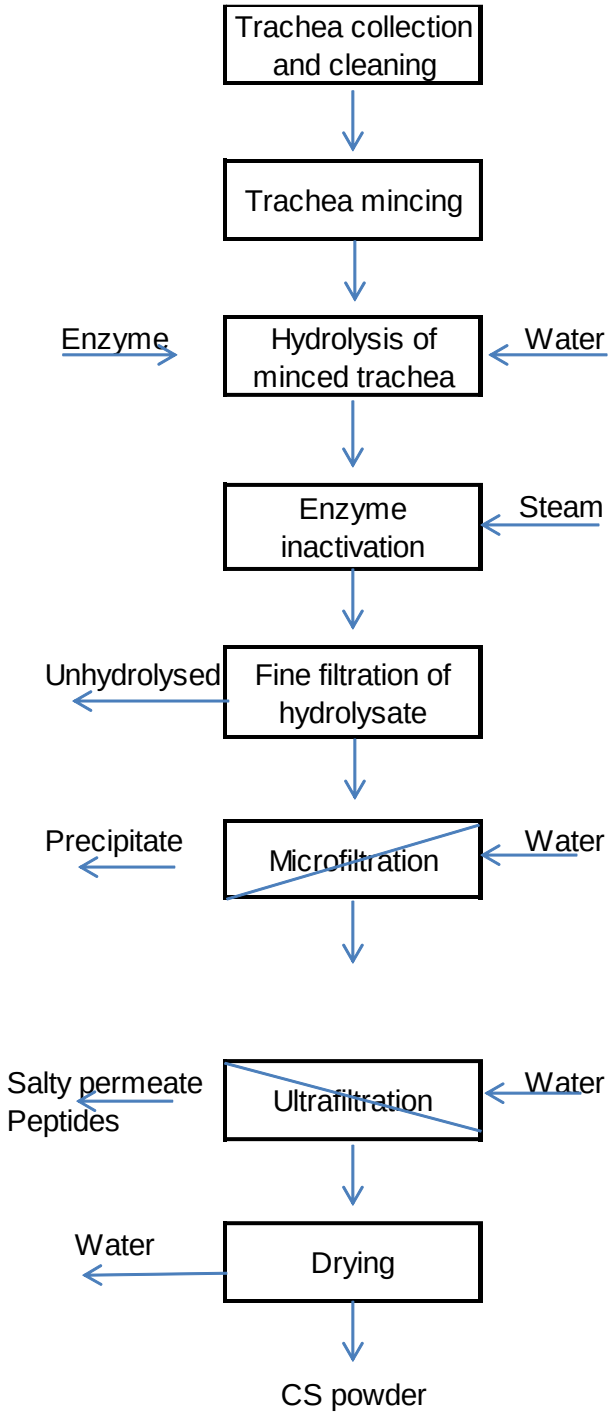

Figure 9.2 Schematic of the conceptual enzymatic and membrane process to extract and separate CS from bovine trachea.

1.1.11 Estimate of cost of production of CS using membrane systems

The details of the estimate of the COP to produce 49 tonnes/year of CS using membranes are detailed in Table 9.2. The COP includes the current income received by an abattoir through rendering at $0.2/kg as an opportunity cost charged as an expense. The COP with the opportunity costs charged as an expense is estimated at $13/kg, but the COP without charging the opportunity costs is estimated at $8/kg. The COP was also estimated assuming a contingency based on adding 50% of direct operating cost. The COP with the added contingency expenditure was estimated at $19.1/kg and without the opportunity cost it was estimated at $11.6/kg of CS produced.

The COP of production for a smaller process plant producing 25 tonnes/year equivalent to trachea from 10,500 animals per day was also analysed. The COP was estimated at $17/kg and without adding the opportunity costs it was reduced to $12/kg. Adding a contingency of 50% to the direct COP resulted in the increase of COP to $25.6/kg and without adding opportunity costs was $18.10/kg of CS produced.

Table 9.2 Preliminary estimate for the COP to produce 49 tonnes per year of CS from bovine trachea using membranes. The cost is estimated on the basis of per batch and total cost per year. The percentage contribution of each cost item based on the total direct COP was also estimated.

Cost of production	$/batch	$/year	% of direct COP
Trachea opportunity cost	1,025	246,000	39.20
Enzyme	270	64,760	10.32
Labour	875	210,000	33.46
Packaging	103	24,600	3.92
Water	49	11,829	1.88
Power (membranes)	59	14,243	2.27
Steam	70	16,913	2.69
CIP	50	12,000	1.91
Spray drying	67	16,102	2.57
Membrane replacement	46	11,114	1.77
Direct COP	**2,615**	**627,559**	
COP/kg	**13**		
Contingency on direct costs (50%)			
Direct COP	3,922		
COP/kg	19.1		
CS production (kg/batch)	205		
CS production (tons/year)	49		

Cleaning–in–place (CIP)

Conclusions on the COP of CS using ion exchange chromatography and membranes

Based on the laboratory data, the COP of producing CS using membranes is lower compared to chromatography, but this should be considered in the overall context of including and comparing the capital costs of the two approaches. A preliminary estimate of capital cost will be made after the next milestone of the project when the laboratory process will be scaled to laboratory preparatory scale.

Both technologies result in a low COP especially considering the major cost components are the opportunity cost of the trachea and labour costs. The chromatographic option is costlier than the membrane option mainly due to the costs

of replacing the resin, assumed to be every 2 years, however, resins used in the dairy industry have lasted for much longer periods.

One of the advantages of the chromatographic approach is that the process to separate BSA from plasma has also identified the same resin, QAE GibcoCel HB3 as the optimum anion exchange resin. This provides opportunities to produce 3 different products (BSA, IgG enhanced fraction and CS) using the same capital investment, hence reducing commercial risks.

The direct COP of CS using both technologies is very low compared to the selling price of the product varying between $80 and $110/kg. Therefore, it is recommended that the technology be further developed to pilot scale to establish scaled up design and operating parameters for the process and validate consistency of product specification. The pilot scale data will also enable the refining of the COP and also establish an estimate of the capital cost of a commercial process using both technologies. The meat processors or value adders will then have information to make decisions regarding the product portfolio and the technology options they intend on pursuing.

Conclusions and recommendations

The conclusions from the work carried out are:

- In the scientific and patent literature, several procedures have been used to extract CS from various cartilage sources including alkaline hydrolysis, exogenous and endogenous proteolytic enzymes and use of mild acids such as acetic acid. The extraction of CS using these methods has been variable and most likely due to the different sources of cartilage and the quality of the cartilage. There are instances where alkaline hydrolysis has been employed but the use of alkaline hydrolysis has the disadvantage of requiring adjustment of pH back to around neutrality, creating a very large salt stream. Alkaline hydrolysis is also reported to deteriorate CS, hence its effect on structure and efficacy is not known. Therefore, alkaline hydrolysis is not recommended when other methods achieve acceptable CS extraction.

- Ethanol precipitation is a widely reported technique in the literature to separate CS from the solubilised cartilage solution. The safety aspects of using ethanol in a commercial production environment, in addition to the added significant expense of requiring special flame proof equipment and electrical wiring, ethanol precipitation is not recommended as a technique to separate and purify CS.

- Treating minced trachea with protease enzyme Alcalase while stirring the reaction mixture resulted in the highest level of extraction and solubilisation of CS.

- QAE Gibco HB3 resin was the most suitable resin to separate CS from the trachea based on adsorption capacity and cost of resin.

- Experimental data suggest a membrane of molecular weight cut-off (MWCO) ranging between 10 and 30 kDa is suitable to separate and purify CS.

- Purity of the CS produced using ion exchange chromatography and dialysis without any optimisation of the process was 81.6 ± 2.6% on a solids basis.
- Both chromatography and membranes resulted in low COP, especially considering the major cost components are the opportunity cost of the trachea and labour costs.

- Direct COP of CS using both technologies is very low ($19.9/kg for chromatography and $13/kg for membranes) compared to the selling price of the product varying between $80 and $110/kg.

- An advantage of the chromatographic approach is that the process to separate BSA from plasma has also identified the same resin, QAE Gibco HB3 as the optimum anion exchange resin. This provides opportunities to produce 3 different products (BSA, IgG enhanced fraction and CS) using the same capital investment, hence reducing commercial risks.

The production of bioactives derived from co-product streams will provide the meat industry with the following opportunities:

- Follow a similar development trajectory to the dairy industry in adding value to co-products and extracting maximum value from input of scarce resources by deployment of cost effective technologies, but novel to the industry.

- Deploying technologies such as ion exchange chromatography using QAE Gibcocel HB3 will enable the production of 3 different product streams contributing towards a suite of bioactive products from the same investment.

- Significantly contribute towards the sustainability of the meat industry that includes environmental, resource utilisation, job creation and job security in regional areas and economic sustainability.

Recommendations

Considering the favourable experimental results, the analysis of COP of CS and the potential gains to the industry discussed above, the following are recommended for further development of the technology for future commercialisation.

- The technology (Alcalase hydrolysis, ion exchange chromatography and membrane separation approaches) should be further developed to pilot scale.
    - Establish scaled up design and operating parameters for the process.
    - Validate consistency of the process and of product specification.
    - Produce prototype samples for customer evaluation.
    - Refine the preliminary COP using data generated at pilot scale.
    - Projected estimate of the capital cost of a commercial scale process using data generated at pilot scale.
    - Evaluate the economic feasibility of a commercial process using data generated at pilot scale.

- Based on the laboratory data, the COP of CS using membranes is lower compared to chromatography, but this should be considered in the overall context after including and comparing the capital costs of the two approaches.

- The patent by Zheng and Lu (2008), a Chinese patent should be scrutinised further if commercialisation of separating CS using a membrane approach is pursued.

- Conduct bioactivity studies (*in vitro* and *in vivo*) to compare the efficacy of current products in the market with the products produced using the proposed technology.

- Development of technology to recover the hydrolysed collagen as a novel bioactive product should be developed as a subsequent stage as this stream will also provide an additional income stream.

These recommendations will provide meat processors or value adders with information to make decisions regarding the product portfolio and the technology.

Reference list

Bali, J.P., Cousse, H. and Neuzil, E. 2001. Biochemical basis of the pharmacologic actions of chondroitin sulphates on the osteoarticular system. *Semin. Arthritis Rheum.* 31, 58 – 68.

Casu, B., Guerrini, M. and Torri, G. 2004. Structural and conformational aspects of the anticoagulant and antithrombotic activity of heparin and dermatan sulphate. *Curr. Pharm. Des.* 10, 939 – 949.

Davies, N.P., Roubin, R.H. and Whitelock, J.M. 2008. Characterization and purification of glcosaminoglycans from crude biological samples. *J. of Agricultural and Food Chemistry.* 56, 343 – 348.

Deal, C.L., Moskowitz, R.W. 1999. Nutraceuticals as therapeutic agents in osteoarthritis. The role of glucosamine, chondroitin sulphate and collagen hydrolysate. *Rheum. Dis. Cli. North Am.* 25, 379 – 395.

Doran, P. M. Bioprocess engineering principles. Academic Press; 1995. pp 235

Douck-Choun, Park Yeun-Suk, Gu Cheong-Il, Ji Seon-Bong and Kim. 2001. Enzyme hydrolysis conditions for preparation of sea cucumber hydrolysates containing chondroitin sulphate. *Food Sci. and Biotechnology.* 10 (6), 686 – 689.

Hamano, T., Mitsuhashi, Y., Acki, N., Yamamoto, S., Tsuji, S., Ito, Y. and Oji, Y. 1989. High-Performance liquid chromatography assay of chondroitin sulphate in food products. *Analyst.* 114, 891-893.

Jo, J.H., Park, D.C., Do, J.R., Kim, Y.M., Kim, D.S., Park, Y.K., Lee, T.K. and Cho, S.M. 2004. Optimization of skate (*Raja flavirostris*) cartilage hydrolysis for the preparation of chondroitin sulphate. *Food Sci. Biotechnol.* 13, 622-626.

Jordan K. M. and Arden, N.K. 2003. An evidence based approach to the management of knee osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT). Ann. Rheum. Dis. 62, 1145–155

Hascall, V.C. and Sajdera, S.W. 1970. Physical properties and polydispersity of proteoglycan from bovine nasal cartilage. *J.Bio.Chem.* 245, 4920 – 4930.

Kiani, C., Chen, L., Wu, Y.J. and Yee, A.J. 2002. Structure and function of aggrecan. *Cell Res.* 12, 19-32.

Levene, P.A. 1925. Hexosamines and Mucoproteins. Longmans, Green and Co. London, UK, 165.

Lignot, B., Lahogue, P. and Bourseay, P. 2003. Enzymatic extraction of chondroitin sulphate from skate cartilage and concentration-desalting by ultrafiltration. *Journal of Biotechnology*. 103, 281-284.

Murado, M.A., Fraguas, J., Montemayor, M.I., Vazquez, J.A. and Gonzalez, P. (2010). Preparation of highly purified chondroitin sulphate from skate (*Raja clavata*) cartilage by-products. Process optimization including a new procedure of alkaline hydroalcoholic hydrolysis. *Biochemical Engineering Journal.* 49, 126-132.

Nakano, T., Ikawa, N. and Ozimek, L. (2000). An economical method to extract chondroitin sulphate peptide from bovine nasal cartilage. *Canadian Agricultural Engineering.* 42 (4), 205 – 208.

Nakano, T., Betti, M. and Pietrasik, Z. 2010. Extraction, isolation and analysis of chondroitin sulphate glycosaminoglycans. *Recent Patents on Food, Nutrition and Agriculture.* 2, 61-74.

Nakano, T., Nakano, K. and Sim, J.S. 1998. Extraction of Glycosaminoglycan peptide from bovine nasal cartilage with 0.1M sodium acetate. *J. Agric. Food Chem.* 46, 772-778.

Silva, L.F. 2006. Isolation and Purification of Chondroitin Sulfate. In, Advance in Pharmacology: Chondroitin Sulfate: Structure, Role and Pharmacological Activity. Ed. N. Volpi. Elsevier, London, UK.

Udenfriend, S., Stein, S., Bohlen, P., Dairman, w., Leimgruber, W. and Weigele, M. 1972. *Science* **178** : 871 – 872.

Volpi, N. and Bolognani, L. 1993. Glycosaminoglycans and proteins: different behaviours in high-performance size-exclusion chromatography. *J. Chromatography*. 630, 390-396.

Volpi, N. 1996. Purification of heparin, dermatan sulphate and chondroitin sulphate from mixtures by sequential precipitation with various organic solvents. *J. Chromatography B* 685, 27 – 34.

Volpi, N. 2004. The pathology of osteoarthritis and rationale for using the chondroitin sulphate for its treatment. *Curr. Drug Targets.* 4, 119 – 127.

Watanbe, H., Yamada, Y. and Kimata, K. 1998. Roles of aggrecan, a large chondroitin sulphate proteoglycan, in cartilage structure and function. *J. Biochem.* 124, 687-693.

Zhang, W. and Moskowitz, R. W. 2007. OARSI recommendations for the management of hip and knee osteoarthritis, Part I: Critical appraisal of existing treatment guidelines and systematic review of current research evidence activity. Osteoarthritis and Cartilage 15, 981-999

Patents

Zhang, L., Han, L., Yu, Q., Chen, Y. and Tian, J. 2010. CN 101904491-A. Production of chondroitin sulphate from cow or sheep by dissolving cartilage powder in hot alkali solution, enzymatically hydrolysing leached liquid, extracting and drying dehydrated material to obtain chondroitin sulphate extract.

Huang, H., Tan, A., Yang, H., Yu, W. and Zhang, K. 2009. CN101348815-A. Preparing chondroitin sulphate, by extracting cartilage with sodium hydroxide and sodium chloride, processing extract with homogeneous pancreas of pig and 2709 alkaline protease, and subjecting processed extract to alcohol extraction.

Wang, Q., Fu, Y., Luo, C. and Yang, M. 2009. CN101358220-A. Method for extracting calcium chondroitin sulphate in shark cartilage.

Zheng, F. and Lu, Y. 2008. CN101280027-A. Extracting method of chondroitin sulfate.

Song, J. and Yan, D. 2007. CN1903889-A. Method of extracting chondroitin sulphate includes using alkali to extract and enzymolyze animal soft bone to obtain filtrate, resin adsorption and purification, using acrylic strong base type ion exchange resin to absorb the filtrate.

Khare, A.B., Houliston, S.A. and Black, T.J. US patent 20040146993A1. 2004. Isolating chondroitin sulphate.

Gérard, J. 2004. WO 2004/046199–A2. Novel method of obtaining chondroitin sulphuric acid and uses thereof.

Tang, Y.I. 2004. CN1486993-A. Preparation of chondroitin sulphate with medium molecule weight.

Ashie, I. and Pederson, B.P. 2005. WO2005037871-A1. Extracting glycosaminoglycan such as chondroitin sulphate, hyaluronate, keratine sulphate from animal tissue, involves hydrolysing tissue with endoprotease and exoprotease and separating glycosaminoglycan from hydrolysed animal tissue.

Josse, G. and Josset, G. 2004. WO2004046199-A3. Production of chondroitin sulphate useful for treating arthritis and ocular disorders comprises hydrolysing bovineor porcine cartilage with Bacillus licheniformis alkaline protease.

Lee, J.H. and Park, J.H. 2002. KR2002096272-A. Obtained method of chondroitin sulphate A involves hydrolysing the organ of oxen by using pepsin, heating to deactivate enzyme, separating obtained one by centrifugation, purifying, dissolving Ito water, salting out dissolved one.

Beserra, De Paula, H.C. and Wilson, De Alencar, J. 2001. BR200105320-A. Production of chondroitin sulphate from bovine trachea consists of cleaning of the cartilage tissues for enzyme based hydrolysis, precipitation and grinding.

Takashi, N., Tadaaki, T. and Tadashi, O. 2000. JP2000273102-A. Method for isolation and purification of chondroitin sulphate.

Tadashi, H., Keiichi, Y. and Takashi, O. 1976. JP51051509-A. Highly pure chondroitin sulphate preparation from animal cartilage- by alkaline or enzymic extraction and purification using strongly basic anion exchange resin.

Appendices

Appendix 1–Literature patent review–methodology

1.1.12 Scientific literature searches

Free text searches were conducted on the following databases:

Commonwealth Agricultural Bureau Index (CABI) contains over 3.8 million records from over 10,000 journals, books, conferences, reports, and other kinds of literature published internationally. Subjects covered include animal and crop husbandry, animal and plant breeding, plant protection, genetics, forestry, economics, veterinary medicine, human nutrition, and rural development. This database has extensive coverage of the meat and livestock industry. It was searched using the following key word search terms:

"chondroitin" was combined with separate* or recover* or fraction* or extract* or purify*.

Food Science and Technology Abstracts (FSTA) is the internationally recognised world's leading Food Science and Technology Abstracts database and it has extensive coverage of post- farm-gate food processing, and ingredients industry.

The term "chondroitin" was hand combed for relevant articles.

1.1.13 Intellectual property searches

Derwent Innovations Index

Intellectual property searches were carried out on Derwent Innovations Index, a respected patent database with worldwide coverage. Derwent covers material back to 1963 although this coverage varies with the country of origin. This variable country coverage means that material that has been registered in a single jurisdiction prior to that jurisdiction being covered by Derwent will not appear in these results. The advantage of Derwent, apart from

its breadth of coverage, is that it collates patents into their families. This means that the user does not have to bring single applications in different countries for the same patent together in order to follow their registration and status in different patent jurisdictions. If a patent was registered later in a covered zone, the patent family approach sometimes mitigates the country coverage variability issue.

Country Coverage by Derwent Innovation Index

Country/Abbreviation	Initial Year of Coverage
Argentina (AR)	1974-1976 only
Australia (AU)	1983 (also 1963-1969)
Austria (AT)	1975
Belgium (BE)	1963
Brazil (BR)	1976
Canada (CA)	1963
China (CN)	1987
Czech Republic (CZ)	1994
Czechoslovakia (CS)	1975-1994
Denmark (DK)	1974
European Patents (EP)	1978
Finland (FI)	1974
France (FR)	1963
Germany (East) (DD)	1963
Germany (DE)	1963
Hungary (HU)	1975
India (IN)	2004
International Technology Disclosures (TP)	1984-1993

Ireland (IE)	1963-1969; 1995
Israel (IL)	1975
Italy (IT)	1966-1969 (section A subjects only); 1978
Japan (JP)	1963
Korea (KR) (South)	1986
Luxembourg (LU)	1984
Mexico (MX)	1997
Netherlands (NL)	1963
New Zealand (NZ)	1993
Norway (NO)	1974
Patent Cooperation Treaty (WO)	1978
Philippines (PH)	1995
Portugal (PT)	1974
Research Disclosure (RD)	1978
Romania (RO)	1975
Russian Federation (RU)	1994 (Russia)
Singapore (SG)	1995
Slovakia (SK)	1994
South Africa (ZA)	1963
Soviet Union (SU)	1963-1994
Spain (ES)	1983
Sweden (SE)	1974
Switzerland (CH)	1963
Taiwan (TW)	1993
United Kingdom (GB)	1963

United States (US)	1963

The terms and international patent classification codes (IPCs) discussed below were used for locating patents in the Derwent Innovations Index (DII) database.

1. **International Patent Classification (IPC) Codes**

IPC codes substitute for keyword or descriptor indexing on patent databases. The following bold highlighted IPC codes were used to identify separation of CS materials. These were combined with "chondroitin" to eliminate other compositions. The relevant codes are shown in their hierarchy of meaning.

C08 - Organic macromolecular compounds; their preparation or chemical working-up; compositions based thereon

C08B - Polysaccharides; derivatives thereof

C08B-037/00 - Preparation of polysaccharides not provided for in groups - see cross reference IPC C08B-001/00 to - see cross reference IPC C08B-035/00; Derivatives thereof

C08B-037/08 - Chitin; Chondroitin sulfate; Hyaluronic acid; Derivatives thereof

C08L - COMPOSITIONS OF MACROMOLECULAR COMPOUNDS

C08L-005/00 - Compositions of polysaccharides or of their derivatives not provided for in group - see cross reference IPC C08L-001/00 or - see cross reference IPC C08L-003/00

C08L-005/08 - Chitin; Chondroitin sulfate; Hyaluronic acid; Derivatives thereof

A61K - Preparations for medical, dental, or toilet purposes

A61K-031/00 - Medicinal preparations containing organic active ingredients

A61K-031/70 - Carbohydrates; Sugars; Derivatives thereof

A61K-031/715 - Polysaccharides, i.e. having more than five saccharide radicals attached to each other by glycosidic linkages; Derivatives thereof, e.g. ethers, esters

A61K-031/737 - Sulfated polysaccharides, e.g. chondroitin sulfate, dermatan sulfate

As most of the records in this code were for applications out of scope it was combined with a set of IPC codes for separation technologies, namely

B01D – SEPARATION was searched in its truncated forms as BO1D* and with some selected more specific codes.

The following free-text forms were also combined with this code to get separation items:

Separate* or sepn or aqueous or solvent or salt or precip* or membrane or filtrate* or resin or chromatograph* or hydrolys* or hydrolyz* or synthesis

2. Free Text Searches

The following free text terms were searched as title terms and hand combed

“chondroitin sulphate*” or “chondroitin sulphate*”or “sodium chondroitin” or “chondroitin polysulphate” or “chondroitin monosulphate” or “chondrotin sulphuric acid*” or “chondroitin sulphuric acid*”.

The same terms were searched as topic terms i.e. could appear anywhere in the record and combined with:

Separate* or sepn or aqueous or solvent or salt or precip* or membrane or filtrate* or resin or chromatograph* or hydrolys* or hydroly* or synthesis.

Appendix 2–Free Amino Nitrogen Assay (FAN Assay)

Free Amino Nitrogen levels increase when proteins undergo hydrolysis and this can be measured by the FAN assay described by Udenfriend *et al.* (1972). Fluorescamine is a non-fluorescent chemical used in this assay, which reacts with the primary amines to form fluorophores. The fluorescence can be detected at 390 excitation and 475 nm emission wavelengths.

In this assay, 0.1 mL of fluorescamine solution (0.1% w/v in acetone) was added to 10 μL of appropriately diluted sample buffered to pH 7.0 with 0.3 mL 0.2 M borate buffer, pH 7.0. The samples were added into a black 96 well plate and the fluorescence level was read using a microplate reader at 390 nm excitation and 475 nm emission wavelengths. The standard curve was constructed with Gly-Gly-Gly standards with concentrations ranging from 0–2.5 mM in milliQ water.

Appendix 3–Blyscan Assay for CS determination

The Blyscan assay is a quantitative dye-binding method for the analysis of sulphated proteoglycans and glycosaminoglycans. The principle is based on the interaction of 1, 9 dimethyl-methylene blue at acidic pH with glycosaminoglycans. The samples were prepared according to the method described in the Blyscan kit. Standard curve was constructed with Chondroitin Sulphate A at concentrations ranging from 0 – 100 μg/mL in milliQ water. The standards and samples were added into wells of a 96 well plate and read at 656 nm using a microplate reader.

Appendix 4–Solids Content Analysis

The total solids content in the hydrolysates were determined using a Mettler Toledo HR73 Halogen Moisture analyser.

Appendix 5–Assumptions used in determining the cost of producing CS using ion exchange chromatography

Number of animals killed per day	20,500
Weight of a trachea (g/trachea)	250
Trachea slurry concentration (%)	20
Trachea solids content (%)	27
CS content in cleaned trachea (g/kg)	50
CS recovery (%)	80
Steam for heating (kg/kg water heated)	0.11
Steam for drying (kg/kg water evaporated)	2.25
Enzyme addition based on trachea solids (v/w%)	0.5
Resin CS binding capacity (g/L resin)	3.5
Elution CV	1
Re-cycled salt molarity (M)	3
Ultrafiltration flux rates ($l/m^2/hr$)(70% of expected flux)	50
Ultrafiltration feed CV	2
Ultrafiltration concentration factor	85
Diafiltration number	5
Diafiltration flux (l/m2/hr) (50% of expected)	38
Power for ultrafiltration (kWh/kL)	3.5
Cost:	
Enzyme ($/L)	39
Resin ($/L)	169
Membranes ($/$m^2$)	100
Water ($/kL)	1.2
Salt ($/kg)	0.55
Packing ($/kg)	0.5
CIP chemicals ($/batch)	50
Spray drying ($/kg water evaporated)	0.056
Power ($/KWh)	0.135
Steam ($/ton)	25
Opportunity costs of trachea ($/kg)	0.2

Column volume (CV); Cleaning–in–place (CIP)

Labour ($/year)	35,000
Number of workers per shift	2
Trachea loading and heating time (hours)	1
Hydrolysis time (hours)	4
Enzyme inactivation time (hours)	1
Coarse filtration time (hours)	1
CIP time (hours)	1
Chromatography (hours)	20
Ultrafiltration (hours)	18.6
Diafiltration time (hours)	1.4
CIP (hours)	4
Chromatography cycle time (hours)	1
Resin life (years)	2
Membrane life (years)	2
Number of hours operated per shift	8
Number of shifts/day	3
Number of operating days per week	5
Number of weeks per year	48
Trachea to be processed (kg/day)	5,125
Batch size (L)	25,625
Enzyme (L/batch)	6.92
Hydrolysis batches per day	1
Expected CS content (kg/batch)	205
Resin volume (L)	2,929
CS content per chromatography cycle (kg)	10.25
CS concentration in the eluate (g/L)	1.75
UF feed volume (L/batch)	117,143
Ultrafiltration area (m^2)	126
Diafiltration area (m2)	130
CS in UF concentrate (%)	14.88
Salt (kg/batch)	514
Water to be evaporated (kg/batch)	1,173

Appendix 6–Assumptions used in determining the cost of producing CS using membrane systems

Number of animals killed per day	20,500
Weight of a trachea (g/trachea)	250
Trachea slurry concentration (%)	20
Trachea solids content (%)	27
CS content in cleaned trachea (g/kg)	50
CS recovery (%)	80
Steam for heating (kg/kg water heated)	0.11
Steam for drying (kg/kg water evaporated)	2.25
Enzyme addition based on trachea solids (v/w%)	0.5
Ultrafiltration flux rates ($l/m^2/hr$)(70% of expected flux)	30
Ultrafiltration concentration factor	22
Diafiltration number	6
Diafiltration flux (l/m2/hr) (50% of expected)	22
Power for ultrafiltration (kWh/kL)	3.5
Microfiltration flux rates ($l/m^2/hr$)(70% of expected flux)	100
Microfiltration concentration factor	10
Diafiltration number	3
Diafiltration flux (l/m2/hr) (50% of expected)	75
Power for ultrafiltration (kWh/kL)	10
Cost:	
Enzyme ($/L)	39
Membranes ($/$m^2$)	100
Water ($/kL)	1.2
Packing ($/kg)	0.5
CIP chemicals ($/batch)	50
Spray drying ($/kg water evaporated)	0.056
Power ($/KWh)	0.135
Steam ($/ton)	25
Opportunity costs of trachea ($/kg)	0.2

Labour ($/year)	35,000
Number of workers per shift	2
Trachea loading and heating time (hours)	1
Hydrolysis time (hours)	4
Enzyme inactivation time (hours)	1
Coarse filtration time (hours)	1
CIP time (hours)	1
Microfiltration and diafiltration (hours)	6
Diafiltration time (hours)	2
Ultrafiltration (hours)	9.57
Diafiltration time (hours)	3.43
CIP (hours)	2
Membrane life (years)	1
Number of hours operated per shift	8
Number of shifts/day	3
Numberof operating days per week	5
Number of weeks per year	48

PART B: Bovine Serum Albumin

Contents

Background and introduction

Background

The sustainability and development of the meat processing industries is constrained by a focus on meat cuts only. Insufficient value is gained from the remaining majority of the animal which is usually processed as render (normally around $0.10/kg). This project is designed to add value to meat processing co-product streams by developing innovative cost-effective separation technologies to produce chondroitin sulphate (CS) and bovine serum albumin (BSA) as bioactive molecules for use as dietary supplements, nutraceuticals, cosmeceuticals or functional food ingredients. The MLA has estimated that the production of 5 major meat derived bioactives could add a net benefit of ~$30m pa to the meat industry. The major impediments to elicit meat industry investment to develop a bioactive industry have been the capital costs of the technology and its related operating costs.

The global BSA market is supplied by employing traditional solvent extraction and heat precipitating methods. In the pharmaceutical industry, high purity BSA is manufactured using a combination of different chromatographic methods. All these methods are expensive due to the use of solvents and the cost of their recovery. The different chromatographic resins used in the pharmaceutical industry are also very costly.

The development and use of cost-effective technology for the production of CS and BSA will make it viable for the meat industry to invest in developing a bioactives industry and help improve the sustainability of the meat industry and provide the impetus for additional export income.

This project will build upon the different separation technologies developed and implemented in the dairy industry for protein separation, the knowledge and experience in enzyme technology employed to digest connective tissues to extract marine CS and employ the knowledge and expertise in using ion exchange chromatography, membrane separation and, thermal and acid/base reaction systems to separate BSA from plasma and CS from connective tissue in a cost effective manner.

This report details the activities and experimental results from the programme undertaken to develop a process to separate BSA from bovine plasma and details of the program undertaken to separate CS will be reported separately.

Introduction – Membrane filtration

Membrane separation is a technology widely used in the food processing industry to concentrate, fractionate and purify components such as proteins in the dairy and soy processing industries and in the beverage industry to concentrate juice. New applications of membrane-based processes continue to emerge as these processes give the food industry three major advantages: food safety, competitiveness and environmental friendliness.

Membrane technology is based on a semi-permeable membrane with a nominal molecular weight cut-off range mounted on a porous support that allows free passage of solutes and fluid through it. The molecular weight cut-off of the membrane determines the size of the molecules that are rejected at the membrane surface or allowed to permeate through the membrane.

Microfiltration (MF) is commonly used to recover macromolecules and retain suspended colloidal particles, and is integrated into both upstream and downstream processes. A large range of MF applications are reported such as pre-treatment steps, removal of small molecules from bigger protein molecules, clarifying suspensions for cell harvesting, and sterilising liquids to remove viruses and bacteria (Saxena, Tripathi, et al., 2009). Ultrafiltration (UF) membranes were designed to provide high retention of proteins and other macromolecules. The UF process has become particularly important for concentrating proteinaceous solutions (Saxena, Tripathi, et al., 2009).

Microfiltration and ultrafiltration are potentially attractive processes for the fractionation of protein solutions in downstream processing of bovine plasma. Both MF and UF offer mild operating conditions and the proteins retain their functional and biological properties.

Review of literature and patents on separation of BSA by membrane filtration

Effectiveness and efficiency of microfiltration and ultrafiltration are strongly dependent on operating parameters such as solution pH, ionic strength, concentration, permeate flux and system hydrodynamics. Several studies have been conducted to demonstrate the importance of these factors for plasma proteins separation/fractionation by selective membrane filtration.

Burns and Zydney (1999) have examined the effect of solution pH on the sieving characteristics of a variety of proteins having different molecular weight and surface charge characteristics. It has been shown that the protein-sieving coefficient attained its maximum value near an isoelectric point (pI) of a protein and decreased at pH both above and below the pI. For example, the sieving coefficient of BSA decreased by more than two orders of magnitude (from 0.22 to 0.002) as the pH was reduced from 4.7 to 3.5 due to the strong electrostatic exclusion of the positively charged protein from the membrane pores. The authors postulated that the nonlinear dependence of the sieving coefficient on pH was due almost entirely to the nonlinear dependence of the BSA charge on solution pH arising from the acid–base equilibria of the various charged amino acids in the protein structure.

The effect of salt concentration on permeability of BSA through an asymmetric 100 kDa molecular weight cut-off polyethersulfone (PES) membrane has been investigated in the study of Pujar and Zydney (1994). The observed BSA sieving coefficients decreased by more than 2 orders of magnitude when the ionic strength was reduced from 0.15 to 0.0015 M due to changes in both membrane and bulk mass transport associated with the decreased electrostatic shielding at low salt concentrations.

Saksena and Zydney (1994) obtained experimental data for the transport of BSA and immunoglobulins (IgG) through 100 and 300 kDa molecular weight cut-off PES membranes in a stirred ultrafiltration device at different solution pH and ionic strength. Under physiological conditions (pH 7.0 and 0.15 M NaCl) the maximum selectivity of a100 kDa membrane for the BSA-IgG separation in the binary model system was only about 2.0. In contrast, BSA-IgG selectivities as high as 50 were obtained with the same membranes when the protein solution was at pH 4.8 and 0.0015 M NaCl. This increase in selectivity was attributed to the electrostatic exclusion of the positively charged IgG, with the uncharged

BSA passing through the membrane. It has been also demonstrated that the membrane selectivity could actually be reversed, with higher passage of the larger IgG molecules, by using a 300 kDa molecular weight cut-off membrane at pH 7.4 and an ionic strength of 0.0015 M NaCl.

The effects of pH and ionic strength on transmission of human serum albumin (HSA) and human immunoglobulins (HIgG) through a 100 kDa PES membrane were studied by Wan, Ghosh, et al. (2002). A pulsed sample injection technique was used in their study. 500 µl of 20 mg/ml HSA and 200 µl of 20 mg/ml IgG were injected into the UF module in the form of a pulse through a sample injector. A selectivity of more than 300 was observed for fractionation of HSA/HIgG with 100 kDa PES membrane at pH 4.7 and a salt (NaCl) concentration of 1.5 mM.

Shukla et al. (2000) have demonstrated the possibility of fractionating proteins with a similar molecular weight such as bovine serum albumin (69 kDa) and bovine haemoglobin (67 kDa) by ultrafiltration. The mixture of 0.1% BSA and 0.01% haemoglobin was used as a model system. Three different membranes, regenerated cellulose, polysulfone (PES) and surface modified polyacrylonitrile (PAN), each with a nominal molecular cut-off of 100 kDa, were examined. The experiments were conducted in dead end, crossflow and vortex flow filtration modes and the separation was studied as a function of feed pH and ionic strength. Under similar system hydrodynamics, the surface modified PAN membrane displayed the highest resolution with minimum membrane fouling. The highest separation factor of 40 was obtained at the pI 6.8 of haemoglobin.

A similar study was conducted by Lin et al. (2008) where the effects of operating parameters on the flux and separation factor of BSA and haemoglobin using PES and PAN membranes (MWCO 100 kDa) in dead-end UF were investigated. Binary mixtures of BSA and haemoglobin at concentrations ranging from 100 to 500 ppm were used as the feed material. The high separation of BSA and haemoglobin was obtained at conditions of lower applied pressure, low ionic strength and solution pH higher than pI of haemoglobin. Under comparable conditions, PAN membrane displayed higher flux and higher separation factor than that of PES membrane.

Feins and Sirkar (2005) developed a new UF technique based on a multi-membrane stack to fractionate BSA and haemoglobin from a binary protein mixture with low concentration of BSA (1.0 mg/ml) and haemoglobin (0.2 mg/ml). Complete rejection of BSA was achieved using three PES 100 kDa membranes under optimum conditions. However, a longer period of operation (~5 hours) was needed for complete recovery.

Higuchi et al. (1991) have reported the separation of BSA and IgG using unmodified and surface modified PES membranes (MWCO 200 kDa) in batch-type ultrafiltration experiments. The mixed solution of BSA and IgG with the concentration of each protein 100 ppm was used as the feed material. The unmodified membrane did not extensively separate the proteins at any pH, unlike the surface modified membrane which rejected almost 100% BSA at pH 8.0. The authors attributed this “reverse selectivity” to the different interactions of the BSA and IgG with hydrophobic and hydrophilic segments on the surface of the modified membrane.

Christy et al. (2002) have demonstrated that separation of plasma proteins could be enhanced by using charged membranes. The sieving of positively charged proteins was much lower in positively charged membranes due to like-charge repulsion. Selection of a buffer pH where the product protein had a positive charge and the impurity proteins had a net neutral charge resulted in low passage of the product and high passage of the impurities. This enhanced separation was greatest at low ionic strengths.

Mayani et al. (2009) examined the use of multi-stage or cascade ultrafiltration systems for continuous fractionation of model proteins, human serum albumin (HSA) and human immunoglobulin G (HIgG). The feed solution consisted of 0.23 g/mL HIgG and 1.0 g/mL HSA. The maximum purity and recovery achieved with three-stage counter-current system for HSA were >98% and 90%, respectively while the corresponding values or HIgG were >64% and >95%, respectively. The trade-off between purity and recovery was also observed with the cascade systems but this was not as critical as with the single-stage system.

Membrane fouling is a critical factor affecting the performance and overall effectiveness of membrane processes. Membrane fouling is a process where solute or particles deposit onto

a membrane surface or into membrane pores in a way that degrades the membrane's performance.

Kelly et al. (1993) has described two major causes of protein fouling of UF membranes: protein adsorption, which is a specific interaction between the protein and membrane, and protein deposition, which involves the formation of an additional protein layer during filtration. The mechanism of protein deposition in these systems is still uncertain, with previous investigations attributing the deposit formation to: (1) protein precipitation or gel layer formation associated with the high concentration of retained proteins that accumulate at the membrane surface, (2) a polymerisation-type reaction in the concentrated protein solution at the membrane, and/or (3) the entrapment of proteins in the small pores of the ultrafiltration membranes.

Membrane fouling can be a strong function of solution pH due to the influence of protein-surface and protein–protein interactions on both the rate and extent of fouling (Burns and Zydney, 1999).

A number of studies have been devoted to the interaction of BSA, the most important protein in blood plasma, with polymeric membranes.

Salgin (2007) has investigated the influence of electrostatic interactions on membrane fouling during the separation of bovine serum albumin (BSA) using 10 kDa molecular weight cut-off polyethersulfone (PES) membrane. Experiments were carried out at different pH values between 3.78 and 7.46 and for different ionic strengths between 0.001 M and 0.1 M. The changes in permeate flux, cake layer resistance, zeta potentials of BSA and PES membranes, and electrostatic interaction energies, were evaluated. At all of the ionic conditions studied, PES membranes are negatively charged. However, BSA molecules are either negatively or positively charged depending on the ionic environment. Whereas the cake layer resistance decreased with increasing pH and ionic strength, the permeate fluxes increased. The calculated electrostatic energy was a minimum at the isoelectric point of BSA. However, at this point, the cake resistance corresponding to fouling at each ionic strength were not minimized.

Wang and Rodgers (2011) have characterised the pressure-dependent permeate flux behaviour of completely rejected BSA. Tangential-flow diafiltration experiments with a 30 kDa regenerated cellulose membrane were performed using BSA solutions at pH between 4.7 and pH 10, and various ionic strength conditions (0.0037-0.15M). It was observed that the pressure-dependent permeate flux behaviour increased with increasing pH (corresponding to the increasing net surface charge of BSA) and decreasing ionic strength (corresponding to decreasing screening effects). At pH 4.7, the pI of BSA, the permeate flux was low, and did not show significant ionic strength dependence. The highest flux was observed at pH 10 and 0.0037M ionic strength, corresponding to the strongest electrostatic contribution among the three pH conditions studied, BSA has the most negative net charge at pH 10, while 0.0037M ionic strength provided the least electrostatic screening effect.

Due to the much larger pore sizes of microfiltration membranes (as much as ten times the size of the proteins) it is very difficult to explain fouling during microfiltration. Kelly et al. (1993) have examined experimental studies on macromolecular fouling of MF membranes and have proposed several explanations: (1) the fouling behaviour can be attributed to the secondary protein adsorption onto the pore walls induced by the high shear rates associated with the solvent flow through the membrane pores; (2) the small changes can occur in the protein structure during filtration and these conformational alterations may lead to the additional protein adsorption on the pore walls; (3) the formation of the protein deposit on the membrane surface can cause decline in flux and protein transmission; (4) prolonged pumping tends to form relatively large protein aggregates in the bulk solution, with the observed fouling behaviour attributed to the deposition of these large aggregates on the membrane surface.

Kelly et al. (1993) investigated fouling behaviour of PES microfiltration membranes (0.15 µm) used for filtration of different commercial preparations of BSA. The researchers found that the fouling time of these BSA preparations was very highly correlated with concentrations of conformationally altered or possibly aggregated BSA. The results suggested that BSA fouling of these microfiltration membranes was associated with the deposition of trace quantities of aggregated and/or denatured BSA and these fouling species served as initiation sites for the continued deposition of bulk protein.

Most of the studies on the membrane-based separation of blood proteins have been conducted using model systems and there are only a few papers dealing with the fractionation of whole blood plasma or blood serum.

Ohno et al. had developed and patented in 1982 the process of separating albumin and γ-globulin proteins in the whole blood serum by ultrafiltration using a 100 kDa molecular weight cut-off polyethersulfone (PES) membrane. The selective separation of proteins was achieved by adjusting the pH of the blood serum protein mixture to 3.8 - 4.7, maintaining total protein and salt concentration in the solution below 40 g/l and 0.6 mole/l, respectively. The higher protein concentration caused clogging of the membrane. The protein selectivity decreased significantly at higher salt concentration.

Fernandez et al. (1999) have developed a process for the concentration and desalination of BSA. In a first step the bovine plasma containing 3.8% sodium citrate was microfiltrated using 0.1 µm membrane. This was followed by ultrafiltration with an inorganic 300 kDa molecular weight cut-off membrane. The retentate was a conserved fibrinogen fraction. Permeate was concentrated using a 15 kDa membrane. Finally a diafiltration process was used to separate salts and low molecular weight compounds. The final concentration of BSA, with a much reduced amount of salts, was 3.9 times the initial concentration.

Project objective – Membrane filtration

The overall aim of the project was to develop a cost effective laboratory scale process to produce BSA at > 80% purity from bovine plasma, and to scale up the process to produce a gram quantity sample at preparative laboratory scale.

The objective of this part of the project was to investigate the membrane-based processes (ultrafiltration and microfiltration) to isolate bovine serum albumin (BSA) from bovine plasma and to select promising technologies and develop laboratory based process to produce BSA at >80% from bovine plasma. A preliminary analysis of the cost of production of BSA was also to be assessed.

Methodology – Membrane filtration

Materials

1.1.14 Membranes and membrane filtration equipment

Four flat sheet ultrafiltration and three tubular microfiltration membranes were selected to compare their ability to isolate bovine serum albumin (BSA) from bovine plasma. All membranes are FDA listed. Properties of selected membranes as provided by the manufacturers are summarised in Table 3.1.

Table 3.1 Specifications of ultrafiltration and microfiltration membranes used for investigating the fractionation of BSA from bovine plasma.

Membrane Type	Membrane Code	Manufacturer	MWCO nominal	Membrane Material	pH range during filtration	Maximum operating temperature (°C)
UF	HFM-180	KOCH Membrane Systems	100,000 Daltons	PVDF (polyvinyl difluoride)		
UF	PM UC100	Microdyn Nadir	100,000 Daltons	Regenerated cellulose	1 - 11	55
UF	LY	Synder Filtration	100,000 Daltons	PES (polyethersulfone)	2 - 10	55
UF	LX	Synder Filtration	300,000 Daltons	PES (polyethersulfone)	2 - 10	55
MF	ET1025001 CG-B-A	PALL corporation	0.1 µm	Ceramic	1 - 14	95
MF	ET1025002 CA-B	PALL corporation	0.2 µm	Ceramic	1 - 14	95
MF	ET1025005 CA-B	PALL corporation	0.5 µm	Ceramic	1 - 14	95

Ultrafiltration experiments were conducted using Amicon stirred cell 8050 (Millipore) with holding volume of 50 ml and nominal membrane diameter of 43 mm. Prior to use the membranes were soaked in reverse osmosis (RO) water to remove the preserving agent. The ultrafiltration membrane was placed at the bottom of the cell. The device was pressurised with compressed air to force liquid through the membrane. Gentle stirring was applied to control the concentration and accumulation of proteins on the membrane surface.

Figure 3.1 Amicon Stirred Cell 8050 components and assembly

A Membralox XLAB 5 tangential flow bench scale membrane unit (PALL Corporation) was used for microfiltration trials.

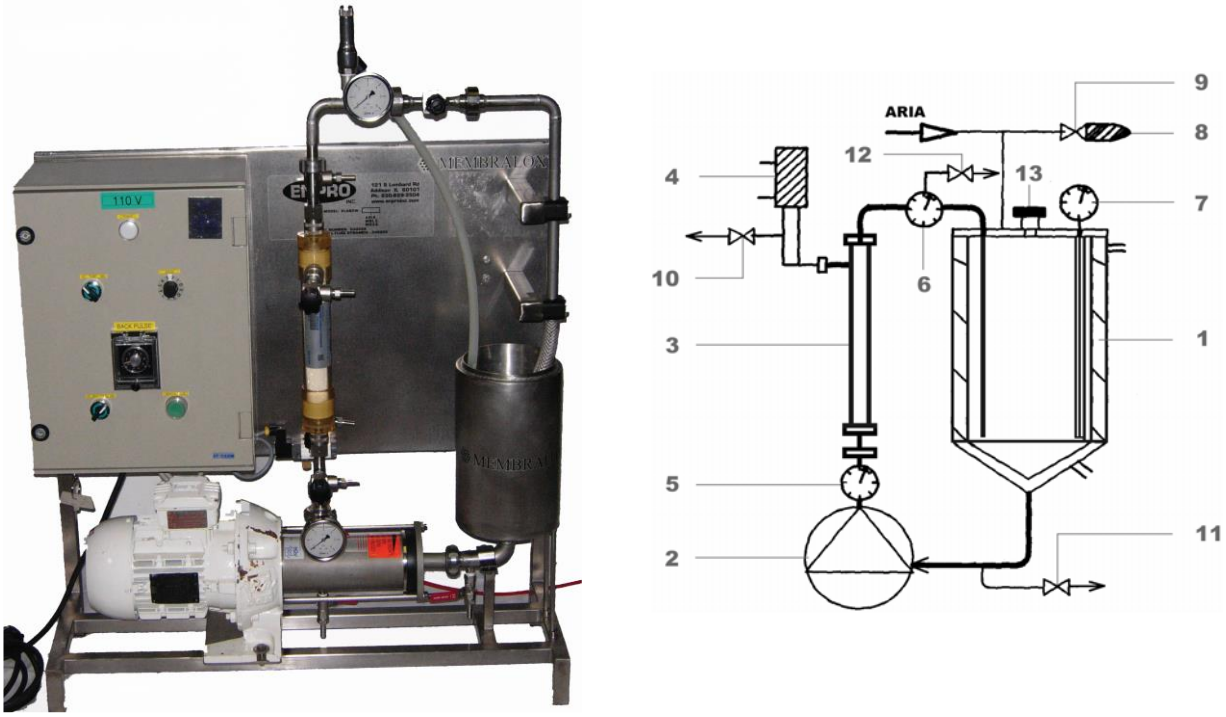

Figure 3.2 Membralox XLAB 5 components and assembly: 1 - jacketed feed tank;

2 - pump; 3 – membrane; 4 - back–pulse device; 5, 6 - pressure gauges; 7 - temperature probe; 8 - muffler; 9, 10, 11 - valves; 12 – air purge valve.

The system consists of manual permeate and retentate sampling valves, back-pulse device, variable speed pump and 3.5 litre feed tank. The module housing holds individual ceramic tubes with the length of 250 mm and the channel diameter of 7 mm. Prior to use all membranes were cleaned according cleaning–in–place (CIP) instructions provided by Pall Corporation.

1.1.15 Chemicals

Chemicals used for preparation of mobile phases in ultra performance liquid chromatography (UPLC) analysis were analytical-grade trifluoroacetic acid (TFA) (Sigma-Aldrich, Germany) and acetonitrile (Optigen Scientific, Australia). Purified powders of bovine serum albumin (BSA) and transferrin were purchased from Sigma-Aldrich (USA) and used as the reference material for proteins quantification.

Methods

1.1.16 Plasma preparation for membrane filtration trials

The frozen bovine plasma used in laboratory scale experiments was obtained from JBS Swift Company (Dinmore, Queensland) and stored at the temperature of –20^{o}C. Immediately prior to use, plasma was thawed at the room temperature and filtered through Whatman (No. 4) filter paper. The filtered plasma had a protein content of 8.17% and solids content of 9.43%. The pH and conductivity of the plasma were 7.78 and 13.6 mS/cm, respectively.

The analytically determined concentration of BSA, IgG and transferrin in the bovine plasma together with the published values for isoelectric point and molecular weight of proteins are presented in Table 3.2.

Table 3.2 Properties and composition of BSA, transferrin and IgG in bovine plasma supplied by JBS Swift Company. Protein content of plasma was determined using UPLC.

	BSA	IgG	Transferrin
Concentration in plasma (mg/ml)	30	22	4.2
Isoelectric point	4.9	5.8-7.3	5.9
Molecular weight (kDa)	68	160	79

1.1.17 Quantification of BSA and Transferrin

The concentration of BSA and transferrin in bovine plasma and processed samples were determined by ultra performance liquid chromatography (UPLC). The UPLC system was equipped with four ACQUITY units –sample manager, binary solvent manager, column heater, and Photodiode Array (PDA) Detector (Waters Corporation, USA). Proteins were separated on UPLC BEH300, C_4, 1.7 µm column. The column temperature was maintained at 50°C. The gradient was applied by using solvent A (0.1% TFA in water) and solvent B (0.1% TFA in Acetonitrile). The total run time was 4 minutes. The proteins were detected at the wavelength of 220 nm. The concentration of BSA and transferrin were calculated from standard calibration curves. The standards were prepared at concentrations 0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml and injected to the column at 2 µl. The UPLC overlaid chromatogram showing transferrin and BSA in bovine plasma and the corresponding standards is presented in Figure 3.3.

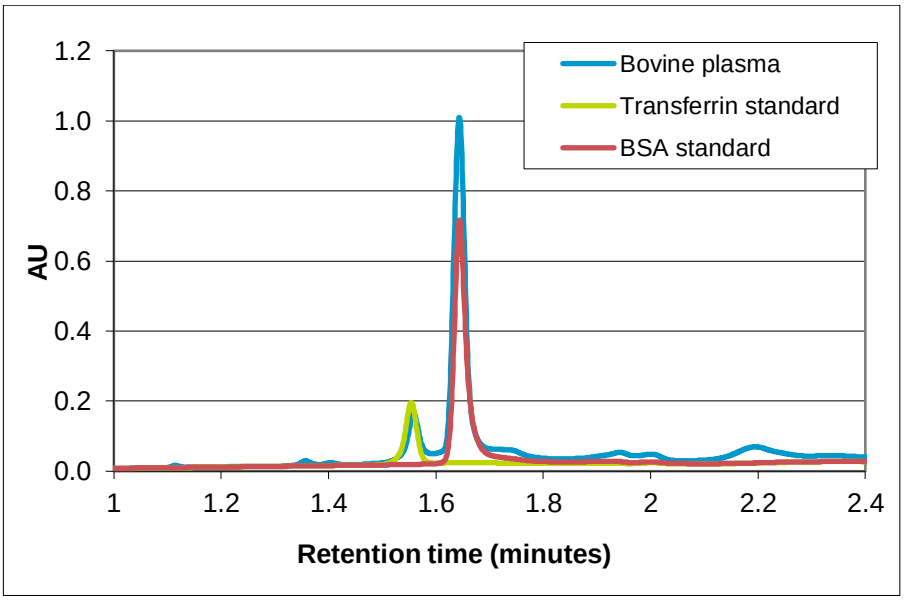

Figure 3.3 Overlaid UPLC chromatogram of bovine plasma, BSA and Transferrin standards

1.1.18 Determination of total protein

The total protein content was determined using the Dumas combustion method with a LECO-FP-2000 instrument which measures the nitrogen content in the samples. The protein content of the samples was estimated by multiplying the obtained nitrogen value by the conversion factor of 6.25.

Results and discussion – Membrane filtration

Separation of BSA from bovine plasma using ultrafiltration

Laboratory scale ultrafiltration trials were conducted to determine the molecular weight cut-off (MWCO) and the influence of membrane material on separation of BSA from bovine plasma. The membranes available in scale for pilot-plant and commercial processing were selected with the aim of scaling up the ultrafiltration process. Three 100 kDa membranes made from polyvinyl difluoride (PVDF), regenerated cellulose and polyethersulfone (PES) and one 300 kDa PES membrane were used in the membrane screening experiments. The effective filtration area of all selected disk membranes was 13.4 cm^2.

After rinsing the membrane with water 50 ml of the plasma feed was added to the cell. The gentle stirring was applied to the cell using a magnetic stirrer. The system was pressurised with compressed air (pressure – 30 psi). During operation 1 ml permeate samples were collected and the time was recorded. Collected samples and the plasma feed were analysed for BSA and transferrin content by UPLC analysis.

The PVDF membrane with 100 kDa MWCO was initially tested. The feed material used for ultrafiltration was the non-diluted plasma. The pH of the plasma was 7.74 and the conductivity was 13.6 mS/cm. The total permeate volume of 30 ml was collected over the period of 4 hours 40 minutes. The average permeate flow rate was low - 97.6 ml/min/m^2. There was a continual decline in flux during ultrafiltration (Figure 4.1). The permeate flow rate was reduced by about 20% and 80% of the initial flux after 1 hour and 4 hours of operation, respectively.

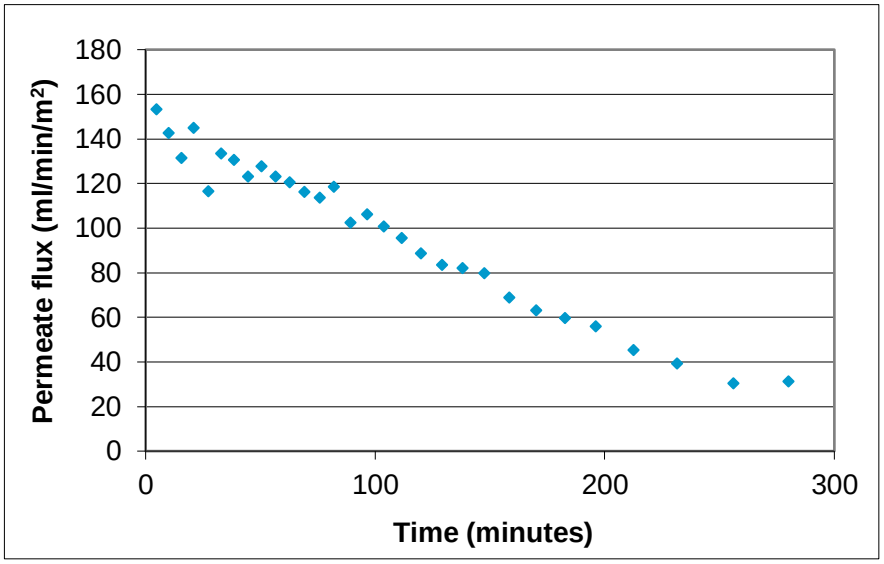

Figure 4.1 Permeate flux during ultrafiltration of the feed plasma (non-diluted, pH 7.74, conductivity 13.6 mS/cm) using PVDF 100 kDa membrane.

Chromatograms obtained by UPLC analysis of permeate samples show very small BSA and transferrin peaks (Figure 4.2) with the areas outside the standard calibration curves. Therefore, it was not possible to quantify proteins as the amount in permeates was negligible. At the end of ultrafiltration the concentration of BSA and transferrin in the final retentate was 2.5 times higher than in the plasma feed. These results clearly demonstrate that PVDF 100 kDa ultrafiltration membrane retained BSA and transferrin and isolation of BSA from bovine plasma was not achieved.

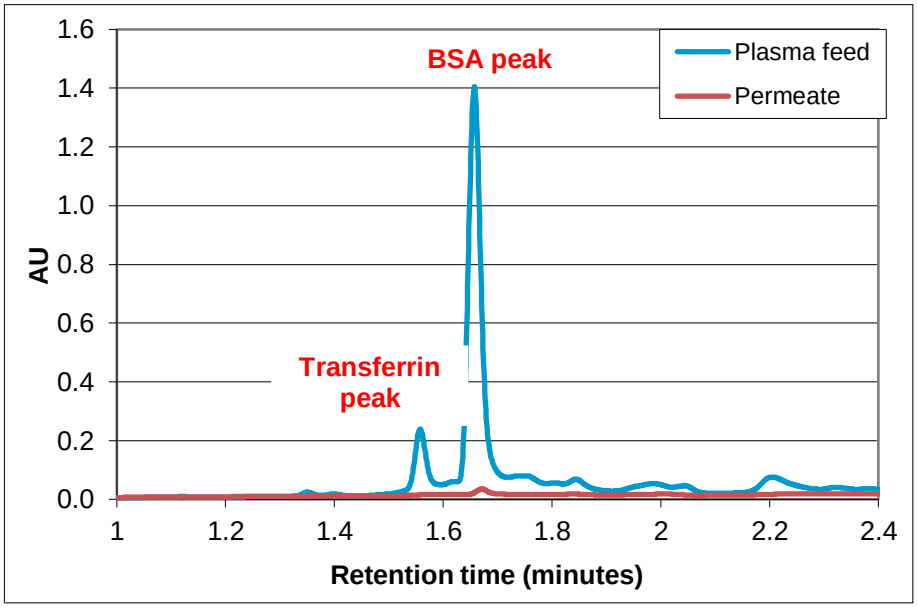

Figure 4.2 The UPLC profile of the plasma feed (non-diluted, pH 7.74, conductivity 13.6 mS/cm) and permeate after 10 minutes of ultrafiltration. For UPLC analysis the feed was diluted 20 times and permeate was used non-diluted. Injection volume was 2 µl.

The rapid decline of permeation flux and almost no transmission of proteins through the membrane is attributed to membrane fouling. The fouling tendency of unmodified and modified PVDF membranes has been examined in the studies of Hester and Mayes (2002) and Boributh et al. (2009). In the protein fouling experiments investigators used BSA as a protein model solution. It was confirmed that the unmodified PVDF membranes had a higher amount of BSA adsorption than the modified membranes due to high hydrophobicity of the PVDF membrane. Since the PVDF membrane surface is hydrophobic, membrane fouling would be a limiting factor for the filtration of protein solutions. It was decided to exclude the 100 kDa PVDF membrane from the next experiments and use the hydrophilic cellulose membrane (100 kDa) and the PES membranes (100 kDa and 300 kDa) which are less hydrophobic and more negatively charged than PVDF membranes.

The selectivity and permeability of PES membranes are strongly dependent on the physicochemical properties of the feed solution (pH, ionic strength and concentration). The effects of these parameters on the ability of 100 kDa PES membrane to separate BSA from the plasma were evaluated by using six different preparations of the plasma feed.

The summary of experiments conducted with 100 kDa PES membrane is presented in Table 4.1.

Table 4.1 Conditions and results from ultrafiltration trials using 100 kDa PES membrane

Trial number	Plasma feed properties			UPLC analysis results
	Concentration of BSA (mg/ml)	pH	Conductivity (mS/cm)	
1	26.34	4.69	13.6	No BSA and transferrin were detected in permeates
2	7.15	7.76	4.6	No BSA and transferrin were detected in permeates
3	6.79	4.67	4.6	No BSA and transferrin were detected in permeates
4	5.32	4.60	0.72	No BSA and transferrin were detected in permeates
5	3.0	4.60	0.37	No BSA and transferrin were detected in permeates
6	1.50	4.60	0.21	No BSA and transferrin were detected in permeates

The concentration of BSA in the feed material ranged from 1.5 mg/ml to 26.3 mg/ml. The conductivity of the feed varied from 0.21 mS/cm to 13.6 mS/cm, the conductivity of plasma. For comparison, 0.16 M NaCl solution has a conductivity of approximately 13.6 mS/cm. In order to reduce the conductivity of the feed, the filtered plasma was diluted with RO water (trials 2 and 3). The diluted plasma in trial 3 was pH adjusted to 4.67. For trial 4 the diluted plasma was dialysed using the dialysis membrane with MWCO 6-8,000 (Spectrum Laboratories Inc.). After dialysis the pH of the plasma solution was adjusted from 7.56 to 4.6. The feed solutions in trials 5 and 6 were prepared by dilution of the feed from the trial 4. In five experiments the pH of the plasma feed was adjusted to 4.6–4.7. After acidification some proteins precipitated and solutions were centrifuged at 20,800*g* for 3 minutes at room temperature using Eppendorf centrifuge 5417R.

The results from UPLC analysis of collected permeates show that under all tested conditions, where the BSA concentration and the feed conductivity varied, both BSA and transferrin were retained in the bulk solution by 100 kDa PES membrane and separation of BSA from plasma was not achieved. Although the plasma feed in the sixth experiment had properties favourable for ultrafiltration using PES membranes (low protein content, low conductivity, pH at pI of BSA making it uncharged) the isolation of BSA was not obtained. These results are not in agreement with the findings of Ohno al. (1982). According to the method of their invention, separation of BSA from bovine blood serum can be achieved using PES membrane with MWCO of 100 kDa by maintaining total protein and salt concentration in the solution below 40 g/l and 0.6 mole/l, respectively. However, the membranes described in the patent were not commercially manufactured and could have characteristics (pore size, thickness, the degree of polymerisation, etc.) different from commercially available 100 kDa PES membranes. Moreover, in the comparative example, presented in the patent, inventors used a commercial 100 kDa membrane ("Diaflo XM-100", Amicon Co Ltd) composed of an acrylonitrile/vinyl chloride copolymer. They reported that only 5% of BSA permeated through the tested membrane and 95% of BSA was rejected. They also observed the gradual decrease in the filtrate flow rate indicating membrane fouling.

In our study the high rejection of BSA by the 100 kDa PES membrane can be explained by adsorption of proteins on the membrane surface due to hydrophobic interactions and/or blocking the membrane's pores with the protein aggregates.

Maruyama et al. (2001) have demonstrated that aggregation of BSA during ultrafiltration can contribute to the fouling of PES membranes. Protein fouling was studied using BSA as a model protein. The BSA solution (2%) was ultrafiltered through the PES membrane with MWCO 6 kDa. The aggregates were collected from BSA gel-like deposits on the ultrafiltration membrane by centrifugation. Analysis of BSA aggregates clearly demonstrated that aggregates consisted of BSA oligomers and the main factor in the BSA aggregation during ultrafiltration was the intermolecular exchange of disulfide through cysteinyl residue (Figure 4.3).

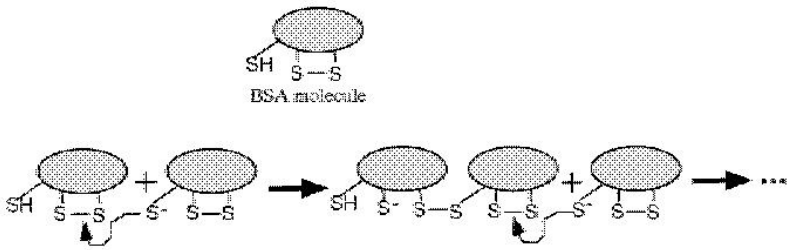

Figure 4.3 Schematic model of BSA aggregation during ultrafiltration copied from Maruyama, Katoh, et al. (2001).

Salgin et al. (2005) have investigated static adsorption of BSA onto 10 kDa hydrophobic polyethersulfone (PES) and hydrophilic cellulose triacetate (CTA) ultrafiltration membranes at the solution pH values of 3.78, 4.78, and 6.80, and ionic-strengths of 0.01M and 0.1M. More BSA was adsorbed on hydrophobic PES membranes than it was on hydrophilic CTA membranes at all the pH and ionic strength values. The highest degree of BSA adsorption on PES membranes was obtained at pH 3.78 whereas the minimum adsorption occurred at the isoelectric point of BSA (pH 4.78). These results were supported by the AFM images of the clean and fouled membranes (Figure 4.4).

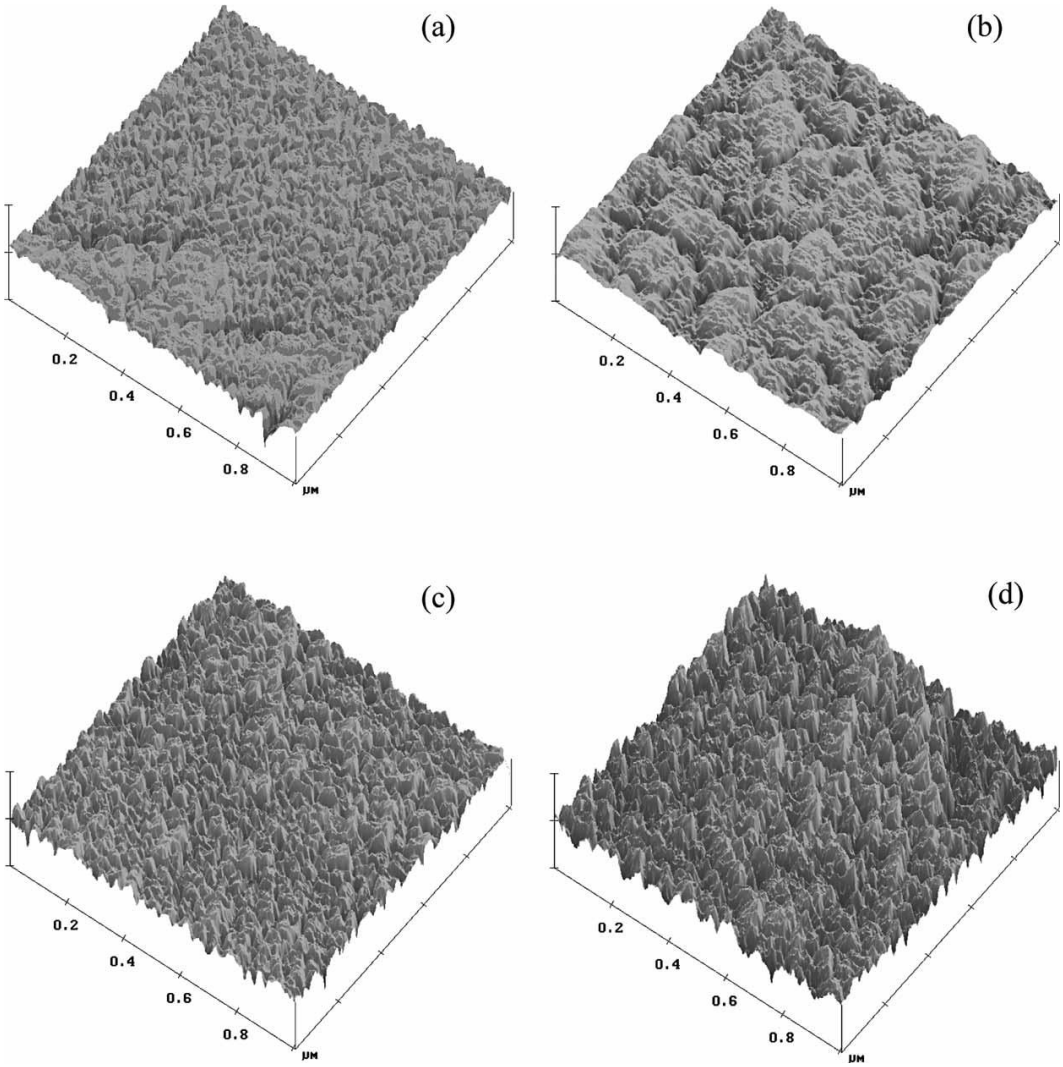

Figure 4.4 AFM images of PES membranes in 0.01M KCl at a) clean membrane; b) protein-fouled at pH 3.78; c) protein-fouled at pH 4.78; d) protein-fouled at pH 6.8. Copied from Salgin et al. (2005).

The hydrophobic interactions between the PES membrane and protein surfaces resulted in a high degree of adsorption (Salgin et al. 2005). The authors also suggested that protein–membrane interactions could cause changes in the structure of adsorbed molecules. On the surface of hydrophobic membranes, the protein appeared long and filamentous, more open and denaturated, which can increase the extent of adsorption. Another reason for the higher adsorption of BSA on PES membranes was the higher surface charges of hydrophobic PES membranes compared with those of hydrophilic CTA membranes (Salgin et al. (2005).

In the next two experiments the selectivity and permeability of the hydrophilic 100 kDa membrane made from regenerated cellulose were evaluated (Table 4.2).

Table 4.2 Conditions and results from ultrafiltration trials using 100 kDa regenerated cellulose membrane.

Plasma feed properties				
Trial number	Concentration of BSA (mg/ml)	pH	Conductivity (mS/cm)	UPLC analysis results
1	5.33	7.76	4.6	No BSA and transferrin were detected in permeates
2	5.32	4.60	0.72	No BSA and transferrin were detected in permeates

The plasma feed was prepared at two different conductivities (4.6 and 0.72 mS/cm), and pHs (7.76 and 4.6). Both feed solutions had the same concentration of BSA (~5.3 mg/ml). Analysis of permeate fractions revealed that BSA and transferrin were rejected by the membrane and separation of BSA from bovine plasma was not achieved.

The experimental data obtained from ultrafiltration trials show that irrespective of the type of membrane material (polyvinyl difluoride (PVDF), polyethersulfone (PES) or regenerated cellulose) membranes with 100 kDa MWCO were not capable to isolating BSA from bovine plasma. Fouling of the membranes was observed at all tested conditions.

The results of the experimental work suggest that a membrane with a bigger pore size may be needed to improve separation efficiency and reduce protein fouling during ultrafiltration. It was decided to investigate a 300 kDa membrane. This size was available only for PES membranes.

The selectivity and permeabilty of 300 kDa PES membrane was evaluated in three ultrafiltration trials. The effects of the feed pH, conductivity and the protein concentration were investigated. Experimental conditions are presented in Table 4.3.

Table 4.3 Conditions and results from ultrafiltration trials using 300 kDa PES membrane

	Plasma feed properties			
Trial number	Concentration of BSA (mg/ml)	pH	Conductivity (mS/cm)	UPLC analysis results
1	5.32	4.60	0.72	BSA and transferrin were detected in permeates
2	5.92	7.56	0.72	BSA and transferrin were detected in permeates
3	1.51	7.84	0.18	BSA and transferrin were detected in permeates

The UPLC analysis of the permeate samples collected during ultrafiltration show that the concentration of BSA and transferrin in the permeate stream was very low. The pH of the feed did not significantly affect protein permeability (Figure 4.4 A). In trials 1 and 2 the plasma solutions had pH 4.6 and 7.56, while the conductivity and protein concentration were similar. At pH 4.6, slightly more BSA permeated through the membrane. The BSA concentration in the 5 ml permeate collected in the trial 1 was 0.76 mg/ml compared to 0.56 mg/ml, which was obtained in trial 2. More diluted plasma was used for ultrafiltration in the trial 3. As the result, less BSA permeated through the membrane. The BSA concentration in the 5 ml permeate was 0.21 mg/ml. The amount of BSA and transferrin in the 15 ml permeate sample was significantly reduced and the degree of reduction was more than 95% for the three tested feed solutions (Figure 4.4 B).

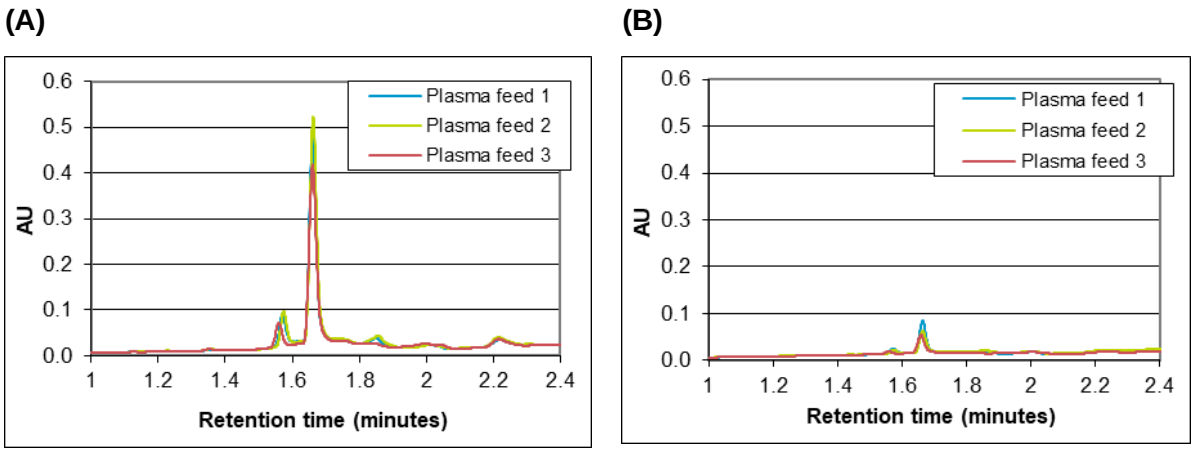

Figure 4.4 The UPLC profile of the 5 ml permeates (A) and the 15 ml permeates (B) collected during ultrafiltration of three different preparations of the plasma feed. For UPLC analysis non-diluted permeates were used. Injection volume used was 2 µl.

The gradual decrease of protein concentration in the permeates indicated membrane fouling. This explanation was also supported by the flux data. The permeate flux rate recorded in trial 3 is shown in Figure 4.5.

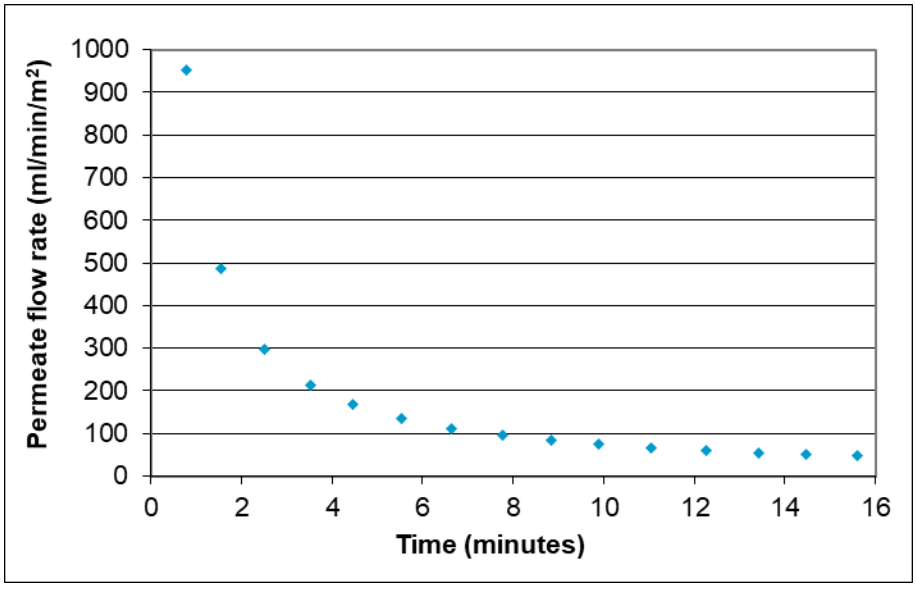

Figure 4.5 Permeate flux during ultrafiltration of the plasma feed (dialysed, diluted 16 times, pH 7.84, conductivity 0.72 mS/cm) using PES 300 kDa membrane.

Although diluted plasma with low protein concentration was used in trial 3, a sharp decline of the permeate flow was observed. The permeate flow rate decreased by 86% and 95% of the initial flux after 5 and 15 minutes of operation, respectively. The rapid fouling may be due to pore blocking. Due to excessive fouling, the amount of transferrin and BSA permeated through the membrane under tested conditions were vey low –2.7% and 3.0% of the amount in the feed.

As it was not possible to separate BSA from bovine plasma using ultrafiltration it was decided to investigate microfiltration membranes for plasma fractionation next.

Separation of BSA from bovine plasma using microfiltration

Laboratory scale microfiltration experiments were conducted using three ceramic microfiltration membranes of 0.1, 0.2 and 0.5 µm molecular weight cut-off (MWCO) with filtration surface area of 0.005 m^2.

The MF membrane with 0.1 µm MWCO was tested initially. The feed material used for microfiltration was the plasma filtered and diluted 4 times with RO water. The conductivity of the diluted plasma was reduced from 13.6 to 4.2 mS /cm. The pH of the feed remained at 7.9. The concentration of BSA, transferrin and IgG in the diluted plasma was 7.4, 1.2 and 5.5 mg/ml, respectively.

The prepared plasma (1575 ml) was placed into the feed tank of a Membralox XLAB5 bench scale filtration unit and the system was pressurised (inlet – 3.0 bar, outlet – 2.6 bar). The first 45 ml of permeate which consisted of water in the system was discarded, then the permeate stream was recirculated back into the feed tank. Samples of permeate (2 ml each) were collected every 5 minutes. After 60 minutes two diafiltration steps were carried out by using 300 ml of RO water in each step. At the end of the second diafiltration the feed conductivity was further reduced from 4.2 to 3.1 mS/cm. Permeates from diafiltration steps

and the final retentate were collected and all samples were analysed for BSA and transferrin concentration.

Chromatograms obtained by UPLC analysis of permeate samples show very small BSA and transferrin peaks with the areas outside the standard calibration curves. Therefore, it was not possible to quantify proteins and their amount in permeates was considered as negligible. These results clearly demonstrate that 0.1 µm ceramic microfiltration membrane retained plasma proteins and their fractionation was not achieved. The suggested diafiltration steps did not improve the membrane performance — no proteins permeated through the membrane. It was also observed that after diafiltration the retentate appeared to be very cloudy compared to the feed material (Figure 4.6).

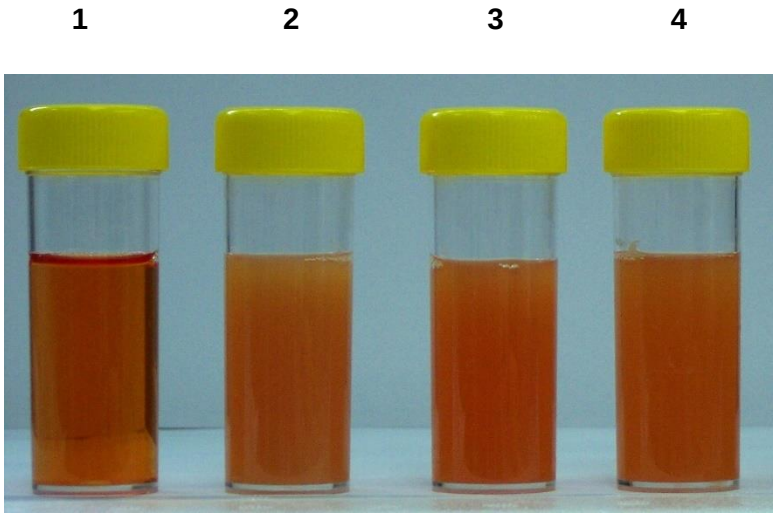

Figure 4.6 The plasma feed diluted 4 times, pH 7.9, conductivity 4.2 mS/cm **(1)** and corresponding retentates from ceramic membranes 0.1 µm **(2)**, 0.2 µm **(3)**, and 0.5 µm **(4)**.

This indicated that aggregation of some proteins was taking place under shearing conditions during microfiltration. The UPLC analysis of the feed plasma and the final retentate did not reveal any changes in BSA and transferrin concentrations (Figure 4.7). This suggested that plasma compounds other than BSA and transferrin were responsible for “cloudiness” of the retentate fraction.

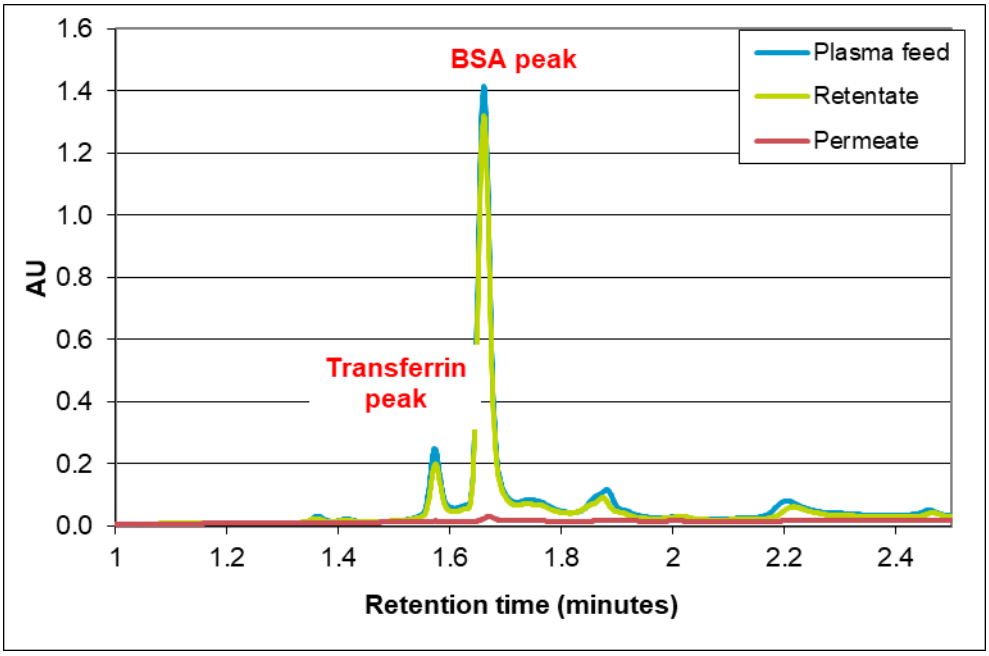

Figure 4.7 The UPLC profile of the plasma feed (diluted 4 times, pH 7.9, conductivity 4.2 mS/cm), permeate after 10 minutes of recirculation and the final retentate.

Microfiltration of diluted plasma using 0.2 µm and 0.5 µm membranes was also investigated. The plasma was prepared in the same manner (dilution factor 4, pH 7.9, conductivity 4.2 mS/cm) and experiments were repeated except that the diafiltration steps were not carried out.

Figure 4.8 compares the permeability of BSA, transferrin and IgG through 0.2 µm membrane (A) and 0.5 µm membrane (B) during recirculation of diluted plasma.

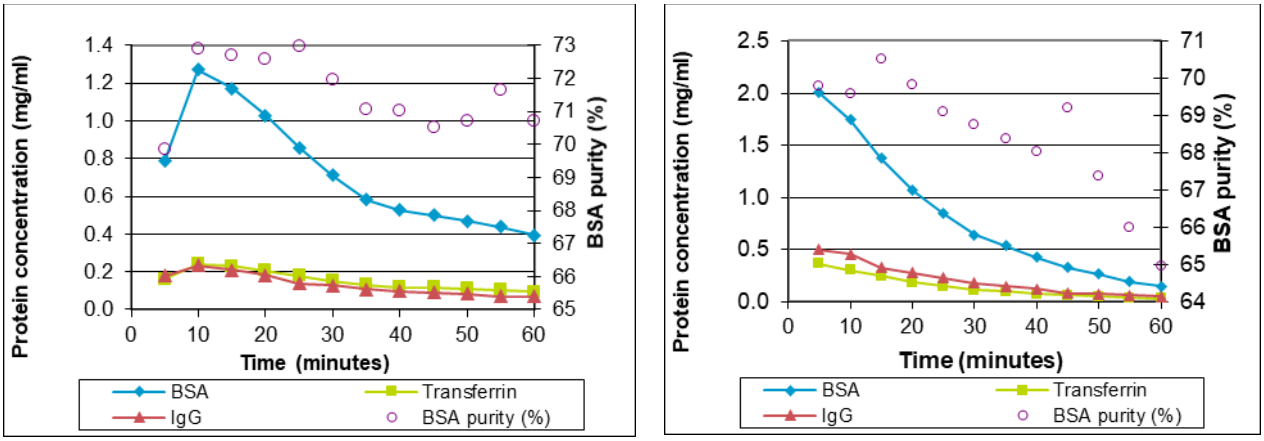

Figure 4.8 Concentration of BSA, transferrin, IgG and BSA purity in permeates collected during recirculation of diluted plasma (dilution factor 4, pH 7.9, conductivity 4.2 mS/cm) using 0.2 µm (A) and 0.5 µm membranes.

The BSA concentration in the plasma feed was 7.4 mg/ml. In the permeates collected after 10 minutes of recirculation using 0.2 µm and 0.5 µm membranes the BSA concentration was 1.27 and 1.74 mg/ml, respectively. These results show that the membrane with the bigger pore size allowed more BSA to permeate. However, within the next 50 minutes of operation the BSA content in permeates decreased significantly and the degree of reduction was 6 times higher when using 0.5 µm MF membrane. The concentration of BSA in the permeates collected after 60 minutes of recirculation using 0.2 µm and 0.5 µm membranes was 0.39 mg/ml and 0.15 mg/ml, respectively.

The BSA content or "purity" calculated as a percent of the total content of three major proteins BSA, IgG and transferrin was ~52% in the plasma feed. In the permeate fractions collected from 0.2 µm membrane the BSA purity ranged from 69.9 to 73.0%. In permeates produced by 0.5 µm membrane the BSA purity was not higher than 70.5% and the BSA content decreased to 64.9% at the end of the experiment. The membrane selectivity determined as a ratio of sieving coefficients for BSA and IgG was also estimated. Sieving coefficient is a ratio of protein concentration in the filtrate to protein concentration in the plasma feed. Both membranes demonstrated low selectivities for BSA–IgG separation. The MF 0.2 µm membrane had a slightly higher selectivity factor of 4.21 ± 0.87 compared to 2.76 ± 0.49 calculated for 0.5 µm membrane.

MF membranes with 0.2 µm and 0.5 µm MWCO showed a continual decline of the permeate flow rate during recirculation of the plasma feed The permeate flow rate was higher when 0.5 µm membrane was used for microfiltration. However, the rate and extent of the flux reduction (~30%) were similar for the two membranes (Figure 4.9).

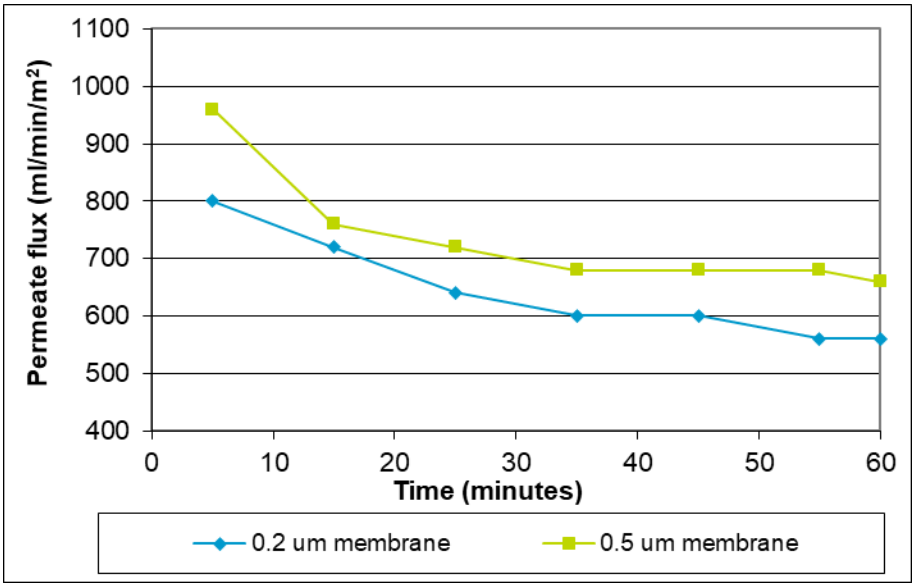

Figure 4.9 Permeate flux during recirculation of the feed plasma (dilution factor 4, pH 7.9, conductivity 4.2 mS/cm) using 0.2 µm and 0.5 µm membranes.

It was observed that the final retentates collected after recirculation had cloudy appearance compared to the clear feed material. The 0.5 µm retentate was cloudier than the 0.2 µm retentate (Figure 4.6). The higher rates of flow achieved with 0.5 µm membrane generated higher shear rates that promoted formation of insoluble particles in the bulk solution during recirculation. The UPLC analysis of the feed plasma and the retentate fractions did not show any changes in BSA and transferrin concentration, suggesting that no precipitation of these proteins occurred during the recirculation process and that the aggregation of other plasma compounds caused the “cloudiness” of the retentate. However, BSA molecules can contribute to the fouling phenomena on or around the membrane resulting in concentration polarisation and the formation of a secondary membrane through electrostatic interactions. The surface charge of BSA was negative at the feed pH 7.9. With the repulsion force, most of the BSA molecules that had been directed towards the membrane surface from the bulk

liquid could not transmit through the membrane because of the gel layer formed by the BSA (Tung et al., 2010).

For the next experiment it was decided to investigate the potential improvement in BSA permeability through the membrane by adjusting the feed pH to 4.55 where BSA is neutral and cannot be attracted or repelled by charged particles. In contrast to what was expected, a strong filtration resistance was experienced and the permeate flux was very low—less than 560 ml/min/m^2. A small amount of proteins was transmitted through the membrane. At the start of plasma recirculation, the BSA concentration in the permeate was 2.0 mg/ml and decreased to 0.15 mg/ml at the end of processing. The fouling of the membrane can be explained by formation of insoluble aggregates. Figure 4.10 clearly shows a thick layer of precipitated material in the final retentate.

1 **2**

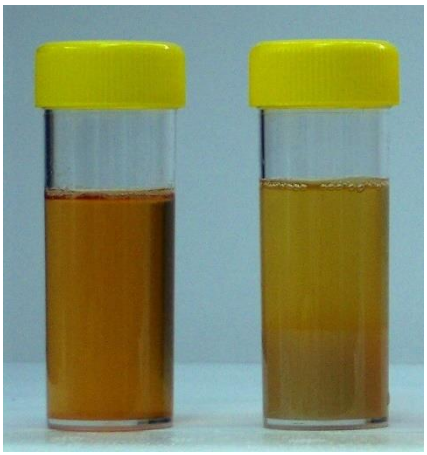

Figure 4.10 The plasma feed DF4, pH 4.55, conductivity 5.37 mS/cm **(1)** and the retentate from ceramic membrane 0.5 □m **(2)**.

The UPLC analysis of the feed plasma and the retentate revealed that 10% of transferrin and 20% of BSA became insoluble after 60 minutes of plasma recirculation. These results suggest that in mildly acidic conditions the native conformation of BSA was destabilised and BSA formed insoluble aggregates under shear forces during filtration. El Kadi et al. (2006) have investigated the ability of albumins to undergo a reversible conformational transition with changes in pH from 7 to 2. They found that the secondary structure of BSA displayed few changes in a range of pH values between 6.5 and 5, the latter value being close to the isoelectric point of the protein (pI 4.9). Below this value, the structural alteration was due to

the repulsive forces acting within the highly charged protein leading to the partial unfolding. The partial unfolding process of the BSA helices resulted in a progressive exposure of the protein surface to the acid aqueous environment, leading to increased hydration. At the same time protein voids and cavities, which in the native protein contribute substantially to the compressibility and to the partial specific volume, were collapsing.

Moreover, the recent study of Bekard et al. (2011) has demonstrated that the hydrodynamic stress generated in simple shear flow was capable of disrupting the tertiary structure and unfolding the α-helical segments of a natively folded BSA. A model of the sequence of events occurring during shear-induced unfolding of BSA is shown in Figure 4.11.

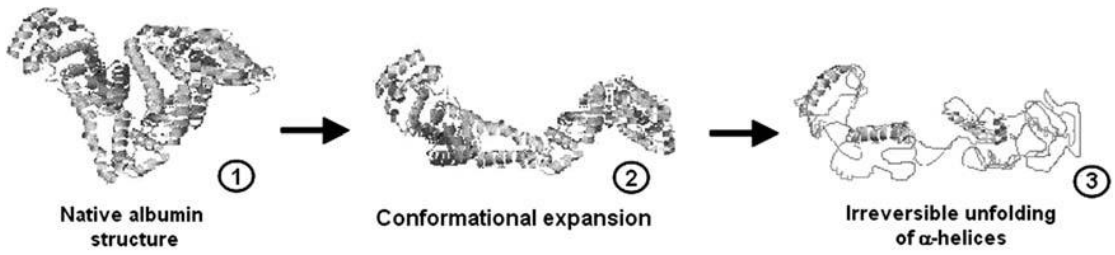

Figure 4.11 Representation of the irreversible conformational changes observed in native BSA during shear exposure (copied from Bekard, Asimakis et al. 2011).

It was demonstrated that flow-induced unfolding of BSA occurred in simple Couette flow where both exposure time and shear rate increased the degree of irreversible unfolding. The shear rates used in this study were comparable to those encountered in bio-processing.

The previous studies based on model protein systems of low concentrations and the current experimental work using complex bovine plasma suggest that the underlying mechanisms governing the fouling behaviour of UF and MF membranes could be different and understanding of the effects of physicochemical characteristics of the bulk protein solution and operating conditions on the initiation and the rate of fouling is very important. However, these investigations are outside of the scope of this project.

Conclusions – Membrane filtration

Laboratory studies were undertaken to examine the feasibility of ultrafiltration and microfiltration as cost-effective and scalable processes to separate bovine serum albumin (BSA) from bovine plasma.

Four ultrafiltration membranes (100 kDa PVDF, 100 kDa RC, 100 kDa PES and 300 kDa PES) and three microfiltration membranes (0.1 µm, 0.2 µm, 0.5 µm ceramic) were evaluated for selective separation of BSA. The effect of the BSA concentration in the feed (from 1.5 to 26.3 mg/ml), solution pH (7.8 and 4.6) and conductivity (from 0.21 to 13.6 mS/cm) on the separation efficiency of the membranes was investigated.

Under all tested conditions selective separation of BSA from bovine plasma was not achieved and all membranes demonstrated very poor flow characteristics due to excessive protein fouling.

Background – Chromatographic separation

Introduction

Chromatographic separation processes are widely used to isolate and purify value added products in food industries. A typical chromatographic production process is robust, easy to scale up, readily automated, easy to adapt to new products and requires no organic solvents. Chromatography is a gentle and reliable technique for purifying proteins on a commercial scale.

Chromatography is recognised as a purification technique capable of obtaining high purity plasma proteins with high yields in blood processing. Industrial-scale chromatographic fractionation and purification methods have been used increasingly in the last few years for human plasma fractionation. This has resulted in the development of a new generation of therapeutic plasma derivatives, especially coagulation factors, protease inhibitors and anticoagulants. Implementation and combination of ion-exchange, affinity and size-exclusion chromatography have allowed the development of new therapeutic products with improved purity and safety.

Review of literature and patents on separation of BSA by Ion-Exchange Chromatography

Chromatographic techniques have been used in blood processing since the 1980's, emerging as an effective method of purifying blood components for therapeutic use.

Burnouf (2007) has reviewed modern plasma fractionation techniques and manufacturing processes of plasma protein products. According to his review all therapeutic albumin preparations are produced by fractionation of cryo-plasma using ethanol precipitation. Albumin recovery of 75% to 85% and purity of 96% to 98% are typically obtained. Some processes combine ethanol fractionation with polishing ion exchange chromatography, which generally improves product purity to 99%, whereas in one production method, albumin is purified mostly by anion exchange, cation-exchange, and size-exclusion chromatography.

Separation of serum albumin based on ion-exchange chromatography has been employed by several investigators. Curling, et al. (1977) described the chromatographic purification method to produce albumin from plasma. A partially purified plasma, free from coagulation factors and the major part of IgG, was separated on an anion-exchanger and then on a cation-exchanger. The separations were carried out by elution with aqueous buffer systems. This technology was developed by the Pharmacia Company which supplies different types of chromatographic resins.

Condie (1979) has invented a process of isolating and purifying IgG and serum albumin from human and bovine plasma. The method involves the initial stabilisation of plasma by treatment with silica. IgG and albumin are isolated by contacting with ion exchange resin (Sephadex QAE-50) and eluted by the adjustment of pH and ionic strength. Albumin fraction could be further purified by passing it through Sephadex SP-50 ion exchanger to achieve high yield and high purity.

Dromard et al. (1984) have disclosed a method to fractionate plasma by contacting a plasma solution with at least one hydrophilic ion exchanger (DEAE Sephadex, DEAE Sepharose, QAE Sephadex, SP Sephadex, CM Sephadex, CM Sepharose, DEAE Trisacryl M, CM Trisacryl M) and at least one partially hydrophobic adsorbent (Phenyl Sepharose CL-4B, Octyl Sepharose CL-4B, Spherosil Q MA) in fixed bed chromatography columns. The process may be used for large-scale recovery of albumin for therapeutic use, giving good yields of high-purity products (~95%). However, prior to ion-exchange chromatography the plasma material has to be treated to remove salts and advantageously euglobulins, which are unstable protein complexes.

Dernis et al. (1990) have developed a process of separating serum albumin from human or animal plasma or plasma fraction by ion-exchange chromatography utilising DEAE Sepharose resin. The starting material was adjusted to protein content of 15-18 g/l, conductivity of 1.5 – 2 mS (using sodium acetate) and pH of 5.0-8.0 (using acetic acid). The plasma solution was injected into an ion-exchange resin chromatography column packed with DEAE-Sepharose resin which absorbed albumin. The bound albumin was eluted with a

buffer solution of lower pH (4 - 4.7). The albumin produced by this method had high purity and stability.

Takahashi et al. (1995) has disclosed a method to produce high purity albumin from crude albumin solutions by treating the feed material with anion-exchangers (Q-Sepharose, QAE Toyopearl, QMA_Sepharosil) then cation-exchangers (SP-Trisacryl and CM-Trisacryl) and subjecting the resultant solution to heat treatment. The recovery of albumin was higher than 90%.

Moure et al. (2004) have developed an anion-exchange chromatography process to separate the main protein fractions of bovine blood plasma using a composite material, with quaternary amino groups, Q-HyperD; and a gel material, Sepharose with DEAE functional groups. The experiments were carried out at semi-preparative scale using citrated and centrifuged plasma at pH 7.5. Of the two materials tested, Q-HyperD exhibited a greater selectivity for the plasmatic proteins, a greater number of separations and defined fractions being obtained.

Project objective – Chromatographic separation

The overall aim of the project was to develop a cost effective laboratory scale process to produce BSA at > 80% purity from bovine plasma, and to scale up the process to produce a gram quantity sample at preparative laboratory scale.

The objective of this part of the project was to investigate ion exchange chromatography to isolate bovine serum albumin (BSA) from bovine plasma and to select promising technologies and develop laboratory based process to produce BSA at >80% from bovine plasma. A preliminary analysis of the cost of production of BSA was also to be assessed.

Methodology – Chromatographic separation

Materials

1.1.19 Ion-Exchange resins

Five chromatographic resins were screened to determine their binding capacity and specificity to isolate bovine serum albumin (BSA) from bovine plasma. Only resins that had been classified as generally regarded as safe (GRAS) were employed. Characteristics of selected resins as provided by the resin manufacturers (GE Healthcare Life Sciences, Gibco Invitrogen Corporation and Dow Chemicals) are summarised in Table 8.1.

1.1.20 Chemicals

Chemicals used for preparation of mobile phases in HPLC/UPLC analysis were analytical-grade as well as chemicals used in SDS PAGE assay – Na_2HPO_4 Disodium Orthophosphate (BDH Chemicals Ltd, Australia); Glycine (Chem Supply Pty Ltd., Australia); Trifluoroacetic acid (Sigma-Aldrich, Germany); Acetonitrile (Optigen Scientific, Australia); tris[Hydroxymethyl]aminomethane (TRIZMA-Base), Ehylenediamine tetraacetic acid (EDTA) (Sigma-Aldrich, Australia); Sodium Dodecyl Sulphate (SDS) (BDH Chemicals Ltd, UK); 2-Mercaptoethanol (Merck, Germany).

Purified powders of Bovine Serum Albumin (BSA), Transferrin and IgG were purchased from Sigma (USA) and Innovative Research (USA) and used as the reference material for protein quantification.

Table 8.1 Characteristics of the ion-exchange resins investigated to separate BSA from bovine plasma.

Resin name	Manufacture	Type	Functional group	Matrix	Protein Capacity (mg/mL)	Shape	Size	pH range
Q Sepharose Big Beads (BB)	GE Healthcare Life Sciences	Strong anion	Quarternary amine	Highly cross-linked agarose	70	Beaded	100 – 300 microns	2 - 12
QAE Gibco HB2	Gibco Invitrogen Corp	Strong anion	Quarternary amine	Reg.Cellulose-hydroxypropyl/cross link	60-80	Beaded	300 – 600 microns	2 - 12
QAE Gibco HB2 High Substitution	Gibco Invitrogen Corp	Strong anion	Quarternary amine	Reg.Cellulose-hydroxypropyl/cross link		Beaded	300 – 600 microns	2 - 12
QAE Gibco HB3	Gibco Invitrogen Corp	Strong anion	Quarternary amine	Reg.Cellulose-hydroxypropyl/cross link		Beaded	300 – 600 microns	2 - 12
Dowex 22	Dow Chemical Company	Strong anion	Quarternary amine	Styrene-DVB		Beaded	300 – 1200 microns	0-14

Methods

1.1.21 Plasma preparation for membrane filtration trials

The frozen bovine plasma used in laboratory scale experiments was obtained from JBS Swift Company (Dinmore, Queensland) and stored at the temperature of –20^{o}C. Immediately prior to use, plasma was thawed at the room temperature and filtered through Whatman (No. 4) filter paper. The filtered plasma had a protein content of 8.17% and solids content of 9.43%. The pH and conductivity of the plasma were 7.78 and 13.6 mS/cm, respectively.

The analytically determined (UPLC) concentration of BSA, IgG and transferrin in the bovine plasma together with the published values for isoelectric point and molecular weight of proteins are presented in Table 8.2.

Table 8.2 Properties and composition of BSA, transferrin and IgG in bovine plasma supplied by JBS Swift Company

	BSA	IgG	Transferrin
Concentration in plasma (mg/ml)	30	22	4.2
Isoelectric point	4.9	5.8-7.3	5.9
Molecular weight (kDa)	68	160	79

1.1.22 Quantification of BSA and Transferrin

The concentration of BSA and transferrin in bovine plasma and processed samples were determined by ultra performance liquid chromatography (UPLC). The UPLC system was equipped with four ACQUITY units –sample manager, binary solvent manager, column heater, and Photodiode Array (PDA) Detector (Waters Corporation, USA). Proteins were separated on UPLC BEH300, C_4, 1.7 µm column. The column temperature was maintained at 50^{o}C. The gradient was applied by using solvent A (0.1% TFA in water) and solvent B (0.1% TFA in

Acetonitrile). The total run time was 4 minutes. The proteins were detected at the wavelength of 220 nm. The concentration of BSA and transferrin were calculated from standard calibration curves. The standards were prepared at concentrations 0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml and injected to the column at 2 µl. The UPLC overlaid chromatogram showing transferrin and BSA in bovine plasma and the corresponding standards is presented in Figure 8.1.

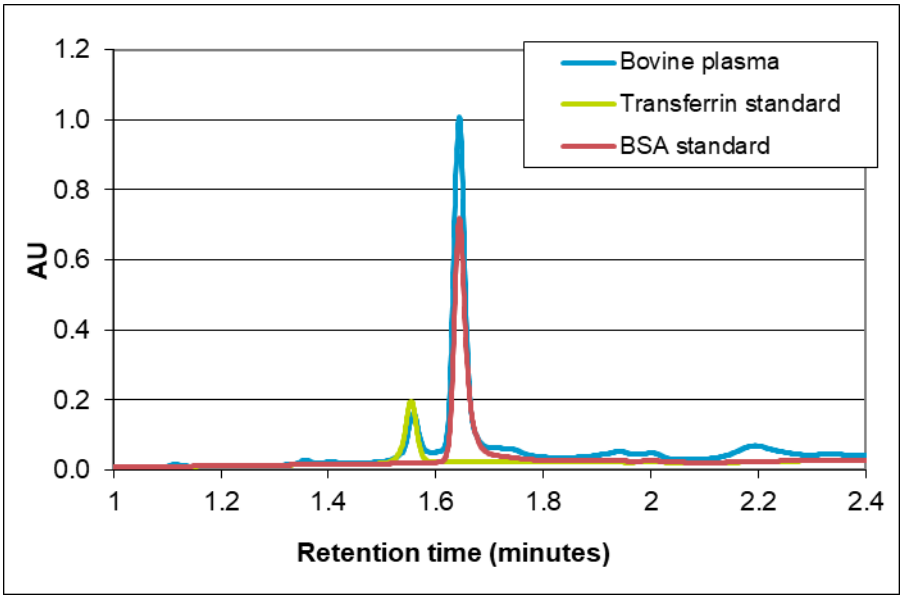

Figure 8.1 Overlaid chromatogram of bovine plasma, BSA and Transferrin standards.

1.1.23 Quantification of IgG

Immunoglobulin G (IgG) were quantified by Protein G affinity chromatography. This HPLC method is an established method and had been shown to be highly reproducible. The HPLC system consisted of Alliance 2795 Separations Module and Photodiode Array (PDA) Detector (Waters Corporation, USA). IgG was separated on Hi-Trap Protein G column (GE Healthcare Life Sciences) by gradient elution using solvent A (20 mM Sodium Phosphate, pH 7.0) and solvent B (0.1 M Glycine, pH 2.7) which were delivered to the column at the flow rate of 1ml/min. Detection was achieved at the wavelength of 280 nm. The IgG concentration in bovine plasma and the samples produced during the trials were calculated using the standard calibration curve. To generate the calibration curve, a series of injections were performed with 2 mg/ml IgG standard (Innovative Research, USA) at injection volumes from 10 to 200 µl.

1.1.24 SDS PAGE Electrophoresis

The SDS PAGE of bovine plasma proteins was performed on Phast System (Pharmacia LKB Biotechnology) under reducing conditions. The samples of bovine plasma and isolated fractions were prepared at total protein concentration ~ 1.0 - 2.0 mg/ml. Samples and standards with molecular weight ranging from 14.4 to 97 kDa were heated with a specified amount of “sample buffer”, containing sodium dodecyl sulphate (SDS) and 2-mercaptoethanol for 2 minutes in boiling water followed by cooling on ice. PhastGel Gradient Gels 8 to 25 (GE Healthcare Life Sciences) were employed to analyse the prepared samples. The gels were run for approximately 40 minutes at 65Vh using an automated program. At the end of the run, the gels were immediately transferred to the development chamber. The gels were developed by an automated method with PhastGel Blue for staining, acidic methanol for destaining and glycerol / acetic acid for preserving.

1.1.25 Determination of total protein

The total protein content was determined using the Dumas combustion method with a LECO-FP-2000 instrument which measures the nitrogen content in the samples. The protein content of the samples was estimated by multiplying the obtained nitrogen value by the conversion factor of 6.25.

Results and discussion – Chromatographic separation

Selectivity and static adsorption capacity of strong anion exchange resins

Based on the isoelectric point (pI) of BSA and the pH of plasma, five resins have been selected to evaluate static adsorption capacity and specificity to isolate BSA from bovine plasma. The selected resins were strong anion exchangers - Q Sepharose Big Beads (BB) ,QAE Gibco HB2, QAE Gibco HB2 High Substitution, QAE Gibco HB3 and Dowex 22.

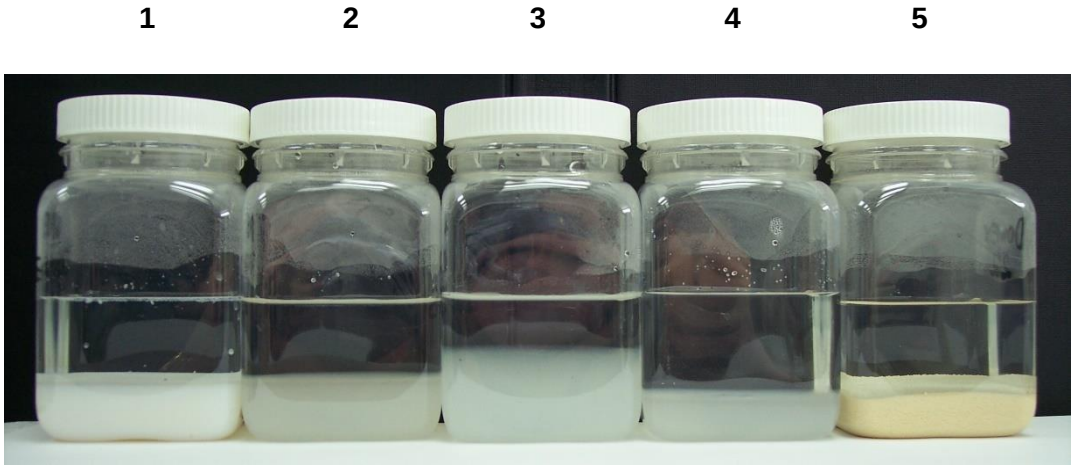

Figure 9.1 Strong anion exchange resins - Q Sepharose Big Beads (BB) - **1** ,QAE Gibco HB2 - **2**, QAE Gibco HB2 High Substitution - **3**, QAE Gibco HB3 - **4**, Dowex 22) – **5**.

The resins are composed of fixed charged functional groups (quarternary amine groups) attached to an insoluble matrix such as agarose, cellulose or Styrene- divinylbenzene. Counter ions of opposite charge are weakly adsorbed to these functional groups. In biological media, ion exchange is achieved by exchanging the counter ions adsorbed to the charged functional groups with charged molecules (e.g. proteins). Anion exchangers attract and exchange negatively charged molecules. The dissociation of the counter ion is dependent on the pH of the solution and the type of exchanger. For strong exchangers, ionisation or dissociation is achieved over a wide pH range (2–12). The interaction of target proteins with the chromatographic stationary

phase depends on many factors such as pH, ionic strength of the feed material, additives, accompanying biomolecules and the chemical structure of the resin.

Static binding experiments were conducted to evaluate adsorption capacity and specificity of five selected resins to bind BSA. Prior to use, all resins were washed and activated according to the manufacturer's instructions. Two ml of resins equilibrated to the required pH was used in each experiment. Excess liquid was removed by suction filtration using a Buchner funnel then the moist resin was transferred into syringes.

Non-diluted plasma and plasma diluted three times with RO water were used as the feed material. The pH of plasma solutions were adjusted to 6.5, 7.8 and 8.5 with 1M HCl and 1M NaOH, then 10 ml of the plasma feed was added to the resin and left in contact under gentle mixing for 1 hour. After 1 hour the protein depleted liquid, the flow through (F/T) fraction, was collected and resin samples were washed with 20 ml of RO water. The proteins bound onto the resins were eluted with 4 ml of 1M NaCl.

The plasma feed, F/T fractions and eluates were analysed for BSA and transferrin by UPLC and IgG by HPLC. The efficiency of BSA adsorption was calculated as the amount of BSA adsorbed and eluted from 1 ml of resin. The potential BSA purity was estimated as the percent of the total protein components (BSA, transferrin and IgG) in the eluate. Figure 9.2 shows BSA adsorption by the resins at different experimental conditions.

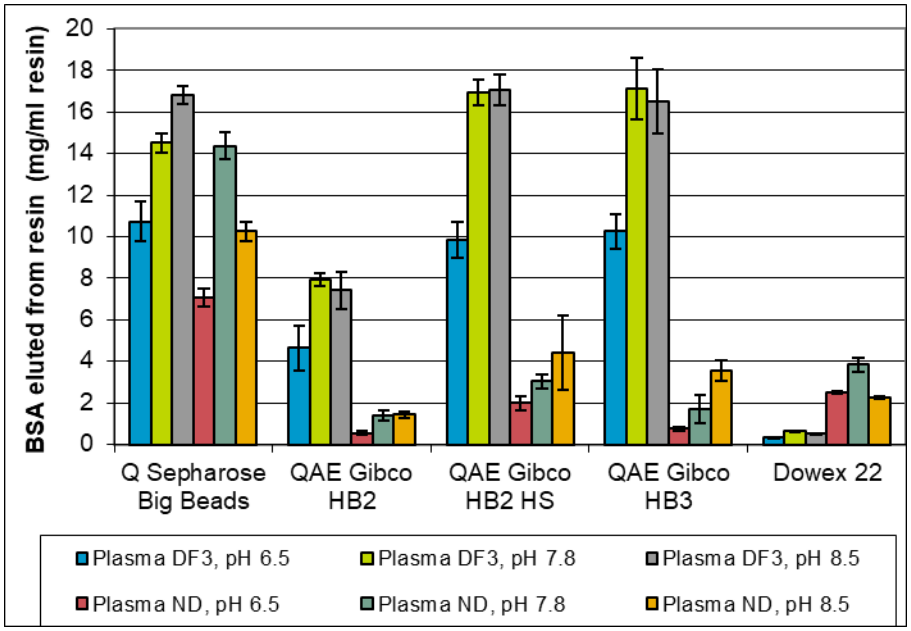

Figure 9.2 BSA static adsorption capacities (BSA eluted mg/mL resin) of strong anion exchange resins when using diluted and non diluted plasma at the pH 6.5, 7.8 and 8.5. DF – dilution factor, ND – non-diluted. Number of replicates – 3. Error bars – standard deviation.

The highest adsorption of BSA (~17 mg/ml) was achieved by using Q Sepharose BB, QAE Gibco HB2HS and QAE Gibco HB3 resins. The maximum binding capacities of QAE Gibco HB2 and Dowex 22 resins were 8 and 4 mg/ml, respectively. Q Sepharose BB resin and all Gibco resins had higher BSA binding when diluted plasma was used as the feed material. Only Dowex 22 resin adsorbed more BSA from the non-diluted plasma solutions. Dilution of plasma resulted in reduced proteins concentration and conductivity of the feed. The effects of protein concentration and ionic strength on BSA adsorption were investigated in more detail and the results are presented in section 5.5.

The BSA binding capacities of all tested resins was reduced as the pH of diluted and non-diluted plasma solutions decreased from 7.8 to 6.5. The isoelectric point (pI) of BSA is 4.9 and at the pH above this value BSA is negatively charged. Bohme and Scheler (2007) determined the effective charge of BSA as a function of pH (Figure 9.3). The graph clearly shows that BSA at pH 7.8 is more negatively charged than at pH 6.5.

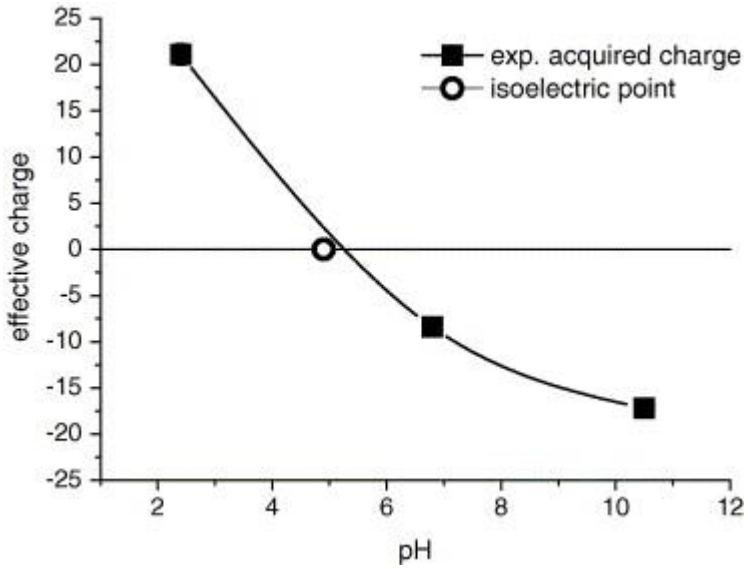

Figure 9.3 Effective charge of BSA as function of pH. Copied from Bohme and Scheler (2007).

Since anion exchangers attract and exchange negatively charged molecules, a decrease in the BSA adsorption capacity by the anionic resins at pH 6.5 was expected. It was also expected a higher BSA binding with plasma feed at pH 8.5 when BSA is more negatively charged. However, the results did not show this trend for all resins. Adsorption of BSA from diluted plasma at pH 7.8 and 8.5 did not vary significantly except for Q Sepharose BB resin which bound 16% more BSA at the higher pH.

Greater adsorption of BSA from non-diluted plasma at pH 8.5 compared to pH 7.8 was observed for QAE Gibco HB2HS and QAE Gibco HB3 resins. QAE Gibco HB2 resin did not show a significant difference in BSA binding at pH 7.8 and 8.5. The amount of BSA adsorbed by Q Sepharose BB and Dowex 22 resins from non-diluted plasma decreased as the pH of the plasma feed increased from 7.8 to 8.5. The reduction in BSA binding capacities of Q Sepharose BB and Dowex 22 resins when using the plasma at pH 8.5 is difficult to explain. Figure 9.4 shows the BSA purity in 1M NaCl eluates.

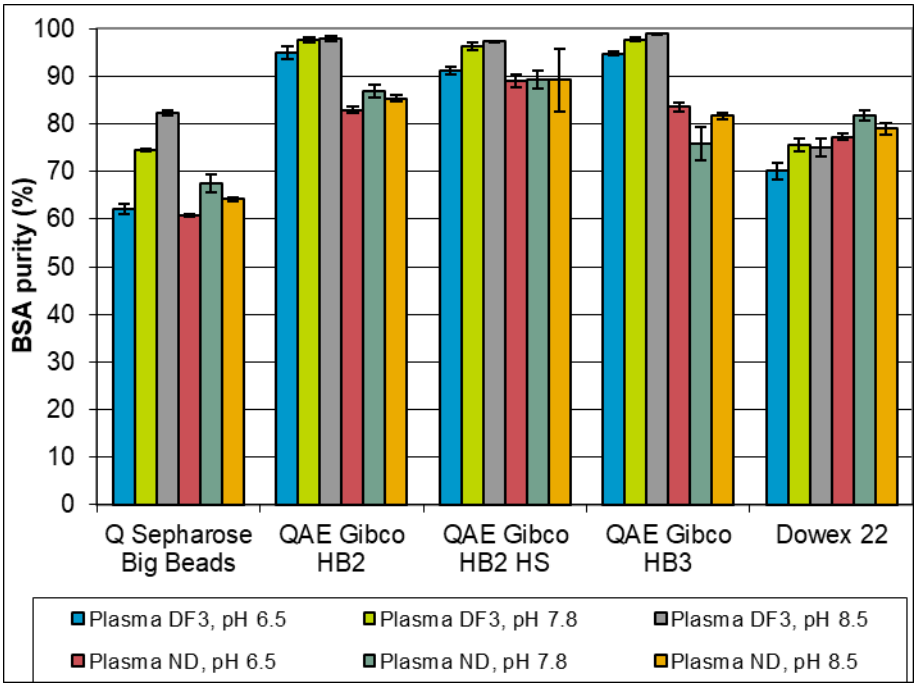

Figure 9.4 BSA composition (% of total BSA, transferrin and IgG content) in 1M salt eluates obtained from strong anion exchange resins when using diluted and non- diluted plasma at the pH 6.5, 7.8 and 8.5. DF – dilution factor, ND – non-diluted. Number of replicates – 3. Error bars – standard deviation.

The fractions with the BSA content more than 95% were produced by Gibco resins when diluted plasma was loaded at pH 7.8 and 8.5. The high purity of BSA (91-95%) was also found in elutes obtained from these resins when using the diluted feed at pH 6.5. When non-diluted plasma was applied on Gibco resins the BSA purity in the desorbed fractions decreased and varied from 75.8% to 89.3%.

Q Sepharose BB and Dowex 22 resins produced BSA fractions with the highest BSA purity of ~82%. These fractions were obtained by contacting the diluted plasma at pH 8.5 with Q Sepharose BB resin and non-diluted plasma at pH 7.8 with Dowex 22 resin.

Overall, among the five anion-exchange resins examined the three Gibco resins were able to produce fractions with estimated BSA purity greater than 95% when using the diluted plasma at pH 7.8 and 8.5. At these feed conditions QAE Gibco HB2HS and QAE Gibco HB3 resins had a BSA binding capacity of 17 mg/ml which was twice as high as the BSA binding capacity of QAE Gibco HB2 resin. Similar BSA binding was also achieved by using Q Sepharose BB resin and diluted plasma at pH 8.5. However, the BSA purity in the fraction desorbed from Q Sepharose

BB was only 82%. In addition, the cost of Q Sepharose BB resin is more than 4 times higher than the price of QAE Gibco resins. As it was not possible to obtain isolates with high BSA content and purity using QAE Gibco HB2 and Dowex 22 resins and because the cost of Q Sepharose BB is much higher than for QAE Gibco resins only QAE Gibco HB2HS and QAE Gibco HB3 were selected for future research in this project.

Effect of plasma dilution on adsorption of BSA by QAE Gibco HB2HS resin

The feed material for this experiment was prepared by dilution of plasma with RO water. Dilution factors ranged from 1 to 5. The pH of the diluted plasma solutions was checked and if necessary adjusted to 7.8 by using 0.1M NaOH or 0.1M HCl. The conductivity of the plasma feed was also measured and recorded. The concentration of BSA in the feed was determined by UPLC analysis. All measured feed characteristics are presented in Table 9.1.

Table 9.1 BSA concentration, pH and conductivity of the plasma feed solutions

Dilution Factor	BSA concentration (mg/ml)	pH	Conductivity (mS/cm)
1.00	29.23	7.76	13.60
1.25	23.02	7.83	10.90
1.50	18.07	7.83	9.46
1.75	16.76	7.85	8.33
2.00	14.54	7.83	7.38
2.50	12.16	7.84	6.37
3.00	9.83	7.83	5.46
3.50	8.60	7.82	4.82
4.00	7.48	7.84	4.19
4.50	6.44	7.83	3.75
5.00	5.95	7.83	3.48

QAE Gibco HB2HS resin was washed and equilibrated to pH 7.8 and 2 ml of the pre-treated resin was placed into syringes. A 10 ml aliquot of the prepared plasma feed was added to the

resin and left in contact under gentle mixing for 1 hour. After 1 hour, the flow through (F/T) fractions were collected and the resin samples were washed with 20 ml of RO water. The bound proteins were eluted with 4 ml of 1M NaCl. The plasma feed, F/T fractions and eluates were analysed for BSA and transferrin by UPLC and IgG by HPLC. The protein analysis results are presented in Figures 9.5 and 9.6.

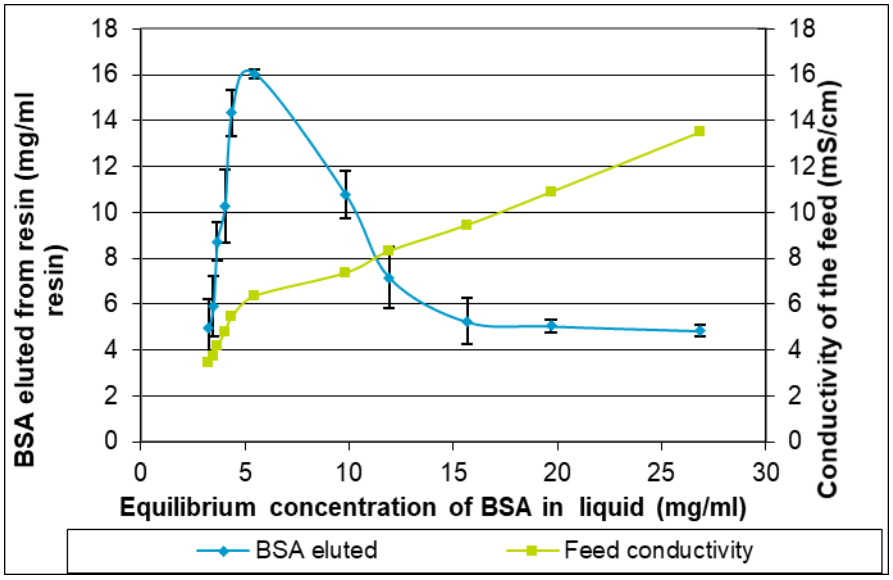

Figure 9.5 Isotherm for adsorption of BSA onto QAE Gibco HB2HS resin and the feed plasma conductivity curve.

The graph in Figure 9.5 shows the BSA static binding isotherm and the feed conductivity curve. The static binding isotherm was constructed by plotting the amount of BSA eluted from 1 ml of resin against the BSA concentration in the flow through solutions after the equilibrium was attained.

With knowledge of the isotherm, the effect of feed concentration on capacity can be estimated and used as a guide in process design. An isotherm can be favourable (concave), and then fitting a Langmuir model to experimental data yields a useful estimate of the maximal adsorption capacity (Q_{max}).

The obtained BSA isotherm did not have a typical concave shape. The BSA binding capacity was sharply increased from 4.9 to 16.1 mg/ml resin when the BSA concentration in the feed

increased from 5.9 to 12.1 mg/ml where the equilibrium BSA concentration in the liquid was 3.3 and 5.5 mg/ml. Further increase of the BSA concentration in the plasma feed did not result in the higher adsorption of BSA by the resin. On the contrary, when the BSA concentration increased from 12.1 to 18.1 mg/ml the BSA binding capacity of QAE Gibco HB2HS was reduced to 5.25 mg/ml. When non-diluted plasma with BSA concentration 29.2 mg/ml was loaded onto the resin the BSA adsorption was even lower — 4.85 mg/ml resin. The reduced BSA binding capacity can be explained by the high ionic strength of non-diluted and slightly diluted plasma solutions. The conductivity of the non-diluted plasma (13.6 mS/cm) is similar to the conductivity of 0.16 M NaCl solution. The results in Figure 9.5 demonstrate the need to reduce the ionic strength of the plasma to improve the binding capacity of the resin.

It is well known that in ion-exchange chromatography salts modulate the binding affinity of the proteins. Since binding affinity decreases as the salt concentration is increased, binding capacity of ion-exchange resins also decreases with salt concentration (Tsumoto et al., 2007). More information on the mechanisms of the salt effects on binding affinity and capacity for various column chromatography and on nonspecific protein–protein or protein–surface interactions can be found in the review of Tsumoto et al. (2007). The results suggest that in order to demonstrate the “true” effect of the BSA protein concentration on binding capacity of the resin the conductivity of the feed solutions should be maintained at the same level. This investigation was undertaken and discussed in section 9.4.

The effect of plasma dilution on the BSA purity in the fractions eluted from QAE Gibco HB2HS resin is shown in Figure 9.6.

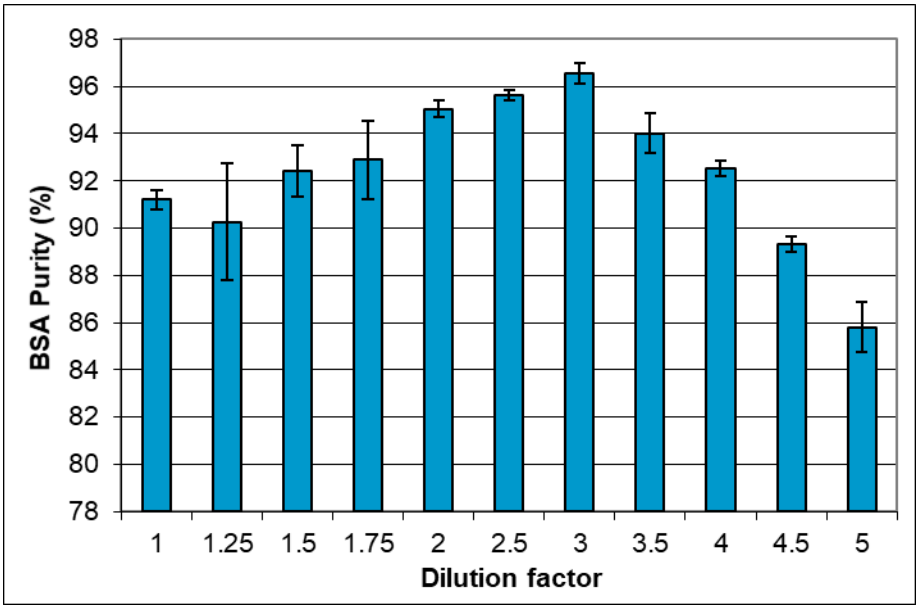

Figure 9.6 Effect of plasma dilution on the BSA purity in the fractions eluted from QAE Gibco HB2HS resin

The highest BSA purity (> 95%), calculated as a percent of the total content of three major proteins BSA, IgG and transferrin, was obtained when using diluted plasma with dilution factors 2, 2.5 and 3. The fractions produced with non-diluted plasma and plasma diluted 1.25, 1.5 and 1.75 times had the BSA purity lower than 95%. It varied from 90.3 to 92.9%. The more pronounced decline in BSA purity was observed in fractions isolated from less concentrated plasma solutions (dilution factors 4–5). When the plasma diluted 5 times was used as the feed material, the 1M NaCl eluate had the lowest BSA composition 85.9%. This fraction also contained 11.7% of IgG and 2.4% of transferrin. The observed reduction of BSA binding in more diluted plasma feeds could be due to increased competitive adsorption from IgG in solutions at low BSA concentrations.

Effect of salt concentration on elution profile of BSA and transferrin from QAE Gibco HB2HS resin

Washed and equilibrated to pH 7.8, QAE Gibco HB2HS resin (2 ml) was placed into syringes. The feed material used in this experiment was plasma diluted with RO water 2.5 times (pH 7.8, and conductivity 6.21 mS/cm). A 10 ml aliquot of the prepared plasma was added to the resin and left in contact under gentle mixing for 1 hour. After 1 hour the flow through (F/T) fraction was collected and the resin sample was washed with 20 ml of RO water. The bound proteins were

eluted with 2 ml of NaCl at the following concentrations: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1M. Fractions were collected separately and analysed for BSA and transferrin using UPLC. The IgG content in eluted fractions was very low and was not possible to quantify. Desorption of BSA and transferrin is shown in Figure 9.7, where percentage of proteins eluted from QAE Gibco HB2HS was plotted against NaCl concentration.

The 0.1M NaCl eluate contained 56.6% of transferrin and only 1.8% of BSA adsorbed on the resin. Increasing the concentration of NaCl to 0.4M resulted in desorption of all transferrin and 70% of bound BSA. Complete elution of BSA occurred at 1M NaCl. Results from HPLC analysis were supported by SDS gel electrophoresis as shown in Figure 9.8.

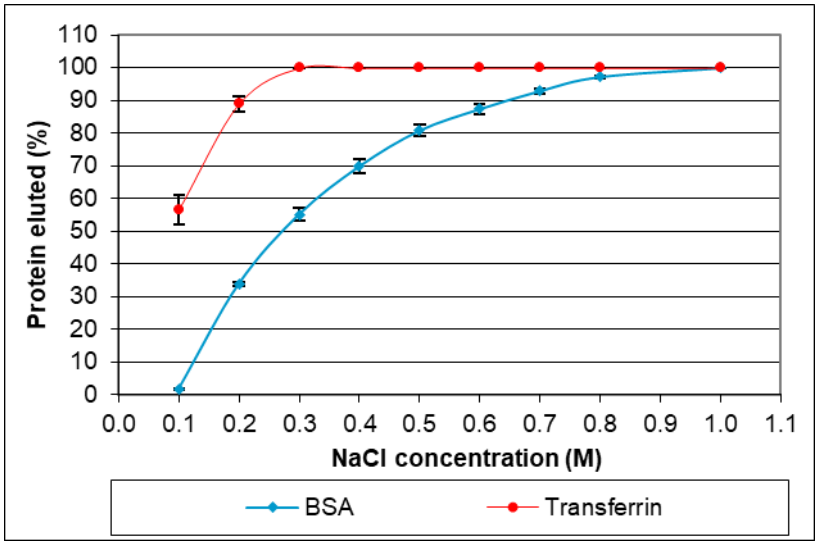

Figure 9.7 Elution profile of BSA and transferrin from QAE Gibco HB2HS resin after loading diluted plasma (DF 2.5) at pH 7.8.

LMW standards	**MW**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
α-Lactalbumin	14.4 kDa →									
Trypsin Inhibitor	20.1 kDa →									

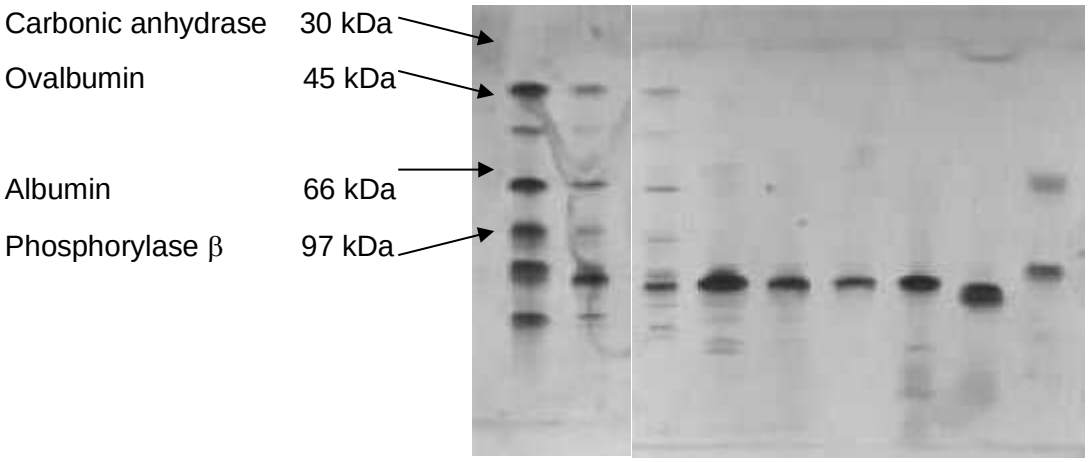

Figure 9.8 SDS PAGE electrophoresis profile of plasma proteins (under reduced conditions). LMW standards (lane **1**), proteins in the feed (lane **2**), 0.1M NaCl eluate (lane **3**), 0.2M NaCl eluate (lane **4**), 0.8M NaCl eluate (lane **5**), 1M NaCl eluate (lane **6**), BSA standard (lane **7**), transferrin standard (lane **8**), IgG standard (lane **9**).

The SDS PAGE profile of the plasma feed (lane 2) shows a series of bands distributed across a molecular weight range (14.4 to 97 kDa), indicating the presence of plasma proteins with a variety of different molecular weights. One band below 66 kDa standard has the high intensity showing that BSA with MW 68 kDa is a major protein in bovine plasma. Transferrin in plasma is shown as a faint band below 66 kDa standard and two IgG bands are visible above 66 kDa and 30 kDa standards. Under reduced conditions IgG is separated into two bands comprising heavy (~50 kDa) and light (~25 kDa) chains (lane 9). The SDS PAGE profile of the fraction eluted with 0.1 M NaCl (lane 3) shows all the bands which are present in the plasma feed (lane 2). In 0.2M NaCl eluate there is one major band with high intensity corresponding to BSA protein (lane 4). The presence of other bands in this fraction becomes less pronounced. When the concentration of NaCl increased to 0.8M and 1M the intensity of the bands representing proteins other than BSA decreased (lanes 5 and 6). In 1M NaCl eluate only one BSA band is visible indicating the high purity of BSA fraction (lane 6).

These results suggest that the purity of BSA in the final isolate could be improved by removing contaminant proteins with the pre-elution step using low concentration of salt (0.1M NaCl).

In laboratory trials undertaken to develop a process for extraction of chondroitin sulphate from trachea (a separate objective of the project) QAE Gibco HB3 resin was showing promising

results in purification of CS. Therefore, it was decided to examine QAE Gibco HB3 properties to isolate BSA from bovine plasma in the next experiments.

Effect of BSA concentration on the binding capacity of QAE Gibco HB3 resin

In previous experiments it was demonstrated that BSA binding capacity of anion-exchange resins was highly dependent on the ionic strength of the feed material. In order to investigate the effect of BSA concentration on the adsorption characteristics of QAE Gibco HB3 resin the feed conductivity was maintained at the same level for each protein concentration. Three BSA static binding isotherms were obtained for three different conductivities of the plasma feed – 13.6, 8.9 and 4.4 mS/cm. For the first binding isotherm 11 feed solutions were prepared by diluting the neat plasma with the solution of NaCl having conductivity 13.6 mS/cm. The dilution factors ranged from 1 to 5. For the second and the third isotherms the plasma was initially dialysed using the dialysis membrane with MWCO 6-8,000 (Spectrum Laboratories Inc.) till the conductivity reached 8.9 mS/cm and 4.4 mS/cm, respectively. After dialysis the plasma was diluted using NaCl solutions with the corresponding conductivities of 8.9 and 4.4 mS/cm. The pH of the diluted plasma solutions was checked and if necessary adjusted to 7.8 by using 0.1M NaOH or 0.1M HCl. The concentration of BSA in all prepared plasma solutions was determined by UPLC analysis. Results are presented in Table 9.2.

Table 9.2 BSA concentration (mg/ml) in the plasma feed for investigating equilibrium isotherms of QAE Gibco HB3 resin and BSA.

Dilution Factor	BSA concentration in the feed (mg/ml)		
	Plasma conductivity 13.6 mS/cm	Plasma conductivity 8.9 mS/cm	Plasma conductivity 4.4 mS/cm
1.00	29.11	26.74	25.58
1.25	22.93	22.36	22.08
1.50	19.22	18.26	18.36
1.75	15.73	15.85	15.34
2.00	14.20	13.92	13.68

2.50	11.23	11.19	10.80
3.00	9.42	9.25	8.98
3.50	8.02	7.72	7.84
4.00	7.08	6.85	6.76
4.50	6.28	6.15	6.16
5.00	5.63	5.61	5.51

Washed and equilibrated to pH 7.8, QAE Gibco HB3 (2 ml) was placed into syringes and 10 ml of the prepared plasma feed was added to the resin and left in contact under gentle mixing for 1 hour. After 1 hour the flow through (F/T) fractions were collected and resin samples were washed with 20 ml of RO water. The bound proteins were eluted with 4 ml of 1M NaCl. The plasma feed, F/T fractions and eluates were analysed for BSA and transferrin by UPLC and IgG by HPLC.

The BSA adsorption isotherms under different ionic strength were constructed from the experimental data obtained from the static experiments and are presented in Figure 9.9.

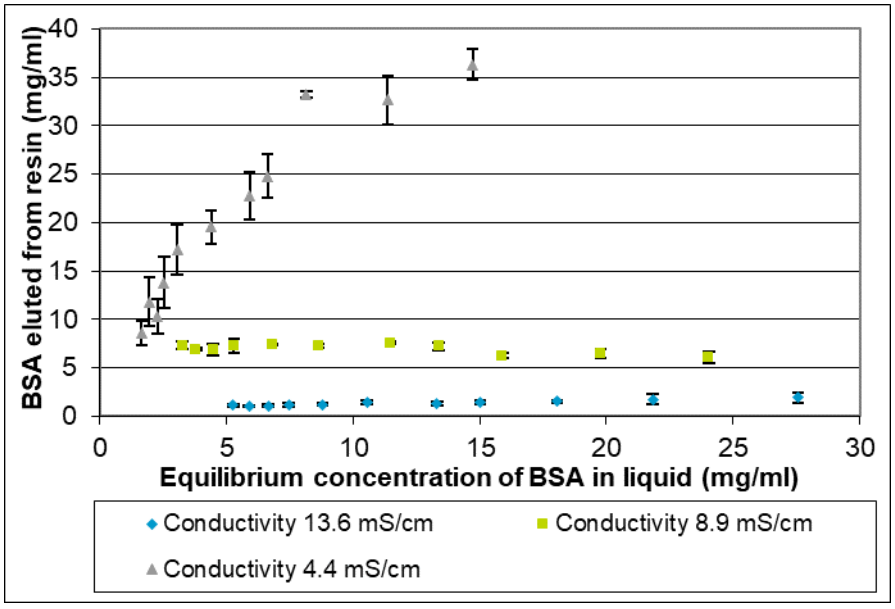

Figure 9.9. Effect of the feed conductivity on equilibrium isotherms of BSA for QAE Gibco HB3 resin.

The BSA adsorption isotherms clearly show that the increased conductivity of the feed material led to the lower binding capacity of QAE Gibco HB3 resin.

Ionic strength has long been recognised as one of the most important factors affecting proteins adsorption onto ion exchangers. Isotherm data regarding the influence of ionic strength on the protein adsorption have been obtained by studies of various investigators. Lan et al. (2001) demonstrated the effect of NaCl concentration on the equilibrium characteristics of BSA adsorbed onto anion-exchange resin Diaion HPA25 (Figure 9.10).

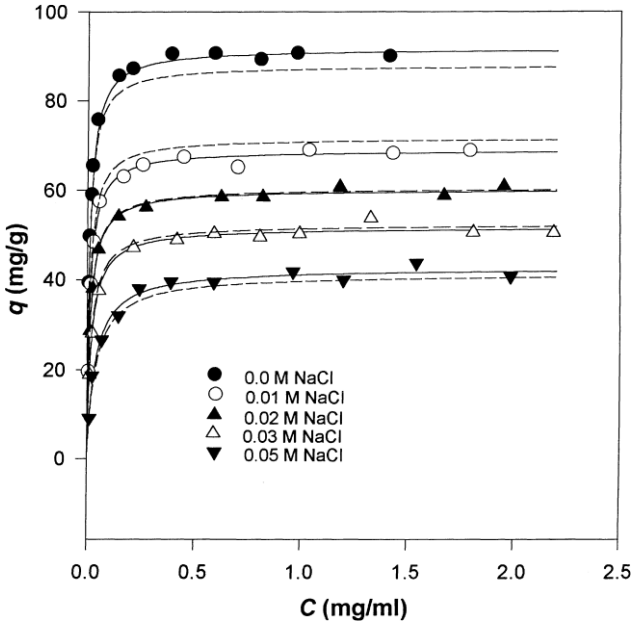

Figure 9.10 Comparison between the experimental isotherms (symbol), best-fit Langmuir isotherms for given individual NaCl concentration (—) and the isotherms predicted by the modified Langmuir model (----) for BSA. Copied from Lan et al. (2001).

These researchers showed that the adsorption of BSA onto Diaion HPA25 obeyed the Langmuir isotherm in the range of ionic strength investigated (0.0–0.05M NaCl). The maximum Diaion HPA25 binding capacity calculated by using Langmuir model was dependent on ionic strength. When the NaCl concentration of the solution increased from 0.0 to 0.05 M, the apparent maximum capacity decreased from 91.69 to 35.6 mg/g. The loss of the capacity of Diaion HPA25 resin was about 61.2%.

Similarly, in our trial the Langmuir model was fitted into experimental data obtained for static adsorption of BSA onto QAE Gibco HB3 resin using plasma solutions at conductivities 4.4 and 13.6 mS/cm which is the equivalent to NaCl molarity of 0.05M and 0.16 M (Figure 9.11 A, B).

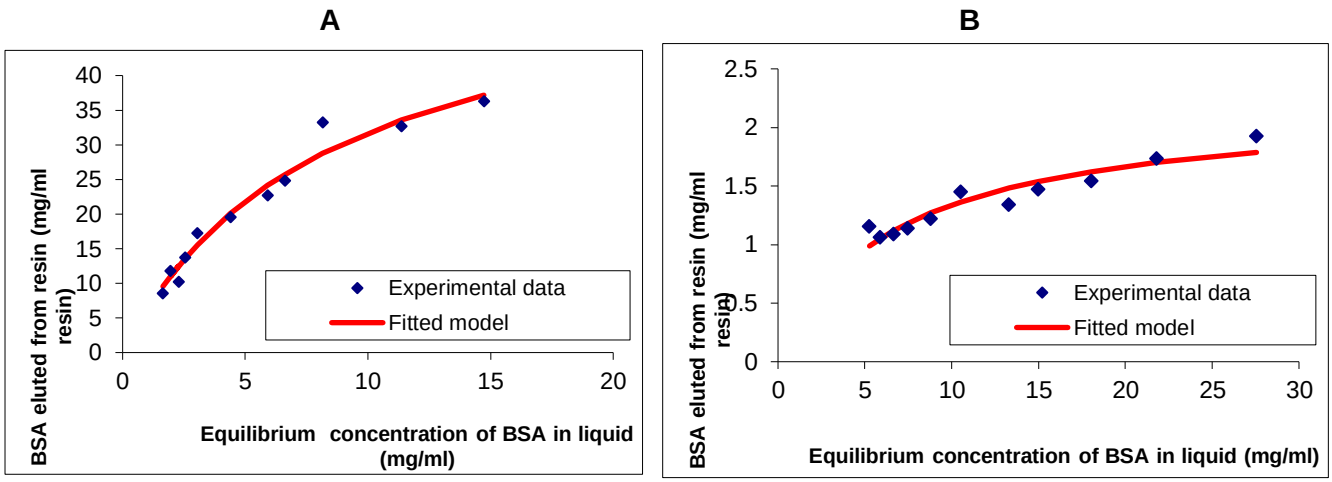

Figure 9.11. Experimental data and fitted Langmuir isotherms for adsorption of BSA onto QAE Gibco HB3 resin using plasma solutions with conductivities 4.4 mS/cm **(A)** and 13.6 mS/cm **(B)**.

The feed conductivity had a dramatic effect on the maximum binding capacity (Q_{max}) derived from the Langmuir model for QAE Gibco HB3 resin. When the plasma conductivity was low (4.4 mS/cm) the maximum binding capacity of QAE Gibco HB3 was 58.4 mg/ml resin. The estimated maximum binding capacity decreased to 2.2 mg/ml resin when the conductivity of the feed increased from 4.4 to 13.6 mS/cm. It was a 25-fold reduction.

The significant decrease of the binding capacity resulting from the increase of ionic strength can be attributed to the interactions between salt ions and BSA molecules and between salt ions and the ion-exchanger. These interactions are very complex and could be considered from the aspects outlined by Lan et al. (2001): (1) the salt counter ions compete against the protein ions for binding sites; (2) the salt co-ions shield the protein ions and the charged binding sites from each other; (3) the change of ionic strength changes the folding and configuration of protein molecules, resulting in variation of the hydrophobic interaction between the protein and the resin

matrix; (4) the increase of ionic strength may cause the network of the resin to shrink, reducing the porosity of the resins and hence the availability of binding sites.

The purity of BSA in fractions produced by QAE Gibco HB3 resin in equilibrium adsorption experiments using plasma at three different conductivities is presented in Figure 9.12.

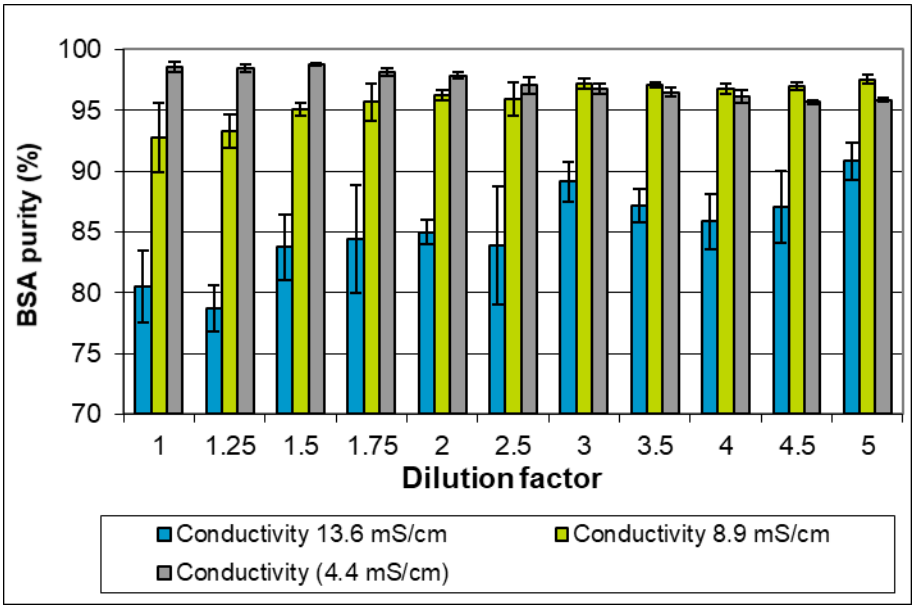

Figure 9.12. BSA purity in the fractions eluted from QAE Gibco HB3 resin when using plasma solutions (DF 1-5) at conductivities 4.4, 8.9 and 13.6 mS/cm.

The lowest purity of BSA (< 90%) was observed in almost all fractions eluted from QAE Gibco HB3 after loading plasma solutions with the conductivity 13.6 mS/cm. Only one eluate had BSA purity 90.8% when 5 times diluted plasma was used as a feed material.

The BSA purity was more than 95% in all fractions isolated from plasma solutions having conductivity 4.4 mS/cm. However, the slight decline of BSA purity from 98.6% to 95.9% was observed with plasma dilution. However, when the plasma conductivity was 8.9 mS/cm the highest BSA purity (97.6%) was achieved with 5 times diluted feed and the lowest (92.8%) with non-diluted plasma.

Estimation of BSA purity in the fraction isolated from diluted plasma using QAE Gibco HB3 resin

The BSA purity of the fraction isolated with QAE Gibco HB3 resin was estimated by conducting the laboratory experiment at a bigger scale. QAE Gibco HB3 resin was washed and equilibrated to pH 7.8. Plasma was diluted with RO water (1:2) and the pH adjusted to 7.8 with 1M NaOH. The conductivity of diluted plasma was 5.71 mS/cm. The sample of diluted plasma (400 ml) was loaded onto 80 ml of the pre-treated resin and left in contact for 1.5 hours with agitation. After 1.5 hours the flow through (F/T) fraction was collected and the resin was washed with excess of RO water. The bound proteins were eluted with 160 ml of 1M NaCl. The eluate was dialysed using the dialysis membrane with MWCO 3,500 (Spectrum Laboratories Inc.). The plasma feed, F/T fraction and dialysed eluate were analysed for BSA and transferrin by UPLC and IgG by HPLC.

The results from protein analysis show that under experimental conditions QAE Gibco HB3 resin had the BSA binding capacity ~17 mg/ml. In the isolated fraction the BSA purity estimated as the percent of the total protein components (BSA, transferrin and IgG) was 97%. The UPLC chromatograms obtained for the plasma feed, 1M NaCl eluate and BSA standard clearly show that the fraction isolated using QAE Gibco HB3 resin consists of only one BSA peak. Transferrin protein and other impurities are not present (Figure 9.13). The purity of BSA in the isolated fraction was confirmed by SDS PAGE analysis of 1M eluate shown in Figure 9.14.

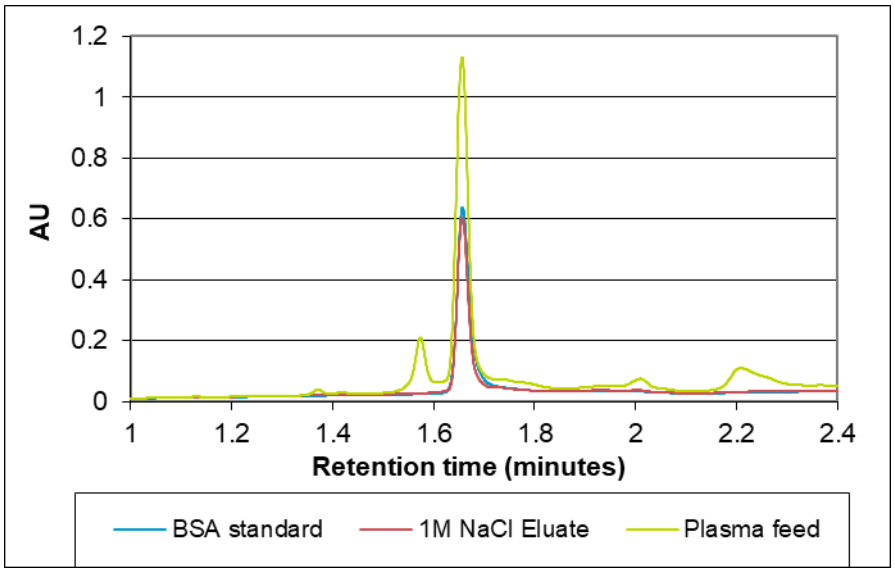

Figure 9.13. The UPLC profile of plasma feed, 1M NaCl eluate and BSA standard.

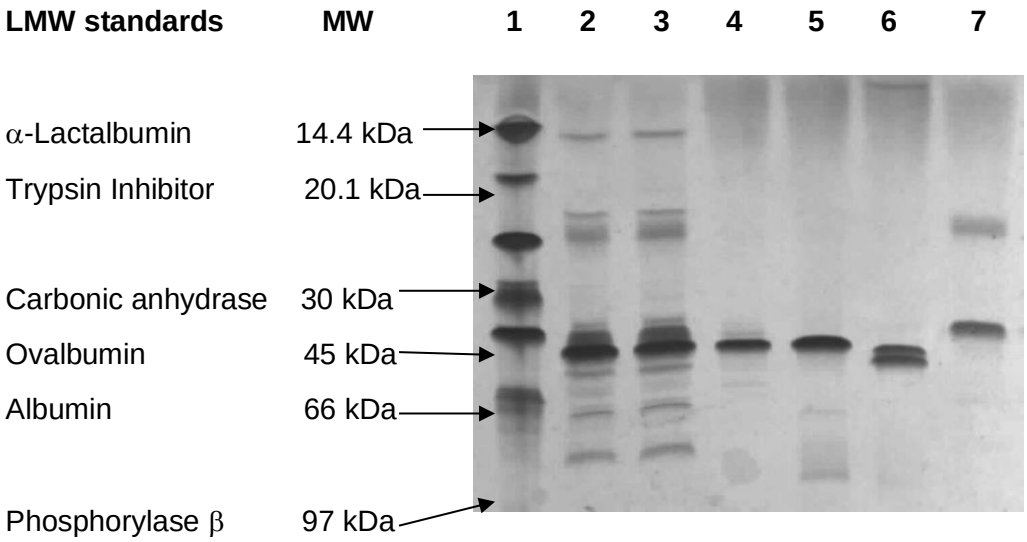

Figure 9.14. SDS PAGE electrophoresis profile of plasma proteins (under reduced conditions). LMW standards (lane **1**), proteins in the feed (lane **2**), proteins in F/T (lane **3**), 1M NaCl eluate (lane **4**), BSA standard (lane **5**), transferrin standard (lane **6**), IgG standard (lane **7**).

The BSA isolate (lane 4) run on SDS PAGE gave a major band representing BSA protein. There are also some very faint bands visible above and below the major band. Similar profile was obtained for commercially purified BSA reference material purchased from Sigma –Aldrich (lane 5) with the certified purity of 96%.

Estimation of IgG content in the F/T fraction (IgG enhanced fraction) from diluted plasma using QAE Gibco HB3 resin

In the F/T fraction, the IgG content was estimated as the percent of the total protein components (BSA, transferrin and IgG) and was 47%, which is similar to the IgG content in colostrum.

Conclusions and recommendations – Chromatographic separation

Five anion-exchange resins were evaluated for static binding capacity and specificity to isolate bovine serum albumin from plasma with various characteristics (protein content, pH and conductivity). Of the ion-exchange resins examined QAE Gibco HB2HS and QAE Gibco HB3 showed the highest BSA adsorption capacity (~17 mg/ml resin) and the highest BSA purity (>95%) when using diluted plasma (DF 3) at pH 7.8 and 8.5, BSA concentration in the feed ~ 11 mg/ml and conductivity 5.76 mS/cm.

It was demonstrated that the resins binding capacity was strongly dependent on the ionic strength of the plasma feed. The higher adsorption of BSA was observed at the low conductivity of plasma solutions. The maximum binding capacity 58.4 mg/ml derived from the Langmuir model could be obtained by applying non-diluted plasma at low conductivity (4.4 mS/cm) onto QAE Gibco HB3 resin.

In order to reduce the plasma conductivity extra processing step is required. It could be achieved with either diafiltration or by dilution of plasma with water. Diafiltration of plasma prior to ion-exchange will increase operating cost associated with ultrafiltration equipment and diafiltation water used for desalting.

Results from the elution trial indicated that the BSA purity in the final isolate could be improved by removing contaminant proteins with pre-elution step using 0.1M NaCl.

The results of the trial also indicate that the F/T fraction has an IgG composition >45%, which is similar to colostrums and will be a non-dairy source of IgG.

Considering the favourable results achieved through the experimental program, it is recommended that:

- Ion exchange technology should be further developed to pilot scale to establish scaled up design and operating parameters for the process, validate consistency of product specification, produce prototype samples for customer evaluation, refine the COP, estimate the capital cost and evaluate the economic feasibility of a commercial process using the developed technologies. This will provide the meat processors or value adders with information to make decisions regarding investments into the future.

- Conduct functionality tests on the BSA fraction to compare the efficacy of current products in the market with the products produced using the proposed technology.

- Conduct bioactivity studies (in vitro and in vivo) of the IgG enhanced fraction to compare the efficacy of current products in the market with the products produced using the proposed technology.

- Develop technology to further purify IgG as an active ingredient and broaden the product portfolio.

Preliminary estimate of cost of production of BSA from bovine plasma

Based on the laboratory scale trials, the production of BSA at a purity >80% (97% achieved) is possible using anion exchange chromatographic. The chromatographic separation is based on adsorbing the negatively charged BSA onto an anion exchange resin thereby increasing the IgG concentration in the F/T fraction.

Based on the laboratory scale data, preliminary estimates for the cost of production (COP) for both BSA and an IgG enhanced fraction as a co-product were carried out. These cost estimates would need to be further refined as preparatory scale samples are produced and more importantly when the laboratory based process is scaled up to pilot scale.

The preliminary estimate of COP of 26 tonnes/year of BSA and 88 tonnes/year of IgG enhanced fraction was based on several assumptions. The details of the assumptions are in Appendix 1 for both products. The main assumptions used in the COP model were:

- The process is assumed to operate continuously 5 days per week with 2 operators for BSA production and 1 operator for IgG production per shift, over 48 weeks of the year.
- Based on experience in the applications of ion exchange chromatography in the dairy industry, the life of the resin and membranes for BSA production was assumed to be 2 years and the life of membranes for IgG fraction was taken as 1 year, assuming the fouling of the membranes would be higher in the F/T fraction. It is expected that ion exchange resins would last longer than the assumed period.
- Since the majority of the abattoirs in Australia do not use a hollow knife system for blood collection, the yield of blood was taken as 8L/animal and the plasma volume was taken as 50%, allowing for potential losses of plasma during separation.
- The red cell fraction produced during plasma separation could be sent for rendering as currently done with blood and the salt permeate from the chromatographic system is handled by the existing effluent treatment systems
- Labour cost was taken as $35,000 per year.

• The current income from rendering blood was taken as $0.15/kg of blood. This was taken as an opportunity cost. The total opportunity cost was recovered from the production of BSA, hence no charge for opportunity cost was made for the production of IgG.

BSA production using ion exchange chromatography

The envisaged processing strategy to separate and purify BSA from bovine plasma employing ion exchange chromatography is shown in Figure 11.1

It is anticipated that hygienically collected blood with added sodium citrate to prevent coagulation will be cooled to a temperature of 4^0C immediately on collection to maintain good hygienic conditions. The blood should be separated using a centrifugal separator into a red blood cell and plasma fraction. The temperature of the plasma should be maintained at 4^0C during storage to maintain the microbial quality of the plasma. The plasma should then be loaded onto chromatographic columns with the temperature maintained between 4–8^0C during processing, using either traditional batch systems or preferably a more efficient simulated moving bed chromatographic system. The eluate from the chromatographic separation, which is the BSA fraction, is then subjected to ultrafiltration using a 20-30kDa molecular weight cut-off membrane to remove salt and concentrate BSA in the solution. The purification of BSA through the removal of salt is achieved by diafiltering the concentrated BSA solution. The concentrated and diafiltered BSA is then dried using a spray drier and packed.

The F/T from the chromatographic column is subjected to ultrafiltration to concentrate the proteins and diafiltration to remove salts. The concentrated and diafiltered IgG fraction is dried using a spray drier and packed.

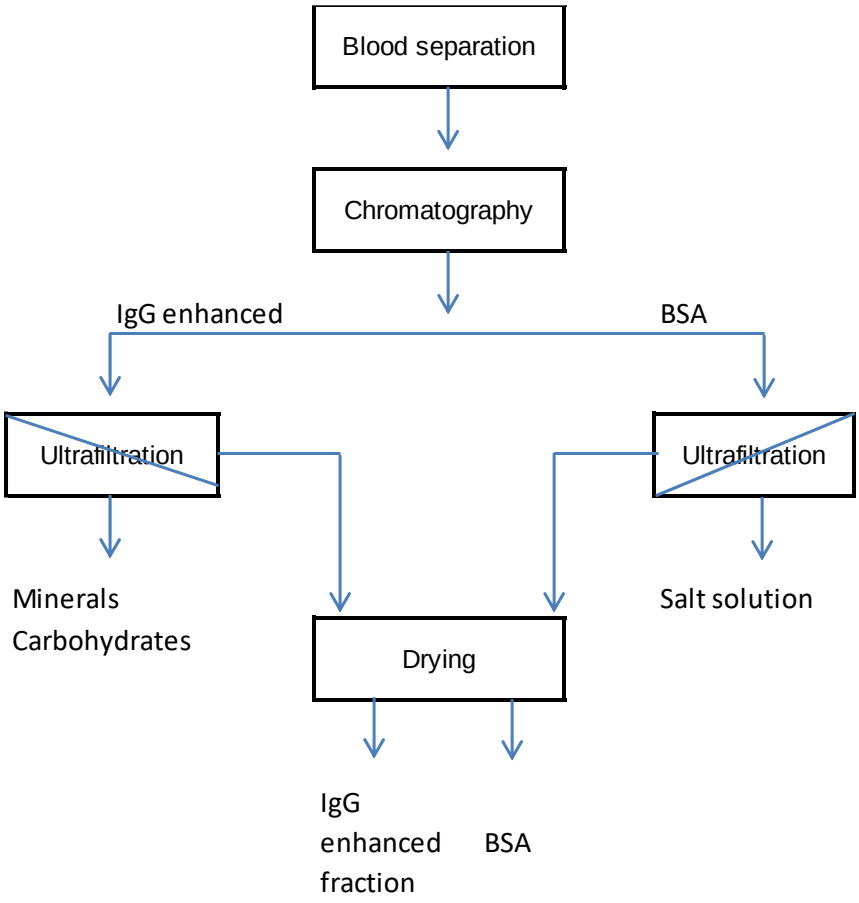

Figure 11.1 Block schematic of the conceptual ion exchange chromatographic process to separate and purify BSA from bovine blood. An IgG enhanced fraction is a co-product of the process.

Estimation of cost of production of CS using ion exchange chromatography

The details of the estimate of the COP to produce 26 tonnes/year of BSA are detailed in Table 11.1 (plasma from 2,000 animals/day). The COP includes the current income received by an abattoir through rendering at $0.15/kg as an opportunity cost charged as an expense. The COP with the opportunity costs charged as an expense is estimated at $26.8/kg of BSA, but the COP without charging the opportunity costs is estimated significantly lower at $15.7/kg of BSA. The COP was also estimated assuming adding a contingency of a further 50% of direct operating cost. The COP with the added contingency expenditure was estimated at $40.30/kg of BSA and without the opportunity cost was estimated at $23.60/kg of BSA produced.

The COP of 88 tonnes/year of IgG enhanced fraction was estimated at $2.16/kg of IgG enhanced product and the COP with the added contingency expenditure was estimated at $3.25/kg of IgG enhanced fraction.

If the COP is taken on the basis of producing the total volume of protein, which is 114 tonnes/year, then the COP of protein is $5.26/kg of protein and with the addition of a further 50% of contingency, the COP is $7.89/kg.

The main cost components in the production of BSA was the opportunity and labour costs and in the production of IgG was the labour and spray drying cost with labour being 55% of the costs.

Table 11.1 Preliminary estimate for the COP to produce 26 tonnes/year of BSA and 88 tonnes/year of IgG enhanced fraction from bovine plasma using anion exchange chromatography. The percentage contribution of each cost item based on the total direct COP was also estimated.

Cost of production (BSA only)	$/batch	$/year	% of direct COP
Plasma opportunity cost	1,200	288,000	41.40
Citrate	104	24,960	3.59
Labour	875	210,000	30.19
Salt	59	14,271	2.05
Packaging	54	12,960	1.86
Water	262	62,846	9.03
Power	15	3,493	0.50
CIP	50	12,000	1.73
Spray drying	23	5,472	0.79
Resin replacement	249	59,647	8.57
Membrane replacement	8	1,945	0.28
Direct COP	**2,898**	695,594	
COP/kg	**26.8**		
Contingency on direct costs (50%)			
Direct COP	4,347		
COP/kg	40.3		
BSA production (kg/batch)	108		
BSA production (tons/year)	26		
Cost of production (IgG fraction)			
Plasma opportunity cost	0	0.00	0
Labour	438	55.43	105,000
Packaging	182	23.11	43,776
Water	9	1.19	2,263
Power	15	1.85	3,499
CIP	15	1.90	3,600
Spray drying	96	12.22	23,143
Membrane replacement	34	4.31	8,163
Direct COP	**789**	189,444	
COP/kg	**2.16**		
Contingency on direct costs (%)	50		
Direct COP	1,184		
COP/kg	3.25		
IgG production (kg/batch)	365		
IgG production (tons/year)	88		
Direct COP of protein (BSA & IgG fraction) ($/kg)	**7.80**		
Direct COP of protein (BSA & IgG fraction) with 50% contingency ($/kg)	11.70		

Conclusions on the COP of BSA and IgG fraction using ion exchange chromatography

Based on the laboratory data, the COP of producing BSA at a purity of >90% using chromatography was cost effective at 26.8/kg. The advantage of the technology is the ability to

produce an additional protein fraction which is enhanced in IgG content (>45%) at a very low COP of $2.16/kg. The overall COP of combined protein was $7.8/kg. The main cost component in the production of BSA was the plasma opportunity cost.

A preliminary estimate of capital cost will be made after the next milestone of the project when the laboratory process will be scaled to laboratory preparatory scale.

Another advantage of the chromatographic approach is that the process to separate CS from cartilage has also identified the same resin, QAE Gibco HB3 as the optimum anion exchange resin. This provides opportunities to produce 3 different products (BSA, IgG enhanced fraction and CS) using the same capital investment, hence reducing commercial risks.

The direct COP of BSA and IgG using chromatography is low with the opportunity cost of plasma being the dominant cost which is applicable to whatever technology is used. Therefore, it is recommended that the technology be further developed to pilot scale to establish scaled up design and operating parameters for the process and validate consistency of product specification. The pilot scale data will also enable the refining of the COP and also establish an estimate of the capital cost of the technologies. The meat processors or value–adders will then have information to make decisions regarding the product portfolio and the technology.

Reference list

Bekard, I.B., Asimakis, P., Bertolini, J. and Dunstan, D.E. (2011). “Bovine serum albumin unfolds in Couette flow.” Soft Matter, in press.

Bohme, U. and Scheler, U. (2007). "Effective charge of bovine serum albumin determined by electrophoresis NMR." Chemical Physics Letters **435**(4-6): 342-345.

Boributh, S., Chanachai, A. and Jiraratananon, R. (2009). "Modification of PVDF membrane by chitosan solution for reducing protein fouling." Journal of Membrane Science **342**(1-2): 97-104.

Burnouf, T. (2007). "Modern plasma fractionation." Transfusion Medicine Reviews **21**(2): 101-117.

Burns, D. B. and Zydney, L. A. (1999). "Effect of solution pH on protein transport through ultrafiltration membranes." Biotechnology and Bioengineering **64**(1): 27-37.

Christy, C., Adams, G., Kuriyel, R., Bolton, G., and Seilly, A. (2002). "High-Performance Tangential Flow Filtration: a Highly Selective Membrane Separation Process." Desalination **144**(1-3): 133-136.

Condie, R. M. (1979). Isolation and purification of gamma-globulin and albumin - by treating blood plasma with silica then with ion exchangers, Minnesota University (Minu). US4136094-A

Curling, J.M., Lindquist, L. D. and Eriksson, S. (1977). "Chromatographic processing of human plamsa-oliot plant study." Process Biochemistry, 12(3): 22-23.

Dernis, D., Burnouf, T. and Demis, D Albumin soln. prepn. by de-lipidisation and chromatography - from plasma or plasma fractions, to obtain stable, high purity solns. of therapeutic use.

EP402205-A1; FR2648048-A1; EP402205-A; AU9056812-A; FR2648048-A; NO9002499-A; CA2018511-A; FI9002843-A; HU54382-T; ZA9004315-A; JP3081290-A; DD296842-A5; CS9002857-A2; AU633925-B; EP402205-B1; DE69024105-E; ES2081952-T3; NO300041-B1; FI101228-B1; JP2965623-B2; US6022954-A; CA2018511-C.

Dromard, A., Exertier, M., Rollin, C., Tayot, J. L., and Tardy, M. (1984). Chromatographic fractionation of plasma - using hydrophobic absorbent and hydrophilic ion exchanger, Rhone-Poulenc Rech (Rhon) Inst Merieux (Inmr) Rhone Poulenc Rech (Rhon). EP121468-A3; EP121468-A2; FR2543448-A1; EP121468-A; FR2543448-A; AU8426264-A; DK8401744-A; ZA8402204-A; JP60023320-A; ES8501635-A; CA1203478-A; US4675384-A; EP121468-B; DE3469132-G; JP93020439-B.

El Kadi, N., Taulier, N., Le Huerou, J. Y., Gindre, M., Urbach, W., Nwigwe, I. (2006). "Unfolding and refolding of bovine serum albumin at acid pH: Ultrasound and structural studies." Biophysical Journal **91**(9): 3397-3404.

Feins, M. and Sirkar, K. K. (2005). "Novel internally staged ultrafiltration for protein purification." Journal of Membrane Science **248**(1-2): 137-148.

Fernandez, S. O., Rodriguez, J. A. and Padilla, A. P. (1999). "Removal concentration and desalination of bovine seroalbumin (BSA) With membrane technology." Desalination **126**(1-3): 95-100.

Hester, J. F. and Mayes, A. M. (2002). "Design and performance of foul-resistant poly(vinylidene fluoride) membranes prepared in a single-step by surface segregation." Journal of Membrane Science **202**(1-2): 119-135.

Higuchi, A., Mishima, S. and Nakagawa, T. (1991). "Separation of proteins by surface modified polysulfone membranes." Journal of Membrane Science **57**(2-3): 175-185.

Kelly, S. T., Opong, W.S. and Zydney, A. L. (1993). "The influence of protein aggregates on the fouling of microfiltration membranes during stirred cell filtration." Journal of Membrane Science **80**(1-3): 175-187.

Lan, Q. D., Bassi, A. S., Zhu, J. X., and Margaritis, A. (2001). "A modified Langmuir model for the prediction of the effects of ionic strength on the equilibrium characteristics of protein adsorption onto ion exchange/affinity adsorbents." Chemical Engineering Journal **81**(1-3): 179-186.

Lin, S. H., Hung, C. L., and Juang, R. S. (2008). "Effect of operating parameters on the separation of proteins in aqueous solutions by dead-end ultrafiltration." Desalination **234**(1-3): 116-125.

Maruyama, T., Katoh, S., Nakajima, M. and Nabetani, H. (2001). "Mechanism of bovine serum albumin aggregation during ultrafiltration." Biotechnology and Bioengineering **75**(2): 233-238.

Mayani, M., Mohanty, K., Filipe, C., and Ghosh, R. (2009). "Continuous fractionation of plasma proteins HSA and HIgG using cascade ultrafiltration systems." Separation and Purification Technology **70**(2): 231-241.

Moure, F., Rendueles, M. and Diaz, M. (2004). "Bovine plasma protein fractionation by Ion exchange chromatography." Bioprocess and Biosystems Engineering **27**(1): 17-24.

Ohno, S., Koyama, K. and Fukuda, M. (1981). Sepn. of serum albumin and gamma-globulin - by ultrafiltration of acidic serum protein soln, Toyo Soda Mfg Co Ltd (Toyj). FR2473523-A1; GB2065129-A; JP56081521-A; FR2473523-A; DE3046565-A; US4347138-A; GB2065129-B; JP87006693-B; DE3046565-C.

Pujar, N. S. and Zydney A. L. (1994). "Electrostatic and electrokinetic interactions during protein transport through narrow pore membranes." Industrial & Engineering Chemistry Research **33**(10): 2473-2482.

Saksena, S. and Zydney A. L. (1994). "Effect of Solution Ph and Ionic-Strength on the Separation of Albumin From Immunoglobulins (Igg) by Selective Filtration." Biotechnology and Bioengineering **43**(10): 960-968.

Salgin, S. (2007). "Effects of ionic environments on bovine serum albumin fouling in a cross-flow ultrafiltration system." Chemical Engineering & Technology **30**(2): 255-260.

Salgin, S., Takac, S. and Ozdamar, T. H. (2005). "A parametric study on protein-membrane-ionic environment interactions for membrane fouling." Separation Science and Technology **40**(6): 1191-1212.

Saxena, A., Tripathi, B. P., Kumar, M., and Shahi, V. K. (2009). "Membrane-based techniques for the separation and purification of proteins: an overview." Advances in Colloid and Interface Science **145**(1-2): 1-22.

Shukla, R., Balakrishnan, M. and Agarwal, G. P. (2000). "Bovine Serum Albumin-Hemoglobin Fractionation: Significance of Ultrafiltration System and Feed Solution Characteristics." Bioseparation **9**(1): 7-19.

Takahashi, T., Ikegaya, K., Mochizuki, S., Nishimaki, H., & Motizuki, S. (1995). Prodn. of albumin prepn(s). of high purity - by subjecting an aq. albumin soln. to anion exchange treatment, cation exchange treatment and heat treatment, Green Cross Corp (Grec) Yoshitomi Pharm Ind Kk (Yosh). EP685491-A; EP685491-A2; JP7330626-A; CA2150612-A; EP685491-A3; US5616691-A; JP3702474-B2.

Tsumoto, K., Ejima, D., Senczuk, A. M., Kita, Y., and Arakawa, T. (2007). "Effects of salts on protein-surface interactions: Applications for column chromatography." Journal of Pharmaceutical Sciences **96**(7): 1677-1690.

Tung, K. L., Li, Y. L., Wang, S. J., Nanda, D., Hu, C. C., Li, C. L., (2010). "Performance and effects of polymeric membranes on the dead-end microfiltration of protein solution during filtration cycles." Journal of Membrane Science **352**(1-2): 143-152.

Wan, Y. H., Ghosh, R. and Cui, Z. F. (2002). "High-resolution plasma protein fractionation using ultrafiltration." Desalination **144**(1-3): 301-306.

Wang, Y. H. and Rodgers, V. G. J. (2011). "Electrostatic contributions to permeate flux behavior in single bovine serum albumin ultrafiltration." Journal of Membrane Science **366**(1-2): 184-191.

Appendices

Appendix 1–Literature patent review–methodology

1.1.26 Scientific literature searches

Free text and keyword searches, (taken from their thesauri) were conducted on the following databases:

Commonwealth Agricultural Bureau Index (CABI) contains over 3.8 million records from over 10,000 journals, books, conferences, reports, and other kinds of literature published internationally. Subjects covered include animal and crop husbandry, animal and plant breeding, plant protection, genetics, forestry, economics, veterinary medicine, human nutrition, and rural development. This database has extensive coverage of the meat and livestock industry. It was searched using the following key word search terms:

"bovine serum albumin" and (recover* or purify* or extract* or fraction)

("blood serum" or blood plasma") and protein* (all as descriptors) and (recover* or separate* or fraction* or purify*)

Albumin* (descriptor) and (recover* or purify* or extract* or fraction)

Food Science and Technology Abstracts (FSTA) is the internationally recognised world's leading Food Science and Technology Abstracts database and it has extensive coverage of post- farm-gate food processing, and ingredients industry from 1969 onwards. This database was searched using the following key word search terms:

"bovine serum albumin" (descriptor) were all hand combed for relevance

"bovine serum albumin" and (Separation or fractionation) and blood

"bovine serum albumin and blood and recover*

"blood protein*" (descriptor) and (recover* or purify*)

blood and protein* and (recover* or purify*)

(blood or plasma) and (recover* or purify*)

(plasma and protein*) and (recover* or purify*)

1.1.27 Intellectual property searches

Derwent Innovations Index

Intellectual property searches were carried out on Derwent Innovations Index, a respected patent database with worldwide coverage. Derwent covers material back to 1963 although this coverage varies with the country of origin. This variable country coverage means that material that has been registered in a single jurisdiction prior to that jurisdiction being covered by Derwent will not appear in these results. The advantage of Derwent, apart from its breadth of coverage, is that it collates patents into their families. This means that the user does not have to bring single applications in different countries for the same patent together in order to follow their registration and status in different patent jurisdictions. If a patent was registered later in a covered zone, the patent family approach sometimes mitigates the country coverage variability issue.

Country Coverage by Derwent Innovation Index

Country/Abbreviation	Initial Year of Coverage
Argentina (AR)	1974-1976 only
Australia (AU)	1983 (also 1963-1969)
Austria (AT)	1975
Belgium (BE)	1963
Brazil (BR)	1976

Canada (CA)	1963
China (CN)	1987
Czech Republic (CZ)	1994
Czechoslovakia (CS)	1975-1994
Denmark (DK)	1974
European Patents (EP)	1978
Finland (FI)	1974
France (FR)	1963
Germany (East) (DD)	1963
Germany (DE)	1963
Hungary (HU)	1975
India (IN)	2004
International Technology Disclosures (TP)	1984-1993
Ireland (IE)	1963-1969; 1995
Israel (IL)	1975
Italy (IT)	1966-1969 (section A subjects only); 1978
Japan (JP)	1963
Korea (KR) (South)	1986
Luxembourg (LU)	1984
Mexico (MX)	1997
Netherlands (NL)	1963
New Zealand (NZ)	1993
Norway (NO)	1974
Patent Cooperation Treaty (WO)	1978
Philippines (PH)	1995

Portugal (PT)	1974
Research Disclosure (RD)	1978
Romania (RO)	1975
Russian Federation (RU)	1994 (Russia)
Singapore (SG)	1995
Slovakia (SK)	1994
South Africa (ZA)	1963
Soviet Union (SU)	1963-1994
Spain (ES)	1983
Sweden (SE)	1974
Switzerland (CH)	1963
Taiwan (TW)	1993
United Kingdom (GB)	1963
United States (US)	1963

The terms and international patent classification codes (IPCs) discussed below were used for locating patents in the Derwent Innovations Index (DII) database.

Separation/ Purification Technologies

1. **International Patent Classification (IPC) Codes**

The following codes for extracting food proteins from blood were searched and hand combed for relevance.

A23J - Protein compositions for foodstuffs; working-up proteins for foodstuffs; phosphatide compositions for foodstuffs

A23J-001/00 - Obtaining protein compositions for foodstuffs; Bulk opening of eggs and separation of yolks from whites

A23J-001/06 - from blood

A23J-003/00 - Working-up of proteins for foodstuffs

A23J-003/12 - from blood

The IPC Codes (highlighted in bold), for separation and purification technologies, were chosen from the IPC hierarchy. They are shown in their hierarchy of meaning. These codes were combined with albumin*

B01 - Physical or chemical processes or apparatus in general

B01D – Separation

B01D-011/00 - Solvent extraction

B01D-011/02 - of solids

B01D-011/04 - of solutions which are liquid

B01D-015/00 - Separating processes involving the treatment of liquids with adsorbents

B01D-015/02 - with moving adsorbents

B01D-015/04 - with ion-exchange materials as adsorbents

B01D-015/08 - Selective adsorption, e.g. chromatography

B01D-017/02 - Separation of non-miscible liquids

B01D-017/038 - by centrifugal force

B01D-057/00 - Separation, other than separation of solids, not fully covered by a single other group or subclass,

B01D-057/02 - by electrophoresis

B01D-061/00 - Processes of separation using semi-permeable membranes, e.g. dialysis, osmosis, ultrafiltration; Apparatus, accessories or auxiliary operations specially adapted therefor

B01D-061/02 - Reverse osmosis; Hyperfiltration

B01D-061/04 - Feed pretreatment

B01D-061/06 - Energy recovery

B01D-061/08 - Apparatus therefor

B01D-061/10 - Accessories; Auxiliary operations

B01D-061/12 - Controlling or regulating

B01D-061/14 - Ultrafiltration; Microfiltration

B01D-061/16 - Feed pretreatment

B01D-061/18 - Apparatus therefor

B01D-061/20 - Accessories; Auxiliary operations

B01D-061/22 - Controlling or regulating

B01D-061/24 - Dialysis

B01D-061/26 - Dialysate solution flow, e.g. preparation, regeneration

B01D-061/28 - Apparatus therefor

B01D-061/30 - Accessories; Auxiliary operation

B01D-061/34 - Measuring ultrafiltrate during dialysis

B01D-061/36 - Pervaporation; Membrane distillation; Liquid permeation

B01D-061/38 - Liquid-membrane separation

B01D-061/40 - using emulsion-type membranes

B01D-061/42 - Electrodialysis; Electro-osmosis

B01D-061/44 - Ion-selective electrodialysis

B01D-061/46 - Apparatus therefor

B01D-061/48 - having one or more compartments filled with ion-exchange ma

B01D-061/50 - Stacks of the plate-and-frame type

B01D-061/52 - Accessories; Auxiliary operation

B01D-061/54 - Controlling or regulating

B01D-061/56 - Electro-osmotic dewatering

B01D-061/58 - Multistep processes

B01D-063/00 - Apparatus in general for separation processes using semi-permeable membranes

B01D-063/04 - comprising multiple hollow fibre assemblies

B01D-063/06 - Tubular membrane modules

B01D-063/08 - Flat membrane modules

B01D-063/10 - Spiral-wound membrane modules

B01D-063/12 - comprising multiple spiral-wound assemblies

B01D-063/14 - Pleat-type membrane modules

B01D-063/16 - Rotary, reciprocated or vibrated modules

B01J - Chemical or physical processes, e.g. catalysis, colloid chemistry; their relevant apparatus

B01J-014/00 - Chemical processes in general for reacting liquids with liquids; Apparatus specially adapted therefor

B01J-019/00 - Chemical, physical, or physico-chemical processes in general; Their relevant apparatus

B01J-019/02 - Apparatus characterised by being constructed of material selected for its chemically-resistant properties

B01J-019/06 - Solidifying liquids

B01J-019/08 - Processes employing the direct application of electric or wave energy, or particle radiation; Apparatus the

B01J-019/10 - employing sonic or ultrasonic vibrations

B01J-019/12 - employing electromagnetic waves

B01J-047/00 - Ion-exchange processes in general; Apparatus therefore

B01J-047/02 - Column or bed processes

B01J-047/04 - Mixed-bed processes

The following codes for separating protein products were also searched.

These were combined with BSA or albumin*or blood or serum or plasma and in some instances these were further restricted with the terms bovine or cattle or cow

C07 - Organic chemistry

C07K - Peptides

C07K-001/00 - General processes for the preparation of peptides

C07K-001/14 - Extraction; Separation; Purification

C07K-001/16 - by chromatography

C07K-001/18 - Ion-exchange chromatography

C07K-001/20 - Partition-, reverse-phase or hydrophobic interaction chromatography

C07K-001/22 - Affinity chromatography or related techniques based upon selective absorption processes

C07K-001/24 - by electrochemical means

C07K-001/26 - Electrophoresis

C07K-001/28 - Isoelectric focusing

C07K-001/30 - by precipitation

C07K-001/32 - as complexes

C07K-001/34 - by filtration, ultrafiltration or reverse osmosis

C07K-001/36 - by a combination of two or more processes of different types

C07K-014/00 - Peptides having more than 20 amino acids; Gastrins; Somatostatins; Melanotropins; Derivatives thereof

C07K-014/435 - from animals; from humans

C07K-014/76 - Albumins

C07K-014/765 - Serum albumin, e.g. HAS

C07K-002/00 - Peptides of undefined number of amino acids; Derivatives thereof

C07K-001/02 - in solution

C07K-001/04 - on carriers

C07K-001/06 - using protecting groups or activating agents

C07K-001/08 - using activating agents

C07K-001/10 - using coupling agents

C07K-001/113 - without change of the primary structure

2. Free-Text searching

All results for “bovine serum albumin” as a title term (206 records) were hand combed for relevance.

The full name of the protein was also searched as a topic term i.e. the term could appear anywhere in the record and combined with the following Derwent Class Codes to highlight recovery forms that were especially relevant to food uses. This approach provided 363 records that were also hand combed for relevance.

D - Food, Detergents, Water Treatment and Biotechnology

D1 - Food and Fermentation

D11 - Baking - including bakery products, flour, doughs, bakery ovens, dough transporting and/or handling equipment, pies and pasta, but not flour milling. (A21).

D12 - Butchering, meat treatment, processing poultry or fish. (A22).

D13 - Other foodstuffs and treatment - including preservation of food, milk, milk products, butter substitutes, edible oils and fats, non-alcoholic beverages, artificial sweeteners, food additives and animal feed. (A23B A23C A23D A23E A23F A23G A23H A23I A23J A23K A23L).

D14 - General foodstuffs machinery - excluding machines which can be classified in D11- 13

The term “fraction V” was also combined with BSA or bovine

Bovine and “albumen” was also combined to cover a common misspelling in foreign abstracts.

NB

Bovine serum albumin is particularly problematic for free text searching because of its wide use in a wide variety of applications such as, its use as a molecule for testing membranes or in production of test assays and kits.

Additional free text searching using more loose and generic forms of the source material and the targeted technologies may have yielded additional material, but this would have required searching though thousands of additional references that when sampled had very low or no relevance. Such additional searches may be necessary for individual technologies that did not seem to yield any or good results from using the seemingly relevant codes.

Appendix 2–Assumptions used in determining the cost of producing BSA from bovine plasma using ion exchange chromatography

# of animals killed per day	2,000
Blood per animal (L)	8
Plasma (%)	50
Total plasma (L/day)	8,000
Plasma dilution factor	3
BSA content of plasma (g/L)	30
IgG content of plasma (g/L)	21
Trf content of plasma (g/L)	4.5
Citrate addition rate (g/L blood)	5
BSA recovery in chromatography (%)	45
Protein content of F/T plasma (%)	1.9
Protein recovery in IgG fraction(%)	80
Steam for drying (kg/kg water evaporated)	2.25
Resin BSA binding capacity (g/L resin)	17
Elution CV	2
Re-cycle salt molarity (M)	2
BSA Ultrafiltration flux rates ($l/m^2/hr$)(70% of expected flux)	40
Ultrafiltration concentration factor	55
Diafiltration number	5
Diafiltration flux (l/m2/hr) (50% of expected)	30
Power for ultrafiltration (kWh/kL)	3.5
IgG Ultrafiltration flux rates ($l/m^2/hr$)(70% of expected flux)	21
Ultrafiltration concentration factor	14
Diafiltration number	4
Diafiltration flux (l/m2/hr) (50% of expected)	15
Cost:	
Citrate ($/kg)	1.3
Resin ($/L)	169
Membranes ($/$m^2$)	100
Water ($/kL)	1.2
Salt ($/kg)	0.36
Packing ($/kg)	0.5
CIP chemicals ($/batch)	50
Spray drying ($/kg water evaporated)	0.056
Power ($/KWh)	0.135
Steam ($/ton)	25
Opportunity costs of plasma ($/kg)	0.15

Labour ($/year)	35,000
# of workers per shift (BSA)	2
# of workers per shift (IgG)	1
Chromatography (hours)	20
BSA Ultrafiltration (hours)	17.8
BSA Diafiltration time (hours)	2.2
CIP (hours)	4
Chromatography cycle time (hours)	1
IgG fraction Ultrafiltration (hours)	14
IgG fraction Diafiltration time (hours)	6
Resin life (years)	2
Membrane life BSA (years)	2
Membrane life IgG fraction (years)	1
# of hours operated per shift	8
# of shifts/day	3
# of operating days per week	5
# of weeks per year	48

Plasma processed (L/day)	8,000
Diluted plasma (L/day)	24,000
Resin volume (L)	706
Expected BSA production (kg)	108
Expected IgG fraction production (kg)	365
Salt (kg/batch)	165

BSA

BSA UF feed volume (L/batch)	28,235
BSA eluate concentration (%)	0.38
BSA UF diafiltration volume (L/batch)	2,567
Ultrafiltration area (m^2)	39.7
Diafiltration area (m2)	38.9
BSA in UF concentrate (%)	21.04
Water to be evaporated (kg)	405

IgG fraction

IgG fraction UF feed volume (L/batch)	24,000
IgG fraction feed concentration (%)	1.9
IgG fraction UF diafiltration volume (L/batch)	6857
Ultrafiltration area (m^2)	82
Diafiltration area (m2)	76
IgG fraction in UF concentrate (%)	21.28
Water to be evaporated (kg)	1,714