A PRELIMINARY STUDY OF THE SOLUBILIZATION OF WOOL AND SEPARATION OF SOLUBLE WOOL INTO PROTEIN CLASSES

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Errata Adrian Jull, MSc thesis

p. 3 para 3, line, 3 **prin**cipally
p. 5 last line ---1992).. only need one full-stop
p. 10 para 4, line 1, **led** not lead
p. 11 Scheme 1.3 - oxidation line 3, **loss** not lose
p. 12 para 2 line 4 This large equilibrium is the --- > This large equilibrium **constant** is the ----
line 7 ----specificity to for disulfide--- > ----specificity **for** disulfide---
p. 13 Eqn 1.7 inverted A should be ⇋
p. 17 para 3, line 9 **trypsin**
p. 20 top line should read as an insert following on from p.19, " Glycerol was added to the protein *solution, a thin sheet was spread on polythene and heated at 80^{0}C and peeled off. Films achieved by Yamauchi et al. using this method were applied to mice subcutaneously, no scarring* or abnormalities were observed in the mice."
p. 33 para 2 line 7 silanate
p. 37 Figure 3.1.6 legend change "the Biuret reaction" to "reduction of Cu^{2+}"
p .43 Fig 3.3 1a A is the graph on the left
1b graphs labeled wrong way round
p. 65 last para, line 3 asparagine
p. 76 line 2 remove full stop
p. 83 line 4 remove superscript minus sign
p. 98 Tris is tris(hydroxymethyl)amino**m**ethane, not ----ethane
p. 103 graphs – see correction to Figure 5.3.4b below
p. 104 para 3 line 10 **kept away** not kept in away
p. 105 para 4 line 3 **until** not till
p. 106 para 2 line 3 **foam** not faom
p. 123 bottom **comparing** not caomparing

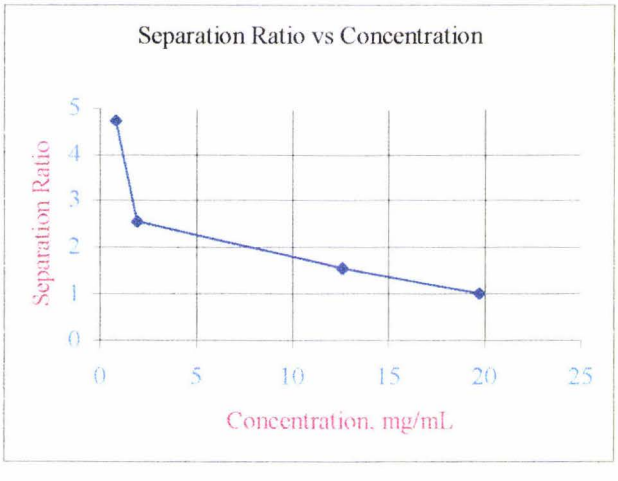

Figure 5.3.4b Degree of separation ratio achieved with concentration change
Dissoluted wool was foamed in a 29 cm x 35 mm ID column using nitrogen gas at a flow rate of 12-14.4 mL/min. A foam fraction was collected after a 10 mL decrease in the foamed bulk liquid volume, (200 mL) occurred. Dissoluted wool of different protein concentrations was titrated with 0.1M phosphoric acid to pH 8.2. Foamed samples were analysed by GPC Sephadex G-15.

ABSTRACT

The processing of natural products into new added value products is of importance to New Zealanders and the economy of their country. Chemical processing of wool into potential new products would be greatly assisted by separation of wool proteins into protein classes. A preliminary study of solubilizing wool protein by reduction and oxidative sulfitolysis of the cystine disulfide linkages was carried out. Oxidative sulfitolysis was used to obtain stable soluble wool protein for further investigation. Assays were developed for analysis of the effectiveness of chemical processing of the wool. To separate dissoluted wool proteins from dissolution reagents ultrafiltration was used to obtain desalted soluble wool protein. Data are reported on foam fractionation conditions of pH, concentration, sparging gas and desalting.

Wool was reduced and solubilized using sodium sulfide. The soluble product was processed under nitrogen and proved unstable in air. Thin films were produced from desalted reduced wool protein solutions by auto oxidation on exposure to air. Sulfonation of wool cystine to S-sulfokerateine proteins routinely achieved stable soluble product of 60 % yield. Films were prepared from the desalted soluble sulfonated wool protein. The most efficient solubilization was achieved by reduction with sulfide ion, with 70+ % yields from a low wool to liquor ratio of 1:10. The lack of stable soluble product hampered down stream processing and oxidative sulfitolysis was chosen for further investigation in this study.

Assays of wool protein proved problematic with interference of dissolution reagents. The bicinchoninic acid assay provided considerably elevated total protein values compared to the biuret and gel permeation chromatography methods, making it unsuitable for wool protein analysis. The biuret assay was found to have variable results. Dissolution of wool by > 50 % produced biuret results in close agreement to total protein values obtained from protein dry mass after dialysis. A method for total protein assay using gel permeation chromatography with UV detection was developed. Gel permeation resulted in separation of protein from the dissolution reagents and detection of the eluting protein peak at 200 nm. Total protein determination by gel permeation was reliable and consistent and was

able to be applied to all the protein solutions analyzed. Excellent correlation was achieved between gel permeation and protein dry mass analysis.

Separation to enable identification of protein classes in the processed material was attempted using capillary electrophoresis and capillary gel electrophoresis. Preliminary investigations established suitable capillary electrophoresis conditions for further study of wool protein. Gel permeation using Sephadex G-75 and Superdex 75 did not yield separation of protein to a standard comparable to that achieved by 1D SDS-PAGE.

Proteins are surface active and therefore represent natural self-foaming agents. Foam fractionation was examined with dissoluted and desalted wool protein. Optimum foam fractionation conditions were established at pH 7.8 - 8.2 and protein 1-3 mg/mL concentration. Protein was precipitated from desalted soluble wool protein using foam fractionation and represents a potential method for separation of wool proteins from solution.

Manipulation of desalted wool protein solutions to surface tension minima defined by pH, holds promise of separation of wool proteins into groups. These groups will share isoelectric points in common and possess similar hydrophobic interactions. Whilst these groups may cut across the established protein classes, products from these groups will possess distinct shared properties. Refinement of the foam fractionation technique utilizing the grouping of surface active wool proteins, holds scope for protein separation with further development.

ACKNOWLEDGEMENTS

First I would like to thank my supervisors Associate Professor David R. K. Harding and Dr. W. B. Bryson for their advice, enthusiastic encouragement and patience during the course of this work. I would also like to thank Rekha Parshot for helping me find my way around the laboratory, her never-ending patience and sunny nature. Dick Poll is thanked for his advice, assistance and interest in the operation of the Capillary Electrophoresis and HPLC instruments. Kate Palmano and Slavica Pavlinic are thanked for sharing their knowledge, encouragement and those essential talks over Monday coffee, they have been much appreciated. The Separation Science Group is thanked for the cooperative and friendly manner in which we worked around each other, and in particular Darren Engelbretson for his humour and sage advice.

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To Giovanna, 'G" my thanks, for helping me to get started again. Thanks are also expressed to the colleagues in the 'other' chemistry laboratory, who accepted my extravagances, and to all the academic staff of Chemistry and Biochemistry disciplines who were always ready to give advice and showed a real interest in my study.

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Finally I wish to thank my wife Liz, and my children Malcolm, Heather, Isobelle and Louise for their encouragement and tolerance of a husband and father who has been pre-occupied with chemistry. It is their wonderful support that has made this study possible, I am deeply indebted to them.

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ABBREVIATIONS AND TERMS

2-AE	2-aminoethanol
AA	amino acid
AcCN	acetonitrile
AU	absorbance units
BCA	bicinchoninic acid
BT	biuret
BSA	bovine serum albumin
BME	β-mercaptoethanol
BV	bee venom
CA	carbonic anhydrase
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
CHY	chromatography
DX	dextran
DTT	dithiothreitol
DW	dissolved wool/dissoluted wool
$\in$	absorptivity
EA	egg albumin
EG	ethylene glycol
μ_{eo}	electroosmotic flow
μ_{ep}	electrophoretic mobility
E_r	enrichment ratio of foamed samples
FE	flame emission
FF	foamed wool dissolution fraction
GPC	gel permeation chromatography
HOAc	acetic acid
HGTP	high glycine-tyrosine proteins
HPho	hydrophobic
HPhi	hydrophilic
HPLC	high performance liquid chromatography
HSP	high sulfur protein
IFP	intermediate filament protein
i/a	interactions
ID	internal diameter
$\in$	molar absorptivity
MW	molecular weight
MWCO	molecular weight cut-off
OSP	oxidative sulfitolysis proteins
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
P.	protein
R_p	percentage recovery of foamed samples
RP	reduced protein
SDS	sodium dodecyl sulfate
sparging	bubbling of a gas through porous sintered glass, ceramic or stainless steel
S_r	separation ratio of foamed samples
-S-S-	disulfide linkage

TDMU	thiosemicarbazide-diacetylmonoxime-urea complex
TGA	thioglycollic acid
TGi	thioglycollate ion
Tris	tris(hydroymethyl-aminoethane)
TTN	tetrathionate
UHSP	ultra-high sulphur protein
UV	ultraviolet
UV/Vis	ultraviolet-visible
W	wool
$W\text{-}S^-$	anion of cysteine sulfhydryl in wool
$W\text{-}SSO_3^-$	S-sulphokerateine
W-SS-W	intact wool fibre

CHAPTER ONE

Introduction

New Zealand has a highly efficient wool fibre production from its agrarian sector. The annual production for the last five years has averaged 203,000 tonnes of wool. The total value of wool exports for New Zealand was $1,207.3M in 1999 with an average of 443 cents/kg for cleaned wool (NZ Yearbook, 1998). A small proportion, less than 10% of the wool production is processed in New Zealand. New Zealand wool is predominantly crossbred medium-coarse 28-46 micron wool. These fibres make good hard wearing carpet wool, but the fibre value is low and the economic return poor. Man-made fibres have made a steady inroad into the textile trade and seen the decline of wool to less than 5% of the world market. There is a need to develop new products and new markets for wool products. Processing of wool opens up opportunities for the development of completely new products as well as enhancement of traditional products. Traditional markets are well supplied with wool and new products that can compete on new markets are required.

This study is a preliminary investigation of soluble wool chemistries and of the possible routes for separation of wool proteins in solution. Solubilization is the first step followed by separation of the protein components. Solubilization of wool using sodium sulfide produces reduced soluble wool that is unstable when exposed to air and is difficult to handle in larger than gram quantities. Sulfonated wool protein is stable when exposed to air, however the yields have been lower than that obtained from reduced wool protein and involve larger liquid volumes and more expensive reagents. Retaining the wool protein in a soluble form involves examining known and new methods of separation. Previous separation has focussed on precipitating the wool protein once extracted from the wool fibre. Precipitation can lead to a material that is difficult to redissolve, hence separation of wool proteins in the soluble form is a desired outcome of this study. An additional objective was to provide an insight into development of processes suitable for industrial scale-up.

1.1 Characteristics of Wool

Wool is dead tissue of epithelial origin. Wool fibre exhibits a cellular structure comprising a central cortex region surrounded by a sheath of flattened cuticle cells as shown in Figure 1.1 (Parry and Steinert, 1995: Dowling and Sparrow, 1991).

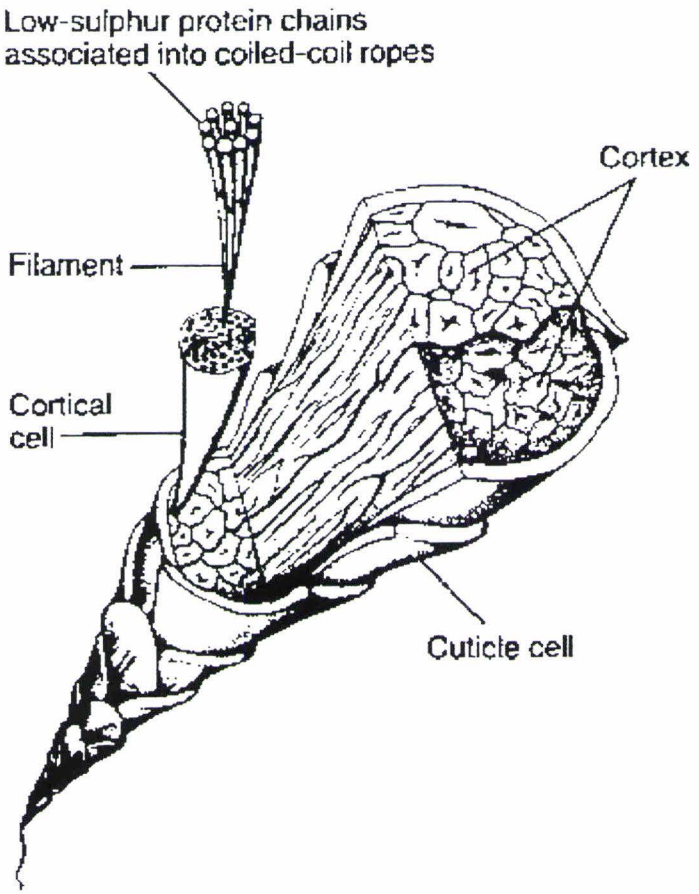
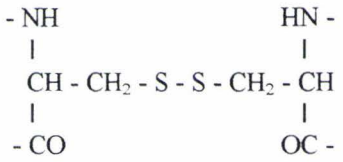


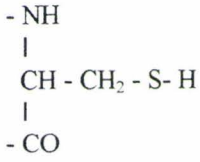
Figure 1.1 Schematic representation of a wool fibre

From Dowling and Sparrow (1991).

The cortex is composed of spindle-shaped cells aligned along the fibre, which enclose filaments of coiled-coils of protein chains. It is estimated that there are over 100 wool proteins in a fibre. The protein chains are highly crosslinked by the disulfide bonds of cystine conferring insolubility in water. Alkaline hydrolysis gives solubility 65-80 % compared to hydrolysis by HCl 6M, 60°C, 2.5 h resulting in 50 % solubility. Wool has a high cysteine content, the most common amino acid in wool at 11 mol %. Most cysteine in wool exists as the disulfide bonded cystine, forming intra-molecular and inter-molecular disulfide crosslinks giving strength, stability and relative insolubility characteristics to wool (Parry and Steinert, 1995).



Cystine



Cysteine

The composition of wool varies with diet, time of year, breed of sheep and the part of the sheep where the wool is produced. Wool protein can be divided into four main classes of protein based on the percentage of key amino acids, Table 1.1.

Protein class	Mass %	Sulfur content %	Molecular mass, kD
Low sulfur	58	1.5 - 2	45 - 60
High sulfur	18	4 - 6	11 -28
Ultra high sulfur	8	8	28 - 37
High Gly-Tyr	6	0.5 - 2	9 - 13

Table 1.1 Wool protein classes, amounts, sulfur contents and molecular masses

Approximate amounts of wool classes, sulfur content and range of molecular masses found in Merino wool. From Maclaren and Milligan, 1981.

The pI of wool proteins is spread across the range 4-7.5, with all wool classes showing a range of pI. The low sulfur wool proteins have some α-helix character, this is not apparent in the other wool classes. A high proportion of the sulfur residues are reported to lie in the terminal regions of the protein molecules (Parry and Steinert, 1995, Yamauchi et al. 1996). Aligned proteins enable cystine linkage to give added strength to the wool fibres.

Within the wool fibre the low sulfur, intermediate filament proteins (IFP) are found predominantly in the microfibrils, whereas the high sulfur proteins (HSP) are found pricipally in the matrix along with the high glycine-tyrosine proteins (HGTP). Washed wool is 85-90 % α-keratin protein. Extraction of soluble wool protein usually occurs from all parts of the fibre structure, resulting in a mixture of all four protein classes.

1.2 Analysis

Determination of the changes occurring during the solubilization process and separation methods is essential. Wool has specific characteristics that limit the range of possible analyses that can be applied to it when determining the total protein and changes in salt/reagent content. Untreated wool is insoluble in water, remaining soluble only in solutions above pH 8 in the absence of chaotropic agents, the presence of the chaotropic agents interfering in subsequent wool analysis. Investigation of possible assay methods was an important precursor to further study of separation methods applied to soluble wool protein.

1.2.1 Analysis by Capillary Electrophoresis

Capillary electrophoresis is a highly efficient and sensitive analytical technique that is able to separate positive, neutral and negative species. Extremely small, μL quantities are required. Separation of species in CE is based on differences in electrophoretic mobilities μ_{ep} , that is the different velocities of the migrating species. The μ_{ep} relates to the solute size and charge of the protein at a given pH. Proteins will change in charge with changes in the pH. If the pI of the proteins differ then separation is possible, Equation 1.1.

$$\mu_{ep} = \frac{q}{6\pi\eta r} \qquad \text{Eqn 1.1}$$

q = sum of charge on protein surface, η = viscosity of the running buffer, r = radius of molecule

If the surface charge on protein changes with pH then the value of μ_{ep} will change and separation can be accomplished. A high potential difference, (12-20 kV), creates an electroosmotic flow μ_{eo} such that all species, positive, neutral and negative are carried through the capillary tube in one direction.

Two processes can act against separation of different proteins, a lack of difference in the charge to size ratio of the proteins and interaction of the proteins with the walls of the capillary tube.

A number of studies have reported good separations of proteins using CE (Chen, 1991, Tehrani et al., 1991, Maa et al., 1991, Baker, 1995, Bushey and Jorgenson, 1989, Lauer and McManigill, 1986, Dolnik, 1995). This encouraged study using CE with fused silica, anticipating that this would provide a useful technique for quick analysis of proteins and for delivery of protein class information.

1.2.2 Analysis by Capillary Gel Electrophoresis

Wool proteins can be separated into their protein classes by molecular mass using 1-D SDS-PAGE slab gel plates (Maclaren and Milligan, 1981). This can be time consuming and provide results that do not always lead to a clear interpretation. CGE is the same sieving process as slab gels, performed in a capillary tube. CGE can achieve very high resolution separation in < 30 min for a 100 cm capillary with theoretical plates as high as 30 million (Li, 1992). Where solutes are similar in their charge to size ratio, CGE separates these effectively on the basis of size, a situation believed to exist for wool proteins.

To perform CGE the capillary is filled with a gel, polyacrylamide (PAG) or linear polyacrylamide gel (LPAG) being the favoured choices. Dextran, polyethylene glycol (PEG), polyethyleneoxide (PEO) and agarose have also being used as gels. The gels have pores that act as sieves retarding higher MW protein molecules more than lower MW protein molecules. A graph of increasing molecular size with time is produced that can be related to known MW markers. Treatment of the capillary wall is necessary to dramatically diminish electroosmostic flow and prevent extrusion of the gel. Zhu et al. (1989) reported that the technique of using gel filled capillaries is inconvenient and does not yield reproducible results.

LPAG was cited in literature as providing improved separation of protein by lowering wall-protein interaction and a cross-linked gel for separation by molecular size (Hjerten, 1985; Manabe et al. 1998; Wu and Regnier, 1992; Cohen and Karger, 1987). Size separation occurs because of the movement of the solute through the 'dynamic pores' of the linear strands of LPAG (Baker, 1995). However the LPAG exhibits absorption in the 200-220 nm wavelength, effectively eliminating it as an option for CGE of wool protein when UV of 200-220 nm is used for detection (Ganzler et al., 1992).. In addition the literature favours

the use of lower pH values to protect the LPAG from degradation effects. Again lower pH is undesirable when dealing with wool proteins creating a problem if LPAG is to be used for soluble wool protein separation.

Solutions of linear, UV transparent polymers offer an alternative to gels bonded to the capillary wall. PEG, dextran and agarose are polymer networks that can easily be introduced with the buffer and provide more reproducible results than those obtained using gel filled capillaries as in Figure 1.2.2 (Zhu et al. 1989, 1990; Iki and Yeung, 1996; Takagi and Karim, 1995; Guttman et al. 1993). The sieving effect of the polymer network is the result of polymer-polymer and polymer-solvent interactions (Guttman et al. 1993).

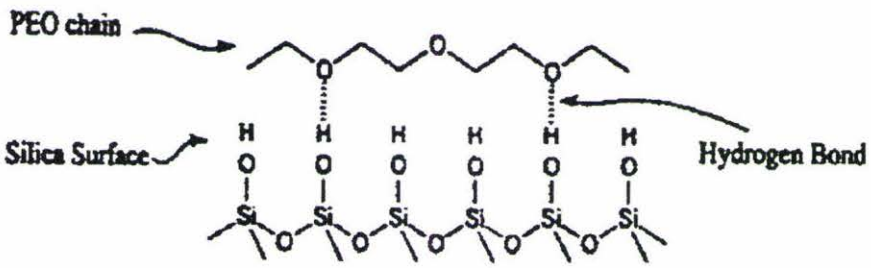


Figure 1.2.2 Interaction of PEO with silanol on capillary walls

From Iki et al. (1995)

Adsorption of PEO to silica was postulated by Iki and Yeung (1996), as preventing dissociation of silanol groups when using higher pH. The coating of the silica decreases the μ_{eo}, producing improved separation. It was hypothesized that the PEG would coat the capillary and provide a sieving medium for the protein. Dextran can be used as a replaceable gel for CGE (Takagi and Karim, 1995; Ganzler et al. 1992). Iki and Yeung (1996) reported good separations using polyethylene oxide as a capillary coating. The application of CE and CGE to wool proteins was to ascertain their suitability as quick analysis systems that would give information on solubilizations and separations of wool protein.

1.2.3 Urea Assay

Wool was solubilized with the aid of large amounts of urea (8M). The distribution of the urea in separation techniques was of interest in this study. Assays have been developed for measuring urea produced as a result of enzyme activity (Mellerup, 1967). The urease assay is very specific to urea, however measurement is of the ammonia produced rather than direct measurement of urea. The thiosemicarbazide-diacetylmonoxime (TDM) assay is a direct measurement of urea. It was designed for analysis of micromol amounts of urea by formation of a coloured complex. Arginase action on arginine produces quantitative amounts of urea (Mellerup, 1967; Geyer and Dabich, 1971). The Geyer and Dabich method was used in a form modified by Patchett (1988), for the assay of arginase activity on arginine.

1.2.4 Salt Content Analysis

The concentration of sodium ions provides information about changes in the ionic content of the solutions being studied. Conductivity of solutions provides information regarding the total ion content of the soluble protein solutions, however more information relating to the concentration and partition of ions in any process undertaken would be helpful. The Corning Flame Photometer is able to detect potassium and sodium ions using a low temperature flame. The sodium ion was the only alkali ion present in reagents used to achieve soluble wool protein and can be measured to 1.0 ppm with confidence. The sodium ion concentration could provide information about specific ion changes that were occurring during separation techniques.

1.2.5 Total Protein Analysis

Numerous methods exist for the estimation of protein concentration by chemical assay. The choice of the particular assay is mainly dependant on the following criteria: the amount of protein available, the presence of chemicals that may interfere, ease and reliability of the assay, the compatibility of the assay method with the characteristics of the protein.

The BCA is a sensitive, stable and quick assay. The BCA is an assay for total protein in the 0.1-100 μg protein range. It is widely used and has less interference than the Lowry assay (Sapan et al. 1999). Protein reduction of alkaline Cu^{2+} to Cu^{+} is dependent on the concentration of protein. The peptide bond reduces the Cu^{2+}-complex intermediate to the purple $Cu(BCA)_2^{3-}$ complex (Braun et al. 1989). Cu^{2+} is reduced by a number of reactants found in protein solutions, particularly tyrosine, tryptophan, cystine and cysteine. Reaction with the side chains of the four reactive amino acids is not temperature dependent and increased temperature development of the BCA ensures that the response being measured is predominantly the peptide bond reaction rather than the above amino acids reducing copper (Weichelman et al. 1988).

The biuret assay is not as sensitive as the BCA, but is more robust, showing fewer mis-readings from chemical interference. The simple assay technique and the suitable range of BT assay made it an assay worth investigating for total wool protein. The BT assay has lower sensitivity 20-100 μg/mL compared to the BCA, Lowry and Comassie-Blue assays of 100 - 1 ug/mL (Jenzano et al. 1986; Sapan et al. 1999; Holme and Peck, 1993). Wool protein solutions were in the range of 0.1-20 mg/mL. Gornall et al. (1948) recommended an amendment using tartrate stabilised reagent to get a reliable assay. The assay is non-specific and applies to all proteins, with little difference being shown between proteins (Holme and Peck, 1993; Sapan et al. 1999; Harris and Angal, 1989).

1.2.6 Gel Permeation Chromatography

To assay the total protein in solution, the concentration changes in reagent chemicals and the changes in concentration of salts in the processes investigated presented specific problems. Keratin is particularly insoluble compared to most other proteins studied. Most assays have been devised around the characteristics of proteins such as bovine serum albumin carbonic anhydrase as essentially soluble proteins present in small quantities. In assessing the changes in the wool protein solutions specific problems must be overcome. Most of the wool protein solutions were in reducing environments, the solutions were frequently pH > 8 and chemicals that show UV interference were often present. In addition many samples needed to be assayed and this precludes some methods that are time and labour consuming.

Short column GPC provides separation of large molecular weight molecules (keratin proteins and peptides MW > 5,000D) from smaller, reagent molecules (MW < 500). Pharmacia lists Sephadex G-15 as having a MWCO > 1500D, and it is often used as desalting medium. In addition a G-15 column coupled with UV detection can be used to determine the total protein by separation of protein from the reaction mix (Hayakawa, 1997; Bollag et al. 1996). The molecular weight range for wool protein has been reported as 9,000 to 60,000D (Maclaren and Milligan, 1981). Any peptides and solubilizing reagents which have MW < 1,500D will elute after the protein peak.

Wool solubilization reagents and products, sulfite, tetrathionate, thiosulfate and Tris have been reported as interfering with the BCA, Lowry and BT assays (Harris and Angal, 1989; Sapan et al. 1999; Bollag et al. 1996). Soluble wool protein has a very low absorbance at 280 nm, lacking sufficient tryptophan (35-44 micromoles per gram) and tyrosine (340-390 micromoles per gram) for significant UV absorbance at this wavelength (Maclaren and Milligan, 1981). In addition, some solubilizing reagents absorb in the 214 nm range, making direct solution measurement impractical. The absorptivity for wool protein was measured at $\epsilon_{\lambda 214}$ 34,300 $cm^2.g^{-1}$ and solubilizing reagents $\epsilon_{\lambda 214}$ 10,400 $cm^2.g^{-1}$. The absorptivity of wool is largely due to the amide bond and is particularly strong in the 190-220 nm range, allowing for increased sensitivity of measurement.

1.3 Solubilization of Wool Protein

1.3.1 Extraction of SolubleWool Protein

Disulfide bond cleavage can be accomplished by reduction or oxidation of the cystine. Reduced cystine, forms thiol anions, WS^- (Eqn 1.2) or undergoes disulfide reduction by sulfite to form S-sulfone, $WSSO_3^-$ (Eqn 1.3).

$$WSSW + 2RS^- \rightleftharpoons RSSR + 2WS^- \qquad \text{Eqn 1.2}$$

$$WSSW + 2SO_3^{2-} + \rightleftharpoons 2WSSO_3^- \qquad \text{Eqn 1.3}$$

W = Wool

Enzymatic dissolution was not investigated in this study, although the action of papain on wool was studied by (Lennox, 1952; Naval and Nickerson, 1958).

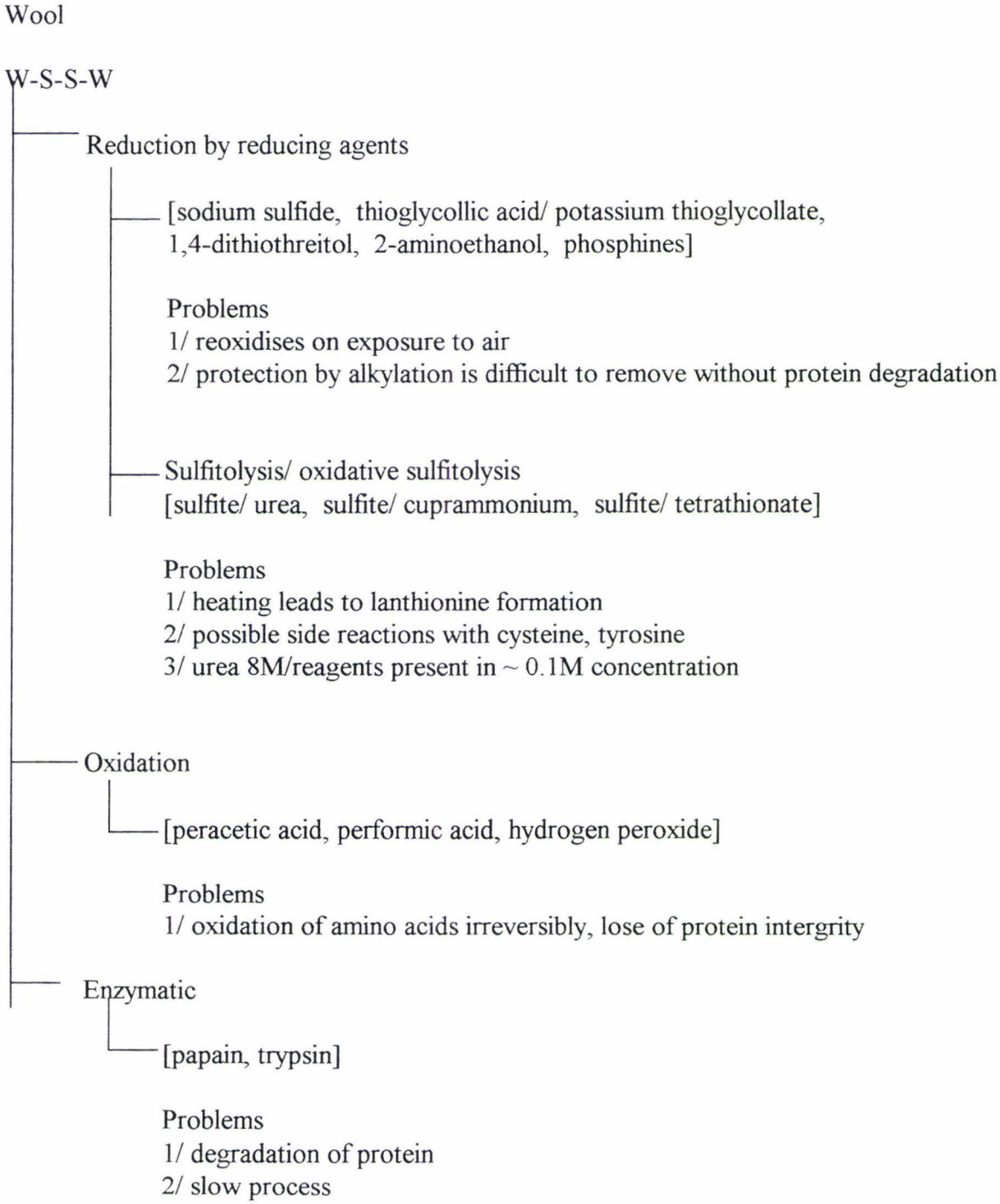
Wool protein was extracted using reducing conditions of alkaline glycollate from native wool (Goddard and Michaelis, 1935). Goddard and Michaelis reported finding two fractions in reduced wool protein that differed in solubility and sulfur content. Extraction of wool requires breaking of disulfide bonds between wool proteins. This was confirmation of the high and low sulfur proteins present in wool. It is now clearly established that there are four classes of proteins in wool.

The Goddard and Michaelis study lead to considerable investigation of solubilization by both reduction using potassium thioglycollate and sulfitolysis using copper ammonia/sulfite solution (Harrap and Gillespie, 1963; Swan, 1961; Koltoff and Stricks, 1951).

The Goddard and Michaelis study lead to considerable investigation of solubilization by both reduction using potassium thioglycollate and sulfitolysis using copper ammonia/sulfite solution (Harrap and Gillespie, 1963; Swan, 1961; Koltoff and Stricks, 1951). These studies showed the versatile routes that were available to achieve soluble wool protein.

Swan (1957,1960) demonstrated that copper ammonia/sulfite reagent was very effective at extracting wool protein achieving an 85+% yield. The difficulties from an industrial point of view with this method are the processing demands of a large wool:liquor ratio of 1:100 and the problems associated with removing copper from the protein product. The reduction pathway has the problem of being highly sensitive to re-oxidation of the cysteine groups (Yamauchi et al. 1996).

Maclaren and Milligan (1981) presented the range of methods in depth, summarized in Scheme 1.3.



Scheme 1.3 Methods of solubilizing wool protein

It has been generally accepted that all wool proteins are soluble and that any observed variation is related to the steric, hydrophobicity and charge effects of neighbouring groups rather than due to any morphological differences (Maclaren and Milligan, 1981). Reduced wool protein is readily oxidized in air reforming disulfide bonds, both as intra- and inter-protein linkages (Maclaren and Milligan, 1989). Thiol groups of reduced wool protein undergo rapid oxidation unless protected by inert atmosphere or excess reducing agent

(Thomas et al. 1983; Kelly 1998). Yields obtained for dissolution vary according to conditions used with alkaline reducing conditions reported as producing yields as high as 87% (Thomas et al., 1983; Maclaren and Milligan, 1981; Wormald, 1948). Most cited solubilizations of wool protein were carried out at alkaline pH, that inevitably leads to degradation of the protein, however some extraction methods i.e. using β-mercaptoethanol can be carried out in neutral pH (Harrap and Gillespie, 1963; Maclaren and Milligan, 1981; Yamauchi, 1996). The shorter time 3 hours needed using reduction is an advantage to the oxidative sulfitolysis requiring 24 hours in limiting alkali damage, however the pH > 10.5 required for reduction will have negative consequences compared to the milder pH > 9 used for sulfonation. Alkaline conditions degrade the protein with hydrolysis of the peptide bonds and conversion of cystine to lanthionine. The protein degradation is more severe with strong alkali, making treatments with pH >11 undesirable. The milder conditions used in oxidative sulfitolysis cause less protein degradation (Maclaren and Milligan, 1981).

DTT used in close to molar equivalence of reagents to wool achieves a high yield solubilization. Cleland (1964) showed that the equilibrium constant for the production of the oxidized form of DTT in the reduction of cystine strongly favours the products, K = 10^4. This large equilibrium is the result of formation of a stable 6-membered ring structure (4,5-dihydroxy-1,2-dithiane). Using a 1:4 ratio of wool to DTT was reported as achieving nearly complete reduction of wool in less than 10 h (Weigmann and Rebenfeld, 1966). The reagent DTT shows specificity to for disulfide bonds of proteins. The use of selenol selenocysteamine as a catalyst in the DTT reaction has been suggested by Singh and Kats (1995) with an ~ 10^2 improvement in the measured rate of reaction.

Oxidative sulfitolysis using sodium tetrathionate (TTN) achieved solubilization albeit at lower yields 56% and more importantly the S-sulfonated cystine group was stable when exposed to air (Thomas et al. 1983).

The oxidative sulfitolysis reaction of ammoniacal copper (II) and sulfite with cysteine was first investigated by (Kolthoff and Stricks, 1951), with the formation of the S-sulfokerateine species. S-sulfokerateine is stable at pH > 7, thus the concentration of WSSW declines from the oxidative action of the copper (II) (Eqn 1.4, 1.5). The overall reaction represented by Eqn 1.6.

$$WSSW + SO_3^{2-} \leftrightharpoons WS^- + WSSO_3^- \quad \text{Eqn 1.4}$$

$$2WS^- + 2Cu^{2+} \leftrightharpoons WSSW + 2Cu^+ \quad \text{Eqn 1.5}$$

$$WS^- + 2Cu^{2+} + SO_3^{2-} \leftrightharpoons WSSO_3^- + 2Cu^+ \quad \text{Eqn 1.6}$$

The sulfitolysis reaction has an equilibrium constant near to unity, thus necessitating a large excess of reagent SO_3^{2-} to get a high proportion of product formed. Oxidative sulfitolysis by TTN in contrast uses the conversion of the thiol anion created in Eqn. 1.4 back to a disulfide that can undergo further reaction, Eqn 1.5.

$$WSSW + 2SO_3^{2-} + S_4O_6^{2-} \forall 2S_2O_3^{2-} + 2WS\text{-}SO_3^- \quad \text{Eqn 1.7}$$

The action of TTN is quite specific, acting only on cystine and cysteine in wool, TTN being a mild oxidant E^o $S_4O_6^{2-}/S_2O_3^{2-}$ = -0.09 V. Some studies have found evidence of protein degradation using TTN, however the production of lanthionine during oxidative sulfitolysis appears to be the result of later hydrolysis reactions as part of the amino acid analysis (Maclaren and Milligan, 1981).

1.3.2. Denaturing Reagent Reaction with Wool

Urea added to reductions using potassium thioglycollate was reported by Harrap and Gillespie (1963) as increasing yield to 85%. Urea added to both reducing and sulfonating environments results in increased yields. Urea breaks the hydrogen and van der Vaals bonding forces between protein molecules assisting the penetration of reagents in solution. Urea decomposes slowly to form cyanate ions. Cyanate can cause degradation of protein, care must be taken in leaving urea in contact with protein (Maclaren and Milligan, 1989; Cole, 1967; Pace 1986; Means and Feeney, 1971).

1.3.3 Physical Disruption of Wool

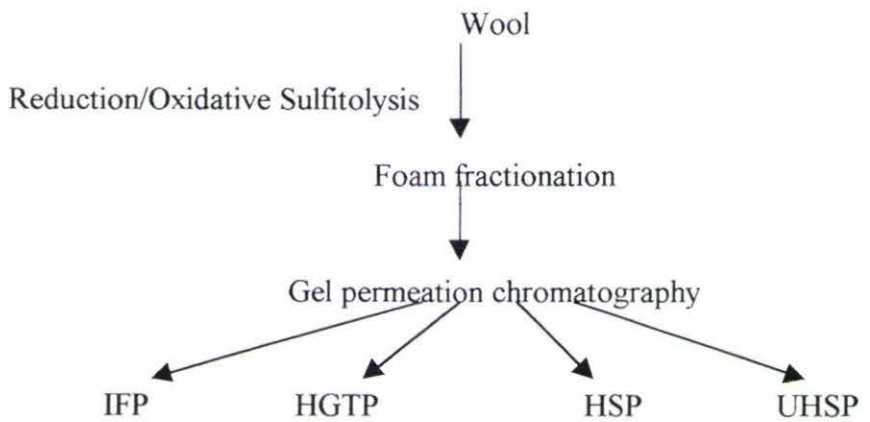
Wool fibres can absorb water up to 30% of total mass, but are insoluble in water. To extract all the protein the internal fibrils of the wool fibre must be penetrated by the dissoluting solution and the soluble protein released. Use of maceration to shatter cells assists the release of protein. Freezing cells to -21 ^{0}C was also found to assist the extraction of protein from loose cells (Albade et al. 1998). Any action that opens the fibre to allow easier passage of reagents to the cystine links will speed up reaction. Urea in high concentrations (8M) has long been recognised as a denaturant of protein, however removal of urea presents problems downstream. In an extraction of protein material from plant cell matter, Abalde et al. (1998) experimented with protein extraction by comparing three methods, sonication at 4°C, freezing at -21°C and thawing at 4°C, freezing in liquid nitrogen and thawing at 4°C. The best extraction was achieved from the freezing at -21°C and thawing at 4°C.

1.4 Separation of Wool Proteins

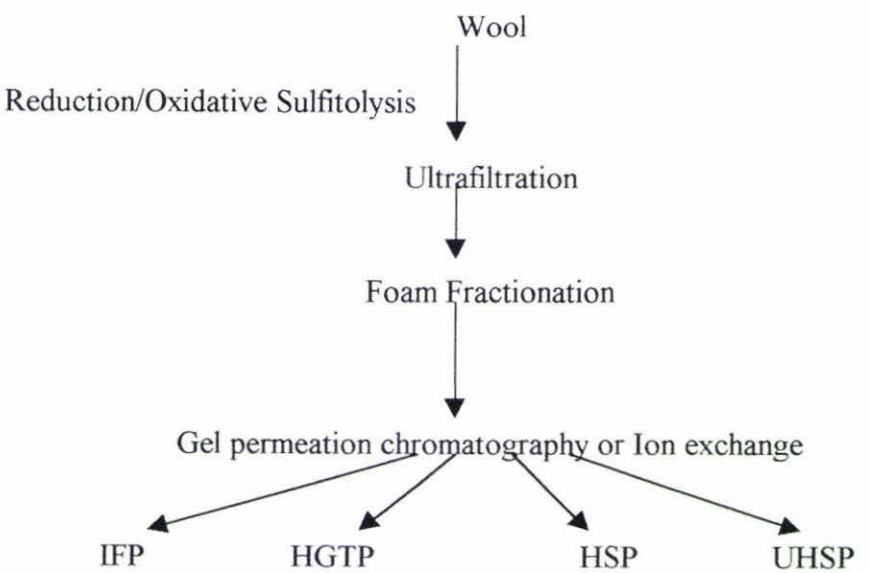
The objective of a separation strategy is to obtain the maximum yield of protein with maximum purity, cost-effectively in a minimum of steps (Harris and Angal, 1989). Each protein has unique properties that can be exploited in separation techniques. The major properties of proteins to exploit in separation are the charge, biospecificity, surface hydrophobicity, pI and molecular mass (Harris and Angal, 1989). In particular proteins are sensitive to pH, temperature, denaturants and ions in solution, these parameters need to be controlled in separation techniques (Grandison and Lewis, 1996).

Wool proteins have been separated on the basis of molecular size, pI and amino acid composition (Gillespie, 1964). The specific properties of the different wool protein classes will confer specific characteristics to that class of proteins that can be used to develop new products i.e. the low sulfur class compared to the high sulfur class. A problem arises with the overlap of characteristics exhibited by the wool protein classes that make it difficult to obtain wool protein of only one class.

Separation schemes are usually designed around principles of, the most general steps first, each step exploits a different property and the product from one technique can be applied to the next step without further manipulation (Asenjo, 1990). Wool protein classes have been separated by precipitation after solubilizing. The disadvantage of this method is that it presents the next step with a solid that must then be redissolved. Two schemes were contemplated as separation strategies, Scheme 1.4a and 1.4b. Each scheme used different properties of wool proteins in each step.



Scheme 1.4a Wool Separation



Scheme 1.4b Separation

The intention was to achieve soluble wool protein of one class that could be used for production of films. Foam fractionation was mentioned in literature as a promising technique with potential industrial value, which was of particular relevance to wool. Foam fractionation was an integral part of the investigation undertaken in this study.

Aims of this study were:

- ❖ to explore the possibility of retaining the wool in a soluble form at each step
- ❖ separate the wool protein from the ionic and urea components with the wool protein in solution
- ❖ separate the wool protein on the basis of wool protein classes

1.4.1 Protein Separation by Foaming - Introduction

Foam concentration is a separation technique in which surface-active proteins are concentrated from dilute solutions by preferential adsorption at gas-liquid interfaces created by sparging an inert gas e.g. nitrogen, through the protein solution (Uraizee and Narsimhan, 1996; Brown et al. 1990). The concentration of protein in foam is the combined effect of interfacial adsorption of the protein and drainage of protein carrying foam (Bhattacharjee et al. 1997).

When bubbles are introduced to a protein solution, protein collects at the gas-liquid interface as a result of the difference in the chemical potential of protein at the interface compared to the chemical potential in the liquid. This difference drives the reaction (Eqn. 1.8) until equilibrium is reached, when the chemical potentials will be equal.

$$\text{Protein}_{(aq)} \leftrightharpoons \text{Protein}_{(g/l)} \qquad \text{Eqn. 1.8}$$

Protein exhibits surface chemical activity and readily accumulates at the interface, thus from the equation it can be deduced that surface tension decreases when protein concentrates at the interface. Proteins exhibit different surface activity as a result of their varied amino acid composition.

The Gibbs energy of the two phases is different by an amount termed the *surface Gibbs energy* which leads to the Gibbs surface-tension equation, Eqn. 1.9 (Atkins, 1994).

$$d\gamma = - RT \sum \Gamma_i \, d \ln a_i \qquad \text{Eqn. 1.9}$$

a_i chemical activity of component *i* at the interface.

Γ_i surface excess of component *i* at the gas-liquid interface

γ surface tension

The surface activity is a result of the particular mix of hydrophobic and hydrophilic functional groups in the protein. The diversity of protein structures and the differences in hydrophobic and hydrophilic domains on the surface of protein makes protein gas-liquid interface behaviour difficult to predict (Hunter et al. 1991). Changes in surface tension are a guide to favourable parameters by which protein may be selectively concentrated.

An amino acid sequence for a high sulfur protein indicated 41 out of 151 residues could be classed as hydrophobic (Maclaren and Milligan, 1981). Most of the hydrophobic residues are found in one half of the sequence, supporting the concept of a hydrophobic part and a hydrophilic segment to the protein. Sequenced wool intermediate filament proteins of the same class show a high degree of homogeneity and can be expected to share similar physico-chemical characteristics (Parry and Steinert, 1995). Liu et al. (1998) noted that the structure of a protein becomes more denatured and hydrophobic when the pH of the solution is close to the pI of the protein. However Liu et al. also found that the optimal pH values for separation of trysin (pH 3.0) and catalase (pH 4.0), are both well away from their respective pI values of 10.7 and 7.0. Determination of the optimum pH for separation by foam fractionation on an individual protein basis is beyond the scope of this investigation.

Lemlich (1972) noted that because of the difficulty of measuring small changes in surface tension γ, and uncertainties in identifying species and evaluating their activity coefficients a_i it has limited use as a quantitative tool in practical situations. An increase in protein concentration leads to an increase in the surface excess, until the CMC is reached, as illustrated by the Langmuir isotherm, Figure 1.4.1 (Lemlich, 1972).

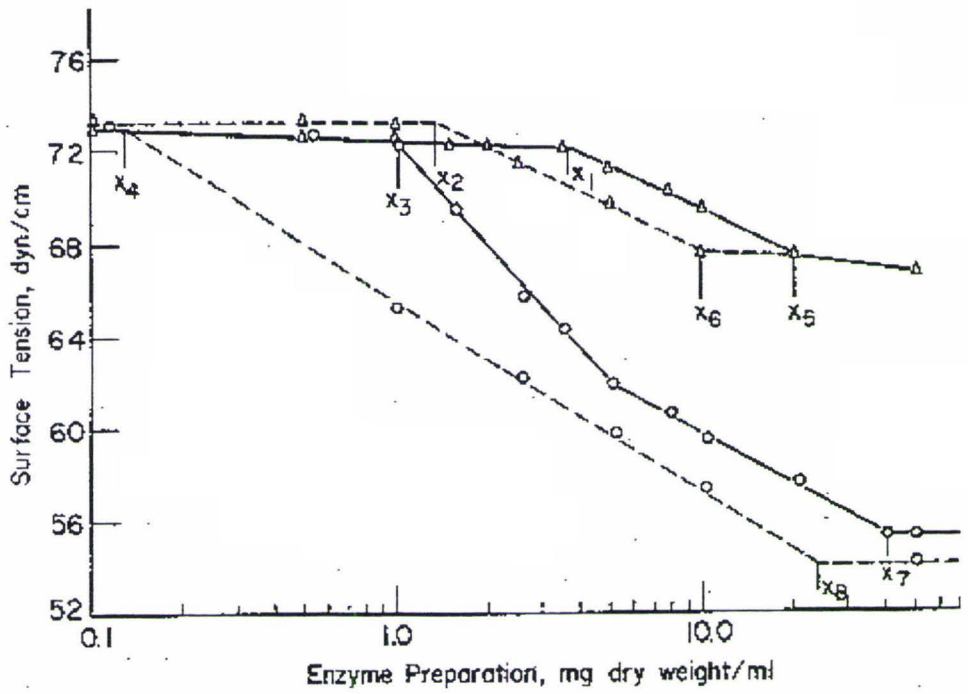


Figure 1.4.1 Surface tension-concentration for catalase and amylase.

Surface tension diagram for catalase and amylase. Amylase in water (△—△); amylase in 10 % $(NH_4)_2SO_4$ (△---△); catalase in water (O—O); catalase in 10 % $(NH_4)_2SO_4$ (O----O). From Lemlich (1972).

Foam fractionation is best performed at concentrations below the CMC, where enrichment is greater. Differences of surface tension indicate points at which fractionation of a protein mixture may be possible. A decrease in the ionic content results in an increase in surface tension and in bubble size. Bubble size is proportional to square root of the surface tension (Liu et al. 1997). Smaller bubbles are more stable, but enrichment is best achieved where bubbles increase in size and thus maximize the drainage of interstitial spaces.

It would be useful to identify the minimum surface tension and hence the maximum accumulation. Liu et al. (1997) found that a minimum surface tension could be established for different proteins by changing the pH. Many, but not all proteins exhibit a minimum surface tension at the pI of the protein. At high pH away from the pI, the surface tension approaches that of water because of the weaker electrostatic attractions. It is likely that for some proteins the hydrophobicity has an important role in accumulation at the surface that overrides the loss of electrostatic attractions. Wool dissolution mixtures being a mixture of reagents and over 100 wool proteins would not be expected to show any clearly defined surface tension pH changes. Any changes of surface tension would however indicate accumulation of proteins of similar surface activity and for example hydrophobicity. It is

surface tension pH changes. Any changes of surface tension would however indicate accumulation of proteins of similar surface activity and for example hydrophobicity. It is assumed that such proteins would share common sequences of amino acids, i.e. be same-class proteins.

Desalting a wool protein solution does not immediately lead to precipitation if the pH is above the pI of all wool proteins. Wool in the soluble denatured form will exist in part as long unwound strands of protein which are exposed for much of their length. Proteins as amino acid polymers have titratable functional groups varying in pK. It is the collective action of these groups that define the pI of the protein (Janson and Ryden, 1996). It is important to recognise the pI as that pH where the sum of the negative and positive charges on the protein is equal. The solubility of proteins in aqueous buffers depends upon the properties of exposed surface groups and the environment in which they reside. No literature cited examined soluble proteins in conjunction with the associated species present in methods used to solubilize wool. This study aims to investigate the foam fractionation of soluble wool protein in the presence and absence of the solubilizing agents as a technique for separation of wool proteins.

1.5 Production of Films and Reconstituted Fibre

Extracted wool protein converted into reconstituted fibre was reported by (Wormall, 1948). Wool protein was reduced with sodium sulfide 25% at 25^{0}C followed by HCl precipitation. The wet washed curd of precipitated wool protein was dissolved in cupra-ammonium solution and extruded to form a reconstituted fibre, however attempts to repeat these experiments recently failed (Kelly, 1998). In an extension of this experiment, Wormall (1948) blended wool protein with casein, producing fibres, but these were deemed to be somewhat inferior to synthetic fibres available commercially.

Reduced wool protein solution exposed to air in a thin liquid layer will readily re-oxidise to form cysteine bonds and 'plastic film'. Yamauchi et al. (1996) reduced wool with a combination of urea, β-mercaptoethanol and sodium dodecyl sulfate (SDS). The resulting solution was dialysed to remove the urea. The SDS was not completely removed, with 5 - 17% remaining associated with the protein in solution. Glycerol was added to the protein

or abnormalities were observed in the mice. The breakdown of the film was slower in mice than by using trypsin. Further work by Kelly (1998) using reduced wool proteins suggests that this is a fertile area for investigation.

The study reported in this thesis has investigated the solubilization of wool protein and the subsequent separation by foam fractionation. Solubilization was performed by reduction of disulfide links to form a thiol and by oxidative sulfitolysis to form S-sulfokerateine. Soluble wool protein was foam fractionated using parameters of pH, protein concentration and ionic/urea content. The wool protein solutions were analysed for urea, ionic species and total protein. A method for assay of total protein using gel permeation chromatography was developed.

CHAPTER TWO

Materials and Methods

2.1 Reagents and Equipment

Scoured, unbleached wool (Romney-crossbred mix, reference number 3J) was supplied by Wool Research Organisation of New Zealand, (WRONZ). Bovine albumin, (BSA, MW 66,000), egg albumin (EA, MW 45,000), carbonic anhydrase (CA, MW 24,000), bicinchoninic acid, bee venom (BV, MW 2,050), dextran 10,000, MW 10 kD, dextran 2,000 kD, polyethylene glycol 3000 and 2-[N-cyclohexylamino]ethanesulfonic acid (CHES) were purchased from Sigma, St Louis, USA. Monopotassium dihydrogen phosphate, dipotassium hydrogen phosphate, potassium chloride, sodium tetrathionate, acetonitrile, 2-aminoethanol were purchased from BDH, Poole, England. Sodium sulfite and sodium sulfide were purchased from May and Baker, Dagenham, England. Sodium azide was purchased from Ajax, Sydney, Australia. United States Biochemical Corpration, Cleveland, USA supplied tris(hydroxymethyl)aminomethane (Tris). Sodium chloride was purchased from Univar, Melbourne, Australia. Sodium tetrathionate (Kurtenacker and Matejka, 1931) was synthesised in the laboratory with iodine and sodium thiosulfate from BDH, Poole, England. Wherever possible all other reagents were of analytical grade. Water was four-bowl Milli-Q grade. The Sephadex G-15, particle size 40-80 μm and G-75 superfine, particle size 40-120 μm were from Pharmacia, Uppsala, Sweden.

The GPC columns were connected to a Waters Associates HPLC system from Milford, USA. This comprised a Model 510 pump, a Model 441 UV absorbance detector with extended wavelength capability and a Rheodyne injection system Contati, USA. The recorder was a Model Omniscribe from Houston Instruments, Austin, USA. A Smart^{TM} System HPLC, Model SMAP50-01-537 from Pharmacia, Uppsala, Sweden, coupled to a NEC Computer, Model MultiSync 3 FG was used for control of chromatographic separationson a 30 cm x 10 mm ID Superdex 75 column.

UV/Vis absorption measurements were made using a Model 8452 Diode Array Spectrophotometer manufactured by Hewlett-Packard USA. A Phillips Morse computer, model PC AT, Amsterdam, Holland was used for the data processing, with printing of spectrophotographs on a Thinkjet printer from Hewlett-Packard.

CE and CGE were performed on a Model 270A Capillary Electrophoresis, Applied Biosystems, Burwood, Australia. The uncoated silica capillaries, 750 mm x 50 μm were supplied by SGE, Houston, USA. The chart recorder, code no. 19-8004-01 and Eppendorf tubes were from Pharmacia, Uppsala, Sweden.

2.2 Routine Operations

- Wool 10 g was cleaned for experimental purposes by gently agitating in the following successive washes. The wool was submerged in 500 mL of water at 60°C for 5 minutes, then drained, then placed in 300 mL isopropyl alcohol at 60°C for 5 minutes, then drained, then placed in 300 mL of hexane at 40°C for 5 minutes, then drained, then finally placed in water 500 mL at room temperature, drained and left to dry in the fume hood overnight.
- Wool fibres were macerated during the OSP and RP studies with a Model T45 Ultra-Turrax, Janke and Kunkel, Breisgau, Germany. Wool was intermittently macerated using a 30 sec maceration every 15 min with magnetic stirring in between macerations.
- Sodium assay was performed on foam samples with a Corning Flame Emission Photometer Model 410, supplied by Ciba Corning Diagnostics Scientific Instruments, Halstead, Essex.
- Conductivity was measured using a conductivity meter, Model CDM83, Radiometer, Copenhagen, Denmark. Measurement of pH was performed with a calomel electrode connected to a Model 501 Digitalionalyser/5, Orion Research, Cambridge, USA. All measurements were at room temperature of 20 ± 2°C. The resting buffer was pH 7.0.
- Surface tension was measured with a surface and interfacial torsion tension balance manufactured by Whites Electrical Instruments, Malvern, England.
- Ultrafiltration was performed either using a Model 8400, 300mL stirred pressure cell and membranes from Amicon, Beverley, USA., or a Model Vivaflow 50 modular tangential flow supplied by New Zealand Medical Supplies, Auckland, New Zealand.

2.3 Ultraviolet/Visible Spectrophotometry

Measurements of the absorption were performed using 4 cm^3 cuvettes with a 1 cm path length. Quartz cuvettes were used for 190-300 nm and plastic cuvettes for 300-560 nm from Starna, Romford, England.

2.4 Dissolution of Wool Protein

Reduced Wool Protein by Sulfide Ion (S^{2-})

The wool was cut into 1 cm lengths. The wool was cleaned as stated in Section 2.2. To 400 mL of (5%) sodium sulfide solution, (25 g $Na_2S.xH_2O$ in 500 mL), was added 40.0 g of washed butt wool. The mixture was macerated for 3 hours in a water-jacketed stainless steel, sealed container that had a continuous flow of nitrogen gas passed over the mixture. Maceration was from three crossed sets of blades spinning at 600 rpm. The wool mix was centrifuged at 10,000 rpm for 30 minutes, the supernatant fluid decanted and kept under nitrogen. The residual wool fibre was twice agitated with water and centrifuged, the supernatant liquid being discarded. The undissolved wool was filtered under suction and oven dried (60°C) to constant mass to allow calculation, by difference of the amount of dissolved wool.

Reduced Wool Protein by Thioglycollate Ion ($HOOCCH_2S^-$)

The wool was cleaned as stated in Section 2.2 and cut into 1 cm lengths. A 0.5M thioglycollic acid was prepared by adding 30.3 mL to 200 mL of water, 20 g potassium hydroxide was added and then titrated to pH 10 with 5M potassium hydroxide. Wool 25 g and urea 180 g (3 mol) was added to 200 mL potassium thioglycollate, the whole made up to 500 mL with water. The mixture was left to soak for an hour prior to being macerated for 3 hours in a water-jacketed, stainless steel, sealed container that had a continuous flow of nitrogen gas passed over the mixture. The mixture was centrifuged 10,000 rpm for 20 minutes. The wool residue was washed twice with 250 mL water, the filtrate being added to the dissoluted wool. The residue wool was filtered over a large 100μm sieve, agitated in a succession of washes with water until the filtrate was clear, followed by a final wash

with acetone. The undissolved wool was filtered under suction and oven dried (60°C) to constant mass to allow calculation, by difference of the amount of dissolved wool.

Reduced Wool Protein by Hydrogen Sulfide Ion (HS^-)

The wool (Texel cross Romney) was cleaned as stated in Section 2,2 and cut with a wool sampler into ~2 mm lengths. To sodium hydrogen sulfide dihydrate 1.03 g (10.6 mmol) was added with stirring 50 mL of water. Wool 4.74 g was added to 50 mL of water and then combined with the sulfide solution to give a total solution of 100 mL. Nitrogen was bubbled through the mix for the first 4 hours, after that the flask was stoppered with additional nitrogen being added whenever the flask was opened. The flask was agitated by magnetic stirrer. After filtering and mixing with varying quantities of polyethylene glycol, 5 mL aliquots of filtrate were poured into petri dishes. A thin film was formed on the plate and left to set in the fumehood overnight.

Reduced Wool by 1,4-dithiothreitol

To 0.6055 g Tris (5 mmol) in 80 mL of water was added urea 48.0 g (0.8 mol) and 1,4-dithiothreitol 0.7712 g (5 mmol) made up to 100 mL with Milli-Q water adjusted with HCl to pH 9.3. The wool was cleaned as stated in Section 2.2. Wool 1.014 g was cut into ~0.5 cm lengths and mixed into the 100 mL solution. Maceration was performed for 30 sec every 15 minutes for the first three hours with nitrogen gas bubbled slowly into the mixture and head space during maceration. Processing was continued with magnetic stirring for 21 hours with slow nitrogen bubbling into the liquor. The dissoluted material was centrifuged 5,000 rpm for 40 min with the supernatant, dissolved wool decanted. The wool residue was agitated in a succession of washes with water until the filtrate was clear. The undissolved wool was filtered under suction and oven dried (60°C) to constant mass to allow calculation, by difference of the amount of dissolved wool.

Sulfitolysis by Copper Ammonium and Sulfite Ion

Obvious detritus was shaken free from the wool before weighing of the air dried, wool fibre. A sample (~1g) was removed at the time of weighing and oven dried to constant weight to establish the percentage of absorbed water in the wool. To urea 96 g (1.6 mol)

dissolved in 150 mL of water was added 50 mL of 0.02M cuprammonium hydroxide and sodium sulfite 12.6 g (0.1 mol). Chopped wool ~1 g (Texel cross Romney) was added to the reagents and magnetic flea stirred for 24 hours at room temperature as per the method in Swan (1960). Undissolved wool was washed in 0.1M citrate buffer followed by dilute acetic acid, (15 mL glacial acetic acid in 400 mL of water) to remove copper. Filtration was through a No.1 Pyrex sintered glass filter. The undissolved wool was filtered under suction and oven dried (60°C) to constant mass to allow calculation, by difference of the amount of dissolved wool.

Oxidative Sulfitolysis by Tetrathionate Ion

Wool from (Romney-crossbred mix, reference number 3J) was cut into ~0.5 cm lengths. Wool (10 g) was soaked in a dissolution mix comprising, urea (108 g, 1.8 mol), sodium tetrathionate (6.12 g, 67mmol), sodium sulfite (5.04 g, 12mmol), tris[hydroxymethyl]methylamine (2.4 g, 67mmol), in 300 mL of Milli-Q water. The wool-reagent mix was kept at 4°C whilst soaking for 24 h prior to maceration. The wool-reagent mix was frozen in a chest freezer with a temperature of -19°C. The sequence that followed was to soak wool at 4°C for 24 h, freeze at -19°C for 48 h, thaw at 4°C for 24 h, this was then repeated before maceration. Maceration in an ice bath was performed as detailed in Section 2.2 for the first three hours, followed by stirring at 4°C for 21 hours. The dissoluted material was centrifuged 8,000 rpm for 40 min with the supernatant decanted from the dissolved wool. The wool residue was agitated in a succession of washes with water until the filtrate was clear. The undissolved wool was filtered under suction and oven dried (60°C) to constant mass to allow calculation, by difference of the amount of dissolved wool.

2.5 Capillary Electrophoresis and Capillary Gel Electrophoresis

All samples used in CE and CGE were filtered using 0.22 μm Type GV filters from Millipore Corporation, Beford, USA., with the addition of a pre-filter membrane when using dissoluted wool samples. Eppendorf sample vials 0.5 mL were used for introduction of all samples, the buffer volumes of the inject and detector reservoirs were 4 and 12.5 mL respectively. A positive polarity was used in all reported CE and CGE data, that is the

capillary inject end was positive and solutes eluted in the order cationic, neutral, anionic species. The polyimide coated fused silica capillary length was 75 cm x 100 μm ID, the distance from injector to UV detector was 50 cm. The polyimide coating was burnt off the capillary to create a UV transparent window of ~ 1.5 cm. This left a carbon residue that was cleaned off with alcohol.

Routine operation of the capillary electrophoretic instrument

CE

➢ Wash	2 min	NaOH 0.1M
➢ Buffer	4 min	as per section 3.1
➢ Sample	0.5 sec	Vacuum
➢ Run	45 min	Buffer

CGE

➢ Wash	3 min	NaOH 0.1M
➢ Buffer	5 min	as per section 3.2
➢ Sample	0.5-1.5 sec range	Vacuum
➢ Run	45 min	Buffer

CGE preparation for PEG/dextran loading.

➢ Wash	20 min	NaOH 0.1M
➢ Wash	15 min	HCl 0.1M
➢ Wash	20 min	water, milli-Q
➢ Gel loading	20 min	PEG 3-5 %/Dextran 10 %

The detector was set with a risetime of 2 and auto-zeroed. The temperature was in the range 25-30°C, except for the dextran which was run at 50°C (Guttman et al., 1993). Voltage was either 12 or 20 kV and the current was kept < 50 μA, to avoid adverse heating effects. No marker was used, urea was present in all dissolution mixtures and used as a neutral marker. The new capillary was washed for 20 min in NaOH 0.1M, 20 min milli-Q water before the first use.

2.6 Flame Emission

A stock solution of sodium ions, 0.1000 g/100 mL (1000 μg/mL) was made from analytical grade sodium chloride (Sigma). The stock solution was diluted using Jencons Sealpette pipettes to give a range of working standards of concentrations from 2 μg/mL to 10 μg/mL (~2-10 ppm). The flame emission photometer was adjusted to give a reading scale of 0-100 for the range 0-10 μg/mL. Ten readings were performed for both standard and sample solutions being measured. The instrument was operated for 5 min. with Milli-Q water to establish a steady baseline. Samples already diluted 1:10 were further diluted by pipetting 50 μL into 10mL total volume.

2.7 Conductivity

Measurement was at room temperature (20 ± 2°C) with calibration of the instrument with potassium chloride (0.1M) at 20 ± 0.1°C (analytical BDH). The temperature of each solution was measured to ± 0.1°C, with temperature adjustment of the conductivity scale on the instrument.

2.8 Surface Tension

Surface tension of wool protein dissolutions was measured using an interfacial torsion balance. The glass dish and chrome surface tension ring of the surface and interfacial torsion balance were washed after each solution measurement, 10 min in 0.1M NaOH, rinsed in water, 10 min in chromic acid solution, rinsed in water and dried in the oven at 80°C. The weight of the surface tension ring was measured after oven drying to a constant dry mass. Ten readings were performed for each solution at 20 ± 2°C. The first two readings were discarded, as they consistently showed a higher reading than the next ten readings.

2.9 Urea Assay

Method was a modified Geyer and Dabich method by Patchett (1988). To prepare the thiosemicarbazide-diacetylmonoxime (TDM) colour reagent, to thiosemicarbazide 0.032g

(3.5 mmol) in 80 mL water was added 2,3-butanedione-2-oxime 0.623 g (61.7 mmol) and the solution made up to 100 mL. The acid reagent was composed of iron II sulfate 0.249 g (0.06 mmol) added to a mixture of 5.67 g phosphoric acid in 4.33 g water giving a 56.7 % w/w of phosphoric acid. The iron/phosphate mixture was diluted by adding 0.1 mL of the mixture to 99.9 mL of H_2SO_4 10 % v/v. The urea standard was made fresh each time an assay was performed by dissolving urea 0.030 g in 100 mL, and taking 10 mL of 0.03 g/100 mL solution and diluting to 100 mL. The stock urea concentration was 0.030 g/L equivalent to 0.5 mM.

Method for establishing standard curve.

	water	Acid reagent	TDM	Total volume added
Standard urea 0-0.5 mL	0-0.5 mL	1.5 mL	1.0 mL	3.0 mL
Sample 0.5 mL	0 mL	1.5 mL	1.0 mL	3.0 mL

The mixed reagents were placed in a 95°C water bath for 30 min, cooled for 10 min in 20°C water bath, before reading by absorbance at 520 nm by UV/Vis spectrophotometer.

2.10 Bicinchoninic Acid Assay (BCA)

Bovine serum albumin (BSA) in milli-Q water was used as the standard protein for all assays. Standard curves were established for each assay using 0 - 100 µL BSA (1.0 mg/mL), in the 0-1.0 absorption unit range. Wool protein solutions were diluted to fall within the absorption range 0.1-0.8 absorption units of the UV/Vis spectrophotometer.

Method for standard curve

- 0-100 µL BSA 100-0 µL water 2 mL BCA reagent Total vol = 2.1 mL

Protein sample, diluted 1:1000

- 100 µL sample no water 2 mL BCA reagent Total vol = 2.1 mL

BCA reagent was prepared by adding 1 part of copper sulfate (4 % w/v) solution to 50 parts of BCA (Sigma, BCA-1 kit, Procedure No. TPRO-562). Typically 51 mL was

prepared for an assay of 20 samples, just prior to addition to samples. The analytical procedure was as follows

- 2 mL BCA reagent was added to the sample (100 μL) and vortexed for 20 sec
- the reaction mixture was incubated at 37°C for 30 min, with gentle agitation then cooled to room temperature, stood for 5 min and read at 562 nm in a plastic cuvette, the blank was water.

2.11 Biuret Assay (BT)

BSA was used as the standard protein for establishing a standard curve with Biuret reagent. BSA concentration was initially made up as 1.0 mg/mL solutions with water, later 2-3.7 mg/mL BSA range was used. The BT reagent was from stock kept in a dark cupboard to avoid reduction of the Cu (ll) by auto-oxidation initiated by light. Wool protein dissolution and foam samples were analysed without dilution. All dissolution samples had been filtered through Whatman No. 1 paper to remove solid fibre. Protein that precipitated in the foaming process was left as part of the total protein assay and in all cases dissolved in the strong alkaline conditions of the BT reagent.

Method

	BSA/Sample	Water	BT reagent	Total volume
Standard curve	0-1.0 mL	1.0-0 ml	1.0 mL	2.0 mL
Samples	0.2 mL	0.8 mL	1.0 mL	2.0 mL

Samples with BT added were vortexed for 20 sec before being left at room temperature for 30 min. The absorption was measured at 540 nm by UV/Vis spectrophotometer in plastic cuvettes.

2.12 Synthesis of Sodium Tetrathionate

To 45 mL of water was added sodium thiosulfate 36.9 g (0.148 mol) with stirring, the dissolved salt was placed in a dropping funnel. Iodine 18.8 g (0.074 mol) was added to 75 mL ethanol. Once dissolved, the iodine solution with a magnetic stirring bar placed in an icebath, sodium thiosulfate was added dropwise over a period of 30 min. The sodium tetrathionate was filtered and washed with ethanol, before drying by suction for 10 min. The product was placed in a dessicator and stored at 4°C. The filtrate was placed in a

freezer at -18°C and the second precipitate collected and treated as above. The literature cites a yield of 30 % (Kurtenacker and Matejka, 1931), expected yield was 40%.

2.13 Ultra-filtration

Dissoluted wool in a stirred cell was ultra-filtrated on a Amicon YM3 (MWCO 3000) membrane. The membrane was cleaned with 0.1M NaOH and stored in 10% ethanol at 4°C to prevent microbial contamination. Tangential flow desalting was carried out using modular units, Viva Flow 50, with polyethersulfone membranes of 5000 and 50,000 MWCO manufactured by Viva Science Ltd, Stonehouse, Gloucester, UK., supplied by New Zealand Medical Supplies, Auckland. These units were treated after each filtration as follows: wash with 250 mL of Milli-Q water; recirculate 0.5mM NaOCl in 0.5M NaOH for 30 mins; drain and wash twice with 250 mL water; flush with 10% ethanol and leave in unit, store at 4°C.

2.14 Gel Permeation Chromatography

GPC was performed using G-15 Sephadex columns of 5 cm x 5 mm ID or 10 cm x 16 mm ID, and on G-75 Sephadex column, 30 cm x 16 mm ID column. The protein sample, 25-100μL, was loaded by manual injection into a diaphragm type, 2 mL sample loading loop, Waters, Milford, MA, USA). The sample was filtered (22 μm), prior to being diluted ten-fold with milli-Q water. A chart speed of 25 cm/hour was used unless otherwise stated. Half-peak width was measured using a Scale Lupe 7x magnifier (Peak). Height and half-peak width were measured in millimetres. Eluent buffer was prepared fresh each day as follows: 400 mL of 0.125M dipotassium hydrogen phosphate buffer (pH 9.0) containing 0.125M potassium chloride was pH adjusted to 9.0 with 0.1M phosphoric acid. The buffer was filtered and added to 100 mL of filtered acetonitrile, and then degassed by water vacuum pump. This gave a final concentration of dipotassium hydrogen phosphate and potassium chloride of 0.1M in 20% acetonitrile buffer. The flow rate was 0.2 mL/min, 0.8 mL/min, and 1.0 mL/min for the 5 cm, 10 cm and 30 cm columns respectively. The pump was flushed out each day with 20% acetonitrile. The columns were stored at 4°C when not in use. Detection was performed at 214 nm for all assays. The protein standards, EA, BSA, CA were prepared in water. Dialysed wool protein was dissolved in 0.1M phosphate/KCl/20% AcCN buffer pH 9.

Glass HPLC columns from Pharmacia, Uppsala, Sweden were packed after dissolving the Sephadex in phosphate/KCl/ACN buffer. Initial trials were conducted with a 50 x 5 mm ID column packed with Sephadex G-15. Total protein analysis was performed on samples using a 100 x 16mm ID column packed with Sephadex G-15. The 300 x 16 mm ID column was packed with Sephadex G-75. A 300 x 10 mm pre-packed column of Superdex 75 was used on the Smart System HPLC, with the phosphate/KCl/ACN buffer.

2.15 Foam Fractionation

Initial foam fractionation experiments on the effect of pH change were performed using a stainless steel HPLC inlet frit placed in the bottom of a measuring cylinder (38 mm ID, 24 cm length). All further foaming was performed in a column of length 29 cm x 35 mm ID fitted with a stainless steel frit and a gooseneck that had an average travel distance of 91 mm. The nitrogen gas flow was controlled by bubbling the gas through water (150 mL) in a round bottom flask (400 mL). The time taken for 50 bubbles, was counted at periods during foaming to ensure a constant flow of gas was maintained to the frit. The flow of gas ranged from 7.9 mL/min to 15.0 mL/min at 20°C and 1 atmosphere. The number of bubbles of gas required to fill a 10 mL measuring cylinder was counted 5 times and averaged to establish the flow rate of nitrogen gas at 20° C and 1 atmosphere pressure.

The foam temperature was 20 ± 2°C during the experimental work. The foamate was collected in a beaker and kept overnight at 4°C to allow for collapse of the foam.

CHAPTER THREE

Analytical Techniques

3.1 Introduction

The three primary objectives of the soluble wool solutions analyses were:

- to determine total protein of soluble wool protein and foam fractions
- to identify changes in wool classes due to processing of the soluble wool
- to determine the extent of change of the ionic component of the soluble wool solutions due to separation methods

3.1.1Capillary Electrophoresis

Capillary electrophoresis (CE) can separate species according to charge and size of molecule based on the differential migration rates of the species in an electric field. Proteins to be separated must exhibit a difference in their size to charge ratios for separation using CE.

Two processes can act against separation of different proteins, a lack of difference in the charge to size ratio of the proteins and interaction of the proteins with the walls of the capillary tube.

- Wool proteins are not globular and a considerable proportion of wool protein is hypothesised as existing in long strands containing part α-helical structures and part undefined structure. Parry and Steinert (1995) noted that intermediate filament protein (IFP), the low sulfur proteins in wool, had a partly helical conformation with all other wool proteins exhibiting no helical character. No alternative conformation has been found for the non-helical parts and wool protein is generally depicted as long chains (Parry and Steinert, 1995). For the solubilization of wool protein a high urea 8M content is used to denature the proteins. It is hypothesised that the α-helices will be strand-like in the solution, thus Equation 3.1, in particular *r*, does not truly apply to linear wool protein molecules. If wool is strand-like it follows that all amino acid groups in wool proteins are exposed to the solvating environment and have the

potential to react to pH changes. This is in contrast to globular proteins that may remain intact with only the surface amino groups being active. Sequences within wool families of proteins are highly conserved, and consequently many wool proteins will have very similar amino acid ratios (Maclaren and Milligan, 1981; Parry and Creamer, 1980; Parry and Steinert, 1995). The similarity of this protein composition will tend to give similar charge patterns along the chain. Comparing two wool proteins of differing length, it follows that a change in chain length is accompanied by a corresponding change in the number of charges exposed along the chain resulting in a very similar q/r ratio and consequently poor separation. This possibility is a major disincentive to the use of CE for wool proteins. The study by Jelinkova et al. (1991) of keratin proteins from rat hairs in studies using fused silica capillaries at 220 nm reported good separation. Thomas et al. (1983) reported separation by electrophoresis of wool proteins noting that HSP and IFP "differ such that they display distinctly different electrophoretic properties". The Jelinkova et al. And Thomas et al. study indicated that despite the potential problems mentioned with keratin protein separation was possible using fused silica CE.

- Protein interaction with the walls of the fused silica capillary can impede separation (Maa et al., 1991; Green and Jorgenson, 1989; Baker, 1995; Li, 1992). The protein-wall interaction provided an incentive for coating of walls as reported in section 3.2.2 of this study. Proteins are complex macromolecules composed of positively and negatively charged groups in addition to hydrophobic and hydrophilic groups (Maa et al., 1991). At pH > 7 the walls of the capillary are composed of a mix of silanols and silonoate ($Si\text{-}O^-$) groups, the ratio being dependent on pH. Interaction between the wall of the capillary and the proteins is inevitable (Maa et al., 1991, Bushey and Jorgenson, 1989, Green and Jorgenson, 1989). A number of methods to disrupt the protein-wall interactions have been examined. Bushey and Jorgenson, (1989) reported favourably on the use of zwitterionic salts in the buffer. Green and Jorgenson, (1989) reported good success with K_2SO_4 at pH 9. In this study no sign of protein being eluted particularly delayed was observed with pH > 8. Lower pH regimes with CE detected no protein at all, indicating low pH had lead to precipitation and/or absorption to the walls of the capillary.

3.1.2 Capillary Gel Electrophoresis

Using a sieving media separation based on molecular size can be achieved using capillary gel electrophoresis (CGE). Separation by size requires a stable media in the capillary tube through which the buffer can flow carrying the separated species past the detector. Coating of the silica capillary wall by polymer adsorption is a simple method. A polymer is introduced to the capillary in a coating solution or as part of the running buffer to provide a dynamic coating. The polymer is adsorbed on to the silica by coulombic forces, hydrogen bonding, and/or Van der Waals forces. Coating polymers considered for this study were PEG and dextran. PEG was available in a range of MW 400-20,000 D and dextran was available up to 2 million D.

Guttman et al. (1993) reported in their studies that temperatures $\leq 30^{\circ}C$ gave the best sieving environment for PEG. Combining the wall coating qualities with the sieving characteristics of PEG reported by Ganzler et al. (1992), seemed to offer an opportunity to achieve capillary coating and a sieving medium at the same time. Dextran can be obtained in very long polymer chains (2 million D, chain) and was an alternative to PEG worth trial as a neutral species for sieving of soluble wool proteins.

An additional complication arises if the preferred buffer affects the capillary wall coating. Guttman et al. (1993) and Ganzler et al. (1992) and Radko and Chrambach (1997) reported success with use of replaceable gels using a zwitterionic buffer of Tris/CHES/SDS 0.1 %.

3.1.3 Buffers for Capillary Electrophoresis and Capillary Gel Electrophoresis

It is essential that proteins in the capillary do not precipitate, and any buffer used is transparent at the 190-220 nm range, for UV detection. Use of buffers at pH > 7 causes the capillary wall to degrade, with solubilization of the surface silica (Baker, 1995). Successive washings with 0.1M NaOH as recommended by many methods will remove silica, such that the walls become pitted and differences appear in the performance of the capillary from one run to another. Strong alkaline solution leaves the walls in silanoate form, giving a negatively charged wall that theoretically will repel the negatively charged proteins if the buffer pH > 8 (Bushey and Jorgenson, 1989). Detection of protein and wool

protein in particular is difficult owing to lack of good chromophore groups in easily accessible spectral regions as noted by Bushey and Jorgenson (1989). These two requirements have restricted the buffers available.

Complications can arise from changing the buffers used, Lauer and McManigill (1986) reported changes in elution order of proteins with change of the pH. In assessing by CE, changes in the pH of the dissolution process must be minimised for valid comparison of the results.

Increasing the ionic strength of a CE buffer helps to counteract the interaction between capillary walls and proteins and increases the $\mu_{eo.}$, but results in an increase in the Joule heating effect, Equation 3.1.

$$E = IR = V/L \qquad \text{Eqn 3.1}$$

E = Electric field. I = current L = total capillary length

The result of doubling the buffer ionic concentration is to almost double the current flow. The current is directly proportional to the heating effect, the heat produced will cause increased heating in the centre of the capillary and separation efficiency declines. In practice it was found best to operate with a current of $< 80\mu A$ to avoid excess heating effect. Zwitterions can be added to the buffer to associate with the negative ions of the protein without adding to the total ionic concentration (Bushey and Jorgenson, 1989). Bushey and Jorgenson showed that zwitterions assist in decreasing wall-protein and protein-protein interactions and allow for higher voltages to be used. Baker (1995) noted that changing the buffer can alter retention and selectivity, different ions in the buffer can interact with the walls and change the μ_{eo}. A consequence of addition of zwitterions is an increase in the viscosity of the buffer, this leads to improved separation by lessening band-broadening that results from convection effects.

Of particular interest were improvements reported by Bushey and Jorgenson (1989) with zwitterionic salts and changes reported by Dolnik (1995) from different electrolytes.

Inclusion of a zwitterion was expected to assist the resolution as well as allow for use of a voltage higher than would be possible if a salt was used.

3.1.4 Urea Analysis

Foam fractionation has been used to separate proteins, but these have all been in solutions containing no urea. Dissolved wool protein solutions have urea in high concentration (6-8M). A fundamental issue is whether foaming will separate protein from urea. A modified method used by Patchett was used for urea analysis of foam fractions as detailed in Section 2.9.

Storing of samples creates a potential error as the urea in the dissoluted wool protein degrades slowly to give cyanate, which can lead to modification/carboxymethylation of the amino groups on the protein (Means and Feeney, 1971; Pace, 1986).

3.1.5 Flame emission

Flame emission was chosen as a method to analyse the changes in the soluble wool protein of sodium, the predominant positive ion present. Sodium ions were the only positive ions added as part of the dissolution reagents. The concentration of sodium ions provided information about changes in the foam liquid.

3.1.6 Bicinchoninic Acid Assay (BCA)

The BCA assay is widely used because of the sensitivity and the decreased effect of interfering chemicals on the assay. The BCA assay is very specific for Cu^{+1} forming a purple complex, $Cu(BCA)_2^{3-}$ (Eqn 3.2) that can be measured at 562 nm Figure 3.1.6 (Smith et al. 1985).

$$Cu(H_2O)_6^{2+} + 2BCA^{2-} + e^- \rightarrow Cu(BCA)_2^{3-} + 6H_2O \qquad \text{Eqn 3.2}$$

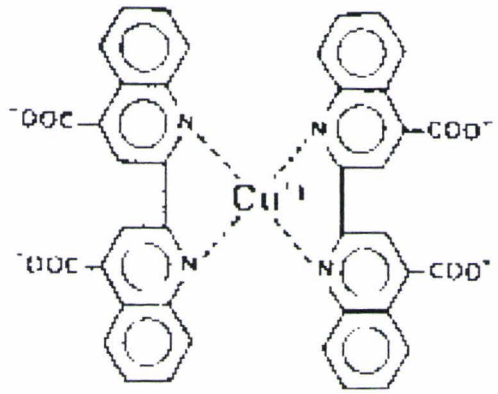


Figure 3.1.6 Cu^{+} complex formed with BCA

Coordination of the Cu^{+} ion with BCA. Cu^{+} is formed by the Biuret reaction.

3.1.7 Biuret Assay (BT)

Biuret complexes Cu^{2+} changing the pale blue copper ion colour to a more intense purplish-violet colour, Figure 3.1.7. The biuret determination of protein with alkaline Cu^{2+} coordinated with the amide nitrogen of proteins has been used for some 80 years (Sapan et al. 1999). The biuret coordination is the basis of the Cu^{2+}-protein-tartrate complex forming the Biuret assay for total protein.

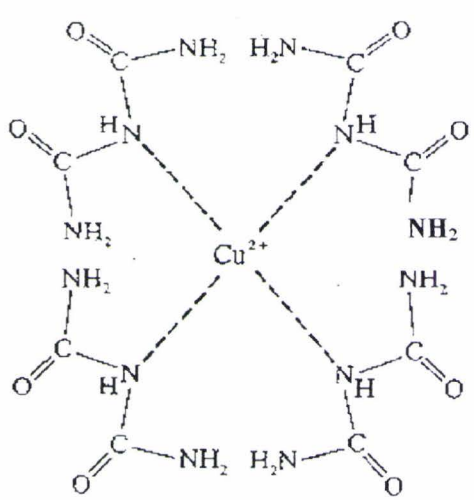


Figure 3.1.7 Biuret complexation of Cu^{2+}

The Cu^{2+} is coordinated to the nucleophilic nitrogens of four biuret molecules. (Holme and Peck, 1993).

In selecting a reference protein as a standard to measure the wool protein against, BSA was the most commonly referred to protein in literature. BSA was used for all of the biuret assays carried out with wool protein. To correlate BSA to wool protein a trial was carried out comparing BSA to human serum albumin (HSA). HSA was reported by Gorshev and Nedkov (1979) to react in a proportionally similar manner to wool protein. Gorshev and Nedhov examined the response of HSA along with human plasma and sheep wool to the biuret assay. The wool was oxidized by peroxide and solubilized in detergent. Sheep wool and human plasma had identical responses to the BT assay, with HSA giving a slightly increased absorbance for the same concentration of protein.

3.1.8 Gel Permeation Chromatography (GPC)

Gel permeation chromatography columns of 5, 10 and 30 cm were investigated for usefulness in separating protein from dissoluting reagents as part of an assay method. Sephadex offers a range of bead sizes and hence separating potentials and has been extensively used for desalting. Separation with GPC using Sephadex gels offers a fast and simple method that results in short elution times.

Solubilized wool generally exhibits precipitation < IEP of pH 8, hence a GPC buffer pH > 8 is needed. Sephadex, polydextran crosslinked with epichlorohydrin to produce a bead 40-120 μm has been reported as stable at pH 9 by Pharmacia. Sephadex G-15 would be expected to exhibit a low hydrophobicity given the high number of hydroxyl groups.

It is possible to separate proteins into broad MW fractions on G-75, which has a range of dextran chain sizes offering molecular weight size separation. Pharmacia states that separation on G-75 requires a 10^4 MW difference, wool proteins range from 9,000-60,000D, consequently separation of the individual proteins was not anticipated.

A Sephadex G-15 (10 cm x 16 mm) column was selected to achieve the objectives of determining total protein concentration in wool solutions in a short time frame and independent of interfering chemicals. Both dissolution and foam fractionation were carried out in experiments lasting up to 24 hours. GPC allowed for samples to be assayed at any time during the experiment and provided immediate feedback on the progress of the

experiment. The column desalts the mixture with the proteins eluting at void volume. The speed and simplicity of GPC allowed for protein assays to be carried out as the reaction proceeded.

3.2 Experimental

The urea, flame emission, conductivity measurement, BCA and biuret assays, and the GPC separation were all performed on soluble wool protein resulting from oxidative sulfitolysis.

3.2.1 Capillary Electrophoresis and Capillary Gel Electrophoresis

The operation of the Applied Biosystems Capillary Electrophoresis Model 270A instrument allowed for four samples to be run over a period of 3-4 hours. Each analysis carried out took approximately 55 min.

A number of buffers are available for CE. Poll (1998) reported good success with 2-AE over a range of separations of protein using CE. To lessen wall-protein interaction 2-aminoethanol (2-AE) was used for early CE. It is transparent at 200-220 nm and combines areas of hydrophobic and hydrophilic interaction in the one molecule.

For comparison of results, pH 9 was chosen for most CE run in the un-coated fused silica capillary. CE was performed in this study at 12-20 kV and current $\leq$ 45 μA, buffers were kept $\leq$ 0.1M concentration to avoid excess heating effects of high voltage and current. CE was performed as in the procedure set out in Section 2.5.

A fused silica capillary was filled using vacuum, with a 3 % solution of PEG, MW 3,000. CGE were performed using 2-AE 50mmol/CHES 0.1M /1 % SDS buffer, pH 9 at 25°C. The temperature was kept $\leq$ 25°C. The same procedure was used to introduce 5% and 10% solutions of dextran, MW 20,000 and 2 million as sieving media to the capillary. A run time of 45 min was used to achieve movement of all the species through the capillary. The operation of the CGE was as detailed in Section 2.5.

3.2.2 Urea

TDM forms a coloured complex with urea, thiosemicarbazide-diacetylmonoxime-urea TDMU, that can be measured at 420-430 nm. Geyer and Dabich examined the colour development with time and temperature as variables and found these to be at a maximum at 20 min and temperature > 90°C.

The concentration of urea being measured was in molar quantities ≤ 6M in the original solutions. To carry out the assay of foam fractionation samples, dilution to one twenty thousandth of the original concentration was required. A dilution of 1:20,000 has the potential for error in the measurement of very small e.g. 10 μL quantities. The small volumes were used to avoid unwieldy volumes involved in handling 20 samples for analysis at each assay. The dilution sequence followed was 10 μL made up to 2.0 mL, vortex, a 5 μL aliquot of the first diluted solution was made up to 0.5 mL in a test tube, vortexed and used as the sample to mix with the TDM and acid reagents. A freshly prepared reference solution of urea 0.5 mmol was used in 0-0.5 mL to obtain a standard curve of absorbance in relation to concentration.

3.2.3 Flame Emission

The method outlined in section 2.6 was followed for all assays. Initial problems with instability in the flame resulted in a calibration relationship that was curved, with no linear section. Instrument instructions recommend a 5 min warm up, however it required at least 15 minutes to achieve the flame stability and produce consistent readings that showed a standard deviation of less than 5%.

3.2.4 BCA

The assay procedure was as detailed in Section 2.10. Conditions chosen for this assay were those suggested by Sigma (1998) of 37°C and 30 min incubation.

3.2.5 Biuret

The assay procedure detailed in Section 2.11 was followed for all analysis. Where the concentration of the protein in solution was expected to be greater than 10 mg/mL a more concentrated BSA solution was used to allow readings in the 2.5-3.5 mg/mL range of the standard curve. The assay can be carried out in the temperature range 20 - 37 °C for 30 min. The biuret colour developed after 30 min is stable for several hours.

3.2.6 Gel Permeation Chromatography

Gel permeation chromatography was carried out using Sephadex G-15, G-75, and a Superdex 75 preloaded column. Separation of wool was performed with a 5 cm x 5 mm ID column packed with Sephadex G-15. This established that separation of protein from the other chemicals in the dissolution mix could be achieved using Sephadex G-15. However the separation of the protein peak from the reagents was incomplete with the 5 cm column, doubling the length of the column to a 10 cm G-15 column gave separated peaks. All subsequent total protein assays of dissoluted wool protein samples and foam fractionation samples were performed using a 10 cm x 16 mm ID column packed with Sephadex G-15.

Several buffers were assessed for transparency at 214 nm. Dipotassium hydrogen phosphate 0.125M solution containing potassium chloride 0.125M adjusted to pH 9.0 was added to acetonitrile in the ratio of 4:1 to form a buffer of pH 9, 0.1M phosphate/KCl/20% AcCN. Protein solutions assayed were in the 1-30 mg/mL range.

The GPC procedure requires the dissoluted wool and foam fraction proteins to be retained in a soluble form. This was accomplished by avoiding a pH close to the iso-electric points of wool proteins (4.5-7.5) by using a buffer pH 9.

The total time taken for a run of dissoluted wool protein on the G-15 column was 28 min at a flow rate of 0.8 mL/min. This could be shortened to 23-24 min by injecting a new sample as the fourth peak of the previous sample was recorded. Peak overlap with this method was not observed. Loading 100μL with an initial dilution 1:10, gave a result within

measurable absorption < 1.0 AU. The filtering and dilution procedure did not delay the assay by more than 10 min in most instances and allowed for analysis as the dissolution or reaction proceeded, an advance on performing either the Biuret or the BCA assays.

The protein concentration for the dissoluted wool protein and the foam fractions was calculated using the following formula:

$$P = h_2/h_1 . b . F/v$$

where, P = concentration of dissolved protein; h_1 = peak height (mm) of the standard protein (WP); h_2 = peak height (mm) of the sample protein; b = amount (mg) of standard protein (WP); F = dilution factor; v = injected volume (μL) of the sample (100 μL).

Sephadex G-75 100 μL samples were diluted 1:10 and injected manually into the Waters HPLC. Detection was at 214 nm and the chart recorder was run at 12.5 cm/60min.

A short trial was performed using Superdex 75 on the Smart™ system. An equipment dysfunction prevented further investigation.

3.3 Results and Discussion

3.3.1 Capillary Electrophoresis

pH

Capillary electrophoresis showed no detection of protein at pH 7. Highly alkaline pH 11 showed a very large absorption by what was presumed to be a negative ion following the protein peak. Acid buffers down to pH 2 gave no detectable protein, wool protein was found to readily give precipitation at below pH 8, so this was an expected result. Distinct differences were observed in CE at pH 9.4 compared to 9.0 and 8.9 Figure 3.3.1a. Buffers in the range pH 9.0 ± 0.2 were used for all CE following this initial investigation, with the best peak resolution observed at this pH as shown in Figure 3.3.1a.

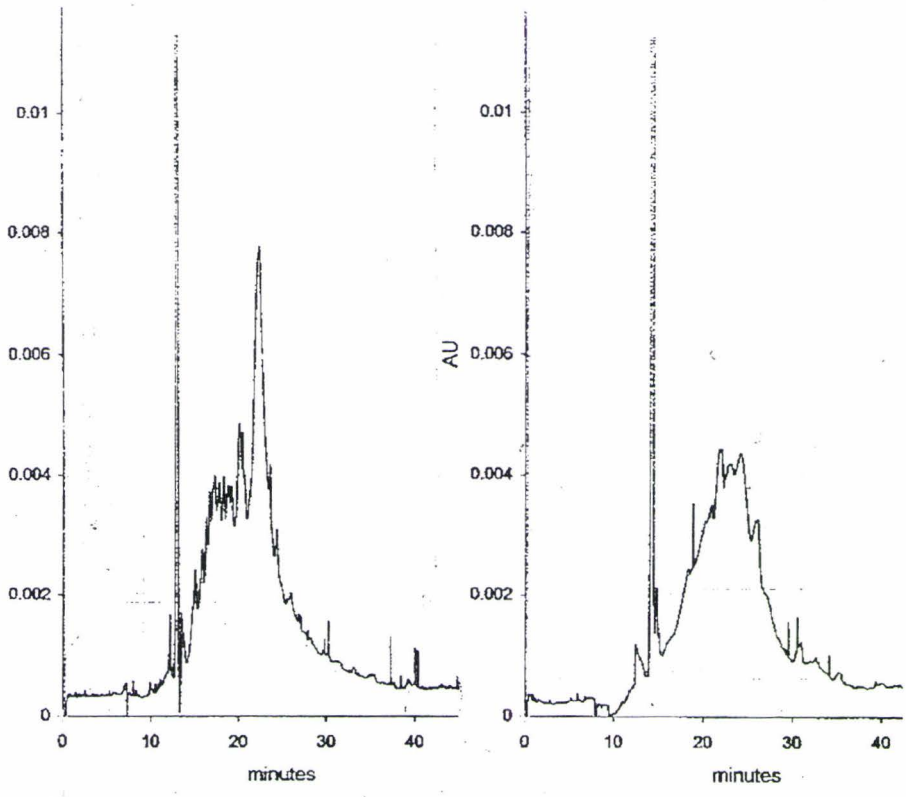


Figure 3.3.1a Buffer pH differences with CE of wool protein dissolutions.

CE were performed with dissoluted wool after 10 h of oxidative sulfitolysis, buffer 2-AE 100mmol, 12 kV, 43 μA, 28°C. Capillary 70 cm, 50 cm to detector, 100μm ID A. pH 9.2 B. pH 9.5

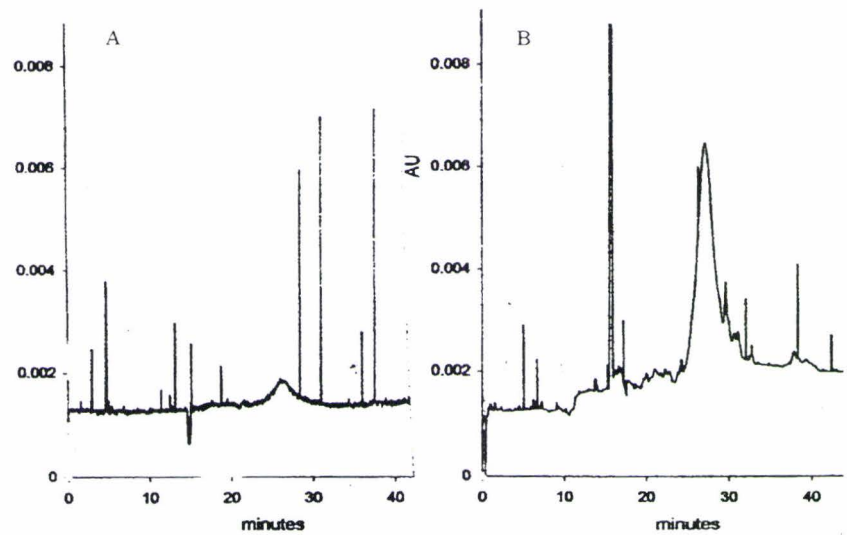


Figure 3.3.1b Absorption of dissoluted wool at 200 nm and 280 nm.

CE were performed with dissoluted wool, buffer 2-AE 200mmol, 12 kV, 40 μA, 25°C, pH 9.0 Capillary 70 cm, 50 cm to detector, 100μm ID. A. 200 nm B. 280 nm. .

200 nm compared to 280 nm

The lack of chromophoric residues at 280 nm in wool protein necessitates the use of 200 nm for detection. The 200 nm detection did have some advantages as illustrated by Figure 3.3.1b. The urea peak (14 min) is a useful neutral marker that is very sharp and pronounced due to the high urea concentration (8M) in the samples. A pronounced peak band is evident in the 200 nm spectrograph. The 280 nm spectrograph does not show carbonyl or amide absorption and hence no urea peak and only a small protein peak from the tyrosine and tryptophan aromatics.

Buffers

Separation of wool protein was not successful using 2-AE as the buffer. Maximizing the parameters of pH and detection wavelength still resulted in undifferentiated peaks. Despite the coalescing of the peaks into a broad band, differences in wool dissolution mixtures at 6 h and at 24 h were observed in the profile of the broad absorption band. Two peaks eluting in the central part of the band were less prominent at 10 h, yet formed major peaks at 24 h. This suggested that some wool proteins were dissolving later than other wool proteins, a circumstance mentioned in Section 4.1. A number of different buffers cited in the literature were examined for their ability to resolve the wool protein using dissoluted wool protein from a single dissolution. All investigations of buffers were carried out at pH 9. Borate (BO_3^-) and borate/CHES (2-[N-cyclohexylamino]ethanesulfonic acid) buffers both showed the same profile of a major broad peak at 25 and 28 min respectively and two minor peaks at 18, 34 and 20, 37 min respectively. Inclusion of CHES with the 2-AE original buffer changed the profile of the wool protein as shown in Figure 3.1.3c. Buffers containing K_2SO_4 produced flat profiles with no detectable absorption, possibly from precipitation of the protein in the capillary. Sulfate ions being associated with stabilization of protein and salting out techniques. Changing the buffer in CE affects the protein-protein and wall-protein interactions and consequently the resulting CE trace as in Figure 3.3.1c.

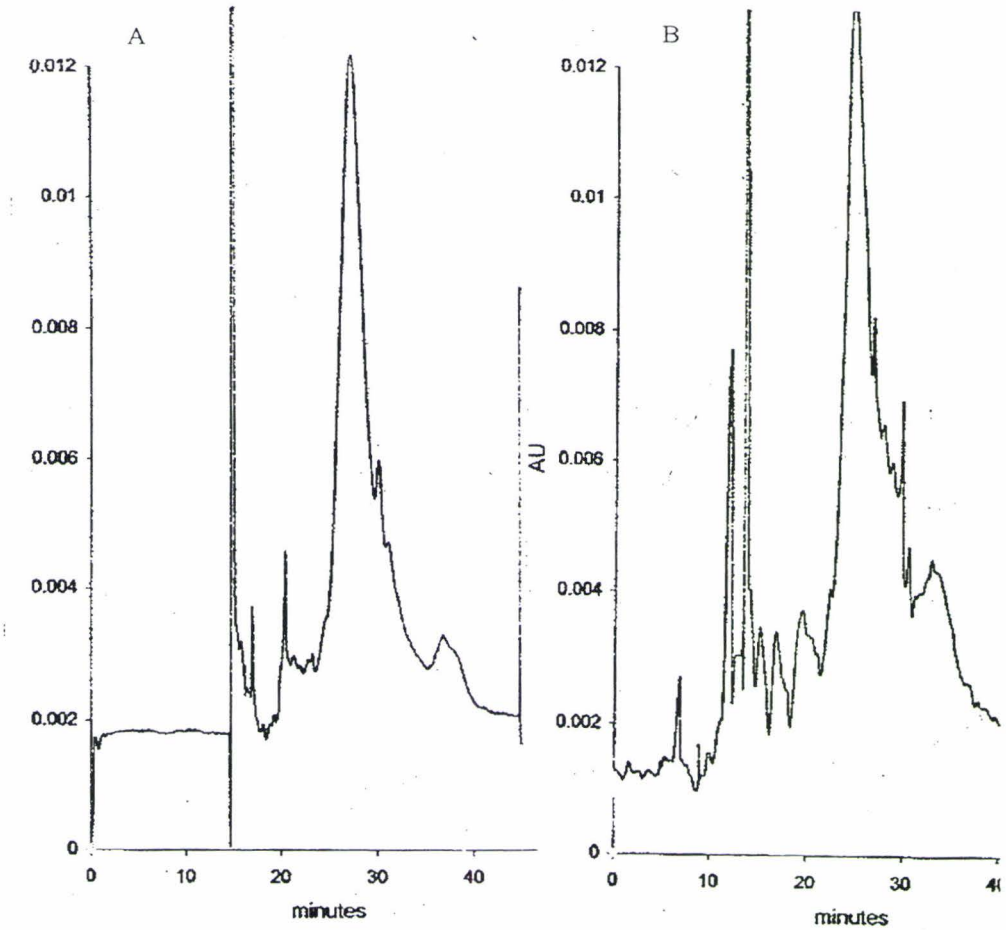


Figure 3.3.1c Different buffers.

CE were performed with dissoluted wool, 12 kV, 35 μA, 25°C, detection was at 200 nm. Capillary 70 cm, 50 cm to detector, 100μm ID. A. borate 50mmol/CHES 0.1M B. buffer 2-AE 50mmol/CHES 0.1M, pH 9.0.

Separation of Dissolution Wool Protein on CE

A Sephadex G-75 column was used to perform separation of dissoluted wool on molecular mass basis. Fractions from the Sephadex G-75 column were run on CE with the first fraction showing a broad peak at 25 min Figure 3.3.1d. The last fraction had no major broad peak at 25 min, but a single spike at 27.5 min. The first fractions eluted from Sephadex G-75 contain the highest molecular mass protein material whereas the latter fractions contain the lower molecular mass protein material.

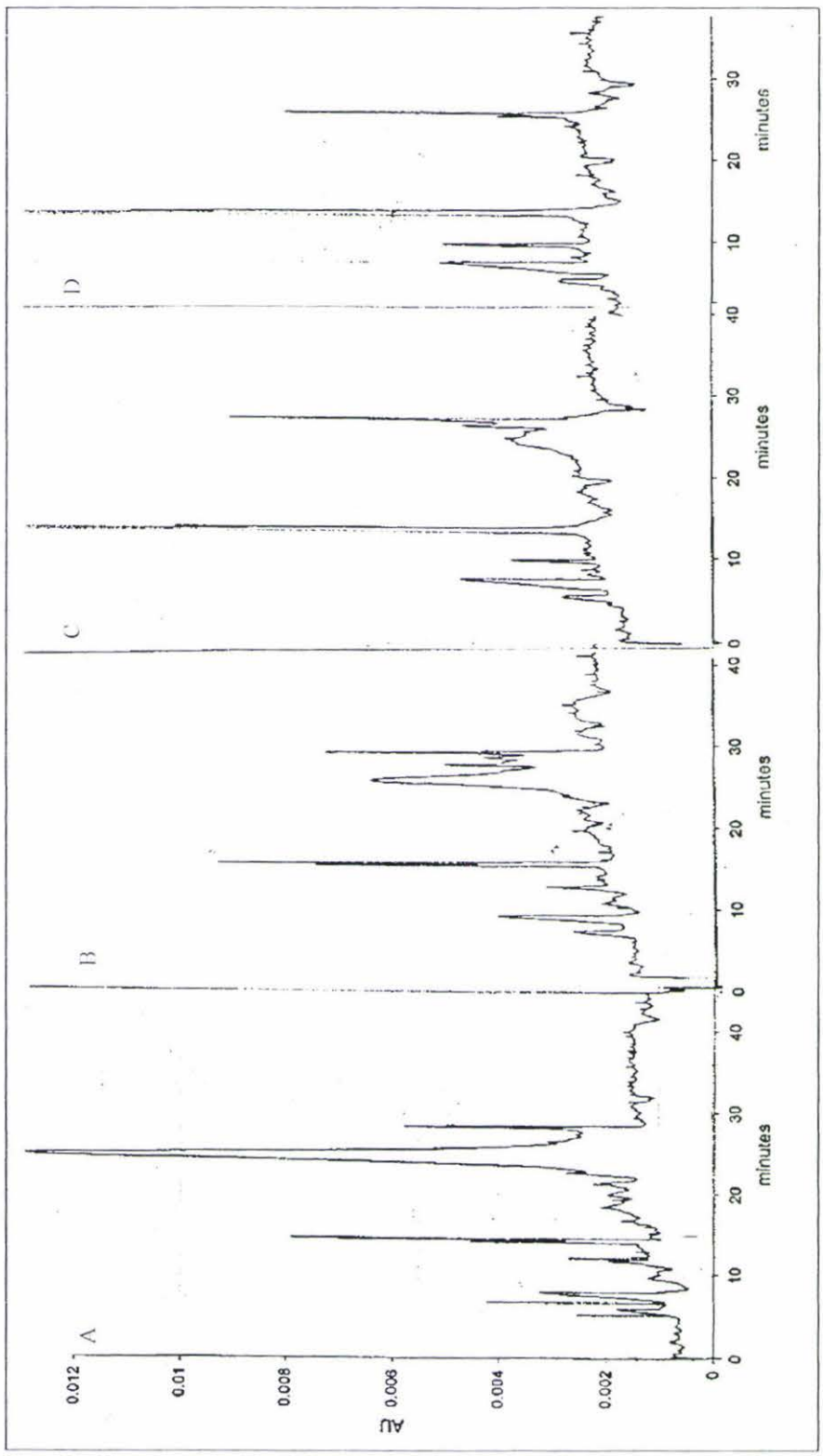


Figure 3.3.1d Separation by CE of wool protein classes.

CE were performed with dissoluted wool that was GPC Sephadex G-75 separated using a buffer NaCl 0.4M/ K_2HPO_4 50mmol. Fractions were collected every 2mL eluted. CE was performed at 12 kV, 36 μA, 29°C, detection was at 200 nm. Capillary 70 cm, 50 cm to detector, 100μm ID., buffer 2-AE 50mmol/ CHES 0.1M, pH 9.0. A. Fraction 4. B. Fraction 7. C. Fraction 10. D. Fraction 12.

Figure 3.3.1d illustrates the change in profile of the CE spectrograph with the different GPC fractions. CE was achieving some degree of separation based on molecular mass, albeit within a very narrow band of 2.5 min.

3.3.2 Capillary Gel Electrophoresis (CGE)

Separation of proteins was attempted using CE, with some indication of peaks being related to protein classes. However literature indicated that CGE could provide improved soluble wool protein separation . A fused silica capillary filled with 3% PEG showed a a very poor response and resulted in a stepped baseline. The noisey baseline may have been caused by the movement of bands of PEG through the capillary carrying entrapped protein. If correct, these bands of PEG would give absorbance outside the normal elution times. A possible explanation was that the buffer at pH 9 was too alkaline for the silica to remain coated and silanoate groups were reformed as the PEG was lost from the capillary. A current of 45 μA was recorded at 12 kV showing that no change in the μ_{eo} had been achieved. Changing to 5 % PEG, CGE gave a series of peaks of low intensity using egg albumin as a protein marker. It was difficult to distinguish the blank run from the protein run.

CGE was performed with a number of wool protein dissolutions and with standard protein markers without any obvious resolution of the signal into identifiable peaks. Egg albumin which has a MW 45 kD, i.e. in the range of wool proteins, was poorly resolved using 5 % PEG MW 20 kD as a preloaded, replaceable gel with the established buffer of 2-AE 50 mmol/CHES 0.1M/SDS 1 % at pH 9. The difference in changing to a buffer composed of Tris 0.1M/CHES 0.1M/SDS 1 % pH 9 with 5 % PEG MW 20 kD as gel was dramatic as illustrated by Figure 3.3.2. Changing the buffer was the most single significant action that caused improvement in the quality of information from CGE operation.

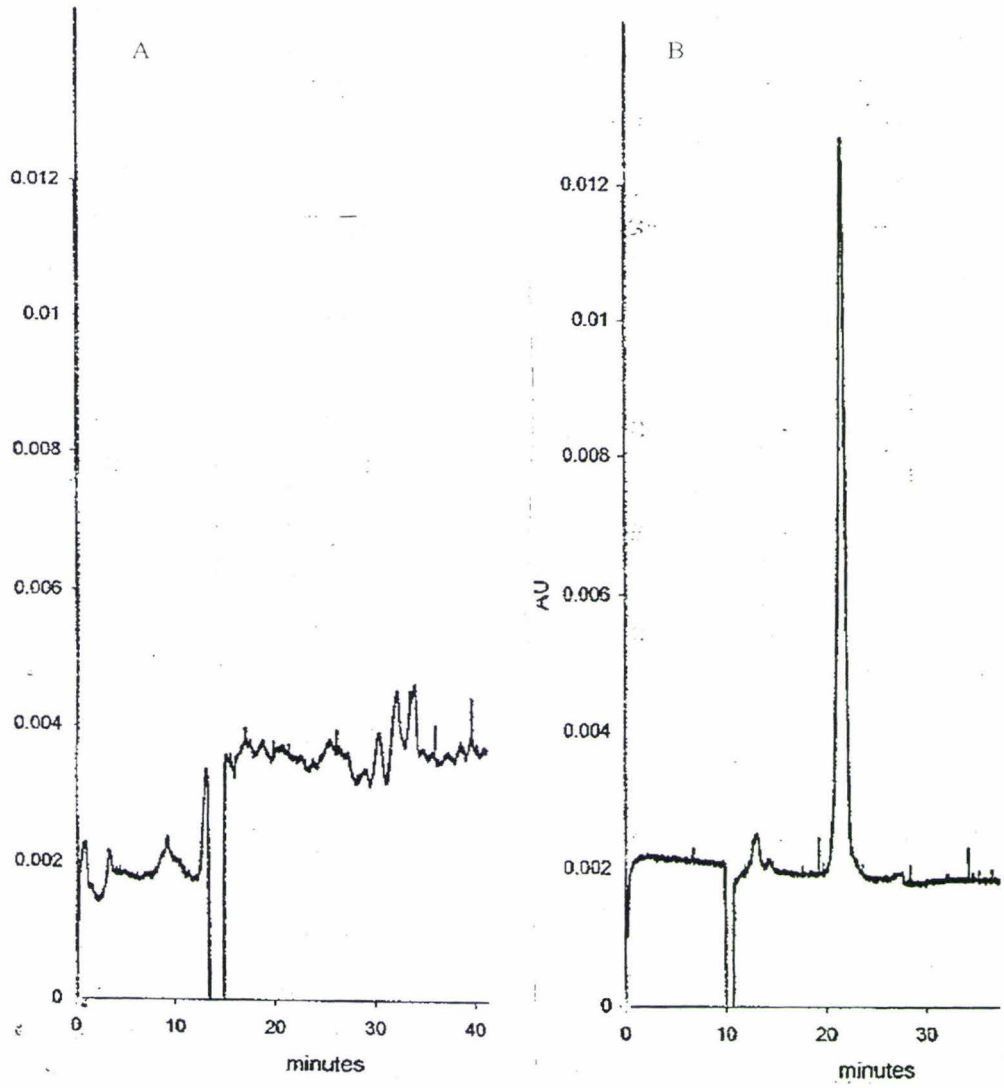


Figure 3.3.2 Buffer effect on CGE using 5 % PEG as replaceable gel.

CGE were performed at 25°C, detection was at 200 nm in a capillary 70 cm, 50 cm to detector, 100μm ID. PEG 5 %. Egg albumin 0.5 mg/mL/ SDS 1 % was the sample in each case. A. buffer 2-AE 50mmol/ CHES 0.1M/ SDS 0.1 %, pH 9.0, 12 kV, 47 μA. B. Tris 0.1M/ CHES 0.1M/ SDS 0.1 %, pH 9, 12 kV, 15 μA.

The single spike for the egg albumin was not evident in the 2-AE/CHES buffer, yet a change to Tris instead of 2-AE made a startling difference. The original buffer in run 158 was carried out at 12 kV and 47 μA. All other conditions were kept constant in the change to the Tris/CHES buffer. The Tris/CHES buffer was operated at 12 kV, but the current immediately dropped to 15 μA. This was despite the change in concentration from 2-AE of 50 mmol to 100mmol of Tris. It is hypothesised that the Tris was not removing the PEG coating on the silica wall and thus a lowered electroosmotic flow resulted.

Repeats of the Tris/CHES buffer showed an increase in the baseline noise, such that within 10 further runs the baseline noise was very evident throughout the CGE run. The cause of the increased baseline noise was not determined. It was expected that using the above buffer would result in an improved separation of the wool proteins. As noted, the baseline noise increased with each run and the dissoluted wool showed a narrow peak compared to the best achieved using CE, as seen earlier in Figure 3.3.1c. CGE of foam fractionation carried out on the dissoluted wool showed changes in the composition of the wool proteins in comparing CGE of the first and fifth fractions foamed. This is reported in detail in Section 5.3.

Replacement of the PEG 5 % in the capillary with new PEG 5 % was carried out and no better peak resolution was achieved with the dissoluted wool. This disappointing result lead to no further investigation of PEG as a dynamic gel. The high pH used for the buffers was believed to be too degrading for the silica walls.

Dextran

A dextran 20 kD oligomer 10 % solution was introduced into the capillary for 20 min after washing of the capillary with NaOH 0.1M for 20 min, HCl 0.1M for 15 min and Milli-Q water for 20 min. The run temperature was set to 50°C as recommended by Guttman et al. (1993) which reported that temperatures of 50°C gave improved separation from more uniformly tangled polymer chains and hence the best sieving effect. The profile of wool protein on the spectrograph was no better than that achieved with PEG 5 %. Although the protein was evident as distinct peaks it lacked the resolution sought for meaningful analysis. Takagi and Karim (1995) used a coated capillary, which was not available for this study. The lack of successful separation appears to be related to the need for a coated capillary if separation is to be achieved.

3.3.3 Urea

A line of best fit for urea 0-0.15 mmol was obtained as shown in Figure 3.3.3. Above 0.15 mmol concentration there was an observed decline in absorbance with higher concentrations. Geyer and Dabich (1971) noted in their study that absorbance declined

from a maximum at 20 min. The higher concentrations of urea were the last to be read in the assays performed and hence had more time to undergo a temperature-labile reaction that causes a decline in absorbance. The change could happen despite all solutions being at 20°C during spectrographic reading. Solutions being measured in foam fractions were all below the 0.15 mmol concentration and were not in the curved part of the graph. No other reason is advanced for the non-linear effect at higher concentrations. Geyer and Dabich (1971) achieved a linear dependence with solutions up to 30 μg/mL (0.5 mmol). It is suspected that the age of the reagents was a major factor in the deviation from a straight line at concentrations above 0.15 mmol as observed in my study.

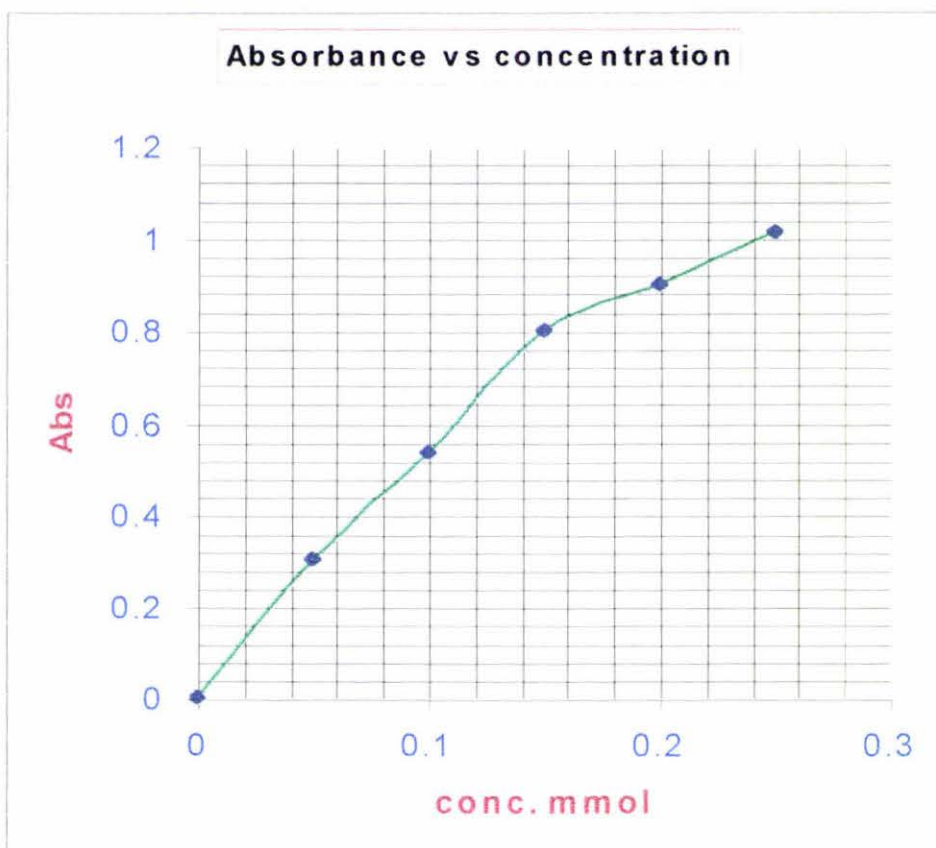


Figure 3.3.3 Standard Curve for TDMU absorbance with urea concentration

Readings were carried out using the UV/Vis spectrograph, plastic cuvettes, measured at 520 nm, 20°C. Urea solutions were from a reference stock of 0.03 g/mL (0.05 mmol). To diluted solution 0-0.5 mL was added 1 mL TDM and 1.5 mL acid reagent. The solution was incubated at 95 °C for 30 min, cooled 10 min at 20°C before reading.

TDM deteriorates with age. Patchett (1988) noted the development of a precipitate in the TDM reagent. He considered the reagent usable if it was less than one month old. The

presence of a precipitate was observed with the TDM reagent used in the urea assays in my studies. The reagent used in this study acquired a slight reddish tinge after a month of storage and was no longer usable. TDM was stored in a dark cupboard at room temperature during the assays carried out in this study. In retrospect TDM would be better made fresh every time it was used in an assay.

For each set of samples analysed a blank was performed, using TDM and acid reagent with no protein or urea present. The blanks gave very confusing results, with absorbance for the blank in one case being greater than the lowest urea solution measured in that assay. The source of these anomalous readings was unkown.

Geyer and Dabich (1971) reported in their study that changes in blanks need to be compensated for with each assay. I believe that compensating for blank absorbance by using the blank values would have given erroneous figures for urea in my data. Stock solutions were used to check each assay and provided a reference point for calculation of urea concentration of samples. Lack of time precluded further study of the blank solution anomaly. The uncertainty created by this was not successfully resolved in this study and more work is required to clarify the problem.

Stock solutions of urea 0.05, 0.1 and 0.15 mmol were analysed with each assay as known reference markers. Where concentrations of known urea solutions were measured these gave values by TDMU absorbance of within 0.005 mmol of the known value Table 3.3.3.

These values from the known markers suggest that the TDM reagent was only contributing a small amount of colour to the total measured colour at 520 nm, in contrast to that suggested by the blanks. The TDMU derived values for urea within foam fractions were indicative of a small decline in the urea concentration with foam fractionation, see section 5.3.

Absorbance	0.3044	0.5664	0.8672
Sample, known concentration, mmol	0.05	0.10	0.15
Calculated concentration, mmol	0.045	0.10	0.16

Table 3.3.3 Known urea concentration compared against measured urea concentration based on TDMU assay.

Readings were carried out using the UV/Vis spectrograph, plastic cuvettes, measured at 520 nm, 20°C. To urea 0.05, 0.10 and 0.15 mmol samples of 0.5 mL volume was added 1 mL TDM and 1.5 mL acid reagent. The solutions were incubated at 95°C for 30 min, cooled 10 min at 20°C before reading. The absorbance measured was converted to a concentration based on the standard curve to give the calculated value.

3.3.4 Flame emission

Assay 1 and Assay 2 show a similar pattern of concentration difference for the foam fractionation sample as shown in Table 3.3.4a. The actual values obtained in each assay varied for the same sample and do not agree. This assay method did not provide reliable data.

Foam fraction Sample	10.0	10.1	10.2	11.1	11.2
Assay 1, mg/mL	23	19.6	29.6	17.6	31.2
Assay 2, mg/mL	13.5	11.7	23.4	18.7	25.7

Table 3.3.4a Comparison of two assays

Foam fractionation experiments 10 and 11 were flame emission assayed after dilution 1:2000, a standard reference curve was carried out for each assay using sodium ion 0-10 μg. Foam fraction 10.1 is bulk liquid before foaming. Fraction 10.1 and 11.1 were foam fractions from experiments 10 and 11, fraction 10.2 and 11.2 were residue left after foaming.

In the foam fractions for experiment 10, readings showed a correlation between the flame emission and conductivity, Table 3.3.4b. The correlation suggests that flame emission or conductivity are valid measures of the ionic content of soluble wool protein solutions.

Foam fraction	10.0	10.1	10.2
Flame emission, Na^+ mg/mL	23	19.6	29.6
Conductivity mS/cm21.0	21.0	17.9	24.9

Table 3.3.4b Conductivity and flame emission comparability

Conductivity was measured at 20°C. sodium ion was assayed by flame emission after dilution 1: 2000. Ten flame emission readings were carried out for each sample. with the average value below 5% standard deviation.

3.3.5 BCA

In performing BCA assays problems were encountered with interfering chemicals. As tyrosine and tryptophan were not present in large concentrations in wool their effect on BCA assay was expected to be limited. Tris 0.1M and urea 6M were also noted as causing interference with the assay, however the concentrations used were recorded as causing only minor problems at those concentrations (Smith et al. 1985). The process used to solubilize wool was oxidative sulfitolysis, a process that will produce a range of ions capable of reducing Cu^{2+}. It is unlikely that many S-H or disufide bonds were still present in the soluble protein. S-Sulfonated wool is labile and $W\text{-}S^-$ would be able to reduce Cu^{2+}, Eqn 3.2

$$W\text{-}S\text{-}SO_3^- \rightleftharpoons W\text{-}S^- + SO_3^{2-} \qquad \text{Eqn 3.2}$$

Braun et al. (1989) noted that BCA assay should be used carefully and only in the absence of even mild reducing agents. Dissolution reagents, sulfite SO_3^{2-} and tetrathionate $S_4O_6^{2-}$ ions will be present in the dissolution mixture in addition to the probable presence of $W\text{-}S^-$. The SO_3^{2-} ion is capable of acting as a reducing agent, as an oxidation, potential E = 0.93 V, Equation 3.3.

$$SO_3^{2-} + 2OH^- \rightleftharpoons SO_4^{2-} + H_2O + 2e^- \qquad \text{Eqn 3.3}$$

$$Cu^{2+} + e^- \rightleftharpoons Cu^+ \qquad \text{Eqn 3.4}$$

The cupric ion reduction E^{o} = 0.153 V combined with oxidation of the sulfite is possible E_{Total} = 1.083 V, and could lead to raised values for protein present. Tetrathionate has a disulfide bond that can react with the cupric ion (Wells, 1962).

Tetrathionate ion

The BCA assay produced raised values for total protein when compared to BT and GPC total protein analyses as shown in Table 3.3.5.

Sample	BCA	Biuret	GPC
OS 2	48.4	10.1	6
OS 3	33.3	4.4	1.6
OS 4	39.9	8.0	5.4
Dissolution mixture	13.6	0.52	0

Table 3.3.5a Comparison of total protein by BCA, BT and GPC

Analysis was performed on three wool dissolutions and dissolution reagent mixture, procedure for each assay as detailed in methods, chapter 2.

The BCA gave very consistent reference curves. A standard reference curve was performed for every assay. Four different reference curves are shown in Figure 3.3.5. This was over a period of six months and shows excellent reproducibility and a linear response across the 0-100 μg range. Fresh BSA solution was prepared each time to avoid any deterioration from storage.

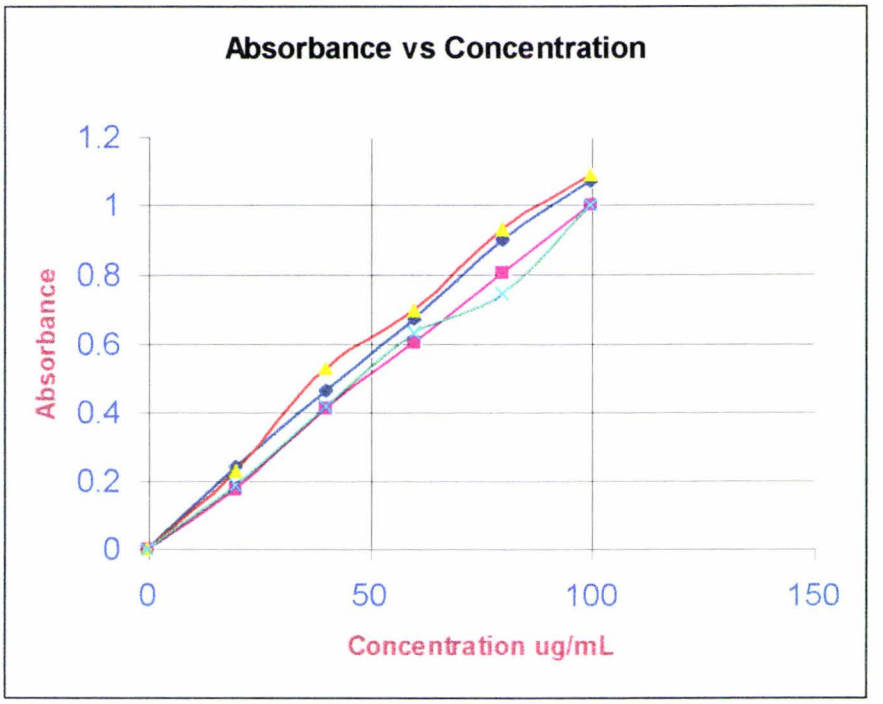


Figure 3.3.5 Absorbance of BCA complex against concentration.

Values obtained for four reference curves plotted on the same graph. The standard was BSA 100 μg/mL dissolved in water with sonication. Incubation was at 37°C for 30 min. Absorbance measured at 562 nm.

The BCA assays for wool dissolutions were very similar despite being several months apart using material that had been kept at -18°C as seen in Table 3.3.5b.

Sample	BCA 4	BCA 9	BCA 11
OS-2	48.4	48.4	51.8
OS-4	39.9	39.9	39.9

Table 3.3.5b BCA assay of dissolution mixes, 2 and 4.

BCA assay was performed on wool dissolutions using BSA as reference. Incubation was at 37°C for 30 min. Absorbance measured at 562 nm. BCA 4, BCA 9 and BCA 11 are assays carried out over a six month period.

The presence of a number of potential reducing agents effectively makes the BCA unreliable as a total protein assay for wool. Further analyses showed that GPC gave the most accurate total protein values and the BT assay was much closer when dissolution was more successful. The BCA was consistently 3-4 times greater than the BT. This result was despite reproducible values obtained with pure BSA used as the reference standard for the

production of a standard curve, see section 3.3.4 (Smith et al. 1985). I believe that the raised total protein BCA assay values are the result of the unreacted sulfite, tetrathionate ion and sulfonated wool reacting with the cupric ion. This discrepancy between the assays was the principal reason for abandoning the BCA assay in favour of the BT and later the GPC assays.

3.3.6 Biuret

The BT assay gave similar responses over the 6 months that assays were carried out, with consistent linear reference graphs as shown in Figure 3.3.6.

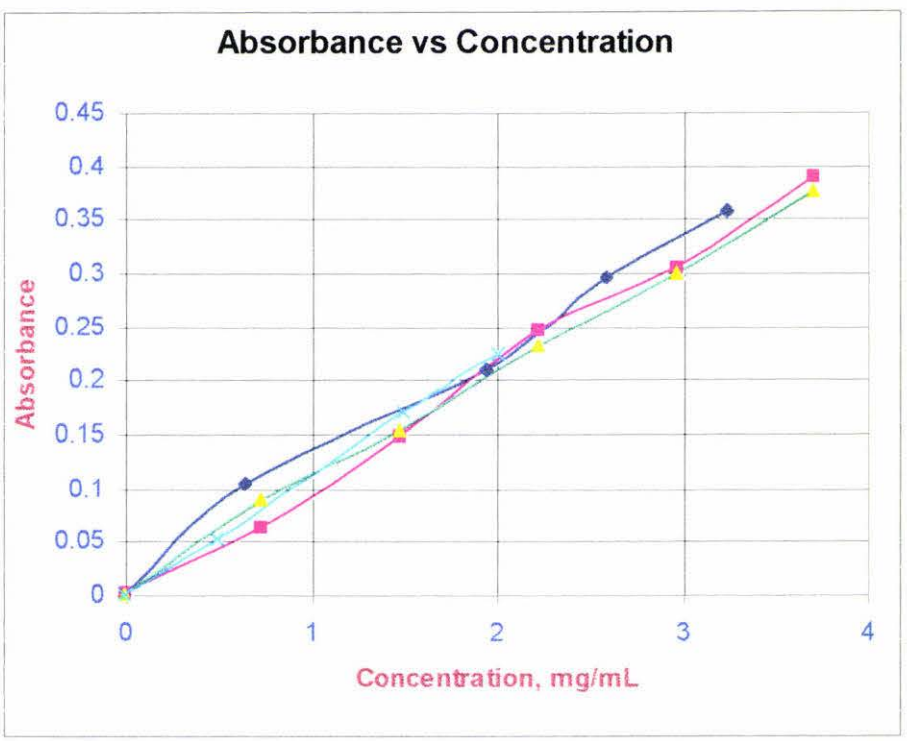


Figure 3.3.6a BT assay standard reference graph for BSA against concentration

BT assay was 0-100 µL of BSA 2.7-3.7 mg/mL, 0-100 µL water with 1 mL Biuret reagent, 25°C for 30 min, measured at 540 nm.

Constituents of protein solutions in general do not cause deviations from the protein-biuret concentration proportionality. Possible interference at high concentrations from Tris, ammonia, amines and glycerol was noted by Sapan et al. (1999). Urea can degrade to form cyanate and then react to form biuret (Means and Feeney, 1971). Biuret formation was not

anticipated given the need for mild acid conditions. To ensure that the dissolution mixture reagents were not influencing the BT reaction, an analysis of the reagents was carried out by the standard addition method of extra reagent to the dissolution mixture and subsequent BT measurement Table 3.3.6a.

	Mass reagent added	Percentage increase of reagent	Protein equivalent from added reagent
Urea	99.6 mg/mL	28 %	0.10 mg/mL
Tris	1 mg/mL	106 %	0.48 mg/mL
sulfite	2.5 mg/mL	74 %	1.1 mg/mL
tetrathionate	2.0 mg/mL	49 %	0.016 mg/mL

Table 3.3.6a Possible interference from reagents in the dissolution mixture.

To a wool dissolution mixture 1.20 mg/mL. that was diluted 1:5 was added the excess reagent in aliquots up to maximum given in the table. The percentage increase is the increase in reagent above that normally in the dissolution mixture. The protein equivalent is the increase in absorbance from the added reagent converted into an equivalent protein value.

Based on the results tabled in Table 3.3.6a, sulfite ion has the most effect on the BT assay, with urea being the next most affecting chemical. Dissolution reagents are partially consumed in reaction with wool and therefore the concentration of any reagent is unknown without assay specifically for that reagent. A BT assay of dissolution reagents gave 0.52 mg/mL protein equivalence, this is in line with later results that showed the BT assay to give slightly raised values compared to GPC. The dissolution reagents were not deemed to cause a significant difference in the assay as indicated in the Table 3.3.6a. Higher values for total protein with BT were believed to be partly due to increased viscosity from the added salt causing an increase in absorbance rather than any increase in protein.

The possibility that unused reagents were responsible for the increase in measured absorbance compared to GPC, was disproved by the assays of reagent addition, which showed no large increase in measured absorbance. No immediate reason is advanced for this anomaly of slightly raised absorbance using the BT assay for total protein.

Initial wool protein dissolutions did not achieve the 50% dissolutions of later processing, and when assayed for total protein were found to overestimate the protein content when compared to GPC values. Later assays of total protein showed good comparability between BT and GPC as shown in Table 3.3.6b.

	OS 2	OS 4	OS 5	OS 8	FF 12.0	FF 12.2	FF 13.2
Biuret Assay mg/mL	10.1	8	25	25.25	26.8	12.38	6.76
GPC assay mg/mL	6.07	5.4	24.1	24.7	25.9	14.73	6.16

Table 3.3.6b Comparison of BT and GPC assays of total protein

GPC was performed using Sephadex G-15 with a flow rate of 0.8 mL/min, 100 µL inject of soluble protein sample diluted 1:10 with water, phosphate/KCl/AcCN buffer, pH 9, UV measurement at 200 nm. BT assay was 1.0 mL of diluted 1:5 protein solution with 1 mL Biuret reagent, 25°C for 30 min, measured at 540 nm. OS were oxidative sulfitolysis wool protein dissolutions and FF were foam fractions.

The earlier OS 2 and OS 4 experiments were both very poor dissolutions and gave an over estimate of total protein. Unused reagent would be present in these two experiments. It is unclear what has been responsible for the over estimation in OS 2 and OS 4 for the BT assay. The foam fraction obtained in experiment FF12.2 was the only solution that gave a higher GPC than BT result. In all other cases the BT was always slightly higher than the GPC.

BSA compared to HSA as reference protein

BSA was used as the standard reference for all BT assays. BSA was compared to Human Serum Albumin (HSA) was performed. HSA and BSA have the same response to the BT assay, Figure 3.3.6b.

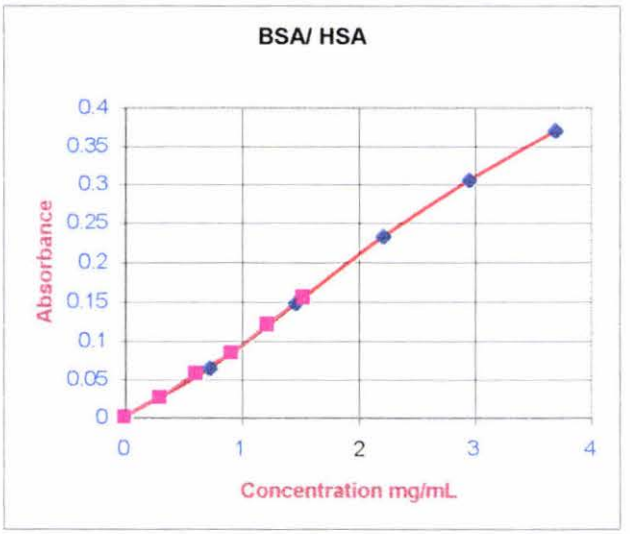


Figure 3.3.6b BSA compared with HSA

BT assay was 0-1.0 mL of -◆- BSA 3.71 mg/mL, and 0-1.0 mL -■- HSA 1.54 mg/mL solution with 1 mL Biuret reagent, 25°C for 30 min, measured at 540 nm.

BSA was not examined in the study by Goshev and Nedkov, but it could be deduced from the response of HSA that BSA will give a higher absorbance at the same concentration as wool protein. This would tend to lead to BT assay giving higher total protein values than GPC as observed in the dual assays performed. The wool in the Goshev and Nedkov study was oxidised in comparison to the reduced state of oxidative sulfitolysis used in this study. In comparing the BT assay and GPC it must be recognised that they are based on different techniques. GPC measures the contribution to UV at 214 nm absorbance of all peptide bonds in the protein plus side chain carbonyls, and double bond entities. BT assay measures the absorbance of 540 nm light by a complex involving the peptide bond. However not all peptide bonds may participate in the complexation reaction. The difference in the total protein values may be due purely to the difference in what the 214 and 540 nm wavelengths measure.

3.3.7 Gel Permeation Chromatography

Buffers

Potassium chloride and acetonitrile (AcCN) were selected as buffer components to diminish hydrophilic and hydrophobic interactions and aggregation (Smyth and Fitzgerald, 1997).

The interactions of charged groups on the protein with the dextran media and interactions leading to protein-protein aggregation were less likely to occur if KCl and AcCN were used. Potassium chloride acts to keep the buffer chaotrophic, acting as an ion-pairing agent to diminish interactions of charged protein and hence keep the protein soluble (Baker, 1985). AcCN dissolves without reaction in water, does not increase the viscosity, has a chaotrophic effect, and reduces hydrophobic interaction, all of which are distinct advantages compared to other organic solvents.

The combination of measuring absorption at 214 nm and pH 9 meant some buffers such as boric acid/potassium chloride were unsuitable. The absorption of potential buffers, Tris, Tris/CHES, 2-aminoethanol, dihydrogen phosphate/KCl was measured over the 190-300 nm range. The pH 9, 0.1M phosphate/KCl/20% AcCN buffer showed low absorption in the 200-220 nm range, with the protein remaining soluble and appearing unaffected by either the phosphate or the salt level. The buffer had a molar absorptivity of $\epsilon_{\lambda 214}$ was 10 $cm^2.g^{-1}$ at 214 nm compared to the dissoluted wool $\epsilon_{\lambda 214}$ for wool protein 34,300 $cm^2.g^{-1}$. The difference ensured that absorption measured was due to protein in the solution and not the GPC buffer.

Superdex 75

This produced a trace with three main peaks at 220 nm. Correlation to known MW markers suggested a major portion of the protein around the 45kD mark, a very large amount of absorbing material in the low peptide/amino acid MW range and a well separated, absorbing salts peak. The wool protein showed a pronounced peak that corresponded to ~45 kD at 280 nm. This could be interpreted as indicating that tyrosine and tryptophan are concentrated in the proteins at this MW. The smaller late peak, is believed to be individual amino acids, as it occurs just past the main peak observed in the 220 nm graph. Regrettably instrument failure precluded further investigation.

Sephadex G-75

While the G-75 would have been expected to show some broad groups of the wool protein classes, the results were disappointing. Two distinct peaks were joined by a broad un-differentiated band Figure 3.3.7a. The time taken (~1 hour) to run each sample was a major disincentive given the number of samples to run. The operating pressure for the 30 cm column could be increased to get a faster elution time, but at pressures greater than 1 Pa compaction of the column was evident. Sephadex media is a soft gel, other size permeation media were not investigated in my study.

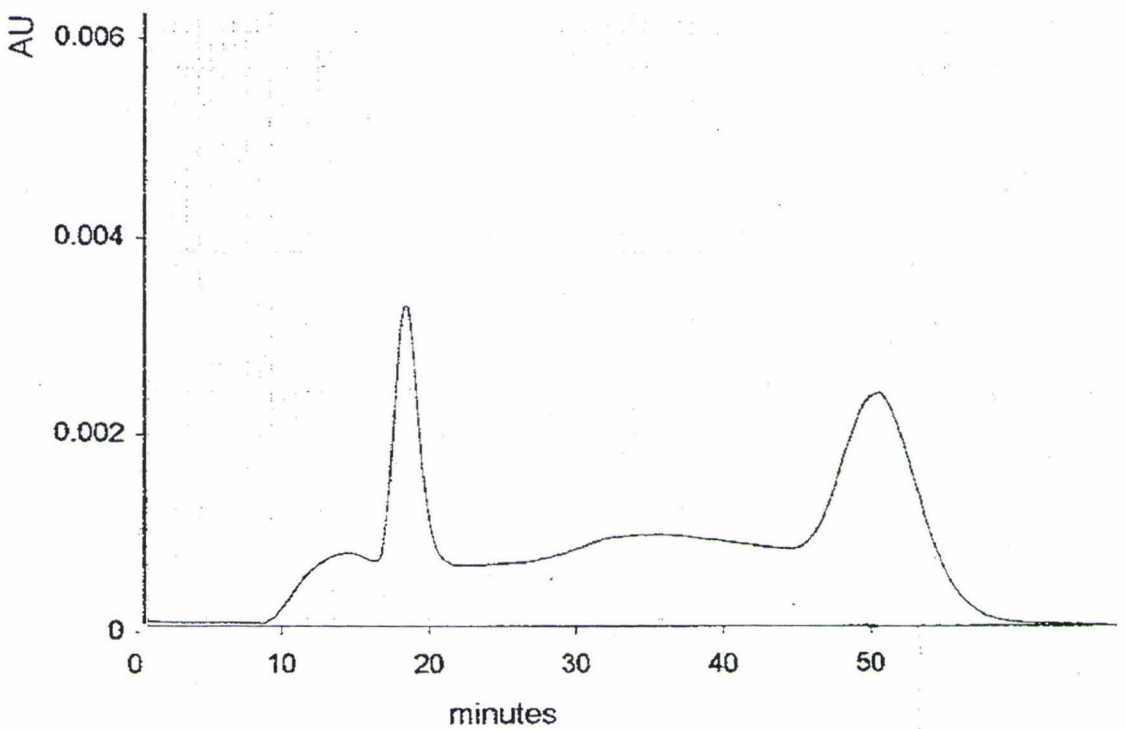


Figure 3.3.7a Sephadex G-75 of dissoluted wool protein.

Dissoluted wool protein 2.3 mg/mL. GPC was performed on Sephadex G-75, using 100μL injection, flow rate with 1.0 mL/min, 0.1M phosphate/KCl/20% AcCN buffer.

Protein Degradation

Samples were stored at 4°C, to limit changes due to cyanate-protein reaction and slow alkali degradation. Maclaren and Milligan (1989) discuss the effects of mild alkali treatment, with the formation of lanthionine and consequent loss of solubility. The rate of protein degradation was unknown in the wool dissolution reactions carried out in this study. Oxidative sulfitolysis shows very little protein damage in 2-D electrophoresis

(Bryson, 2000). The risk of micro-organism growth was considered to be minimal at pH 9 and urea (6-8M), no indications of biological decay were detected in samples assayed.

The original dissolution solution used had urea, sulfite, tetrathionate ions and Tris, all of which absorb at 214 nm, Table 3.3.7a. Tetrathionate has a high absorption and it was critical that this was separated from the protein for total protein measurement.

Reagent	Tris	urea	tetrathionate	sulfite
Absorptivity, L./g.cm	0.52	0.038	22.94	1.43

Table 3.3.7a Absorptivity for wool dissolution reagents at 214 nm.

Reagents were dissolved in water intially at the concentration found in the dissolution mixture and then diluted to give an absorbance reading of less than 1. All readings were performed in a quartz cuvette.

The Sephadex G-15 gave clearly defined peaks for BSA, EA, CA and BV protein. The protein peak eluted at 6.7 ± 0.1 min (chart recorder 25 cm/60 min) when dissolved in water, Table 3.3.7b. The dissoluted wool protein and foam fractionation protein elution times of 6.8 ± 0.1 min were consistent for all samples.

Molecular Weights		66,000	45,000	29,000	2050	
Protein	Dialysed Wool	Bovine Serum Alb	Egg Albumin	Carbonic Anhydrase	Bee Venom	Soluble Wool
Elution Time, min	6.7	6.7	6.7	6.6	6.8	6.7

Table 3.3.7b Elution times for protein samples

Buffer, 0.1M phosphate/KCl/20% AcCN buffer, pH 9, flow rate 0.8 mL/min, Sephadex G-15.

Identification of the Peaks from Sephadex G-15 GPC

Dissoluted wool and foam fractions consistently presented four distinct peaks with G-15. The elution time for a series of proteins was compared to establish that the first peak observed in Figure 3.3.7b was due to wool protein. The proteins shown in Table 3.3.7b

exhibited similar elution times. Bee Venom MW 2050 D, eluted in the same time as the larger proteins in contrast to the triglycine which eluted at 10.8 min and a mixture of single amino acids of serine, valine, glutamic acid and alanine eluted at 12.0 min. The second peak eluting at 11.6 min Figure 3.3.7b, could contain small peptides or individual amino acids from the dissolution process. The Superdex 75 supports this contention with a peak well separated from the main protein peak, in the region where species of MW 100-500D would be expected to elute.

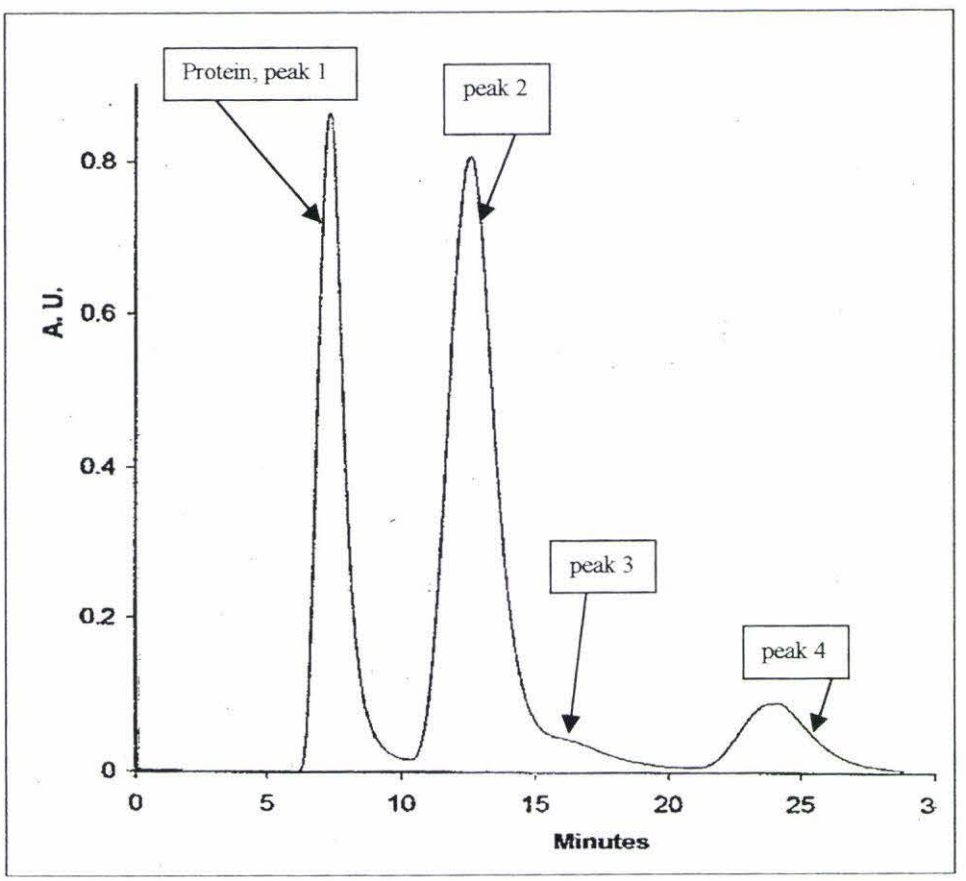


Figure 3.3.7b GPC of dissoluted wool using Sephadex G-15.

Sephadex G-15, 0.8 mL/min, 100 μL inject of dissoluted wool sample diluted 1:10 with water, 0.1M phosphate/KCl /20% AcCN buffer, pH 9.

The BCA assay indicated that only the first and second peaks contained protein material. However the positive protein result in peak two may be attributable to dissolution reagents reacting with BCA. The BCA assay indicated protein concentrations of 14.7 mg/mL in fraction 1 and 3.6 mg/mL in fraction 2, with fractions 3 and 4 gave nil protein values. The BT assay of the original sample was determined at 18.7 mg/mL. Addition of the fractions 1 and 2 gave 18.3 mg/mL which compares well to the BT assay. Both the BCA and BT are

assays for the amide bond and will react to any amide bond, whether in a protein or a small peptide. An elemental analysis of the first and second peaks was inconclusive possibly due to the presence of the buffering salts. Protein was not expected in the third and fourth peaks given the larger elution time of 15 ± 1 and 22.5 ± 1 min respectively for these broad peaks in Sephadex G-15. A BCA assay on the third and fourth peaks showed no protein present. GPC was performed on individual ions used in the dissolution mixture. BSA was added to each ion to give a constant marker. The elution times for the ions has allowed tentative assignment of peaks 2 and 3, Table 3.3.7c. Some reagents were used up by the solubilization process, tetrathionate forms S-sulfokerateine. Tetrathionate disproportionates to form thiosulfate ion, a reaction that is self-catalysed by the presence of thiosulfate, thus leading to an increasing concentration of thiosulfate. The second and third peaks were composites of several absorbing species, a key species being thiosulfate, which absorbs particularly strongly at 214 nm. No assignation was determined for the fourth peak.

Ion	Elution Time (min)	Peak Assignation
sulfite	12.6, 15.1	peaks 2 & 3
thiosulfate	11.6	peak 2
Tris	13	peak 2
urea	16.2	peak 3
tetrathionate	19.8	no peak

peak 1	6.8	protein peak
peak 2	12	sulfite/thiosulfate
peak 3	16.1	urea
peak 4	24	unassignable

Table 3.3.7c Elution times for the reagent ions and peaks performed with G-15.

Ions and the four peaks observed in the wool dissolution mix. OST 8. All GPC flow rate of 0.8 mL/min. 100 μL inject of sample diluted 1:10 with water, 0.1M phosphate/KCl /20% AcCN buffer, pH 9.

Using the G-15 Sephadex column, consistent, reproducible results in peak height, peak area and elution time were obtained. Peak area for 10 consecutive loadings of the same sample of dissoluted wool gave a mean value of 542 ± 5.5 mm^2. A set of 10 runs, 5 each day gave a peak area difference of 1.3% between the means for each day (535 ± 15.7, 542 ± 8.1 mm^2). The consistency engendered a high level of confidence in the readings. The

reproducibility of the GPC meant that seldom were more than two runs performed for a sample. The elution time for protein was reproducible over a range of proteins and over time as illustrated in the Table 3.3.7d.

	Run 16	Run 92	Run 157
Elution time, min.	6.78	6.84	6.84

Table 3.3.7d. GPC elution times for the first protein peak of wool dissolution extracts.

Sephadex G-15 GPC of dissolution mixes using flow rate of 0.8 mL/min, 100 μL inject of dissoluted wool sample diluted 1:10 with water, 0.1M phosphate/KCl/20% AcCN buffer, pH 9.

Protein Reference for GPC

Three proteins, BSA (MW 66,000), EA (MW 45,000) and CA (MW 24,000) plus dialysed wool were assessed for use as a reference for determination of the protein concentration to peak area ratio in mg/mL, Table 3.3.7e.

Protein	BSA	EA	CA	Dialyzed Wool
Protein: peak area ratio	0.0215	0.0225	0.0177	0.0291

Table 3.3.7e Protein mass to peak area measured using Sephadex G-15 GPC.

GPC at flow rate of 0.8 mL/min, 100 μL inject , BSA, EA, CA diluted 1:10 with water, dialysed wool dissolved in running buffer, 0.1M phosphate/KCl /20% AcCN buffer, pH 9.

Differences observed in the protein mass to peak area ratio can be accounted for by the differing number of carbonyl groups in the proteins, which vary according to the total number of amino acids, and amount of glutamine and aspaagine in the protein. Different protein wool classes have different amino acid composition, and concentration or elimination of a protein class will change the absorbance being measured. Ideally a known sample of the protein being measured is the best reference for establishing the mass to peak area ratio (Harris and Angal, 1989; Holme and Peck, 1993). The dialysed sample of S-sulfonated wool protein that had been freeze-dried and re-dissolved constituted a reference

that was closest to the dissoluted wool proteins being measured. This value was used for all total protein calculations using GPC.

The BT assay showed good agreement with the G-15 GPC protein result, Table 3.3.7f. The BT assay was reported as showing less interference from dissolution chemicals than the BCA assay (Sapan et al. 1999).

	OST 5	OST 7	OST 8
GPC, mg/mL	26.6	24.1	24.7
Biuret, mg/mL	23.3	25.0	25.3

Table 3.3.7f. Total protein assayed by GPC and by BT.

GPC conditions, Sephadex G-15 using flow rate of 0.8 mL/min, 100 μL inject of dissoluted wool sample diluted 1:10 with water, 0.1M phosphate/KCl /20% AcCN buffer, pH 9. BT assay was 0-100 μL of BSA 2.7-3.7 mg/mL, 0-100 μL water with 1 mL Biuret reagent, 25°C for 30 min, measured at 540 nm.

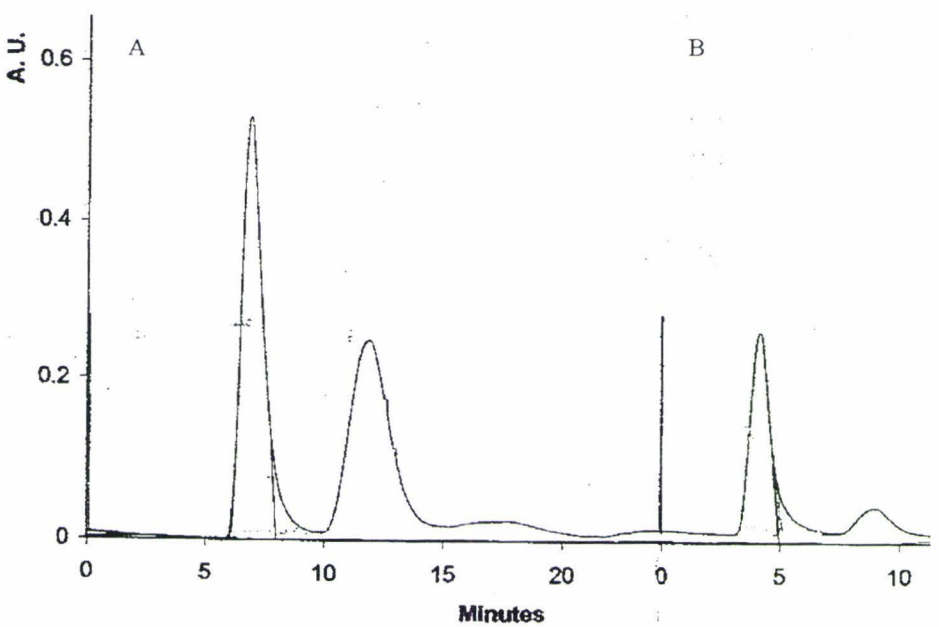


Figure 3.3.7c. GPC of wool protein solutions showing low absorbance and peak tailing

GPC was performed on Sephadex G-15 at flow rate of 0.8 mL/min, 100 μL inject of sample diluted 1:10 with water, 0.1M phosphate/KCl /20% AcCN buffer, pH 9. A. Foam sample of dissoluted wool 0.84 mg/mL showing first and second peaks. B. Filtrate after ultrafiltration desalting.

Measurement of the first peak recorded with G-15 GPC revealed peaks that were asymmetric. All peaks showed a small degree of tailing suggesting some diffusion effects on the column. If the first peak was small due to low protein levels, the error inherent in the measurement of peak area was increased as the peak tail fits less into the triangle of measurement used for all measurement, Figure 3.3.7c A, B. The method of measurement was described in Section 2.14 and is based on the height times the half height width. To reduce the potential error, material was loaded on the column in sufficient concentration to ensure that the first peak was between 0.300-1.0 AU. Where the sides of the peak slope were more than 10° from vertical, inexact measurement of the half peak width could result.

3.4 Conclusion

The uncoated fused silica capillary performance was consistent and showed reproducibility, both in peak time and in the shape and number of peaks on the spectrograph. There is a need to improve the CE parameters to get individual peaks. More study of the effect of the parameters of buffer, zwitter ions, and solution co-ions is needed. CE is still developing and future work may open opportunities for better operation and hence better results relevant to the work carried out in this study.

Uncoated fused silica capillaries do not perform adequately at pH > 8 due to the silanol being in a silanoate form. Coating by inclusion of polymers PEG or dextran in the buffer is not tenable with pH > 7. Fused silica walls of the capillary may have been degenerated by the alkaline conditions used in this study. Wool proteins present a particular problem with their insolubility at pH < 8 and the similarity of the proteins in the dissoluted wool. The aim of separation of protein by size was not achieved in this study. The best buffer used was the Tris/CHES combination. Further study needs to be carried out with a covalently coated capillary before CGE of wool proteins can be dismissed as an untenable method for size characterisation and analysis. The success of researchers in this field suggests that with the correct parameters separation can be achieved with further, perhaps considerable study.

It is possible that a more suitable urea assay for such high urea concentrations could be developed.

Further work is required with flame emission to return a result of more certainty.

The BCA assay is reliable and consistent for BSA and presumeably for other low cystine content proteins. The BCA assay was not accurate for wool dissolutions. From my study I would not recommend the BCA assay for use with wool dissolution mixtures because of interference from potential reducing agents.

The Biuret assay is suitable for total protein analysis of soluble wool protein solutions. The BT assay gave reliable results for well dissoluted wool protein. The total protein values using the BT assay were generally 1-5 % higher than those obtained by GPC. The BT was time consuming and cumbersome to use with the small number of samples (4-8) from either the dissolution process or foam fractionation experiments.

Sephadex G-15 performed well as a desalting procedure. In light of the inadequate separation of wool protein classes that was achieved, further analysis of the wool protein classes cannot be recommended using Sephadex G-75.

The resolution of the permeation chromatograph was not as informative about wool protein classes as 1-D SDS-PAGE.

GPC using Sephadex G-15 proved to be reproducible over several hundred runs. Wool proteins remained soluble in a 0.1M phosphate/KCl/20% AcCN buffer, pH 9. No detailed investigation was carried out on different buffer strengths or on suitability of alternative size exclusion chromatographic media. If this method was contemplated for routine analytical laboratory use further investigation aimed at better resolution of all proteinaceous material, and wool protein class separation is warranted. A shorter assay time should be achievable, certainly desirable. Use of short higher quality columns as used in Smart systems may provide the separation desired.

CHAPTER FOUR

Extraction of Keratin

4.1 Wool Dissolution - Introduction

Wool is insoluble in water and is very slow to degrade in the natural environment. Any attempt to achieve soluble wool proteins will require breaking of the disulfide bonds as described in Section 1.3.1. An aim of this study was to examine the different solubilizing methods and assess their value for industrial use. The study was confined to consideration of reductive processes and did not examine oxidative or enzymatic methods. Oxidative sulfitolysis was chosen for consideration in more depth after a preliminary investigation of different reductive methods.

Maceration is an essential process for the efficient extraction of wool protein. The retention of soluble protein in the wool fibres after dissolution was noted by (Harrap and Gillespie, 1963) and represents a potentially diminished yield of soluble protein. The extraction of these proteins as well as the penetration of reagents into the wool is considerably enhanced by severe disruption of the wool fibre by maceration.

4.1.1 Reduced Wool Protein by Sulfide (S^{2-}) and Hydrogen Sulfide (HS^-) Ion

Disulfide bonds are reduced by monothiols, but the equilibrium constants are near unity, necessitating the use of large excess of monothiols to force the reactions to completion (Means and Feeney, 1971). The mechanism as reported by Means and Feeney proceeds by two sequential nucleophilic reactions with the cystine, giving mixed disulfide intermediates (Eqn 4.1, 4.2).

$$WSSW + RS^- \rightleftharpoons WSSR + WS^- \qquad \text{Eqn 4.1}$$

$$WSSR + RS^- \rightleftharpoons WS^- + RSSR \qquad \text{Eqn 4.2}$$

The reaction is pH dependant, the pKa of S^{2-} is 12.89, making conditions for dissolution with sulfide very alkaline. A high pH or prolonged time at raised pH leads to protein degradation and an increase in lanthionine and lysinoalanine (Maclaren and Milligan, 1989; Means and Feeney 1971). Thiol groups of reduced wool proteins undergo rapid oxidation unless protected by inert atmosphere or excess reducing agent (Thomas et al. 1983; Kelly 1998).

4.1.2 Reduced Wool Protein by Thioglycollate Ion

Gillespie (1964) reported extractions of wool protein at high yields of 91% using urea-thioglycollate. The dissolution reaction gives a mixture of cysteine and wool-thioglycollate disulfides (Eqn 4.3).

$$WSSW + HS\text{-}CH_2\text{-}COO^- \rightarrow WS^- + WS\text{-}S\text{-}CH_2\text{-}COO^- + H^+ \quad \text{Eqn 4.3}$$

$HS\text{-}CH_2\text{-}COO^- K^+$ potassium thioglycollate

Reaction by thiols requires a pH > 10 which introduces the potential for alkaline protein degradation.

4.1.3 Sulfitolysis and Oxidative Sulfitolysis

The focus of the solubilization investigation was narrowed to oxidative sulfitolysis after preliminary studies of both reduced and oxidised extraction methods. Difficulties in maintaining a reducing environment while processing the dissolution mix favoured the sulfonate derivatised product. Soluble wool protein in the form of S-sulfokerateine is stable in air and can be kept for several months without apparent deterioration.

This pathway to soluble wool has lead to the cuprammonium solubility test, which assays the degree of chemical modification of wool (Maclaren and Milligan, 1989). Swan (1961) reported yields of 70-80% solubilization in 24 hours using cuprammonium-sulfite in urea (8M). The S-sulfokerateine can be precipitated by isoelectric precipitation or by salting with ammonium sulfate.

$$RSSR + 2SO_3^{2-} + S_4O_6^{2-} \rightarrow 2S_2O_3^{2-} + 2RS\text{-}SO_3^- \quad \text{Eqn 4.4}$$

A product of this conversion is the thiosulfate ion. The production of thiosulfate from TTN has implications for the stability of the tetrathionate, which is decomposed to trithionate and pentathionate, by catalytic action of thiosulfate (Kurtenacker and Kaufmann, 1926).

Oxidative sulfitolysis was reported in the literature with varying yields, 40-80%. Lower yields may relate to the stability of the TTN ion rather than the oxidative sulfitolysis reaction. The S-sulfokerateine shows stability as the anion in an alkaline environment up to pH 11, with intact protein classes apparent from radio-active labelled SDS-PAGE (Kelly, 1998). As a protecting group, S-sulfokerateine is easily removed by thiols. Solubilization and reconstitution of wool proteins is thus attainable without modification of the wool protein.

4.1.4 Tetrathionate Synthesis

TTN was reported as thermally unstable and unable to be stored for more than a few weeks (Means and Feeney, 1971; Heslop and Robinson, 1963).TTN was synthesised according to Eqn. 4.5. (Wells, 1962).

$$2S_2O_3^{2-} + I_2 \rightarrow S_4O_6^{2-} + 2I^- \quad \text{Eqn. 4.5}$$

Reaction occurs as the iodine in ethanol is added slowly to the thiosulfate. Several possible mechanisms have been proposed, based on the triiodide ion and possible formation of iodo-thiosulfate intermediate (Brasted, 1961; Gould, 1955). Due to the instability of the TTN ion, reaction is carried out at neutral pH, TTN decomposing in alkali according to Eqn 4.6. However decomposition in neutral solutions also occurs as in Eqn. 4.7 (Kurtenacker and Kaufmann, 1926). TTN can also undergo numerous sulfur species rearrangements making fresh preparation necessary.

$$2S_4O_6^{2-} + 6OH^- \rightarrow 3S_2O_3^{2-} + 2SO_3^{2-} + 3H_2O \quad \text{Eqn. 4.6}$$

$$2S_4O_6^{2-} \rightarrow S_3O_6^{2-} + S_5O_6^{2-} \qquad \text{Eqn. 4.7}$$

4.1.5 Wool Treatment Prior to Maceration

There is potential for disruption of the wool fibre by the absorbed water becoming frozen and hence fracturing the fibre. A more broken and open structure should allow better penetration of reagents and better extraction of proteins trapped in the wool fibres. Proton magnetic resonance studies show that all the water in wool is mobile and not bound solely to hydrophilic groups in the protein or lose to move. If the amount of water in this mobile system can be increased then reagents will move more readily through the fibres.

Air dried wool has a variable water content of ~9-11% which is related to humidity changes (Maclaren and Milligan, 1981).Exposed to 100% humidity, wool can hold up to 30% in weight of water (Maclaren and Milligan, 1981).

4.1.6 Urea, Cyanate and Wool Damage

Urea decomposes slowly to form ammonium cyanate. Cyanate can chemically modify amino groups of proteins (Eqn 4.8) (Maclaren and Milligan 1981).

$$P\text{-}NH_2 + NH_4NCO \rightarrow P\text{-}NH\text{-}\underset{\underset{O}{\|}}{C}\text{-}NH_2 + NH_3 \qquad \text{Eqn 4.8}$$

P = protein

$$(NH_2)_2CO \overset{\text{heat}}{\leftrightharpoons} NH_4^+ + OCN^- \qquad \text{Eqn 4.9}$$

urea cyanate

Sulfhydryl, imidazole, tyrosyl and carboxyl groups of proteins also react with cyanate, but do not form stable products. Urea decomposes on storage to form ammonium cyanate Equation 4.9, Pace (1986) reports that a 8M urea solution will contain 17 mmol at equilibrium at pH 7.

4.2 Experimental

Maceration of wool fibres was performed for most dissolutions. Two methods were used to achieve macerated fibres, a high speed spinning set of blades (WRONZ) used for sulfide and thioglycollate experiments, and an Ultra-Turrax chopping head operated as described in Section 2.4.

Dissolution experiment were carried out as detailed in the methods in Section 2.4.

4.3 Results and Conclusions

A preliminary investigation of the methods of solubilizing wool was carried out using sulfide, thioglycollate, 1,4-dithiothreitol, and cupra-ammonium as reduction processes and tetrathionate as oxidative sulfitolysis.

4.3.1 Sulfide Reduction

Wool was reduced by sodium sulfide in 3 hours using a macerator giving a 67% yield of dissoluted wool protein. A water-jacketed, 25°C, closed vessel with nitrogen gas passing through the head-space ensured that re-oxidation was kept to a minimum during dissolution reaction. Tangential flow filtration of 8 circulations of the filtrate through a 1 kD filter gave a pH 12 after 40 min operation.

This represented a very small lowering of the pH from the starting material. Lowering the pH < 10 resulted in protein precipitation. Despite the high pH, a slight smell of H_2S was evident after 40 min suggesting that some hydrogen sulfide hydrolysis was taking place, pKa of HS^- is 7.02. A disadvantage of tangential flow filtration was the foaming that occurred at the filtrate outlet as it exited into the re-circulating solution. The tangential flow filtration was carried out in the air, without any N_2. This aeration as it progresses through the filter membrane negates the efforts made to get an inert environment during the dissolution reaction, allowing some re-oxidation. A closed system would have avoided some of the opportunity for re-oxidation of the soluble wool protein. Thin films made from reconstituted wool protein and up to 5% polyethylene glycol (PEG) poured into Petri

dishes showed a progression to softer and more pliable films as the PEG percentage increased. In addition it was noted that the greater the percentage of PEG, the less translucent the film became. PEG is a plasticiser and may provide cross-linking that is longer and hence more flexible than the cystine linkage of pure reconstituted wool (Tillin et al. 1977). Flanagan (1998) found that wool protein reduced by Na_2S that had been freeze-dried, was soluble as a 10% solution after storage for one day, but failed to re-dissolve after storage for one month in the fridge or dessicator. This suggests that slow re-oxidation has happened when stored. An alternative explanation is that the formation of lanthionine crosslinks has prevented re-solution of the wool protein. Efforts to repeat the experiment without the macerator and using NaSH failed to achieve significant dissolution, yield < 10%. NaSH did not give the reactivity required and the reaction mixture needed addition of hydroxide to achieve the pH > 11 of the S^{2-} solution in the experiment above.

SDS-PAGE of the S^{2-} dissolution mix indicated that protein groups were widespread across the molecular weight range of 14 to 60 kD, rather than discreet groups. The ratio of dissoluted wool protein to liquor is a favourable 1:20 compared to other dissolution techniques with higher ratios of wool to liquor. Further work undertaken at WRONZ indicates that re-oxidation and hence loss of the ability to cross-link is a major problem in reconstituted protein material (Kelly, 1998).

4.3.2 Thioglycollate Reduction

Three hours maceration of wool provided a foamed mixture that easily separated to undissolved wool fibre and filtrate. A pink colour was detected in the solution. This was attributed to a reaction of the iron in the stainless steel and the thioglycollate ion (Kelly, 1998b). The residue filtered readily over 100μm wire. This was in contrast to earlier dissolutions that had clogged paper filters. Solutions were however very viscous as a result of the 8M urea. Any scale up of dissolution will require a simple and rapid separation of the residue wool fibres. The pH was lowered to pH 4.4 with an acetic acid/acetate (1.5M) buffer. Precipitation started at pH 8.5 and continued with the major precipitation occurring between pH 5.5 and 4.5 the latter corresponding to the iso-electric point range of the type I intermediate filament proteins. The protein was very globular in gross appearance and curd-like, it required minimal centrifuging to settle. Salting of the supernatant left after iso-

electric precipitation to pH 4.4 provided a small amount of precipitate that was globular and readily floated on the surface. Analysis on 1-D SDS-PAGE showed all major wool classes present with no specific separation by isoelectric precipitation. The salted precipitate had good purity of HSP and UHSP, but was 7% of a theoretical 26% yield.

The ratio of wool to liquor for thioglycollate extraction was 1:20. While extraction by thioglycollate gave a yield of 46%, the ratio of moles of reagent to wool is high. Wool protein 12 g required a large liquid waste volume containing urea 8M, acetate buffer (500mL 1.5M), and ammonium sulfate 60 g for precipitation. Waste disposal would be a predictable environmental problem, should this process go to industrial scale. Recovery of the dissolved reagents may be possible, but again the dilution factor and volumes involved would add to costs. Thioglycollate dissolution is I believe uneconomic unless the end product has unusually high value. The preferential dissolution of different wool protein classes by thioglycollate is not seen to offer extraction and fractionation in one step (Kelly, 1998a).

4.3.3 Reduced Wool Protein by 1,4-dithiothreitol (DTT)

To attain a reference of dissoluted wool for comparison of different methods, wool was reduced using DTT in the presence of urea 8M with an apparent dissolution of most of the material after 24 h. Analysis of the product by CE gave confusing results with the reagent and the dissoluted material showing similar traces on CE. Subsequent to this a wool dissolution using oxidative sulfitolysis was carried out with good yields. Oxidative sulfitolysis was chosen as the method to study further and in view of the need to work with material similar to the reference sample, freeze-dried extracted wool protein obtained by oxidative sulfitolysis was used in preference to the DTT material. No further work was carried out using DTT.

4.3.4 Sulfitolysis by Copper Ammonium and Sulfite Ion

Experiments on the dissolution of wool produced 56-60% yields of dissolved wool protein if 1 g wool per 200 mL of reagent mix was used. This yield increased marginally to 64% if 2 g of wool was used in 200 mL of reagent mix. The residual wool fibres proved difficult

to filter, with No. 1 Whatman paper unable to drain the viscous liquid. Trials of 8M urea compared to 4M urea achieved yields of 60% and 30%. confirming the observations by Swan (1957) "that high concentrations of urea were needed to get penetration of the reagents". Protein was precipitated from the dissoluted wool mixture (1g wool in 200 mL mixture) using ammonium sulfate with a 50% recovery of dissoluted material. Removing all the copper (II) required extensive washing with water while on the filter. The reconstituted protein had a grey, brittle, hard texture that softened rapidly on wetting. In the dry form it adhered strongly to glass, but was easily removed from plastic Petri dishes. No purity was established for the reconstituted protein.

To effect precipitation of 0.6g protein, 65 g of ammonium sulfate was used, the recovery of this from the filtrate liquid mixed with urea and sulfite reagents was not feasible and would represent a huge cost and waste problem.

4.3.5 Oxidative Sulfitolysis using Tetrathionate Ion

The S-sulfokerateine formed in oxidative sulfitolysis is very stable and can be stored for weeks without any visual deterioration or any change as measured by GPC for total protein. The oxidative sulfitolysis extraction uses 1:20 of wool to liquor ratio, with a mass ratio of 0.6:1 TTN to wool. Sigma (1999) gives the price of TTN as $230/100 g. The use of TTN remains an expensive procedure at A$ 1.38/1 g of wool processed. Using an industrial scale production of TTN would reduce the price considerably, but the ratios of liquor to wool and reagent to wool are still high compared to other processes. The high urea level is a problem if it is unable to be recycled. Urea presents a low value bulk fertiliser as waste material and could be expensive to dispose of and to transport.

4.3.6 Tetrathionate Synthesis

Difficulty encountered in getting soluble wool protein using the laboratory potassium tetrathionate ($K_2S_4O_6.2H_2O$) (TTN) plus a delay in supply of TTN lead to sysnthesis of TTN in the laboratory.

Synthesis of TTN gave a yield varying from 26-66%, and differing quality as determined by colour of the product and the reagent reactivity on wool dissolutions. During the addition of thiosulfate to the iodine, the colour of the mixture faded from the dark iodine colour to a pale yellow, several batches gave a stronger yellow colour and this was subsequently attributed to sulfur formation. As the solubility of sodium iodide is 184 g/100 mL water, the volume of the mixture could ostensibly be reduced by 80% before precipitation of sodium iodide occured. In practice the volume decrease was found to be counterproductive with the formation of considerable amounts of sulfur and sulfate resulting. TTN is thermally unstable and rotavap reduction of the mixture at 20°C created decomposition conditions and production of a mix of trithionate, pentathionate, thiosulfate, sulfate and sulfur. The first precipitate formed on reaction was used in preference to the second precipitate formed after refrigeration for 24-48 h. The second precipitate had a more crystalline appearance and when used for dissolution the yield of dissolved wool protein was reduced from the average 56% down to 11%! This suggested that it contained considerable amounts of other sulfur anions. The first product obtained was white and on dissolving had no traces of insoluble sulfur in contrast to the second precipitate which always showed some sulfur present and had a yellow colour which increased with time despite storage at 4°C, also noted in the synthesis by (Kurtenacker and Kaufmann, 1926).

A refinement of the synthesis was devised and a yield of TTN in the 40-50% range was achieved with reliable dissolution of wool following its use.

- Reaction carried out in an ice bath, 0-1°C.
- Dropwise addition of cooled thiosulfate, completed within 30 min.
- Minimal solution needed to get the thiosulfate to dissolve in water with concentration of 0.33 mol/ 100 mL, and iodine in ethanol 0.099 mol/ 100 mL.
- Immediate filtration and suction drying with transfer of precipitate to dessicator stored at 4°C. (Freeze drying was used as an alternative option to the dessicator).
- TTN stored at 4°C or below and used within three weeks.
- Cool filtrate to -18°C for 48 h., filter precipitate and re-crystallise with ethanol/water.

4.3.7 Water Content of Wool

To account for the individual differences in water content, wool was always sampled prior to reaction and the dry wool mass adjusted accordingly. Average percentage and standard deviation for moisture in wool was 10.1 ± 0.99 % for 10 samples, Table 4.3.7. The included moisture in wool is insufficient to give fracture of fibres on freezing. Uptake of water is postulated to occur if wool is soaked in water and/or urea before freezing, thus increasing the chances of physical shattering of the wool. Wool soaked and then left to drain can retain up to 30% of its mass in water (Maclaren and Milligan, 1981).

Sample No.	1	2	3	4	5	6	7	8	9	10
% Moisture	11.6	11.4	9.9	9.8	11.1	10.8	9.2	8.6	8.9	10

Table 4.3.7 Percentage of moisture in air dry wool.

Wool, Romney-crossbred mix, reference number 3J WRONZ, kept at room temperature and exposed to air was sampled during a 12 month period. Samples of ~ 1 g were dried in the oven at 80°C for 24 h to a constant weight.

4.3.8 Pre-maceration Treatment of Wool

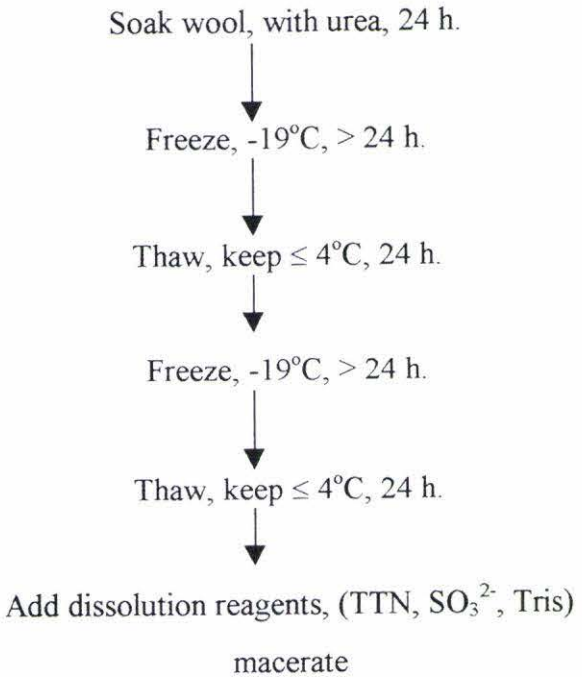
Two treatments of cleaned wool prior to maceration were compared,

a. freeze water-soaked wool, thaw, soak in urea 4M and water

b. soak in urea 4M and water

The effect was to increase the yield of protein extracted by oxidative sulfitolysis from 21% to 32%. The wool fibre soaked and frozen for 24 h (a) showed little visual change with no evidence of swelling in contrast to that seen when soaked in urea (b).

A further trial with a freeze regime repeated twice on soaked wool, then solubilized as per the oxidative sulfitolysis process with 2M urea gave a low yield of 10%, emphasizing the critical nature of the urea for expansion of the fibre and penetration of reagents. An improvement on total yield from 32% to 51% was made by following the regime in Scheme 4.3.8. Most dissolutions cited used urea as a denaturing agent. Considerable swelling and softening of the wool fibre was observed with increasing urea concentration.



Scheme 4.3.8 Treatment of wool prior to oxidative sulfitolysis

Wool from Romney-crossbred mix, reference number 3J WRONZ, was treated as shown above prior to conventional work up by oxidative sulfitolysis, TTN method, Section 2.4.

The freeze-thaw regime was carried out in 24 h blocks, but the same effect could be accomplished in a shorter time with a freeze of 1 h and a thaw lasting 3-4 h. Keeping the urea cold during the soaking lessened the likelihood of cyanate damage to the wool protein. The fibres were visibly swollen when soaked first in urea and were more easily broken up by the Ultra-Turrax machine, giving a smooth lose pulp within the first 1-2 min of maceration starting.

Adding all the reagents and urea prior to freezing gave an improved yield from ~50% to 61%, this yield was the best achieved for oxidative sulfitolysis during this study, Table 4.3.8. It was anticipated that the initial protein concentration of the solubilizing solution would be higher due to the urea swelling the wool fibre, allowing penetration of the reagents, which react during the thaw periods. The final yield would be expected to be the same, reached however in a shorter time. To explain the anomalous higher final yield, it is postulated that wool fibre having undergone some oxidative sulfitolysis in the thaw phase, has a more open structure and better overall extraction is thus achieved. In comparing residual fibres after dissolution, it was noted that experiments B, C, D gave finer, more paste-like material than in the case of the urea only pre-treatment.

Wool Treatment	A	B	C	D
Percentage Dissolution	51	61	59	58

Table 4.3.8 Percentage dissolution of freeze/thaw regime

Four experiments A, B, C, D, using Romney-crossbred mix, reference number 3J WRONZ, were carried out prior to conventional oxidative sulfitolysis, TTN method. Experiment A involved soaking with urea, followed by a freeze regime, reagents being added after the freezing regime. Experiments B, C, D involved soaking with urea and reagents before carrying out the freezing regime.

A trial using wool that had been soaked according to experimental procedure A, Table 4.3.8, indicated that dissolution released 25% of the soluble protein obtained within 20 minutes.

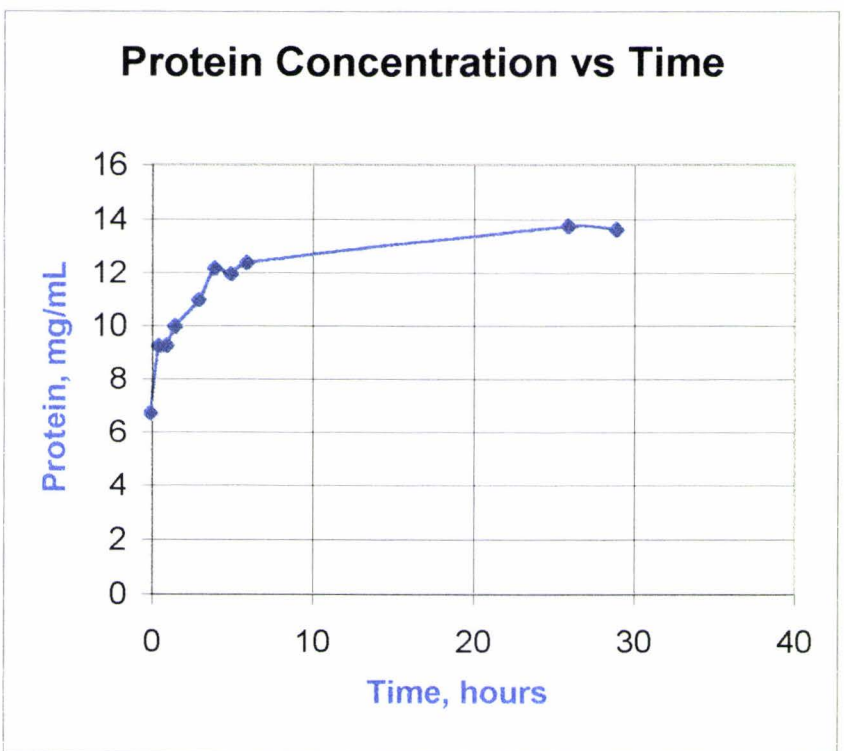


Figure 4.3.8 Protein concentration change with time during solubilization.

Dissolution carried out using oxidative sulfitolysis with TTN/sulfite on 10 g of Lincoln wool. Protein concentration determined on Sephadex G-15 GPC, 0.8 mL/min, 100μL injection, 0.1M phosphate/KCL/20% AcCN buffer.

Release of protein after 2 hours was constant for the next 18 h, this effect would support the differences reported by Maclaren and Milligan (1981) in the reactivity of cystine bonds. The last 3 hours saw an increase in protein release, at this stage the maceration procedure

was repeated and this would appear to have helped in release of protein from the fibres. In view of the increase on restarting maceration, it is anticipated that maceration over a longer period such as 0-8 h would have given better protein release.

The sequence followed in experiment D, Table 4.3.8, was sampled at intervals up to 29 hours, plotted in Figure 4.3.8. Protein was present at time 0 hours as a result of the action of reagents and wool during the thaw phases of pre-treatment of the wool. After 4 hours, 89% of the total protein extracted was in a soluble form.

The freeze, thaw regime advances the extraction of protein from the wool fibres, sufficient that maceration for 30 sec every 15 min over 5 h results in a yield of over 90% of the total extracted dissoluted protein. Further time spent processing the wool does not give significant gains, to offset the costs of operation of machinery.

4.3.9 Urea, Cyanate and Wool Damage

The concentration of cyanate can be expected to be low at the start of the solubilization if the urea is freshly made as recommended by (Geyer and Dabich, 1971). However the process adopted of soaking and then freezing followed by thawing will prolong the time the urea is in contact with the protein and hence the opportunity for protein damage from cyanate reaction. Heating the urea solution can also increase the production of cyanate. To counteract cyanate reaction with wool and decomposition of the tetrathionate all solubilizations were carried out in an ice bath. Urea can have cyanate removed by pouring through an anion exchange. The degree of cyanate formation from urea is very low in the time span the wool is exposed to urea, and does not warrant further treatment to remove the cyanate ion.

4.3.10 Cost Analysis of Solubilization Reagents

On cost sulfide is the cheapest option at $0.06 of sodium sulfide reagent per 1 g of wool processed. The comparison of costs assumes that the reagent is the major cost of chemical and that other reagents such as urea can be recovered and recycled, an assumption that has not been investigated in this study.

Reagent	sodium sulfide	dithiothreitol	cuprammonium /sulfite	thioglycollate	tetrathionate/sulfite
Ratio of wool to liquor by mass	1/10	1/100	1/200	1/20	1/30
Ratio of wool mass to reagent moles	100g/0.26mol	100/0.5	100/10	100/2	100/0.2
Cost of reagent $/g of wool processed	$0.06	$8.65	$2.71	$0.97	$1.13
Urea added	none needed	8M	8M	8M	8M

Table 4.3.10 Comparison of wool to liquor, wool to reagent, reagent cost per gram of wool.

The table compares the five main dissolution methods. (a) Wool mass to mass of liquor volume is compared as performed in the experiments. (b) The wool masses to reagent moles are compared per 100 g of wool used. (c) The cost of reagents per gram of wool processed is compared. Yields varied, for the purposes of this comparison yields were assumed to be equal. Reagent used for comparison in cuprammonium/sulfite is sodium sulfite as major reagent.

The cost of the other reagents added in each solubilization method were not included, hence the table is not exhaustive. Combining the low cost of sulfide reagent with the wool to reagent ratio of 1:0.6, plus the apparent lack of need for urea favours the reductive extraction of wool protein. Reduction also gives the best wool to liquor ratio of 1:10.

4.4 Conclusions

In the authors view there is no clear best technique for solubilization. If intact protein classes are required then alkaline degradation and instability to air make reduction by sulfide less suitable. Milder reductive techniques all have greatly increased costs and wool to liquor ratios. Yields for some solubilizations are high, but the ratio of wool to reagents is in many cases vastly more than stoichiometric proportions as shown in the ratio of mass of wool to moles of reagent in Table 4.3.10.

The problems to be overcome with sulfide reduction are the readiness of the kerateine group to re-oxidize and the high pH used. Oxidative sulfitolysis delivers intact protein that is stable in air, but has high ratios of reagent to wool and liquor to wool, that create large

cost barriers. The removal of reagents and urea is a downstream problem for the oxidative sulfitolysis method. The problems encountered with the reoxidation of sulfide reduced proteins also applies to the thioglycollate method.

Problems were encountered in the cuprammonium/sulfite reaction that preclude its use on a commercial scale. The residual undissolved wool was hard to filter. The ratio of wool to liquid is 1:200, requiring unsustainable amounts of liquid in any scaled up process. Oxidative sulfitolysis using cupra-ammonium/sulfite is suitable only as an extraction method for small scale analysis of wool structure and has limited industrial value. Usage in biomaterial manufacture of medical implants or similar high valued purpose-specific end-use may make this method worth further investigation.

Maceration appears essential to any wool protein solubilization, with 4 hours of maceration appearing adequate for removal of over 90% of the dissolvable protein. A freeze-thaw programme involving soaking in all the reagents is recommended to aid the separation of protein from the fibre and shorten the dissolution time. No study was carried out on the freeze-thaw regime with sulfide reduction, as the yield for this method is already 84%. The prolonged attack on amino acids and peptide bonds by the alkaline conditions may be too severe if intact proteins are required. More study is required if reduction is chosen as the technique to ascertain the appropriateness of such a regime.

An operating temperature of $\leq 4^{\circ}C$ is advantageous with oxidative sulfitolysis using TTN, given the instability of TTN and the presence of urea. A lower temperature may be desirable with other methods, reducing the protein damage from prolonged alkali treatment.

All processes using oxidative sulfitolysis require urea and hence are susceptible to cyanate degradation of protein. The impact of urea degradation on the wool is worthy of investigation only if prolonged exposure to urea is anticipated.

Separation of the macerated fibre from the solubilized wool mixture was a problem in the laboratory scale operations. Centrifugation was the method used in the studies reported here. On an industrial scale, centrifugation or a rotating drum filter may offer a solution to

the problem (Krijgsman, 1992). Neither method of separation of liquid-solid was investigated in this study. The presence of urea in the soluble wool mixture with the attendant swelling of the fibres makes a very viscous mixture that resists drainage. Adding water reduces the swelling and helps plasmolyse the protein from the fibres, but increases the total liquid volume as well as decreasing the concentration of protein. Investigation of the value of plasmolysis instead, or in addition to fibre separation needs to evaluated further.

Based on this study the sulfide method appears to offer opportunities for development within a reducing environment, ahead of the oxidative sulfitolysis method.

CHAPTER FIVE

Foam or Bubble Separation Techniques

5.1 Introduction

Wool protein classes can be separated by isoelectric precipitation and salting-out. However the quantities of salt used to accomplish the precipitation combined with the wool protein product being a solid are detractors to this method. The aim of investigating foam fractionation was to retain the product in a soluble form for on-going processing. Separation of non-foam components from proteins was one of the prime objectives of this study of foaming. The initial rationale for performing foam fractionation was that it may provide a method for the extraction and concentration of protein from the solubilized wool protein solution. Foaming of desalted mixtures was undertaken as part of this study.

5.1.1 Protein Foam Fractionation

Protein surface activity is dependent on the protein's physico-chemical characteristics of size, charge and hydrophobicity. These factors combined with solution conditions of ionic strength, pH, presence of salts and other additives lead to a complex situation. Noble et al. (1998) noted that the most critical characteristic for separation of mixed protein solutions appears to be the closeness of the pI of the proteins. Noble et al. (1998) based their study on very different origin proteins with similar pI values. Wool proteins possess pIs 8 - 4.5, ranging over the protein classes (IFP, HSP, UHSP, HGTP), hence foaming is not expected to give clear separation of wool class proteins. However the type IFP's are grouped in a relatively narrow size and pI band, therefore they possibly could be separated under conditions favouring their characteristics. Hydrophobicity is also a key factor in foaming. Wool proteins from the same class, may differ in pI, but may share a similar hydrophobicity response. Hydrophobicity may offer a characteristic that enables separation to be carried out in foam fractionation.

Foaming can increase the denaturing of protein. This is a desirable action when foaming dissoluted wool, particularly as one of the desired outcomes of foaming is the desalting of the dissoluted wool. During foaming there is a risk of precipitation in the foam as a result

of the salt and urea concentration decreasing as the protein concentrates at the gas/liquid interface. Precipitation of protein is less likely to occur when the protein is denatured (Janson and Ryden, 1989). There is a balance for the protein of loss of denaturing salts and urea with the denaturing effect of the foam liquid/gas interface.

The creation of stable foam allows for the concentration of protein. Liquid carried in foam up a column drains from the adjoining bubble films into the plateau border regions at the intersection of the three bubbles, Figure 5.1.

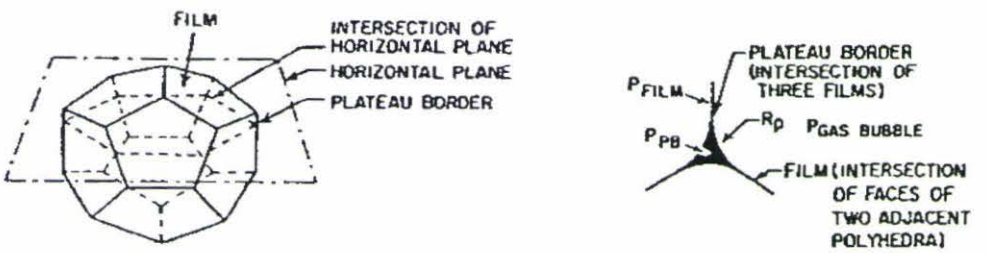


Figure 5.1.1 Idealized bubble cross-sectional view

Bubble boundary showing the adjoining films of three bubbles. Liquid between the bubbles drains into the intersecting plateau border. Bubbles are hypothesised to be regular polyhedrons with relatively flat adjoining surfaces and curved plateau borders (Brown et al. 1990)

The intersecting plateau borders are at a lower pressure than the pressure at the film surfaces pressure, thus liquid is drained rapidly (a few milliseconds) from film surfaces into the plateau borders. This accumulated plateau border liquid drains by gravity back to the bulk liquid via adjacent plateau borders and coalescing bubbles (Brown et al. 1990). Interstitial liquid carried over with the foam is an important non-foam component (Lemlich, 1972). The liquid will lower the enrichment and may affect the equilibrium at the gas-liquid interface as a result of ionic-protein interaction (Liu et al. 1998). Ionic components will also be involved in non-specific adsorption, in the complex mixture of dissoluted wool, this adsorption interaction will be difficult to predict.

The performance efficiency of foam fractionation can be measured in terms of the enrichment of the foam, separation of the protein from the residual liquid, and percentage recovery of protein from the bulk solution (Brown et al. 1999). These values are defined as follows:

$$\text{Enrichment ratio} \quad E_r = \frac{\text{protein concentration in foam } C_f}{\text{protein concentration in initial bulk liquid } C_i}$$

$$\text{Separation ratio} \quad S_r = \frac{\text{protein concentration in foam } C_f}{\text{protein concentration in residual bulk liquid } C_r}$$

$$\text{Percentage recovery} \quad R_p = \frac{\text{mass of protein in foam } m_p \times 100}{\text{mass of protein in initial bulk liquid } m_i}$$

An additional concept, the separation yield (Y) combines separation of protein by foam and the volume of liquid required to achieve the separation.

$$\text{Separation Yield} \quad Y_s = \frac{\text{concentration of foamate X volume of foam liquid}}{\text{concentration of bulk liquid X volume of bulk liquid}}$$

The generation of preferential adsorption of protein at a gas-liquid interface is a prerequisite for a satisfactory partition of proteins by foaming (Liu et al. 1997). A study by Brown et al. (1999b) found that foaming gave good enrichments and recovery, but very little separation of different types of proteins, rather concentration of the mixture itself. Lemlich (1972) identified a number of interacting factors that influence the selective adsorption and concentration at the gas-liquid interface, draining of the foam as it ascends, residence time in the bulk liquid, pH and physico-chemical characteristics of the protein.

Uraizee and Narsimhan (1996) showed that operation of successful foaming technique required manipulation of the following key parameters.

- pH: protein adsorption with foaming is often at a maximum around the pI of the protein.
- Gas velocity: increasing the gas flow gives an increase in recovery, but lower enrichment.
- Foam height: as foam travels up the column, bubbles drain and coalesce, hence achieving better enrichment with increasing height of column.
- Bubble size: enrichment increases with increasing bubble size, larger bubbles collapse more readily leading to foam enrichment. Large bubbles present less surface area for protein adsorption in the bulk liquid and carry less mass and hence lead to lower percentage recovery.

- Bulk liquid concentration: increasing the concentration of the bulk liquid leads to a decline in enrichment and a slight improvement in percentage recovery until the critical micelle concentration is reached and no further enrichment occurs.
- Column size: gravity determines the optimum ratio of height to width for a column, height versus width of column shows an asymptotic relationship.

5.1.2 Factors Influencing Foam Fractionation

pH

The surface tension reaches a minimum at the point where pH ≅ pI of the protein. At the pI the sum of charges on the protein will be neutral, the protein is at its most hydrophobic point and has its lowest solubility in aqueous media (Krijgsman 1992). Surface tension reflects the adsorption of protein at the gas-liquid interface, however Liu et al., (1997) found that some proteins exhibited different behaviour. Surface tension reached a minimum at the pI for BSA, but remained at a plateau as the pH increased past the pI for hemoglobin. Similar surface tension-pI anomalies were reported by Hossain and Fenton (1998) using a range of milk proteins. Casein, α-lactalbumin and β-lactoglobulin showed a change of surface tension with pH, yet sodium caseinate, BSA and chymotrypsinogen A were observed to have only small changes with pH change. Brown et al. (1990) noted that the pH effects on proteins when foaming, may be masked by the change in bubble size as the pH moves away from the pI. Large bubbles are associated with higher surface tension and decreased adsorption of protein. It was also reported by Brown et al. (1990) that concentration and ionic strength can have an overriding effect with maximums for E_r being observed further from the pI as the concentration of protein increased to 5 mg/mL. In batch foaming operations the pH may change as time progresses and the change in pH will affect protein adsorption. This can be monitored and the pH adjusted if desired. Foam is generally more stable at the pI of the protein, hence foam may not coalesce as readily when foaming occurs at the pI of the protein. The lack of caolescence may lead to lower enrichment at or near the pI of a protein. Proteins vary in their stability at their individual pIs. Some proteins will aggregate at their pI, creating additional problems in foaming. There does not appear to be an easy rule as to which pH is best for foaming, rather experimental work is required for each protein to determine its specific best working pH.

Gas Flow Rate and Bubbles

The residence time of bubbles in the bulk liquid affects the enrichment and separation ratios. Enrichment $E_r > 60$, can be achieved, but due to the low gas flow required to achieve high E_r, the R_p will be very low. Studies show that the partitioning of the protein between the bulk liquid and the bubble interface takes a finite time to equilibrate (Lemlich, 1972; Uraizee and Narsimhan, 1996; Prokop and Tanner, 1993). A bubble generated at the base of the liquid (20 cm deep) in a column was observed to take an average of 3 sec to reach the foam/liquid interface at the 20 cm mark. Lemlich calculated that lactic acid dehydrogenase took 68 sec to achieve equilibrium in an initial solution of concentration of 3.69 mg/mL. Retaining a bubble in bulk liquid for 68 sec was not practical in the experimental apparatus used, however using a tall column did increase the time spent by the bubble in the bulk liquid and hence improved the partitioning of the protein. Maximizing the time of residence of the bubble in the bulk liquid is important to approach the equilibrium partitioning conditions. Placing a short internal cylinder around the sparger inside the column with the consequent re-circulation of the bubbled bulk liquid before bubbles reach the foam/liquid junction has been shown to improve E_r (Liu et al. 1997). Consideration should be given in any industrial scheme to a bubble loop system. Bhattachajee et al. (1997) examined casein and BSA solutions and reported that the foam surface concentration increased linearly with bubble bulk liquid residence time over a 5 sec time span. Their graphs all show experimental results that could be interpreted as suggesting a slowing of concentration increase with time. From an equilibrium perspective, a decreasing mass of protein will transfer to the surface of the bubble as time progresses and the surface concentration approaches equilibration.

Gas flow affects the time spent in the bulk liquid and the size of the bubbles. High gas flow results in larger bubbles. Increasing the gas flow decreases the adsorption time, decreasing the E_r. Chai et al. (1998) using sweet potato extract in a 1 L cylindrical column found a distinct change in foam stability with unstable foams forming with superficial gas velocities > 2 cm/sec. At high superficial gas flows the turbulence and back mixing creates high shear forces that lead to coalescence in the foam column. Fast travel of foam up a column gives insufficient time for drainage and desired enrichment. The same pattern observed by Chai et al. was reported by Brown et al. (1990) albeit at lower gas

velocities. Hossain and Fenton (1998) reported four milk proteins as showing a decrease in enrichment with increasing gas velocities, however two milk proteins examined showed no change. The behaviour cited by Hossain and Fenton appears to be contrary to that recorded by other authors in the field.

Sparging Gas

Sparging involves the passage of a gas through the small apertures in a frit submerged in a liquid. The gas used for all foaming experiments except one was nitrogen. Compressed air was used to check for differences in performance of wool dissolution mix. Liu et al. (1998) found that over a range of pH (3-9) there was a distinct difference in enrichment between air, nitrogen and carbon dioxide. Air was deemed to be the least effective and this was attributed to oxidation effects, although air caused considerable variation with pH change. Carbon dioxide was the most effective sparging gas and the buffering effect of the dissolving carbon dioxide was perceived to be influencing the protein recovery. In the case of wool proteins no major difference was anticipated given the use of a blocked thiol in the form of S-sulfokerateine.

Foam/Bubble

The surface area of the plateau border is 1/1000 of that of the films, but the plateau border is the repository of most of the entrained liquid. Given the low surface to volume ratio of plateau borders, it is assumed that once the bubble leaves the bulk liquid, negligible protein adsorption occurs (Bhattacharjee et al. 1997). Brown et al. (1990) and Hossain and Fenton (1998) noted that increased protein concentration in the bulk liquid leads to higher levels of protein being adsorbed, but also gives a more stable foam, thus decreasing the drainage from coalescence. High protein adsorption lowers the film surface tension, which assists the drainage of liquid to the plateau border zones from the increased pressure gradient between film and plateau border. Consequently higher protein concentration i.e. 25/30 mg/mL could be conducive to better drainage from plateau borders. There is a balance between the stable foam giving increased drainage from pressure differences and the foam coalescence giving enrichment. The smaller bubbles provide a larger overall surface area for adsorption and hence attain better adsorption levels. Bigger bubbles drain better leading to higher protein concentrations. The ideal is for small bubbles in the liquid

that readily breakdown to give large bubbles that drain well in the foam stage (Chai et al. 1998). Brown et al. (1990) noted that it was found necessary to clean the sparger after every foam fractionation, due to the build up of protein at the surface.

Bulk Liquid Concentration

An increase in the concentration of protein in the bulk liquid, creates a more stable foam. Noble et al. (1998) found that E_r declined from 10 to 2, for an increase in concentration of BSA from 0.08 to 0.5 mg/mL. Conversely the R_p increased from 60% to 90% over the same range. A more stable foam and lack of coalescence leads to more entrained liquid in the foam. The same pattern of increasing R_p and decreasing E_r was observed for β-casein (Brown et al. 1998) and for BSA (Uraizee and Narsimhan, 1996). The foaming of a mixture of proteins presents additional problems. Sarkar et al. (1987) noted that at higher concentrations extraneous proteins other than the desired protein, concentrate in the foam, leading to poor purification. This is of significance with respect to foaming of a dissolved wool solution, which contains similar protein families, family members and isoforms within the four main protein classes of very different composition and structure.

Dilution prior to foaming of the starting bulk liquid solutions has been cited frequently in literature as providing improved protein enrichment for foam fractionation, with values of E_r of > 60 (Hossain and Fenton, 1998; Brown et al. 1990; Brown et al. 1999a; Uraizee and Narsimhan 1996; Noble et al. 1998).

Refoaming

Foaming is a technique with potential for protein separations. Re-foaming of a foamed solution may allow for increased protein concentration and reduction in the salt concentration of the foamed product. No cited literature references were found relating to re-foaming or to observed changes in the salt content of bulk liquids and foams.

Desalting of Bulk Liquid Prior to Foaming

Studies of foam fractionation cited in the literature relate to specific protein solutions that have been dissolved in water or dilute buffers. The wool dissolution process involves using 6-8M urea, 0.1M Tris, 0.1M sodium sulfite and 0.1M TTN resulting in high salt concentrations.

Of interest was the concept that the surface activity of the protein was affected by the ionic content of the dissolution mixtures. Almost all of the protein solutions cited in the literature were made from pure protein dissolved in water, hence free of salts and possible protein-ionic interaction. The high urea concentration up to 8M created a solution that was very viscous. The high viscosity dissolution mixes were slow to circulate past the filter membranes. An additional difficulty lay in the protein insolubility as the pH decreases. The effect of desalting on the pH of the protein solution was investigated. Brown et al. (1990) reported that E_r increased as the ionic concentration decreased. Conversely the R_p declined as the ionic concentration decreased, with a dramatic improvement in R_p at ionic strength > 0.1M.

5.1.3 Salt, Proteins and Desalting

Ultrafiltration by a stirred pressure cell and by tangential cross flow was examined with the products subsequently foamed. The efficiency of the two systems was assessed in terms of time, protein recovery and effective desalting. Desalting by ultrafiltration is a slow process and depends on the ability of the ultrafiltration membrane to discriminate between molecules of differing mass and shape and in some cases is aided by molecular properties. Both systems relied principally on pore size of the holes in the membrane for sieving of the dissolution molecules and retention of the larger protein molecules. The membranes used were not equal in their MWCO sieving capacity and so a definitive comparison was not made between tangential flow and pressure cell filtration for wool proteins.

For dissolution mixtures at pH ≥ 8 the proteins present a net negatively charged surface. When these proteins are suspended in an electrolyte solution they attract positive ions to form the 'Stern layer' of counter ions. Next to this is a more diffuse 'Gouy-Chapman layer'

of mobile counter ions (Krijgsman, 1992; Janson and Ryden, 1996). As protein molecules approach each other the double layer, Stern and Gouy-Chapman layers will repel at the same time as the hydrophobic areas will be attracted causing alignment of the hydrophobic zones of the proteins. Precipitation is a result of the interaction of these forces. Adding salt to the protein solutions restructures the water around the proteins and allows the hydrophobic regions to combine inter-molecularly, resulting in precipitation. Salt levels in desalted solutions were too low for any salting out effects and the predominant influences become the charge on the protein and the increased hydrophobic interaction at low urea levels. Urea is a bifunctional hydrogen donor that disrupts the intra and inter molecular hydrogen bonding in protein. Low urea levels will allow the inter-molecular hydrogen bonds to form, promoting aggregation of the protein molecules (Esaka et al. 1995, Green and Jorgenson, 1989).

Foam fractionation uses the chemical surface activity of proteins to get accumulation at the gas-liquid interface. When protein molecules are removed from the solution and brought into close contact the hydrophobic interaction will overcome any localised electrostatic repulsion on the protein to get precipitation. The gas-liquid interface offers a unique additional option for aggregation of the protein molecule. Lining up of the protein molecules at the interface would enhance the hydrophobic bonding opportunities. Ionic components in desalted solutions are in low concentration and as the pH declines will place many overall neutrally charged proteins adjacent.

5.1.4 Foam Fractionation on an Industrial Scale

Foam fractionation has been successfully used in laboratory scale operations to separate protein from starch (Prokop and Tanner, 1993). Industrial separation of protein has been a goal of foaming technique for quite some years, but has meet limited success due to the lack of knowledge about protein solution properties (Liu et al. 1997).Lemlich (1972) noted that multi-component separations can sometimes be achieved using tall columns at very low gas flow rates. Selection of molecular parameters for protein differentiation by foam fractionation is not possible by theoretical considerations alone (Liu et al. 1997). However, more progress will be achieved if more data of a fundamental nature is available, particularly in terms of the gas-liquid interface and the complex influence of ionic strength and pH on surface tension of proteins (Prokop and Tanner, 1993). Noble et

al. (1998) notes that there are currently few reported studies of protein recovery/separation from multi-component solutions using foams. There is a need for more research in the area of foaming if it is to realise its potential in the field of separation and recovery of proteins in biotechnology. As an increasing emphasis is placed on the production of bio-engineered products, separation from other components will be an integral part of the total process (Brown et al. 1999a and 1999b).

5.2 Experimental

Experimental methods were followed as detailed in Section 2.14. To carry out the wool protein foam fractionation, experimental parameters were kept within a narrow range where appropriate.

- Precipitation of wool protein occurs at pH < 8, thus pH ≥ 8 was used with dissoluted wool-foam experiments except the experiments in Section 5.3.1. Precipitation at pH < 8 was particularly apparent if the concentration of protein was > 1 mg/mL.
- Gas flow rates were set at a low level, 6.7 - 14.1 mL/min to maximize the E_r, with foaming continued over several hours to improve the R_p.
- Foam fractionation experiments were usually performed in a 30 cm x 35 mm ID column of 300 mL capacity, unless otherwise stated. These dimensions were close to optimum for laboratory scale operation as reported by Liu et al. (1997). Thus at the onset of foam extrusion from the spout and starting with 200 mL of bulk liquid, the volume ratio of bulk liquid to foam was 2:1. The ratio did not include the spout attachment which increased the foam volume by ~ 30 mL.
- The sparger was cleaned in NaOH 0.1M after every experiment and tested for uniformity of bubbles before use.
- No literature cited parameters for viscosity, temperature or ionic strength, so no best practice was established. The temperature was kept in the 20-25°C range. Ionic strength and viscosity varied with each experiment.

5.2.1 Operation of the Foam Column

No foaming agent was used with the dissoluted wool, as it foamed readily at concentrations ≥ 1 mg/mL. The dissoluted wool has a detergent like response when aerated, indicating a mixture of hydrophobic and hydrophilic amino acids. Foam was

generated by passing nitrogen gas through the stainless steel sparger at the base of the glass column. The bubbles collected at the liquid-foam surface. The continued production of bubbles forced the foam up the tube and into the gooseneck in a continuous flow to a collection beaker. The foamate was fractionated and the fractions analyzed for total protein using GPC and/or the BT assay.

The gas flow can be measured by one of two methods. Measurement of gas volume introduced per unit time (mL/min) to the bulk liquid was used for all experiments done in this study. Alternatively some studies have used the superficial gas velocity. This is the number of bubbles passing through a unit cross-sectional area of foam column per unit time multiplied by the volume of the bubbles. The superficial gas velocity thus allows for coalescence of bubbles and is a measure more of the foam production than the gas flow introduced to the liquid. However for practical reasons gas flow was used in this study.

Foaming fractionation studies were batch operations with a consequent reduction in the volume of bulk liquid and a continual change in the bulk to foamate volume ratio. Using the 300 mL foaming column, the starting bulk liquid volumes were kept in the range 100 - 200 mL, except in the pH trials. Foaming typically progressed until the bulk liquid volume was reduced by half. The E_r, S_r and R_p ratios changed as foaming progressed. The concentration of protein in the bulk liquid reduced with time as bubble adsorption of protein progressively removed protein from the bulk liquid. The concentration of protein in the foamate also changed with time, depending on variables used in each experiment. In this study only batch operations were performed and no attempt was made to introduce new feed flow to the bulk liquid or maintain the volume of the bulk liquid. In an industrial application a continuous feed flow system would be the desirable for both cost and efficiency reasons.

5.2.2 pH Controlled Foam Studies

The pH adjusted protein dissolution mix was foamed, with 5-6 mL needed to produce foam to the top of the cylinder before collection. The volume of bulk liquid used for these experiments was 60 mL excluding the sparger volume. Foamate was collected in approximately 10 mL fractions till the bulk liquid volume had decreased to 25 mL. The time taken for foamate collection varied, depending on the ease of foaming. Foam

separation at pH 9, 8 and 7 used a wool protein concentration of 6.9 mg/mL. Foam separations at pH 6 and 5, used a protein dissolution mix concentration of 10.1 mg/mL. The BT assay was used for all protein assays in this study of the effect of pH change on foaming performance. Total protein was determined in latter experiments by GPC.

5.2.3 Refoaming

A dissolution mixture, (200mL) with a protein concentration of 19.2 mg/mL was foamed in a 30 cm x 35 mm column for 260 min resulting in a residual bulk liquid of 80 mL, (experiment No.1). The starting pH of the dissolution mix was 8.6. A flow rate of 13.2 ml/min was maintained for the duration of the foaming. Re-foaming, (experiment No. 2), used the collapsed foamate (92 mL), collected in (experiment No. 1) at a pH of 8.4. The flow rate was 12.7 mL/min and foaming continued for 188 min, resulting in 54 mL of collapsed foam. In the foaming and re-foaming experiments the foam collected was treated as a single sample for each experiment.

5.2.4 Total Protein Analysis

Total protein analysis of the foam fractionation experiments was by GPC, Sephadex G-15 and in some cases, biuret assay. The GPC assay and biuret assay methods and development are described in detail in Section 3.3.6 and 3.3.7.

5.2.5 Desalting by Ultrafiltration

The units were cleaned in a 50 mmol sodium hydroxide, 50 mmol sodium hypochlorite solution, rinsed and stored in 10% ethanol at 4°C, with no observed deterioration.

5.3 Results and Discussion

5.3.1 Stability of Stored Soluble Wool Protein

Wool dissolution mixtures foamed in this study were obtained by oxidative sulfitolysis. These dissolution mixtures appeared to show little visual change in the several weeks they were held for study at 4°C. However several studies performed using foam fractionation

with mixtures held longer than 1 month, were observed to have distinct ammonia odours and showed raised pH 8.4 to 9.3. The measured pH changed quickly with foaming, becoming less alkaline, suggesting the loss of ammonia and hence some of the alkalinity.

5.3.2 pH Controlled Studies

pH trial	Initial Protein Mg/mL	Foam Fraction	pH	Surface Tension N/m 10^{-3}	Biuret mg/mL	Enrichment	Separation
pH 9	7	1	8.91	41.7	7.9	1.14	2.19
		2	8.63	47.2	7.7	1.12	2.14
		residue	8.66	55.8	3.6		
pH 8	7	1	8.21	41.7	16.2	2.35	4.50
		2	8.19	48.6	6.4	0.93	1.78
		residue	7.99	56.2	3.6		
pH 7	7	1	7.33	40.1	19.6	2.84	5.60
		2	7.28	45.9	9.3	1.35	2.66
		residue	6.88	53.7	3.5		
pH 6	10	1	6.23	37.1	13.1	1.30	2.02
		2	6.21	44.5	11.9	1.18	1.83
		3	6.33	48.7	14.1	1.40	2.17
		residue	6.01	52.4	6.5		
pH 5	10	1	5.45	32.9	20.8	2.06	2.67
		2	5.45	37	21.5	2.13	2.76
		3	5.27	46.4	14.6	1.45	1.87
		residue	5.00	50.5	7.8		

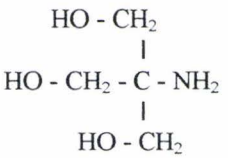
Table 5.3.2 Foaming and pH Change

Wool dissolution mixture was foamed at successively lower pH levels. Two dissolution mixtures were used for foaming, pH 9, 8 and 7 from the same dissolution (total protein 6.9 mg/mL) and pH 6 and 5 from a second dissolution mix (total protein 10.1 mg/mL). The dissolution solution was titrated to the desired starting pH with 0.1M HOAc. Foam fractions were collected after sequential 10 mL decreases in the bulk liquid volume, the starting bulk volume was 60 - 70 mL. The biuret assay was used to analyse total protein, including any precipitated protein in solution.

Foam produced from bulk liquid of pH 9.1, was at pH 8.6, yet all other lower pH bulk liquids gave raised pH levels by ~ 0.2 above the starting pH, see Table 5.3.2. Tris was the buffer used in the dissolution mixture and has a degree of hydrophobicity that may have lead to its inclusion in the bubble film. Inclusion of Tris, pKa 8.1 (Sigma, 1999) into the foam would move the pH of the sample towards the pKa of Tris.

A trend of pH movement toward the Tris pKa value of 8.1 was noted in the pH 6-9 range, with foaming experiments performed in this study. The residual bulk liquid left over after

foaming, remained at a pH close to that of the original sample. Proteins will act as weak buffers having both acidic and basic residues and may also be involved in buffering action when protein adsorption occurs at the bubble interface.



Tris tris(hydroymethylaminoethane)

To avoid alkali damage to protein, higher pH levels were not foamed, thus no data is available for higher pH values. Soluble wool protein initially at pH 9.4 that was stored for more than 6 weeks before foaming gave a distinct ammonia smell on foaming and had noticeably raised pH levels of 9.6. Urea degrades over time to eventually produce ammonia. Ammonia is a volatile gas and may concentrate in the foam gas. Degradation of amines will also release ammonia. The pH of the foamate has not received any attention in the literature examined, but is of significance if separation from salts and buffering agents is one of the desired outcomes of foam fractionation. Determination of salt content before foaming is reported in section 5.3.6.

Concentration of the protein occurred for all pH levels, bar the result for the second foam fraction at pH 8. This result does not appear to fit the pattern displayed by the other results and may have been due to inconsistencies in the Biuret assay. Enrichment ratios were highest in the pH 7-8 region, with the first fraction of pH 7 achieving an E_r of 2.84 and the first fraction of pH 8 achieving an E_r of 2.35. Lowering of the pH by the addition of HOAc caused an increase in the murkiness of the protein solution as protein precipitated. The protein will precipitate as the pH approaches the pI of the protein, for wool over pH 8-4.4. Wool has a range of proteins with pI covering pH 8-4.4 range. Increasing opaqueness was observed with pH decrease. The pH 5 solution had a milky appearance and the increase in E_r recorded may have been due to particle flotation by the foam rather than soluble protein accumulation at the gas/liquid interface of the bubble. The S_r showed more clearly the change that is occurring in foaming with the greatest separation occurring in the pH 8 and 7 foaming experiments. The E_r and S_r values suggest that a pH 7-8 range would be the best for further study of foaming and consequently most foam trials were conducted in this pH range. The desire to avoid precipitation was a major

factor in favouring pH 8 for performing further foaming experiments. It may be desirable to consider carrying out experiments in the 8.5-9.5 range to avoid any precipitation.

5.3.3 Air Compared to Nitrogen for Sparging

A comparison of air and nitrogen as sparger gases with dissoluted wool showed no significant difference. In this case, no difference was expected because oxidation of the cysteine sulfahydryls to form disulfide bridges within or between adjacent proteins was blocked by cysteine being in the S-sulfonate form. Liu et al., (1998) using air as the sparger gas, noted that the E_r was double for active catalase at pH 7 compared to the E_r at pH 4. In addition carbon dioxide gas was found to produce twice the E_r compared to nitrogen across the pH range 4-8. Liu et al., used reduced protein material and they attributed the differences with air as sparging gas to oxidative effects. They discussed the buffering effects of CO_2 which produced an acidic environment closer to the maximum for protein concentration. A comparison of foaming in air and nitrogen was performed at pH 8.2. A 58 cm x 45 mm column was used for this study. The column had a volume capacity of 600 mL total. The E_r were recorded for large volumes of foamate. The 400 mL initial bulk liquid resulted in a small enrichment. There is a small 8% increase in S_r when using nitrogen was used as the sparging gas, but is not seen as significant Table 5.3.3.

Gas	Enrichment	Separation	Percentage Recovery
Air	1.25	1.19	17%
Nitrogen	1.25	1.30	14%

Table 5.3.3 Comparison of E_r, S_r and R_p using Air and Nitrogen

Starting protein concentration of dissoluted material was 11.3 mg/mL, air or nitrogen at a flow rate of 35-40 mL/min was bubbled in a 58 cm x 45 mm ID column through a sintered glass sparger. Dissoluted wool (450 mL) was foamed for 1 hour. The foam sample collected was allowed to collapse before determining total protein by GPC.

The total bulk liquid volume was 420 mL, whilst the foamate liquid volume using air or nitrogen was 23 and 24 mL respectively. Consequently the yield reported is low for the comparative study. Possible total yields are discussed later in this chapter. Sparger gas was not investigated further in view of the insignificant differences observed with wool in

the S-sulfonated form. The foaming column was particularly tall at 58 cm and it was anticipated that higher S_r and E_r would be achieved using a taller column (Sarkar et al. 1987; Uraizee and Narsimhan, 1996; Brown et al. 1999). Several factors operated against attaining better protein separation. The bulk liquid occupied over two thirds of the column and may not have allowed for adequate drainage, a key component of S_r and E_r. A complicating factor was the rate of flow of gas. This proved difficult to control and the gas tended to exit the sintered glass sparger in larger bubbles and at a faster flow rate 35-40 mL/min compared to the flow rate of 11-13 mL/min delivered by the stainless steel sparger used in other experiments reported in this chapter. During foaming, both trials showed considerable re-circulation of the bubbles before reaching the surface of the liquid, indicating that flow rates were too fast. The extended time spent in the liquid would have been expected to increase the protein adsorption on the bubble. As the trials of nitrogen and air were carried out using the same column and protein material, bubble size was not considered a discriminating factor. Small bubbles expose a large surface area for adsorption. Efforts were made in later experiments to control the bubble size via the gas flow rate and hence maximize the surface available for adsorption of protein.

5.3.4 Foam Enrichment with Dilution

My study on wool proteins did not achieve results showing large enrichments with dilute protein solutions. Improved enrichment and separation with wool proteins were nevertheless achieved at lower protein concentrations of 0.8-3 mg/mL. Four soluble wool solutions of differing protein concentration were compared to determine the effect of dilution on foaming of wool protein solutions. The soluble wool solutions were titrated with 0.1M phosphoric acid to pH 8.1 ± 0.1, each solution had a similar ionic concentration as evinced by the conductivity range for the four dissolutions of 17-22.5 mS/cm. The nitrogen gas flow rates used were 11.9-14.4 mL/min. The first fraction of foam collected was used to assess enrichment. Separation and percentage recovery were based on 3 hours of continuous foaming. The total protein was determined for all solutions by GPC using Sephadex G-15 as described in Section 3.3.8.

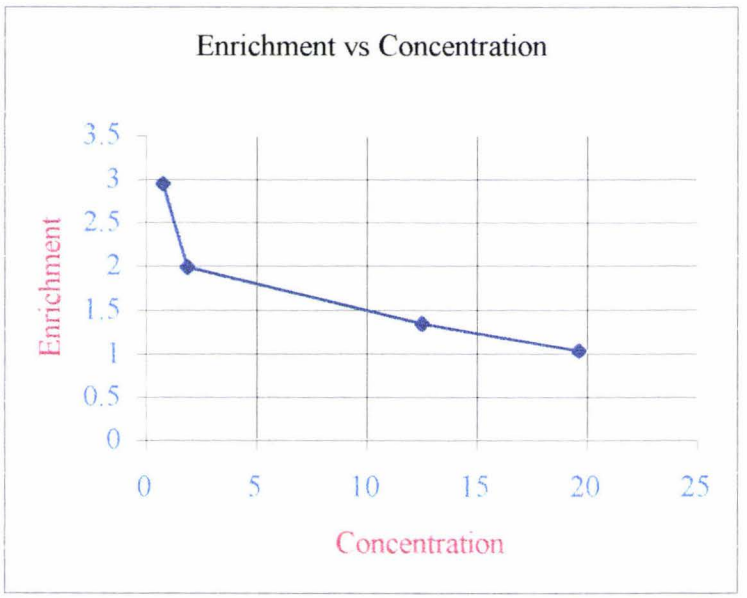


Figure 5.3.4a Variation of enrichment ratio with concentration change.

Dissoluted wool was foamed in a 29 cm x 35 mm ID column using nitrogen gas at flow rate 12-14.4 mL/min. A foam fraction was collected after a 15 mL decrease in the foamed bulk liquid volume, (200 mL) was performed. Dissoluted wool of differing concentrations was titrated with phosphoric acid 0.1M to pH 8.2. Foamed samples were analysed by GPC Sephadex G-15.

Enrichment E_r, did increase with dilution of the protein solution as shown in Figure 5.3.4a. The limit on foaming with dilution is the ability to form stable foam. The most dilute solution, 0.84 mg/mL, showed signs of unstable foam with spaces of 10-20 mm appearing in the foam column as the foam moved up the column. This indicates a high degree of coalescence and consequent enrichment of the foam sample. At the same time a reduced recovery R_p, will result from the severely reduced flow of foam out of the column. A low concentration protein solution has a large bubble surface area to interact with and reach equilibrium. The limit to concentration is the rate of mass transfer of protein, by diffusion, from the bulk liquid to the interface of the bubble before it leaves the bulk liquid. Higher concentrations are limited in their enrichment by the number of bubbles and the size of the bubbles as the CMC is approached. The number and size of bubbles will determine the total surface area available for protein interfacial interaction. The surface tension, a measure of the surface excess of a component in the most concentrated protein solutions foamed, gave values $\geq$ 32.9 dynes/cm. Although multiple layers and consequently an increased concentration of protein are possible within the micelle, the lack of concentration above the observed maximum of 27 mg/mL suggests that this is not

happening in the wool dissolution mixtures. Lack of higher concentrations than 25-30 mg/mL lead me to believe that this represented the CMC.

The urea may be providing an alternative mechanism for alleviation of hydrophobic stress in the protein. Increased protein concentration has been achieved with the use of surfactants as reported by Noble et al. (1998). Colloidal gas aphrons comprising microbubbles 10-100μm diameter surrounded by a detergent shell were created by high speed stirring of the protein solution. The high level of urea used may preclude multiple layering in protein solutions. The effect of detergents on the chemical activity of wool proteins was not explored in this study but is worthy of investigation.

A wool dissolution of 0.1 mg/mL failed to foam adequately, creating a layer of foam < 1 cm high in the column, that constantly coalesced, thus establishing a lower limit for foaming of wool dissolutions. The filtrate of an ultra-filtrated, 1.4 mg/mL protein solution of low ionic and urea concentration, would not foam adequately. Suggesting that an ionic or urea component is needed for foaming to occur at low concentrations. More study is required to quantify this observed phenomena. As the bulk liquid concentration approaches the CMC, foaming did not lead to enrichment, an E_r of 1.34 was attained with 12.6 mg/mL compared to an E_r of 1.03 for the 19.2 mg/mL wool protein solution. The highest wool protein concentration achieved in a foaming fraction was 23 mg/mL.

Separation S_r, a measure of the concentration of the foamed sample compared to the concentration of residual bulk liquid showed the same pattern as enrichment, Figure 5.3.4b. As with enrichment the best separation was achieved in the lowest concentration. The separation in Figure 5.3.4b refers to a much longer period of sample collection than for the enrichment, had separation been based on the first 10 mL sample of foam collected the values would have been higher. Separation declines with time. For the least concentrated solution a 25% reduction of liquid in the bulk liquid volume took 4 hours of foaming, as against the 70 min taken to achieve the 25% reduction for the most concentrated solution.

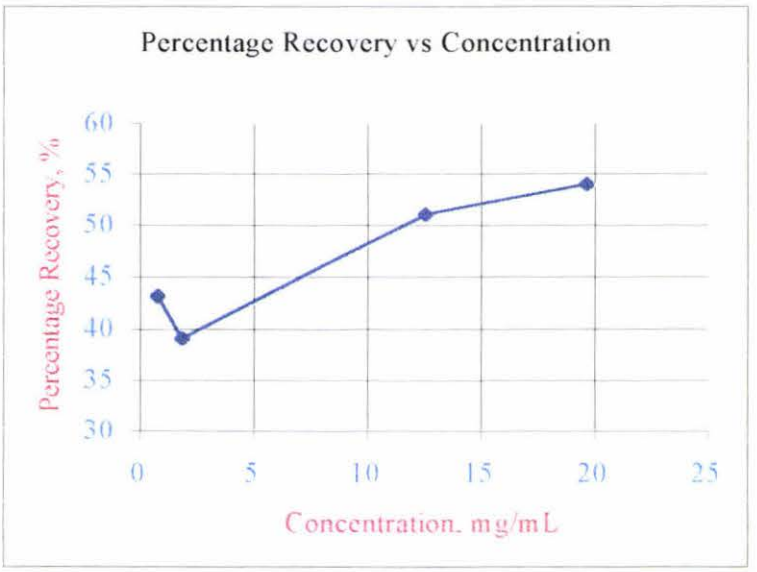


Figure 5.3.4b Degree of separation ratio achieved with concentration change

Dissoluted wool was foamed in a 29 cm x 35 mm ID column using nitrogen gas at a flow rate of 12-14.4 mL/min. A foam fraction was collected after a 10 mL decrease in the foamed bulk liquid volume, (200 mL) occurred. Dissoluted wool of different protein concentrations was titrated with 0.1M phosphoric acid to pH 8.2. Foamed samples were analysed by GPC Sephadex G-15.

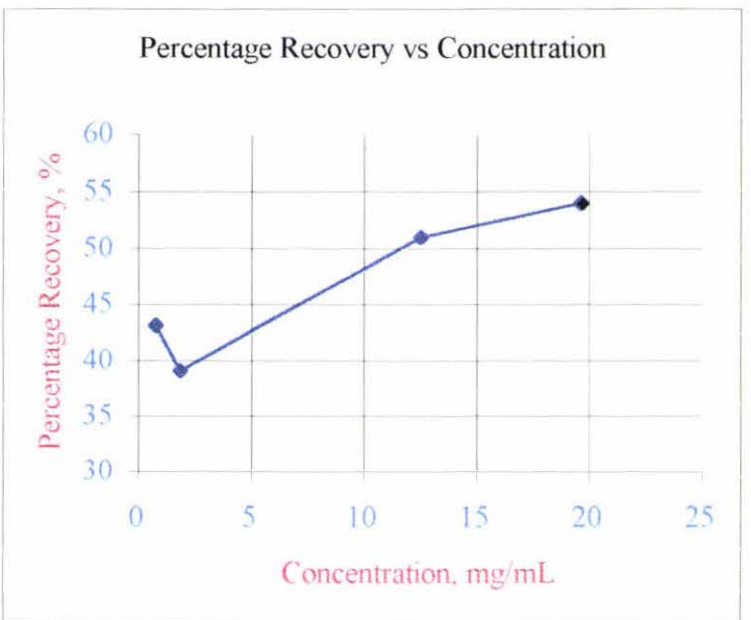


Figure 5.3.4c Percentage recovery achieved with concentration change.

Dissoluted wool was foamed in a 29 cm x 35 mm ID column using nitrogen gas at a flow rate of 12-14.4 mL/min. Foamate was collected from bulk liquid (200 mL) for 3 hours. Dissoluted wool of different concentrations was titrated with 0.1M phosphoric acid to pH 8.2. Foamed samples were analysed by GPC Sephadex G-15.

In Figure 5.3.4c R_p was best at the highest concentration tested. However this does not take into consideration what the foam volume of liquid compared to the residual bulk liquid.

Results achieved in this study with dissoluted foam were similar to those achieved by Uraizee and Narsimhan (1996), Noble et al. (1998), confirming that wool dissolution mixtures perform in a manner similar to that of individual and binary protein solutions cited in the literature. This study of the effects of dilution was centred on samples that contained all the ionic and molecular reagents in the wool protein solution. Further studies were carried out on ultra-filtrated wool protein solution and are reported in section 5.3.10.

5.3.5 Urea Concentration of Foam Fractions

The assay used for urea, a modified Geyer and Dabich method (Patchett, 1988), required dilution of the samples to be tested to 1/20,000 concentration of the original solution. Large errors caused by the deterioration of the colour reagent were apparent in performing this assay and although some results were in line with expected results, others in the same assay gave widely differing values as reported in section 3.3. Because of the variation encountered in performing this assay no result could be accepted with confidence in the actual values attained. Regrettably no firm conclusion was able to be drawn from the results. A literature reference noted that the diacetyl monoxime-semicarbazide reagent used for colour development was not stable in light (Mellerup 1967), although others (Patchett, 1988) described it as stable for up to a month if kept in away from light. It is likely that this was the cause of varying readings, a distinct colour being noted in the reagent bottle, despite being kept in a dark environment. As a result of this experience making up the diacetyl monoxime-semicarbazide reagent daily, is a recommendation for future assays.

CGE analyses were performed on foamate fractions obtained in the experiments on foaming and on the effects of pH variation. These analyses were not very informative regarding determining the ability of foaming to separate wool protein classes, with a lack of baseline separation of the multiple peaks, as in Section 3.3.2. In all dissolution mixes the urea spike was a useful neutral marker in CGE. The foamate analyses did show a

reduced peak for the urea position indicating a decrease in the level of urea in the foamate. These findings are in agreement with the urea determinations via the Geyer and Dabich method. The CGE technique was not pursued further. However it did provide some qualitative information regarding changes in the level of urea in foamate compared to bulk liquid during foaming. As noted in section 3.2 further research into improvement of the CGE technique could provide useful information in relation to foaming studies.

5.3.6 Sodium Ion Concentration

Foam studies cited in the literature do not mention any change in salt concentrations with foaming. Sodium was the only alkali metal contained in the dissolution mixture derived from reagents, sodium tetrathionate and sodium sulfite. Sodium measurement thus was a useful ion to assess the efficiency of the foaming for removal of the ionic component of the dissolution mixture. No specific interaction between protein and an ion appears to be occurring (see Section 3.3.4). A slight reduction in the sodium ion content was observed as reported in Section 5.3.7.

Foamed protein at pH $\geq$ 8 will be predominantly negatively charged and will attract oppositely charged ions to the surface of the protein in the formation of the 'Stern layer' of counter ions (Krijgsman, 1992). It was speculated that positive ions, i.e. sodium ions would be preferentially taken up in the foam as a means of neutralizing the essentially negative proteins as the protein concentrated at the gas-liquid interface of the foam. Sodium was present in concentrations of 0.001M from the reagent salts, whereas at pH 8 the concentration of hydrogen ions would be 1×10^{-8}, considerably less than the sodium ions. The exact nature of ion-protein interaction was not pursued in this study.

5.3.7 Re-Foaming

No reference to refoaming was encountered in the literature. Foam fractionation was carried out on 200 mL of soluble wool protein of 19.6 mg/mL. Foaming was carried out till the bulk volume was 92 mL. Protein concentration has increased, albeit only by 13% from 19.6 to 22.6 mg/mL in the re-foaming as shown in Table 5.3.7a.

	Protein concentration, mg/mL	Enrichment, E_r	Percentage Recovery, R_p
Foaming Experiment No. 1	19.6	1.0	61%
Re-foaming Experiment No. 2	22.6	1.2	63%

Table 5.3.7a Protein changes on re-foaming.

GPC using Sephadex G-15 was used to analyse the protein concentration changes. Foam experiment analysis was based on 102 mL of collapsed foam and the re-foamed experiment analysis was based on 54 mL of foam collected. Dissolution of wool protein used 8M urea, 0.1M TTN, 0.2M sodium sulfite, 0.1M Tris.

The bulk liquid protein concentrations for foaming and re-foaming were very close at 19.2 and 19.6 mg/mL respectively. In performing the re-foaming the same column was used as for experiment No. 1. However the ratio of bulk liquid 200 mL to foamate ~ 100 mL in column (ratio 2:1) was reversed in the second experiment with bulk liquid 92 mL to foamate 210 mL (ratio 1:2.3). The longer foamate column portion would give more time for drainage and coalescence, both of which produce an improvement in protein enrichment. This factor was not appreciated at the time and may have been the sole reason for the E_r and R_p improvement observed.

Although the gas flow rates were similar, the production of foam was not equal. The foam collected was allowed to collapse and this figure was used to determine the rate of liquid formation from faom for each experiment. Experiment No.1 had a collapsed foam liquid production rate of 0.46 mL/min compared to experiment No. 2, which had a foam liquid production rate of 0.29 mL/min. Slower foam production can be related to the length of the column that the foam moves up. A longer travel for the re-foamed material would result in more opportunities for bubble collapse during passage up the column as the foam liquid rates indicate, the gas flow rates being almost equal.

Changes in the ionic concentration may assist E_r. Conductivity is a measure of the total ionic content. Foaming in experiment No. 1 has reduced the ionic content, table 5.3.7b. The conductivity value for the collapsed foam liquid in experiment No. 2 suggests rather surprisingly that re-foaming has lead to an increase in the ionic content. It is difficult to explain this result, which could perhaps have been seen as some anomaly, but the pattern was repeated in the flame emission results.

Conductivity in mS/cm	Bulk Liquid,	Foamed Collapsed Liquid	Residual Liquid
Foaming Experiment No. 1	21	17.9	24.9
Re-foaming Experiment No. 2	17.9	23	n.m.

Table 5.3.7b Conductivity of foamed and re-foamed solutions.

Conductivity of collapsed foam was measured at 20 ± 1.0 °C. No figure was available for the residual liquid for experiment No. 2. (n.m.)

The very reasons presented for increased enrichment by drainage and coalescence should have lead to a decrease in the ionic content. Proteins are charged, particularly at the pH used for most foaming experiments and could be contributing to the conductivity. Each mg of protein adds a conductance of 1.5 μS/cm at pH 8, the pH at which the foam fractionation was carried out. The conductivity readings of foam solutions were in the 10-25 mS/cm range and so were not affected by any changes in the concentration of protein.

Analysis by sodium flame emission produced results that mirrored the conductivity measurements. A small decrease in sodium ions with foaming appears to have occurred with experiment No. 1, Table 5.3.7c.

Flame Emission, [Na] mg/mL	Bulk Liquid,	Foamed Collapsed Liquid	Residual Liquid
Foaming Experiment No. 1	13.5	11.7	23.4
Re-foaming Experiment No. 2	18.7	25.7	n.m.

Table 5.3.7c Sodium content of re-foamed wool protein solutions.

Flame emission was performed on diluted samples of solutions. No figure was available for the residual liquid for experiment No. 2 (n.m.). The tabulated values are those calculated for the undiluted samples.

The drop in ionic content would support this as being interpreted as a drop for all ions in solution. Experiment No.2 however, produced an increase in the sodium ion content of the foam. A sample of the bulk liquid in experiment No. 2 was taken for analysis, this should have given results identical to the foam produced in experiment No. 1. The

difference in results of supposedly the same sample material does not give a high level of confidence in the values gained. A sample was taken of collapsed foam, experiment No.1 before the same material was refoamed a second sample was obtained and read as the bulk liquid for experiment No. 2. The reason for the differences in measured values of the same solution are unknown. Calibration of the instrument by standard solutions of sodium ion gave a linear relationship to concentration. The standard solution values obtained were consistent for several runs of the flame photometer. To draw any major conclusions based on the flame emission data would be unwise given the variance observed. Flame emission analysis of foam samples produced from foaming a diluted wool dissolution mixture (0.84 mg/mL) indicated that the [Na^+] had increased in the foam from 15.6 mg/mL to 23.6 and 19.4 mg/mL for the first and second 10 mL samples of foam. It is possible that there is an interaction between the negatively charged protein and the positive sodium ion at the gas-liquid interface. All proteins in solution will possess an electric double layer, (Stern layer) and (Gouy-Chapman layer) around the surface (Krijgsman, 1992). This effect could carry positive ions along with the protein into any foam formed. Wool proteins will be mostly negatively charged at pH > 7 and so attractive to sodium ions, however without further analysis and confirmation of the sodium concentration in the foam it is purely speculation.

The solution chosen for foaming and re-foaming was stored for one month at 4°C. This factor may have influenced the results obtained. Any effects of decomposition of urea or damage to protein from the urea derived cyanate ion on the foaming process is unknown. No assessment was made of the integrity of the protein in the solution.

Samples of the foam/refoam experiments were analysed by GPC and Biuret for total protein, with agreement in trends observed with slightly lower values recorded for the Biuret assay Table 5.3.7d. The change in the length of the foam section of the column was judged to be the major factor in the enrichment found from re-foaming. Longer columns will lead to enrichment, the limit being the balance between the bubble coalescence and the movement of foam up the column sufficient to deliver enriched foam out the top of the column. The results of experiments No. 1 and 2 are contrary to expectations and the area of re-foaming needs to be reassessed.

	Expt. No.1	Expt. No.1	Expt. No.1	Expt. No.2	Expt. No.2
Foam Fraction	Bulk Liquid	Foam	Residual Liquid	Foam	Residual Liquid
GPC mg/mL	19.2	19.6	18.9	23.5	22.6
BT mg/mL	17.3	17.5	16.4	18.9	18.7

Table 5.3.7d Comparison of GPC and BT assays of re-foaming experiment

An investigation of different protein concentration solutions and dilution of the foamed mixture before re-foaming is recommended, in addition to development of specific tests for ionic content appropriate to wool proteins.

5.3.8 Surface Tension and Foam Fractionation.

The surface tension change of two different wool protein dissolution mixes was examined with results that are confusing and have no consistent pattern Figure 5.3.8a. Plots a. and c. show some similarities below pH 8, but diverge above pH 8.

A rigorous cleaning of the chrome ring of the tensiometer was carried out after readings at each pH. As the pH decreased the ring was found to acquire a protein deposit. A minima is evident in Figure 5.3.8 at pH < 7 and plots STa and STc share a minima in the pH 7.8-8.3 range. At pH < 7 protein precipitation was very evident. Normally a protein will exhibit one zone of low surface tension representing the pH where maximum accumulation of protein will occur at the interface, spread over a pH range ~ 0.5. Wool dissolution mixtures could potentially have many minima. The large number (over 100) wool proteins will exhibit minima but these are likely to overlap and be unable to be resolved by the surface tension meter. From Figure 5.3.8 plot STb, two sharp minima (pH 8.4 and 7.6) were evident as possible pH zones where foaming could be performed and lead to enrichment of specific proteins. The plot STb data does not appear to match well to the other two sets of data. No particular reason is advanced to explain the difference.

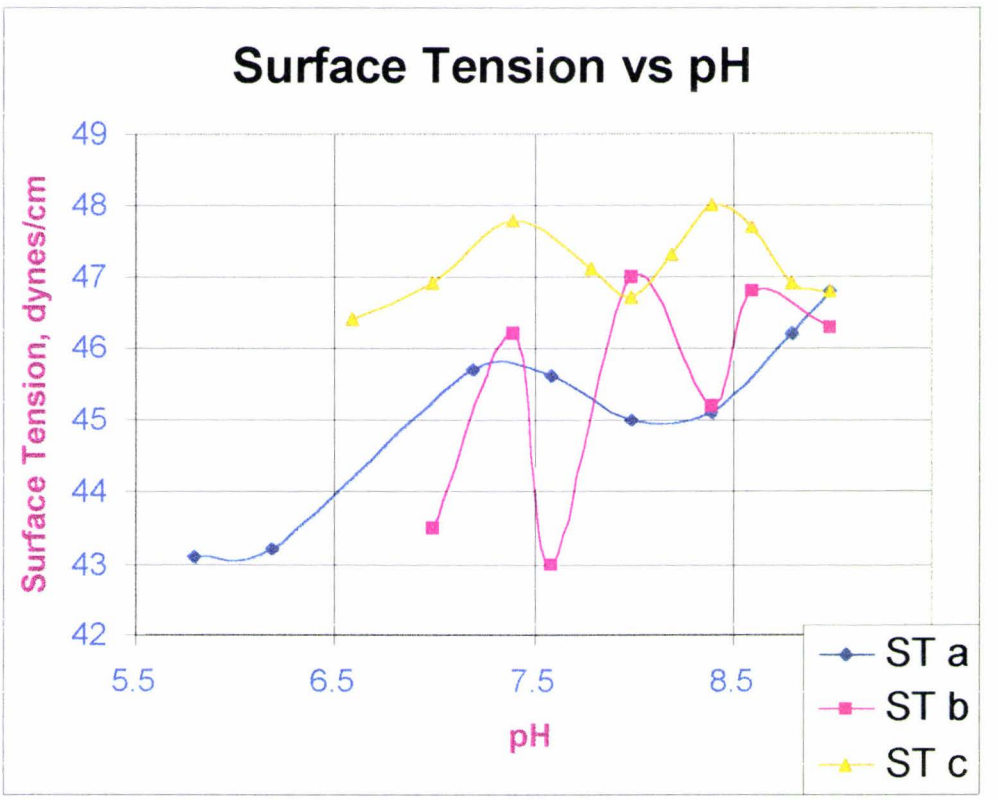


Figure 5.3.8 Surface tension against pH for soluble wool protein

Surface tension of wool dissolution mixture in decreasing pH was measured using a Whites Electrical surface and interfacial torsion balance. The surface of the liquid was tensioned with a chrome ring on a hanging balance arm. Readings were done a minimum of 5 times to ensure a SD of less than 0.5%. All readings were done at room temperature 20±1°C. This graph is the average of three sets of readings. Plots STa. and STb. were of the same dissolution mixture of 26 mg/mL. Plot STc. was a new dissolution mixture of 18.7 mg/mL concentration, of a weaker ionic concentration.

Foaming bulk liquid (total protein 6.9 mg/mL) at pH 8 produced foamate of pH 8.2 with a surface tension of 41.7 dynes/cm and an E_r of 2.35, the E_r was over double the result achieved at pH 9 see Table 5.3.1. There appears to be a surface tension minimum below pH 7.0, foaming at pH 7.3 produced a surface tension of 40.1 dynes/cm for foamate and a higher E_r of 2.84. This was the highest E_r experienced in the pH experiments. Foaming experiments at pH 5 and 6 had the lowest surface tensions for foamate recorded at 32.9 and 37.1 respectively. The E_r was better at pH 5 than pH 6. These enrichment values are based on the biuret and any precipitated protein will be part of the protein determination. Dissolution mixtures where the pH < 7 were very cloudy and protein was precipitating in increasing quantities as the pH dropped. In solutions of pH 7, 6 and 5 the protein caused a cloudy appearance and aggregation in the solution was evident with a very low surface tension indicative of a high level of surface activity at these pH. The original objective was to foam fractionate and achieve a soluble protein product in the foamate, and in a suitable form for on-going reconstitution experiments. To keep the wool protein soluble

most dissolutions were foamed in the region of pH 8.0-8.4. This was indicated as a minima for surface tension and hence likely to facilitate protein separation from the solution. Foam will carry over both, soluble proteins at the bubble interface and precipitated protein. In all cases observed, the surface tension of the first foam sample produced was the lowest surface tension recorded for that foam fractionation, even though the protein concentration may not have changed, the surface tension did with later fractions. The same pattern of the first foamate being the lowest surface tension also applied across the pH range 5 - 9, as seen in Table 5.3.8.

pH	5	6	7	7.8	8.4	8.6	9
Surface Tension, 1st Foamate	32.9	37.1	40.1	40.5	40.8	45.6	41.7
Surface Tension, 2nd Foamate	37	44.5	45.9	49.5	54.1	54.1	47.2

Table 5.3.8 Foam Fractionation and Surface Tension

The table represents a number of different dissolution mixes at pH 5-9, which vary in protein concentration. The surface tension of the starting material was thus be different in each dissolution. Foamate was collected for each dissolution mix foamed at the designated starting pH. The first and second foamate samples were usually 10 mL each. Surface Tension was measured in dynes/cm.

It is possible that different proteins are separating in the first foamate compared to later foam fractions. Some indication of this is seen in the 1-D SDS-PAGE discussed in section 5.3.11. More investigation is required to afford an explanation of this phenomena.

5.3.9 Time Taken for Foaming

The rate at which foam is produced is influenced by several factors. The gas flow rate, the dimensions of the column, the concentration of protein and the bubble coalescence rate have a direct effect on foaming. Principally the gas flow rate and the concentration of protein will determine the R_p. Foam production rates varied, but for most experiments foamate production rate was recorded in the range 0.18-0.4 mL/min in the 30 cm x 14.5 mm column. Some very fast rates were observed, in these cases the E_r and R_p were very low, these are noted in Section 5.3.2 on sparger gases. This would be expected as drainage and coalescence would be reduced. Similar results were also reported for higher gas flows by Liu et al. (1997) and Chai et al. (1998). As foaming proceeds the amount of

protein remaining in the bulk liquid diminishes and the foam produced becomes less stable, a greater degree of coalescence occurs with a corresponding decrease in the rate of foam production. Loss of foam production was not apparent in the experiments performed until the bulk liquid was less than two-thirds the original bulk liquid volume and protein concentrations had fallen from initial concentrations of around 3 mg/mL to around 0.5 mg/mL. The low protein concentration points to a lack of stability leading to loss of foam. The exceptions were desalted solutions that precipitated and then would not foam. A typical time-bulk volume foaming experiment is illustrated in Figure 5.3.9, the gas flow rate was constant and the foam rate is essentially linear.

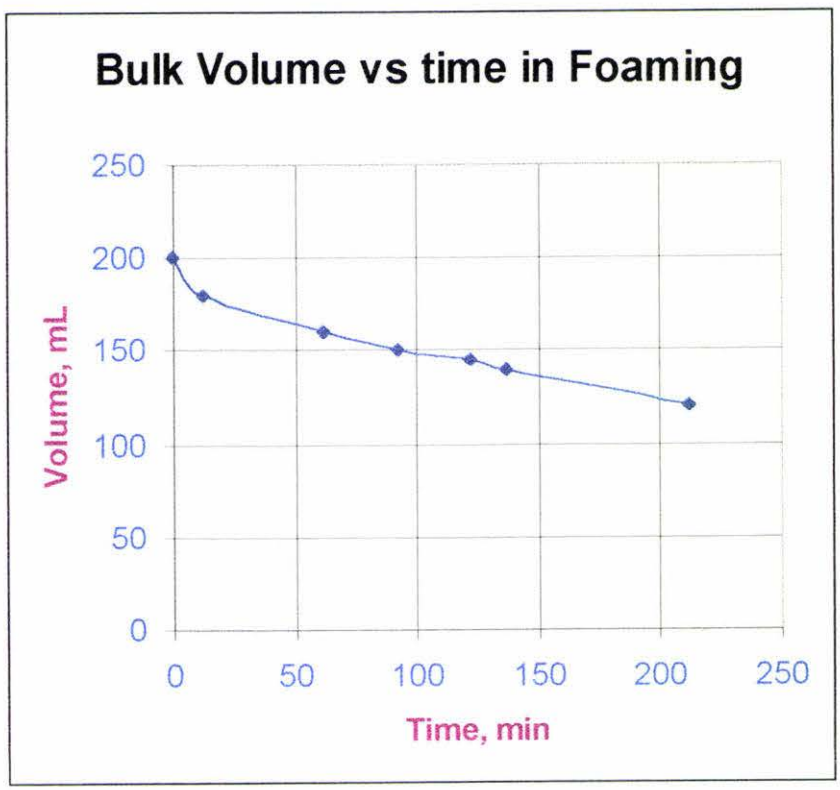


Figure 5.3.9 Time against the volume of bulk liquid.

Protein concentration by foaming at a nitrogen gas flow rate of 13.5 mL/min. This experiment was performed in a 30 cm x 35 mm ID column with stainless steel sparger fitted at the base. The wool protein solution was a concentration of 9.6 mg/mL. The temperature was 20 ± 2 °C.

The initial drop was caused by the foam build-up in the tube and drainage is low compared to the loss of bulk liquid to make foam. Most foam experiments were completed within 3 hours, with the bulk liquid volume reduced by half in that time. No absolute figure can be used to define the foam flow rate because of variables in the experiment. For industrial scale up it is envisaged that the critical factor will be the gas flow rate and that as there are

no moving parts nor any heating, the length of time foaming will be the major cost factor in foaming. Staff supervision of foaming could be significant if the time is doubled by reduction of the gas flow rate, although such supervision would be minimal.

5.3.10 Desalting Wool Protein Solutions

Desalting of bench scale solutions was accomplished by ultra-filtration either using a stirred cell or a tangential cross-flow filtration system. Wool dissolutions desalted in this manner had low urea and salt contents.

Dialysis tubing was also used to obtain some desalted protein for use as a benchmark protein in GPC analysis of total protein. A dissolution mix, 10 mL, took 3 days of stirring in 5 L of water changed twice to achieve a separated protein product. The protein obtained on freeze-drying the dialysed solution yielded 16.5 mg, equivalent to 1.65 mg/mL in the original dissolution mixture. The dialysed and freeze-dried protein value compared favourably with the GPC analysis of 1.67 mg/mL present in the dissolution mix. The conductivity of the dissolution mix changed from 20 mS/cm prior to dialysis to 0.12 mS/cm after dialysis indicating a very low ionic content in the dialysed product. However the time and large water volumes involved make dialysis untenable for any industrial scale use with wool protein solutions.

Ultra-filtration uses a membrane with pores of a pre-determined size. Although the manufacturer will define the MWCO for a membrane, it will have a range of pore sizes (Asenjo, 1990). Laboratory scale membranes are available in smaller ranges than those used in industrial grade operations. The stirred pressure cell was found to be very slow, taking 3 hours to reduce a 3 mg/mL solution from 300 to 150 mL under a nitrogen pressure of 200 kPa. A newer cross flow filter, Vivaflow 50 unit, became available and was used to desalt dissolutions. The cross flow filter was a sealed unit and comprised a polyethersulfone membrane and 5 kD and 50 kD pore size units were available for use. An assessment of these units was carried out to establish possible usefulness for industrial scale up. The 5 kD membrane filtered very slowly, 10 mL of filtrate from a 440 mL dissolution mixture was achieved after 40 mins at 2 bar pressure. In addition a malfunction of the units due to a manufacturing fault thwarted further attempts to use the 5 kD units. No further operations were carried out using the 5 kD units. Figure 5.4.10a

depicts the arrangement used for the desalting experiments. Units can be linked in series or in parallel. High viscosity and high protein concentration are not ideal conditions for effective functioning of the membrane.

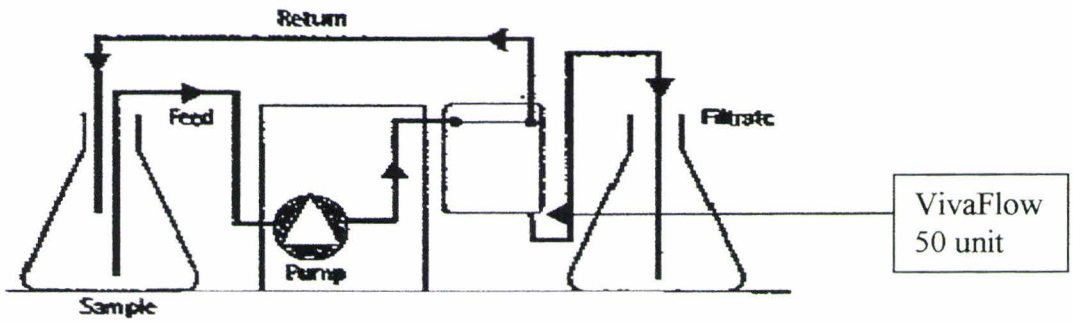


Figure 5.3.10a Arrangement of VivaFlow 50 cross-flow filter.

The unit was used in this configuration for the desalting, with water added to top up the retentate volume in the sample flask. Maintenance of the sample volume was ceased in order to concentrate the retentate. (Vivascience, 1999)

The cross flow filter required a peristaltic pump operating at 200 mL/min and the units were designed to desalt 500 mL solutions. The cost of pumping would be considerable in any industrial plant. The unit was designed to operate at 2 bar pressure. The cost of the filtration membrane was $117 and their operation life span was unknown. The polyethersulfone membranes are reported to be quite robust and resistant to alkaline conditions Vivascience (1999).

GPC of the retentate solutions and the filtrate showed a high retention of wool protein. The retentate showed a decrease in protein concentration from 9.5 mg/mL at time 0 min to 9.1 mg/mL after 125 min of pumping, a loss of 6 % Figure 5.3.10b. The filtrate gave a small protein peak of 0.23 mg/mL, indicating that some protein had passed through the filter Figure 5.3.10b. Wool is reported to have combined protein fractions of 32 % by mass below 40 kD (Maclaren and Milligan, 1981). Asenjo (1990) has noted that fouling will fill the pores of cross flow filters and this effectively closes the pores down to a smaller size and hence the pores will act as lower MW sieves. I believe that this explains the apparent retention of almost all the protein during a 46 % reduction in salts as measured by the area of the second peak, seen in Figure 5.3.10b plot B.

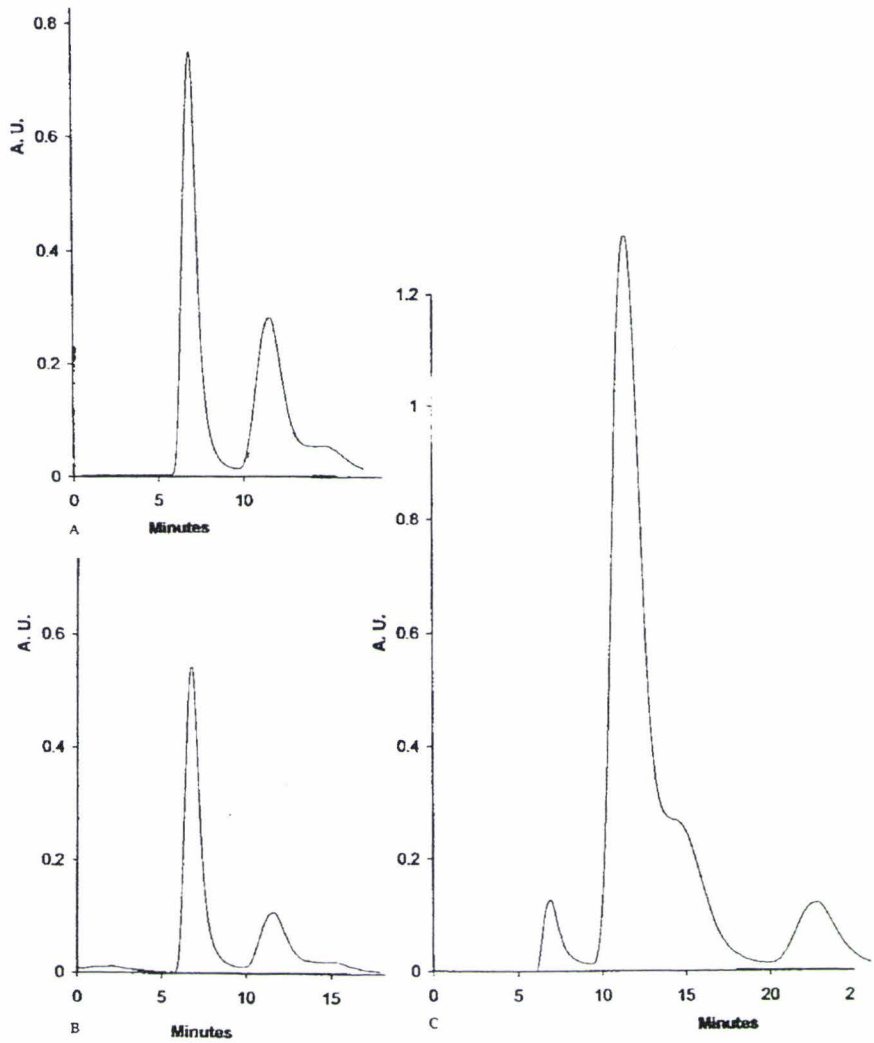


Figure 5.3.10b Protein and salt concentrations in retentate and filtrate during ultrafiltration

5.4.7b (A) Dissolution mix at time zero. (B) Retentate after 125 min and 250 mL filtrate collection. (C) Total filtrate solution at 125 min. A dissolution mix of protein 9.6 mg/mL was cross-flow filtered on a 50 kD membrane in a Vivaflow 50 unit. GPC Sephadex G-15 was performed using 0.1M phosphate/0.1M KCl /AcCN 20% buffer, pH 9, flow rate 0.8 mL/min.

The Table 5.3.10 shows the decline of salt concentration, during which the protein retention was 96%. In a second experiment, a dissolution protein mix of 14.7 mg/mL initial protein concentration was concentrated to 25.5 mg/mL. At this concentration a light milky appearance was evident in the final solution suggesting that some aggregation of proteins was occurring. If all protein had been retained in the retentate then the concentration for the volume reduction would be expected to be 30 mg/mL.

Time, min	0	25	50	75	125
Area of salt peak, mm^2	185	163	143	122	85

Table 5.3.10 Salt reduction over time by cross-flow filtration.

A VivaFlow 50 filtration unit of MWCO 50 kD was used to filter a 9.6 mg/mL dissolution mix. Samples exiting the filtrate outlet were analysed on GPC Sephadex G-15, the area of the second peak represented salts found in the dissolution mix of low MW. The retentate was kept in the 400-380 mL volume with addition of water.

Only a small amount of protein was evident in the filtrate and the apparent loss of protein from the solution, may have been due to a protein build up on or in the membrane surface. The membrane was expected to operate to exclude only proteins above 25kD (Vivascience, 1999), thus low MW protein should appear in the filtrate. It is possible that as the HSP and HGTP are below MW of 30 kD that this form of filtration could be used to separate these proteins from the IFP (low sulfur proteins). The Vivascience technical data sheet suggests selecting a membrane > 50 % below the desired MWCO, therefore membrane of 10 kD ought to be adequate. The lack of protein passing through the 50 kD membrane suggests that fouling was a major problem. It is unknown how quickly fouling of the membranes occurs and what effect fouling will have on the MWCO or the type of protein that passes through the membrane. Asenjo (1990) notes that the fractionation capacity of an ultra-filtration membrane may be time dependent due to fouling. The age of the protein solution may also be a factor in fouling of membranes. After 125 min of ultrafiltration analysis of total protein by GPC showed a very small protein presence of about 0.6 mg/mL in the filtrate Figure 5.3.10b plot B.

This low amount of protein could mean that fouling was happening within minutes of use and only a small amount of protein was able to pass through the filter. Alternatively the low amount of protein going through the filter could be due to some of the 6 % of wool protein that is below 20 kD filtering through. No further analysis was carried out to determine the exact nature of this protein present in the filtrate. The time taken to reduce from 430 to 205 mL was 110 min giving a reduction rate of 2 mL/min. There was a small decline in the pH from 8.48 to 8.17 suggesting that the Tris buffer was passing freely into the filtrate and was not being retained by the protein. Conductivity of the retentate did not change significantly, remaining at 20.1 mS/cm during the filtration. The total volume, i.e. the retentate and filtrate combined was constant, hence salts and pH should remain

constant in both solutions if there is no interaction between the protein and ionic component.

There is insufficient data from this study to eliminate or recommend this process as suitable for industrial scale desalting or concentration of protein solutions. If membranes can be regenerated to perform to original specifications by chemical cleansing, then there is potential for a dual use of the cross-flow membrane to achieve desalting and concentration. Cross-flow membranes did handle larger volumes more quickly and more effectively than the very slow stirred pressure cells.

5.3.11 Foaming Desalted Wool Proteins

The solubility of proteins depends upon the properties of exposed surface groups and the environment in which they reside. This was particularly apparent with dissolution mixtures that were foamed at pH < 8, without large-scale precipitation because of the urea present in the mixture. This was in contrast to the foam fractionation of desalted wool protein solutions which resulted in precipitation.

A desalted wool protein solution of 9.2 mg/mL concentration was titrated with 0.1M HOAc to pH 6.32, giving a final volume of 213 mL. The slightly milky solution was foamed in nitrogen gas flow 14.5 mL/min for 40 min. Immediately on pouring the bulk liquid into the column with the sparging gas on, a precipitate appeared at the sparger surface and in the foam formed at the top of the bulk liquid. The total protein concentrations of the filtered bulk and residual liquids were assessed by GPC, Table 5.3.11.

	Bulk liquid, prior to foaming	Residue liquid in column	Filtered residue liquid
Protein concentration, mg/mL	9.15	3.86	2.82

Table 5.3.11 Protein concentration of desalted foamed fractions.

Total protein of the foam fractionation was carried out after 40 min of foaming. GPC Sephadex G-15 was performed using 0.1M phosphate, /0.1M KCl /AcCN 20% buffer, pH 9, flow rate 0.8 mL/min. Residual liquid in the column had some protein precipitate mixed in it, filtering gave the third result containing just residual liquid with protein in soluble form only.

The foaming rapidly gave a white floating precipitate mixed with a small layer of foam 10 mm thick, at the bulk liquid surface. The precipitated protein yield was 57.8 % of the protein that was present in the bulk liquid before foaming. Foaming occurred at pH 6.32. The purity of the foaming induced precipitated protein was established by GPC analysis. The precipitate was collected, washed, dried in a dessicator and then re-dissolved in the pH 9 GPC buffer of 0.1M phosphate/0.1M KCl /AcCN 20 %. Gel permeation chromatography gave a value of 2.2 mg/mL of protein present in a solution of the solid. The solution was made from a sample of 22 mg dissolved in 10 mL of water, thus the sample was 100 % protein as assayed by GPC. Figure 5.3.11 plot A was the GPC trace obtained for the precipitated protein.

There is only a faint perturbation of the baseline following the protein peak indicating that there were no salts present and the high purity of the protein precipitated. Foaming was continued for a further 35 min with no obvious change to the protein precipitate. The residual liquid, pH was 5.31 when foaming was discontinued. Protein principally accumulated in an aggregated mass at the bulk liquid-foam junction on the surface of the liquid.

Protein also collected around the sparger as long thin streams of protein precipitating at the point of contact of the sparger gas with the bulk liquid. Precipitated protein was assayed by 1-D SDS-PAGE to establish the protein classes present. The 1-D SDS-PAGE indicated some separation of the protein classes. The first foam fraction of precipitated protein, exhibited all protein classes represented with strong bands in the 46-66 kD range and in the 30-35 kD range. The third foam fraction of precipitated protein showed a strong band at 46-66 kD, but lacked the intensity of the 30-35 kD band.

This was direct evidence of protein class differences in foam fractions. Removal of successive fractions at a predetermined surface tension may be a method of separating the most surface-active proteins. This is a concept worthy of further investigation if different surface tension zones can be related to the different classes of wool protein.

The time spent foaming could easily have been shortened, as there was no visual change after the first 5 min when the bulk liquid had become relatively translucent as compared to the slightly opaque appearance at the start of foaming.

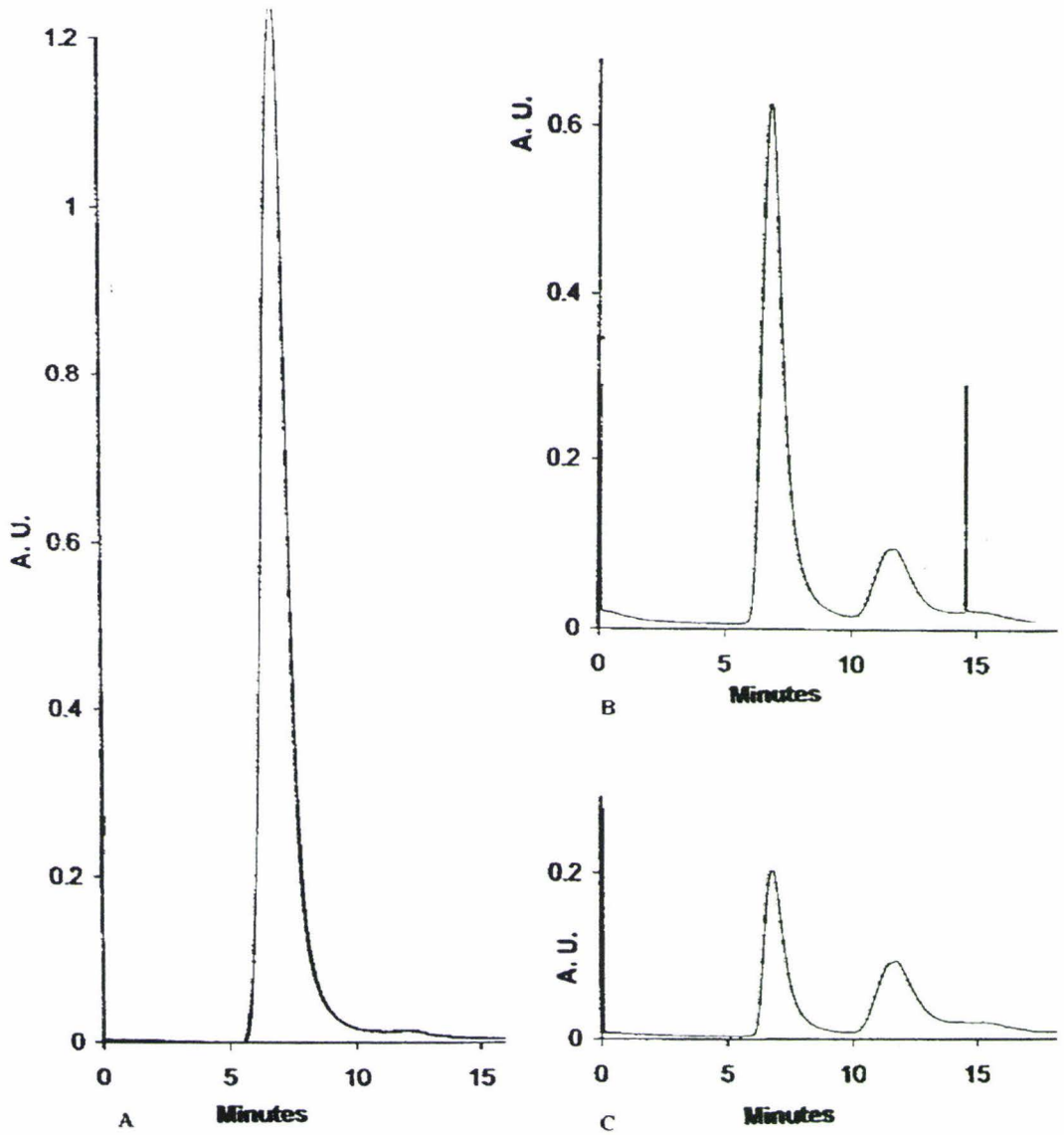


Figure 5.3.11 GPC of desalted foaming.

A. 2.2 mg/mL of precipitated protein dissolved in GPC buffer, 100 μL injection. B. Initial bulk liquid diluted 1:10 in water, 100 μL injection. C. Residual liquid diluted $1/10^{th}$ in water, 100 μL injection. GPC Sephadex G-15 was performed using 0.1M phosphate /0.1M KCl /AcCN 20% buffer, pH 9, flow rate 0.8 mL/min.

In previous non-desalted foaming experiments in this study there has been a problem with unsatisfactory separation of the protein from the salts in the dissolution mixture.

A second desalted foaming was carried out after the residual liquid of pH 5.31 was decreased by titration to pH 4.64. A small amount of precipitated protein collected on the surface or was suspended in the bulk liquid. Foam was so unstable that it was unable to form at the surface of the bulk liquid. The protein in a precipitated form proved more

difficult to extract from the liquid, and dried to form a thin pale brown layer on filter paper. The final residual liquid had a total protein concentration of 2.32 mg/mL compared to 2.82 mg/mL at the start of the attempted foam fractionation, suggesting that the foam fraction at pH 4.64 represented a meagre 0.50 mg/mL removed from the bulk liquid in the second foaming at pH 4.64. The precipitate obtained represented 5 % of the total protein in the bulk solution at the start of the foaming.

A fresh solution of desalted wool protein measured by GPC at 25.56 mg/mL was titrated to pH 6.5 with phosphoric acid 0.1M and foamed with nitrogen gas of flow rate 12.6 mL/min. White precipitate in the solution was just apparent at this pH and after 2 min foaming the bulk liquid had cleared. The conductivity of the bulk liquid was 12.39 mS/cm. The foam-precipitate was collected and left overnight at 4°C to collapse. The result was a clear gel-like material in the bottom of the beaker. Acid can deprotect the S-sulfonate anion and allow reformation of the disulfide linkages. The formation of a gel-like residue may have been due to the acid conditions allowing disulfide bridges to form. This material had the visual appearance of the films that were first poured using reduced wool protein solutions that were left to set in air. No analysis has been carried out on this material.

Using the minima in surface tension of pH 8.0 ± 0.2 did not give precipitation. The lower pH of 6.2 was associated with an immediate protein precipitation. With refinement this method could be used to precipitate protein from desalted solutions. The advantage of this method over the conventional method of adding acid to solubilized wool protein solutions would be a liquid waste that was low salt and more easily recycled, plus perhaps a partly separated protein class precipitate. CGE performed on decreasing pH showed some changes in the position of the peaks with different pH foam fractions, which supports some differentiation of the protein classes. Analysis by 1D SDS-PAGE indicates that precipitation has occurred across the wool protein classes with all MW bands present. However the bands of protein are very pronounced in the ~ 30 kD region, representing the UHSP. The 1D SDS-PAGE of the filtrate remaining after the initial precipitation showed strong IFP bands, but was much fainter in the HSP, UHSP and HG-TP. Thus partial separation would appear to have occurred with the desalted foam precipitation.

5.5 Conclusions

This study of foaming of wool protein has not achieved the objectives of separation and concentration of the proteins. Multiple protein mixtures require more research to determine the ideal parameters for separation of proteins as well as determining the best method for separation of protein from the non-protein constituents of the solution.

- The foam fractionation performed on dissolution mixtures did not provide a satisfactory method for separation of protein. Dilution of the dissolution mixture did provide enrichment of the protein content by foaming. The ionic or urea component did not appear to achieve a significant decrease when diluted dissolution mixtures were foamed. However the concentration of the protein on foaming diluted protein samples did enhance the protein to salt ratio.
- The uncertainty of the assays in urea and flame emission prevented a definitive resolution of the effect of foaming on urea and ionic components. Conductivity values indicated a reduction occurred in the salt contents of foamed samples, but results were not consistent and further confirmation by analyses is required. It would be desirable for more work to be carried out on developing a urea assay that is quick, reliable and copes with the very high concentrations encountered in wool dissolution mixtures. Flame emission assay of sodium ions was not successful, more study needs to be carried out to determine the reasons for the variable results. There were no references in the literature to foam fractionation of protein solutions that include ionic species and urea. This area needs to be investigated in depth to provide much need data.
- No significant difference between sparger gases of air or nitrogen was detected in these experiments when foaming of wool protein after oxidative sulfitolysis was carried out. Further investigation may be warranted using other gases like CO_2, as well as use of a sparger that delivers a smaller bubble to the bulk liquid.
- Foam fractionation does not appear to be worth pursuing for concentration of protein with solutions that are > 3 mg/mL, as little was achieved in terms of protein enrichment.
- Re-foaming of solutions more concentrated than 10 mg/mL was not found to be an advantage, with only slight increases in the protein concentration of foamed samples and slight decreases in the ionic and urea content. Re-foaming of dilute solutions was not investigated in this study, but merits consideration for future study. There is a need

for more data on the link between surface tension, pH and precipitation with desalted wool protein solutions.

- Considerable amounts of protein were left in the residue liquid after foaming. Recovery of 60 % of protein was achieved under optimal conditions of pH of 8.43 and gas flow of 12 mL/min, concentrating a wool protein solution from an intial concentration of 12.6 mg/mL to 16.9 mg/mL Analysis of total protein levels suggests that the first foam fraction achieved the best protein enrichment, this enrichment was the result of foaming < 20 % of the bulk volume.
- Surface tension changes are indicative of pH levels suitable for foaming to achieve precipitated protein from a low salt and urea solution.
- Tangential cross-flow filtration is a viable method for desalting of wool protein mixtures. A number of uncertainties still exist about the extent of fouling, its effect on the sieving process and the long-term viability of the membranes used.
- All the experiments in this study were conducted using batch methods. Continuous flow systems warrant investigation using the best methods achieved in this study.
- Desalted wool protein solution that had foam induced precipitation presented an option that should be considered in future investigation. The possibility exists that with appropriate manipulations of parameters it is possible to achieve separation of protein in much the same way as iso-electric focussing, but without addition of large acid volumes. The protein precipitate gained from foaming of desalted solutions was easily re-dissolved in a 0.1M phosphate/KCl buffer of pH 9. GPC of protein resulting from foaming of desalted solutions indicated that the precipitate from the foaming was over 99% pure protein.
- A novel approach for future investigation is the oil refining model. As the foam moves up the column taking fractions out at different foam heights may yield different protein compositions, analogous to fractional distillation. No reference to this idea has been found in the literature.

CHAPTER SIX

Conclusions and Future Work

6.1 Conclusion

The objective of this study was to investigate solubilization of wool protein and attempt to separate wool into the protein classes. New end-uses of wool protein will provide a better return to the agricultural sector. A broadened base of economic development confers advantages to New Zealand. Soluble wool protein has been investigated for over 50 years, but a feasible low cost separation scheme is needed for the development of products derived from soluble wool. To date such a scheme has not been available. If separation of wool protein can be achieved, then the development of downstream products will be pursued by the industrial sector more vigorously.

Preliminary investigation in this study involved wool protein solubilization by reduction and by oxidative sulfitolysis methods. Oxidative sulfitolysis produced a stable product for further processing. The S-sulfonated product of oxidative sulfitolysis was selected for investigation in more depth because it provided a stable product in sufficient yield for use with laboratory scale operations. Foam fractionation was chosen as a separation method for the soluble wool protein, because it offered a non-destructive technique, that was low cost and had been shown in studies to achieve separation and concentration while still retaining the protein in a soluble non-wool form (Uraizee and Narsimhan, 1996).

Capillary electrophoresis of soluble wool protein achieved partial resolution of protein peaks. An uncoated capillary delivered reproducible spectrographs using a zwitterionic buffer composed of 50 mmol 2-aminoethanol/ 0.1M CHES at pH 9. The capillary electrophoresis of gel permeation samples provided incomplete information regarding the wool protein classes, but did provide evidence of separation of wool proteins using capillary electrophoretic methods. Alternative buffers may achieve a better resolution of peaks. It is conceivable that a pattern might be at least qualitatively useful for caomparing different wool methods.

Capillary gel electrophoresis achieved separation of wool proteins and interfering salts, but the gel was insufficiently stable to achieve reproducible results. Retention of the gel in the capillary was a challenge for pH greater than 7 buffers. The best resolution achieved by CGE with a capillary filled with 10% dextran and using 0.1M Tris/ 0.1M CHES/ 0.1% SDS buffer of pH 9. Coating of the capillary walls with UV 200-220 nm transparent polymer may afford the protection needed for the silanol groups on the fused silica capillary. Reference proteins such as egg albumin produced spectrographs showing sharp peak resolution.

The urea assay did not exhibit reliable results. A variation of up to 20% between assays of the same sample was noted. The sodium ion reference solutions showed consistent values from assay to assay, but the wool protein samples were not reproducible and exhibited variation between assays for the same sample. Sodium reference values were established by using a solution of sodium ions without any dissoluting reagents present in the reference solution. The samples were diluted 1:2000 for the assay, despite the dilution there would appear to be an interference from reagents present and/or in the wool protein solutions.

The BCA assay was found to be unsuitable for determination of total protein in soluble wool protein with interference from the wool dissolving reagents. Total protein was analysed with reproducible results by the biuret assay where the solubilization was greater than 50%. Raised total protein values were obtained when using the biuret assay with wool dissolutions that had less then 40-50% solubilization. The inference from the raised total protein values with decreased solubilization, is that the interfering reagent is used up in the wool dissolution reaction and is below detection with the better solubilization percentage. Agreement of total protein values between the biuret and gel permeation chromatography assays was achieved for wool dissolution and foam fractionation solutions. Biuret can be recommended for use as a total protein assay for wool protein solutions where dissolution is more than 50% yield.

Gel permeation chromatography using Sephadex G-15 provided a method for assay of total protein. The GPC performed very consistently, providing reproducible results with the reference proteins BSA, EA, CA and with soluble wool proteins. Samples of solubilized wool proteins and samples of foam fractions separated into a single protein peak and

reagent peaks for each sample analysed by Sephadex G-15. The protein peak was used to establish the total protein present in samples, using dialysed freeze-dried wool protein as the reference protein for calculation of the concentration of protein present in solution. Dialysed, freeze-dried wool protein samples showed excellent correlation to the mass as measured by the protein peak produced by GPC of dialysed wool. The buffer 0.1M phosphate/ 0.1M potassium chloride/ 20% acetonitrile of pH 9 maintained the wool protein in soluble form as it was desalted by the column. Alternative buffers were not investigated but it is possible that better resolution could be achieved with a buffer that reduced some of the tailing effect observed with the Sephadex G-15 column. The column used was 10 cm long, a shorter column could be contemplated with a Sephadex G-25 gel, a more discriminating gel medium, or syringe microcolumns.

Reduction of wool produced soluble wool protein yields of 67%. The reduced product was unstable in air, auto-oxidizing readily unless an inert atmosphere (N_2) was maintained. The short solubilization time of 3 h, the low cost of reagents at $0.06/g of wool processed and the low wool:liquor ratio 1:10, suggest that further investigation of the reduction method is warranted. Development of a more stable reduced wool material would be beneficial to ongoing processing of the wool protein.

Oxidative sulfitolysis of wool protein using TTN achieved yields of 60%. S-sulfonated soluble wool was stable in air and stored at 4 ^{o}C without apparent deterioration for periods up to two weeks. Using tetrathionate as the key reagent for oxidative sulfitolysis was relatively expensive at $1.13/g wool compared to the reagent cost by sulfide reduction. The wool:liquor ratio of 1:30 combined with the longer solubilization time 8-24 h and the higher cost does not favour oxidative sulfitolysis by TTN for large scale soluble wool protein. The necessary inclusion of urea 8M in the processing was a major disadvantage and created problems in the downstream processing of the soluble wool protein. Electrolytic oxidative sulfitolysis offers an alternative pathway for production of a sulfonated product that uses lesser amounts of reagent.

Maceration dramatically improved solubilization in terms of yield and time taken for both reduced and oxidized methods. A 3 h maceration by intermittent maceration by Ultra-Turrax resulted in the extraction of 90% of the total yield obtained of soluble protein using

the oxidative sulfitolysis method. Adoption of a freeze-thaw regime for wool soaked in reagents prior to maceration achieved increased yields and shortened the time required for processing. Changes due to pre-soaking and freeze-thaw regimes require more assessment before being used as part of any processing. Alkaline degradation was a potential threat to the protein integrity in both main solubilization methods investigated. Any shortening of the time in processing is advantageous and warrants further investigation as part of the solubilization process.

Both reduction and oxidative sulfitolysis produced a range of wool protein classes. Neither method can be recommended over the other. The type of product aimed for will determine the method of solubilization that will provide the feedstock of soluble protein for production of that product.

Isoelectric precipitation achieved some separation of wool protein classes. The large salt additions and large liquid volumes involved in using acid precipitation and salting out of wool proteins as a separation process deem alternative methods worthy of investigation.

Foam fractionation concentrated diluted dissoluted wool protein. Yields, separation and increased enrichment were maximized by low gas flows. Nitrogen and air were investigated as foaming gases with not major difference detected when used with sulfonated wool protein. If reduction was used to achieve solubilization then an inert gas such as nitrogen woulf have to be used. Analyses of foam fractions for urea and salt content, whilst not reliable did indicate that only minor reductions were being achieved with foam fractionation. It may be that ions are being carried over in the foam in association with the protein, improvements to the drainage and coalescence of bubbles whilst in the column may improve the separation of the soluble wool protein from the ions and urea. There is a dearth of literature citing changes to ionic content in foams and thus would seem an area worthy of research.

In this study of foam fractionation did not offer a method for the separation of solubilized wool protein. Wool protein solutions at pHs below 8 showed an increasing tendency toward precipitation with decreasing pH. Desalted wool protein solution precipitated protein when foamed at pH 6.3, a pH that normally results in only a small precipitation.

The wool protein in solution on foaming was concentrating at the gas/liquid interface and aggregating into solid protein. Foaming acts to favour the accumulation of protein at the surface of the bubble. The amount of acid added to the desalted solution was considerably reduced compared to the normal isoelectric precipitation methods used. The solid wool protein that was obtained by foaming desalted wool solution readily re-dissolved after freeze-drying and storage at $4^{\circ}C$. Some separation of the wool protein classes was achieved in foam precipitation of the desalted wool protein solutions based on 1-D PAGE-SDS analysis. The UHSP appear to have been left in solution. It was the production of precipitated protein without large quantities of acid addition or salt that suggests that foam fractionation would be warrant further investigation. The technique may be applicable to a proteins as well as wool proteins .

6.2 Future Work

The separation of soluble wool proteins has centred in the past on the properties of pI and molecular size. Investigation of protein interactions that may lead to separation by new methods would be beneficial and provide information about the characteristics of the proteins that might assist separation. Wool protein classes exhibit specific characteristics that enable separation. The challenge is in utilizing those characteristics to achieve separation in a few steps, at low cost, on a large scale, efficiently.

The use of urea for denaturing is widespread and an improved urea assay appropriate to high concentrations in use with wool solubilizations would help in determining the effect of separation steps on the urea concentration in solution. The result of separation steps on the ionic content was not adequately tracked by flame emission or conductivity in this study. Improvements and examination of alternative methods of assay of ions in the solutions is needed.

The solubilization of wool is achievable. However in view of the high cost and problems of desalting of the oxidative sulfitolysis processes and the instability of the reduced product, enzyme solubilization must be considered in any future investigation. Enzyme solubilization was not examined in this study, nor was the study of timed proteolysis. It is possible that using an enzyme with high specificity to achieve disulfide bond scission would

allow extraction of certain classes of wool protein from the wool and consequently lead to a somewhat different method of separation.

If oxidative sulfitolysis of wool is deemed the best pathway to achieve soluble wool protein, then electrolytic oxidative sulfitolysis is worthy of further investigation.

Foam fractionation of reduced wool protein solutions may provide a pathway for separation. However the cheapest gas available, air, is not applicable if reduced wool protein is to be considered as a solubilization pathway. As only S-sulfonated protein was investigated her in detail there is a need to examine separation by foam fraction of other solubilized wool proteins. The desalting of soluble wool protein followed by foaming of the acidified wool protein resulted in a solid protein that deserves further investigation as an alternative to the traditional ioselectric and salting out processes currently used. Desalting by electrodialysis is used in the dairy industry and may offer a better pathway than ultrafiltration or dialysis in large volumes of liquid.

No investigation was carried out in this study of the usefulness of ion exchange or of covalent chromatography as separation methods. Covalent chromatography could provide a pathway for separation of the wool proteins. The most desirable functional group that is available for formation of stable covalent bonds with wool proteins is the thiol. Reduced soluble wool protein may be able to be bound to a solid phase thiol with the formation of disufide linkage. This may offer a method of separating wool proteins based on sulfur content, a characteristic that defines the protein classes. The specificity of covalent chromatography warrants further investigation.

Efficient, low cost and large scale separation of wool proteins on industrial scale remains a goal yet to be achieved. The special characteristics of wool proteins must however lead to a method that enables separation of wool classes to be achieved.

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