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

This thesis was presented in part fulfilment of the requirements for the degree of Master of Science in Chemistry at Massey University

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Errata Adrian Jull, MSc thesis

p. 3 para 3, line, 3 **principally**
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 $\leftrightharpoons$
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°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**methane**, 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

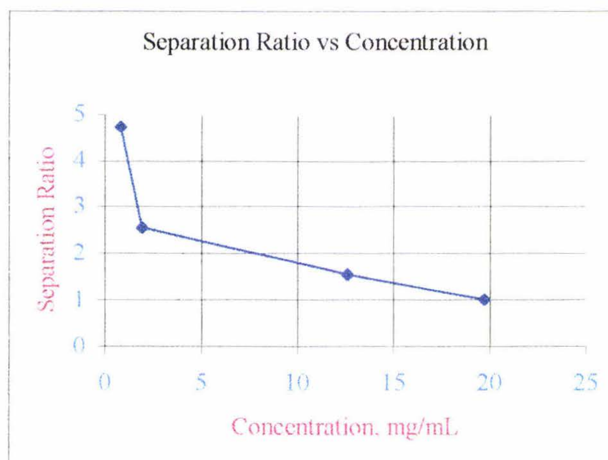

Figure 5.3.4b Degree of separation ratio achieved with concentration change
Dissoluted wool was foamed in a 29 cm x 35 mm ID column using nitrogen gas at a flow rate of 12-14.4 mL/min. A foam fraction was collected after a 10 mL decrease in the foamed bulk liquid volume, (200 mL) occurred. Dissoluted 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.

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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
$\underline{P}$.	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).

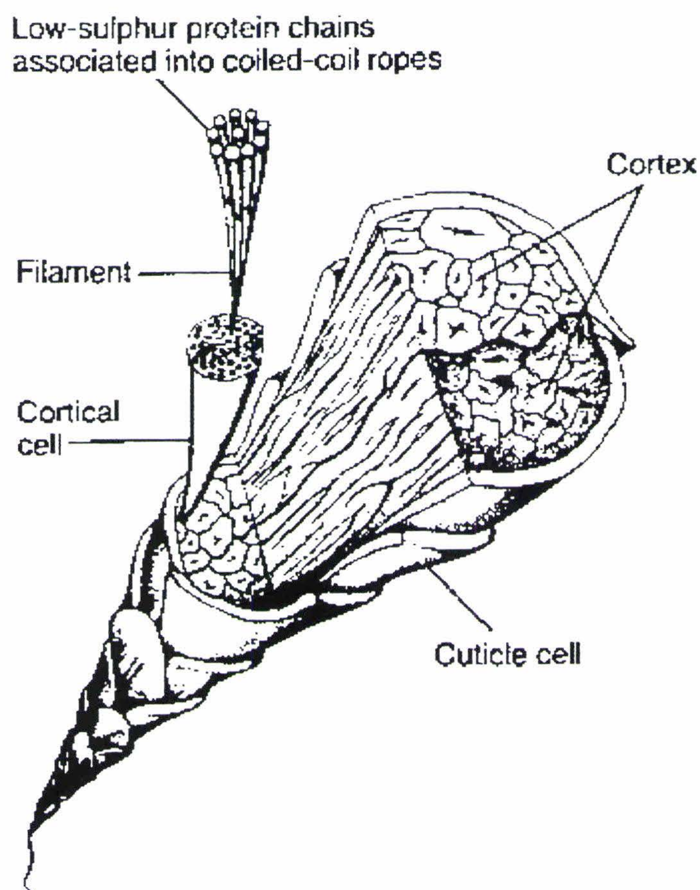
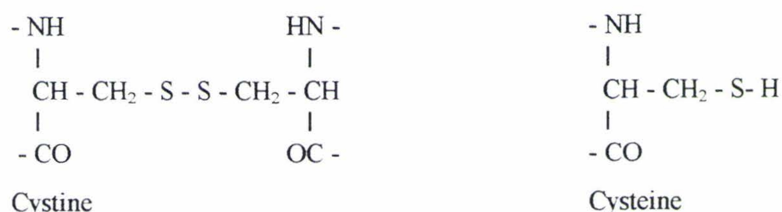


Figure 1.1 Schematic representation of a wool fibre

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).



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).

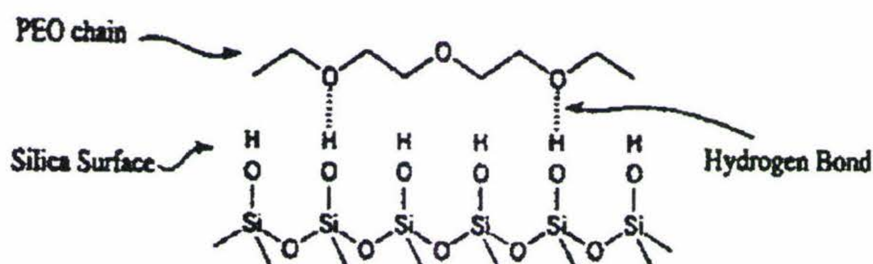


Figure 1.2.2 Interaction of PEO with silanol on capillary walls

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).

$$WSSW + 2RS^- \leftrightharpoons RSSR + 2WS^- \quad \text{Eqn 1.2}$$

$$WSSW + 2SO_3^{2-} + \leftrightharpoons 2WSSO_3^- \quad \text{Eqn 1.3}$$

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).

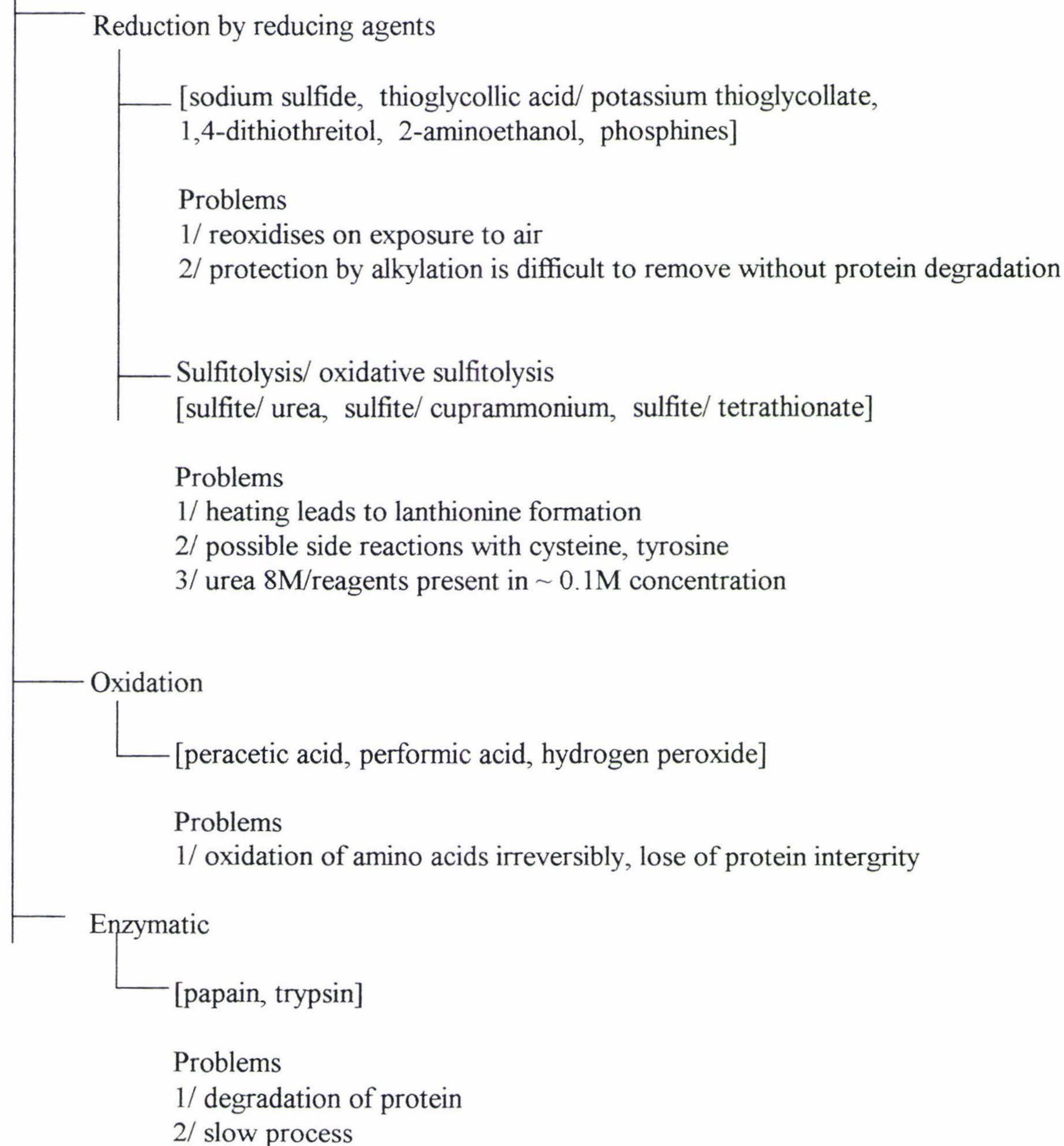
Wool protein was extracted using reducing conditions of alkaline glycollate from native wool (Goddard and Michaelis, 1935). Goddard and Michaelis reported finding two fractions in reduced wool protein that differed in solubility and sulfur content. Extraction of wool requires breaking of disulfide bonds 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

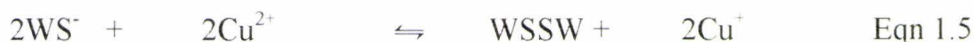
(Thomas et al. 1983; Kelly 1998). Yields obtained for dissolution vary according to conditions used with alkaline reducing conditions reported as producing yields as high as 87% (Thomas et al., 1983; Maclaren and Milligan, 1981; Wormald, 1948). Most cited solubilizations of wool protein were carried 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 $\sim 10^2$ 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

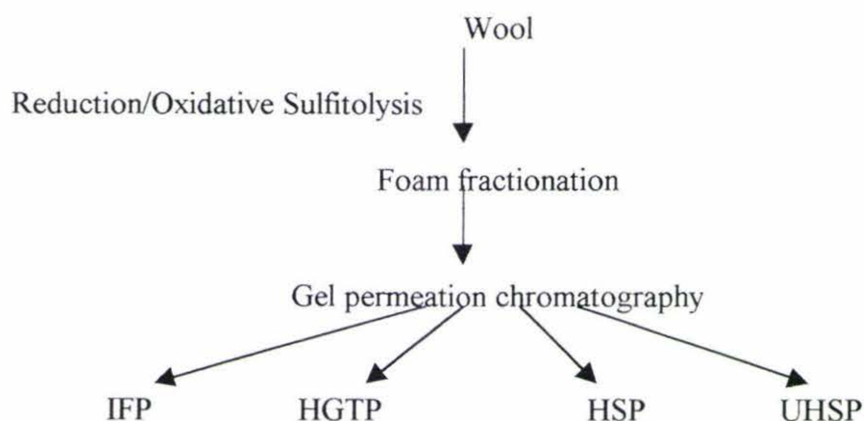
Wool fibres can absorb water up to 30% of total mass, but are insoluble in water. To extract all the protein the internal fibrils of the wool fibre must be penetrated by the dissoluting solution and the soluble protein released. Use of maceration to shatter cells assists the release of protein. Freezing 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

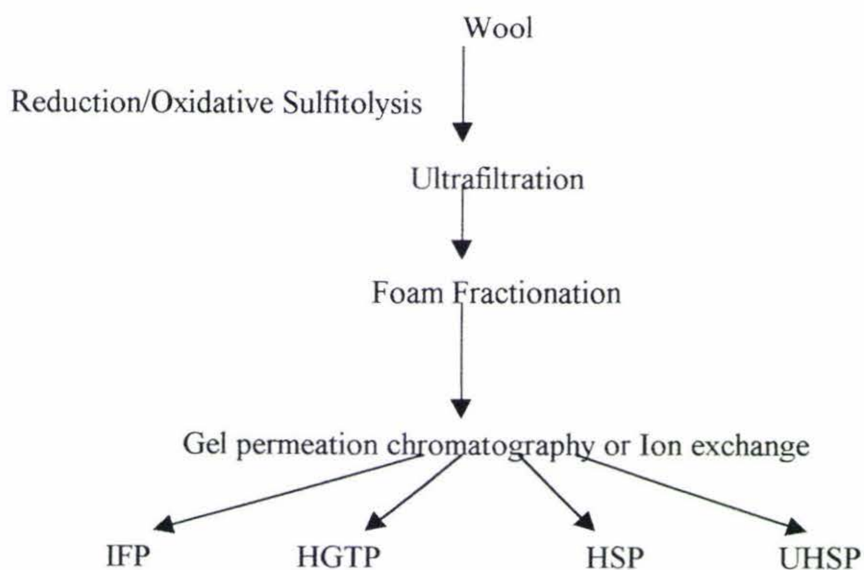
The objective of a separation strategy is to obtain the maximum yield of protein with maximum purity, cost-effectively in a minimum of steps (Harris and Angal, 1989). Each protein has unique properties that can be exploited in separation techniques. The major properties of proteins to exploit in separation 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).

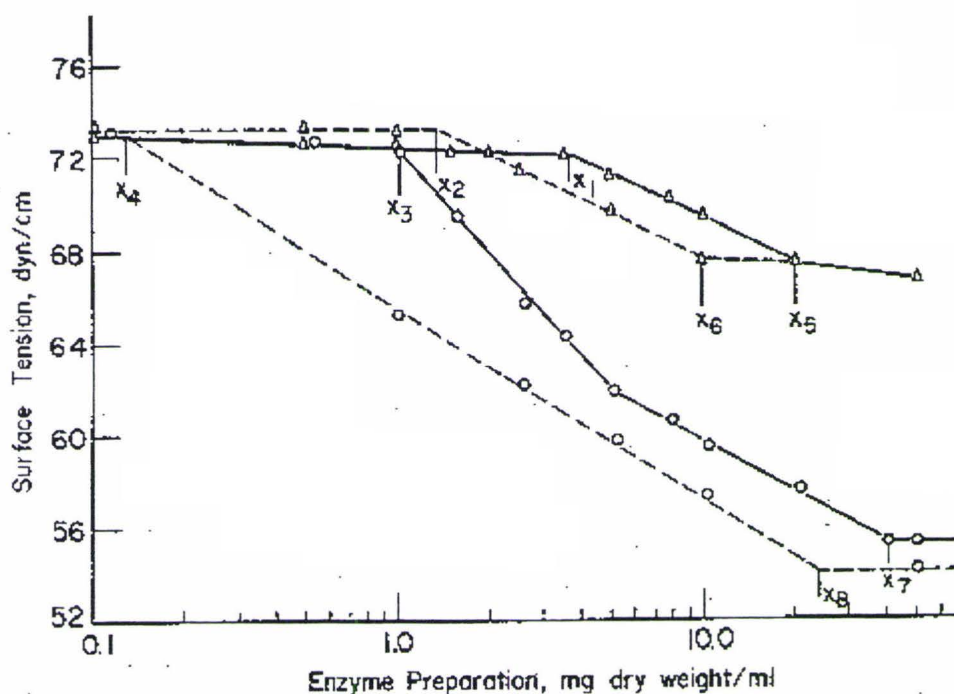


Figure 1.4.1 Surface tension-concentration for catalase and amylase.

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 25^0C 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.