DEXTRAN ENZYME IMINE COMPLEXES: A PRELIMINARY STUDY

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ABSTRACT

A model system involving the formation of protein-dextran complexes has been investigated with a view to improving existing methods of drug administration. Activation of the dextran was achieved by periodate oxidation to give levels of 7%, 21% and 56% activated glucose moieties. The protein-dextran complexes were investigated with the prospect of obtaining sustained release of proteins from the dextran in an unmodified form. Covalent conjugation of proteins to carbohydrate polymers is known to confer stability on the protein. The proteins in this study were bound to the dextran through imine bonds. The proteins investigated were lysozyme, trypsin, amylase, alcohol dehydrogenase and catalase. The selection covered a range of molecular weights and varying enzymatic activities.

As might be predicted, the speed of complex formation was shown to be greater at the 21% level of activation compared to the 7% activation of dextran in all cases studied.

Lysozyme, the smallest protein, readily formed complexes at all three levels of activation. At the 56% level the resulting complex had an extremely high MW, greater than 1MDa. The extensive binding between the dextran and lysozyme molecules resulted in a complex that was inactive and showed no signs of releasing any lysozyme, active or inactive. At the lower levels of activation, complex was formed with relative ease. Upon conjugation lysozyme exhibited only minimal activity. Release of a lysozyme-like species with normal lytic activity was observed.

Precautions were taken to minimise possible autolysis in the trypsin study. Once complexed it was postulated that autolysis would be prevented or minimised. Similarly the 56% level of activation appeared to be too high to obtain a viable complex for facile trypsin release. Sustained release of a trypsin-like protein was observed with complexes at the 7% and 21% levels. SEC and SDS-PAGE, in conjunction with a positive BAPNA assay gave support to the released species being trypsin-like. While complexed to the dextran trypsin showed no signs of activity. Released trypsin-like species and unreacted trypsin showed similar tryptic maps from a synthetic peptide, the peptide was designed to show distinctive fragments.

α-Amylase, twice the MW of trypsin and over three times the MW of lysozyme, formed complexes with ease at both 7% and 21% levels of activation. Conjugation to dextran did not effect the activity of α-amylase. Over time the release of an α-amylase-like species from the complex was observed.

Alcohol dehydrogenase and catalase are both high MW proteins. Complex formation was observed for each protein. Subsequent experiments showed that upon release the proteins appeared to dissociate, most probably into their subunits. It is also possible that the dimers and monomers bound to the dextran. The main advantage of conjugation in this case appeared to be to confer stability on the proteins. The ADH-complex exhibited enzymatic activity.

At 7% and 21% activation levels the lower MW proteins formed complexes with dextran that exhibited release of a protein species. The higher MW proteins were possibly stabilised when conjugated to dextran, but dissociated upon release. Investigations have shown that the level of activation chosen affects the extent of binding and therefore the functions of the resultant complex. Thus activation levels can be manipulated depending on the desired result. While lower dextran activation levels appeared to be more suited for smaller MW proteins, there were indications that the larger MW proteins could form beneficial complexes at higher activation levels. Results indicated that conjugation to periodate activated dextran could be extended to further proteins with the possibility of therapeutic or commercial applications.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES AND SCHEMES

LIST OF ABBREVIATIONS

AAA	amino acid analysis
Ab	antibody
ADH	alcohol dehydrogenase
BAPNA	N-α-benzoyl-DL-arginine-ρ-nitrolanilide HCl
BPNPG-7	blocked p-nitrophenyl maltoheptaoside
CD4	cell surface glycoprotein receptor for HIV
DMSO	dimethyl sulphoxide
DOR	double oxidised residues
FMOC	fluorenylmethoxycarbonyl
GI tract	gastro-intestinal tract
GP120	glycoprotein-120
HPLC	high performance liquid chromatography
FPLC	fast performance liquid chromatography
LLST	laser light scattering technique
met-hGH	recombinant methionyl human growth hormone
MWCO	molecular weight cut off
$NaBH_4$	sodium borohydride
$NaBH_3CN$	sodium cyanoborohydride
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
mPEG	monomethoxypoly(ethylene glycol)
PEG	polyethylene glycol
PNP	purine nucleoside phosphorylase
rhGH	recombinant human growth hormone
rIGF-1	recombinant human insulin-like growth factor
rIL-2	recombinant human interleukin-2
rtPA	recombinant human tissue plasminogen activator
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electropheresis
SEC	size exclusion chromatography
SPPS	solid phase peptide synthesis
Tris	tris-(hydroxymethyl-)aminomethane
TFA	trifluoroacetic acid
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone

Abbreviations used for amino acids:

Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic acid	Asp
Cysteine	Cys
Glutamic acid	Glu
Glutamine	Gln
Glycine	Gly
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tyrosine	Tyr
Tryptophan	Trp
Valine	Val

Asx	asparagine and aspartic acid
Glx	glutamine and glutamic acid

CHAPTER ONE

INTRODUCTION

The world abounds with biological processes that require the confluence of a number of biologically active materials. Important parameters include specific life times and defined concentrations. Natural processes are very efficient in accomplishing this. These parameters have been manipulated by man to improve and commercialise some processes, for example the use of α-amylase to convert starch to sugar for the baking industry. In biomedicine when these bioprocesses fail to achieve their 'normal' task, scientists have investigated the prospect of delivering synthetic materials to specific sites in the body to 'correct' the malfunction.

The actual delivery of biologically active materials is an important aspect of current biomedical research. In the case of the therapeutic drugs current methods include oral ingestion (solid tablets and liquids), and subcutaneous and intravenous injections. Research aimed at improving the existing methods of drug delivery include encapsulation of the drug, non-reversible covalent bonding to a polymer, and sustained release from a polymer. Sustained release of biologically active materials is especially important when the drug is a protein. Leading on from this research, the slow release of enzymes bound to polymers should also find industrial applications.

1.1 Drug Delivery

Large quantities of proteins with pharmacological activity are produced as a result of recent advances in recombinant DNA technology (Oliyai and Stella, 1993). The therapeutic application of many of these proteins is often limited by the need for high, frequent or multiple dosage required to maintain effective drug levels which can lead to toxicity (Battersby *et al*, 1996; Kim *et al*, 1980). The ability to obtain a controlled delivery of therapeutic proteins would therefore greatly improve the existing methods of drug administration.

Investigation into encapsulation with copolymers, and non-reversible covalent bonding of a protein to a carrier such as polyethlene glycol, PEG, has been undertaken. In addition to investigating these two techniques, Genentech Inc., a South San Francisco based company, has also studied the potential for increasing the biological half life of a protein by the administration of a soluble, reversible protein-carrier conjugate. Their interest in this field came about after they observed the formation of a specific imine adduct

between acetaldehyde and the amino terminal group of met-hGH, recombinant methionyl human growth hormone. Imine formation occurred with good yield and could subsequently be hydrolysed with ease to produce an unmodified met-hGH. Because of the unexpected degree of stability of this imine adduct, further investigations into other imine linkages were undertaken with the view of developing sustained release products (Hancock and Battersby, 1991).

In 1970 the first report of controlled release of a macromolecule from a biocompatible polymer was reported. This involved the release of cyclazocine, an analgesic, from a poly(lactic acid) implant (Dang and Saltzman, 1994). It was however another seven years before the technique was applied to other proteins (Langer *et al*, 1996), showing that slow release mechanisms could be used for other large molecules. Polymer-based-release systems for small peptides developed rapidly in the 1980's.

1.2 Controlled Release of Drugs

For the ideal system of drug delivery there should be enough of the drug available to be effective, but not enough to cause toxic side effects (Kim *et al*, 1980). Using conventional methods this would involve the repeated administration of small quantities of the drug which is often impractical, in certain cases impossible and certainly unpleasant for children. A preferred approach would be to provide a 'protected drug supply' with release of the drug over time. This would be optimised if the release rate of the drug could be controlled by a prodrug system. Simple prodrug systems were first reported with oral dosage tablets over two decades ago (Kim *et al*, 1980). Once in the gastro-intestinal tract (GI) the polymeric materials within the tablet are slowly dissolved. The polymer acts as a barrier for dissolution in the stomach.

Carriers for drugs, including therapeutic proteins, can be divided into two broad groups, physical carriers and chemical carriers (Kim *et al*, 1980). Physical carriers control the release rate of the drug by diffusion through a polymer matrix. Chemical carriers control the rate of release through cleavage of the bonds formed between the drug and the carrier. The advantage of a chemical carrier is that it can decrease any toxicity of bioactive agents while increasing therapeutic efficiency.

The main aspects to consider for the prodrug action include dissolution or degradation at the point of administration of both the drug and the carrier (Kim *et al*, 1980). An intact prodrug will show modified absorption, distribution and excretion characteristics

compared to a simple drug. Due to the size difference between the released drug and the complex, distribution between different tissues will vary.

The design of controlled release systems that are applicable to existing drugs may be cheaper than searching for new drugs (Leong and Langer, 1987; Li *et al*, 1987). Controlled release systems should ideally increase the half life of the drug, decrease its clearance rate, prolong its activity and target it to a particular location. Diseases caused by enzyme deficiencies can be better treated by more efficient targeting, as can cancer therapies.

1.3 Encapsulation

Encapsulation is one of the main drug delivery systems studied in the last ten to fifteen years that offers potential for a slow release mechanism. It involves enclosing the protein within a microsphere (Langer *et al*, 1996). This technique has proven successful with small peptides and other small molecules, although not so much with proteins. Conditions required to form the microspheres usually result in denaturation of the protein because the technique involves dissolving a protein-polymer mixture in an organic solvent (Johnson *et al*, 1996; Langer *et al*, 1996). To achieve milder conditions required for proteins, double emulsion methods have been investigated (Cleland and Jones, 1996). These prevent direct protein-organic solvent interactions which may lead to chemical modification, denaturation and aggregation of the protein (Johnson *et al*, 1996).

Release of the protein from microspheres occurs in three stages: initial burst, diffusion controlled release and erosion controlled release (Cleland and Jones, 1996). Major areas of concern include minimising the initial burst, maximising the protein concentration and obtaining a continuous (one phase) release. *In vitro* experiments involving encapsulation of rhGH have been performed by Johnson *et al* (1996). They involved complexing the rhGH with zinc which causes dimerisation to occur stabilising the protein for subsequent encapsulation. Animal studies showed that the encapsulated protein maintained biological activity, and resulted in steady levels of the protein in the serum over a month. Immune responses were not a significant problem.

1.4 Non-reversible Covalent Bonding

Covalent attachment of proteins to polymers has also been used as a means of improving the therapeutic properties of proteins. The polymer acts as a protector against proteolytic attack, renal clearance and immunological response (Monfardini *et al*, 1995). The polymer at the focus of these studies was monomethoxypoly (ethylene glycol), mPEG:

CH_3-O(CH_2-CH_2-O)nH (mPEG)

Several proteins have shown improved therapeutic efficiency when bound to the mPEG including CD4-IgG, recombinant human interleukin-2 (rIL-2) and human growth hormones, hGH (Chamow *et al*, 1994; Knauf *et al*, 1988; Clark *et al* 1996). The biggest benefit seems to come from the longer clearance rate. In the case of hGH there was a decrease in the binding affinity and bioactivity, but the benefits of the longer clearance rate out weighed these other factors (Clark *et al*, 1996).

Clinical studies have shown that mPEG modified adenosine deaminase used in the treatment of severe combined immunodeficiency disease is therapeutically effective (Chamow *et al*, 1994; Hershfiled *et al*, 1987). This condition which effects children, is caused by an adenosine deaminase deficiency and requires bone marrow transplants. Admistration of a modified mPEG enzyme is seen as an improved alternative because of its efficacy, safety and convenience.

A technique has also been developed to ensure that the activity of the protein is maintained upon binding to the mPEG polymer (Caliceti *et al*, 1993 and 1994). It involves the immobilisation of an active site inhibitor onto an insoluble resin. An enzyme is then bound to the inhibitor, which blocks the active site from mPEG during binding ensuring the active site is not obscured. The PEG-enzyme complex is then released. This method has been used with urokinase which was shown maintain its thrombolytic activity after binding (Caliceti *et al*, 1994). *In vitro* experiments showed that the enzyme was more stable, while *in vivo* experiments demonstrated that it had an extended circulatory lifetime.

1.5 Sustained Release of rhGH from Dextran

An *in vitro* study with recombinant human growth hormone (rhGH) showed that it was possible to bind the protein to oxidised dextran and then observe release of unmodified rhGH (Battersby *et al*, 1996). This was determined by N-terminal sequencing and tryptic mapping. From *in vivo* studies performed on rats at Genentech, it was found that the complexes formed between rhGH and activated dextran, when injected into rats, resulted in prolonged weight gain. At the same time normal bolus injections of rhGH given to control rats resulted in normal immediate growth. In this way it was shown that the rhGH-dextran complex maintained a higher level of growth hormone in the blood over a 1 to 2 day period.

Although this initial release of the rhGH only covered a two day period, it points to the possible extension of this technique by either varying the activation level of the dextran or by using differing MW dextran polymers, or both. The complex itself was formed readily and was stable at 4^0C for at least ten days (Puchulu-Campanella, 1991-1993). It could be stored for longer periods at -70°C without loss of activity. Protein release was studied by comparing reduced (with sodium cyanoborohydride) and non-reduced complexes. It was shown that release requires a non-reduced bond, presumed to be an imine. Overall it was found that the adduct formed between the rhGH and oxidised dextran was biologically active, and the presence of rhGH in rats was shown to be prolonged. When rhGH is released in the bloodstream, formation of non-specific complexes between dextran and other proteins was not found to be a problem.

Several other proteins have been studied for their ability to form complexes with the dextran (Puchulu-Campanella, 1991-1993). These include rIGF-1 (MW 7 655Da), CD4 (MW 40 962Da), rtPA (recombinant tissue plasminogen activator, MW 59 042Da), and GP120 (glycoprotein-120 MW 120 000Da). All were found to have the ability to bind to oxidised dextran. From the kinetics of complex formation, it was found that rIGF-1 (the smallest) was the fastest, while GP120 (largest) was the slowest at complex formation. CD4, a cell surface glycoprotein receptor for HIV, is twice the molecular weight of Met-hGH. Kinetic studies showed complex formation to be slower for CD4 which is thought to result, at least in part, from steric hindrance caused by the carbohydrate attached to the protein. IGF-1, recombinant human insulin-like growth factor-1, had the fastest kinetics for complex formation. Release studies for IGF-1 showed a drop in the complex concentration, but no signs of the free protein. Size exclusion chromatography (SEC) showed that upon release, the protein had been hydrolysed into smaller fragments which had longer retention times. GP120 was the largest protein studied (MW 120,000) and it

was also shown to formed a complex with the dextran. Thus the formation of imine adducts between proteins and organic polymers may be a general phenomenon.

1.6 Periodate Oxidation of Dextran

This present investigation seeks to extend the Genentech study. It involves the investigation of complex formation and release of enzymes from Dextran T-40. Dextran is a synthetic polymer made up of repeating glucose units, consisting of 95% α-1,6 links, and the remainder being α-1,3 linked moieties (Battersby *et al*, 1996). The dextran itself can be purchased in varying sizes starting at 2kDa. Dextran T-40 covers a reasonable range of molecular weights, but averages 40 000 Da (Figure 1.6.1).

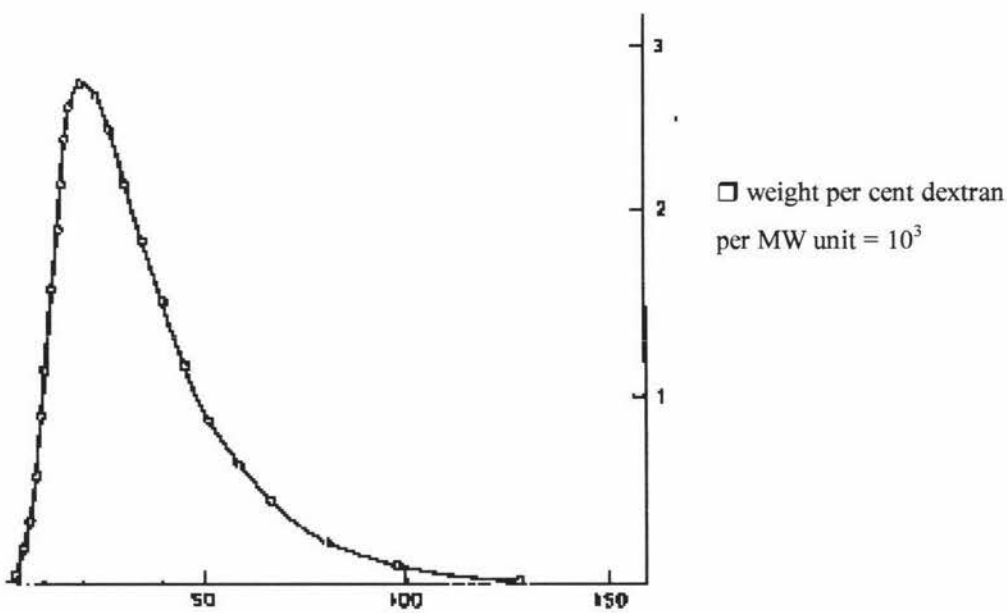


Figure 1.6.1 Molecular weight distribution by gel filtration of Dextran T-40
(Courtesy of Pharmacia)

Aldehyde functionalised dextran (oxidation of the glucose units within the dextran) can be produced by oxidation of dextran with sodium periodate. Dextran contains monomers with vicinal (adjacent) diols that form aldehyde groups by reaction with periodate.

The first step in periodate oxidation involves the formation of a single oxidised intermediate (Figure 1.6.2). This then forms either the desired double oxidised residue (after loss of formic acid) or a hemiacetal. Periodate oxidation of Dextran T-40 has been investigated in both buffered and unbuffered conditions (Puchulu-Campanella, 1991-1993). This led to the finding that under buffered conditions there is a more rapid decay of single oxidised intermediates due to the instability of hemiacetals, and there is greater formation of the double oxidised residues in Dextran T-40. It is the double oxidised residues that are more desirable for reaction with nucleophilic agents. The presence of hemiacetals reduces the accessibility of aldehydes for reaction with the nucleophiles. Protein amino groups can react with aldehydes to produce imine bonds. Therefore the oxidised dextran reacts with ε-lysine and the N-terminal amino groups in proteins.

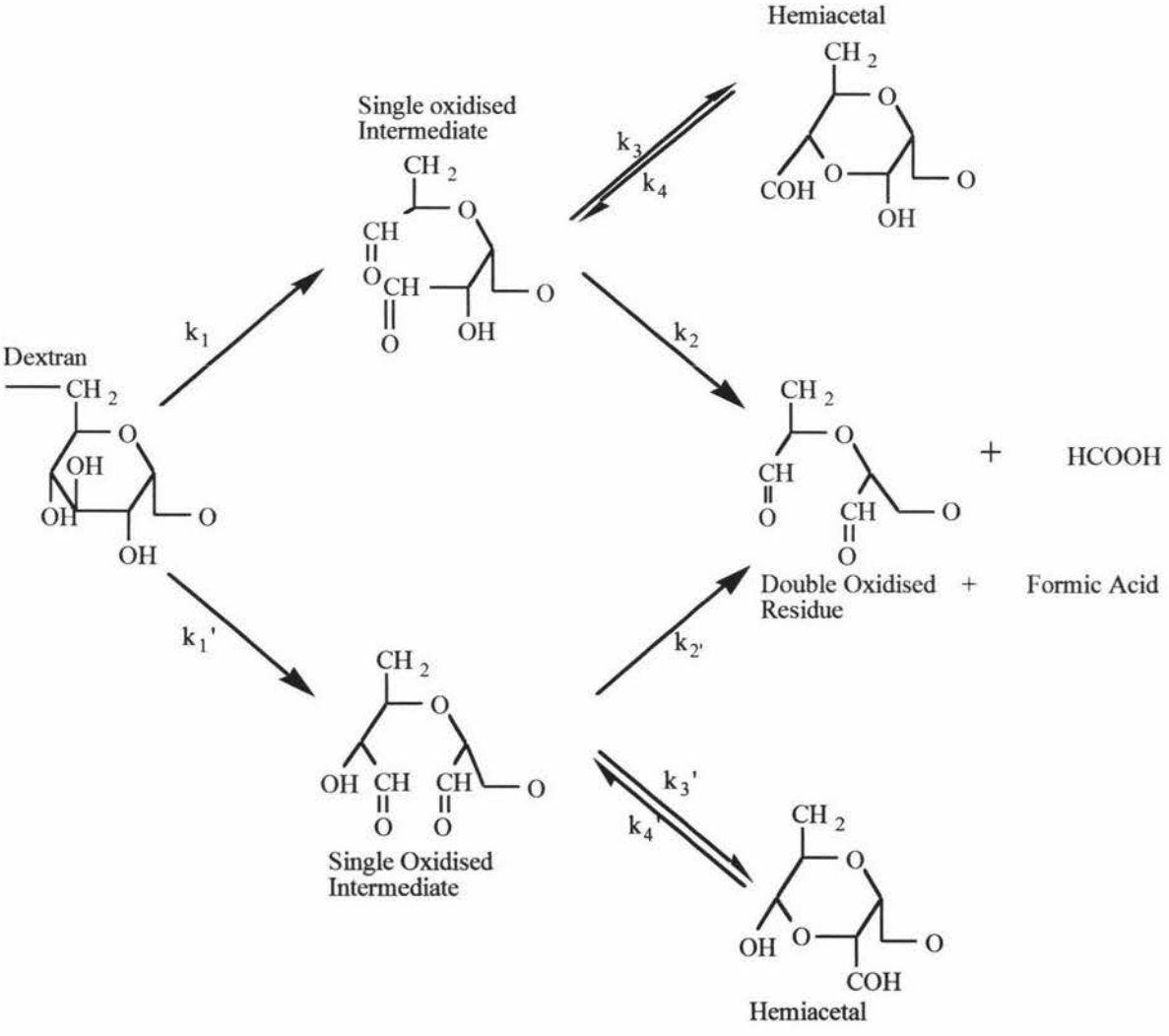


Figure 1.6.2 Periodate oxidation of Dextran (Battersby *et al*, 1996)

The level of activated, or oxidised, dextran molecules can be expressed as a percentage of glucose units that have been oxidised by partial sodium periodate oxidation of dextran (Puchulu-Campanella, 1991-1993). The assumption is made that 2moles of oxidant are consumed per glucose residue. This calculation is performed by taking aliquots of oxidised dextran and titrating with iodine following the addition of arsenite.

$$H_2AsO_3^- + IO_4^- \rightarrow H_2AsO_4^- + IO_3^-$$

$$H_2AsO_3^- + I_2 + H_2O \rightarrow H_2AsO_4^- + 2I^- + 2H^+$$

The arsenite reacts with the unreacted periodate and the excess of arsenite is then titrated with iodine. Moles of periodate consumed are determined from the difference between the amount of arsenite added and the amount of iodine required to take the titration of to the end point. 1mg of dextran is equivalent to 6.25μmole glucose. Therefore the amount of glucose present can be calculated. As it takes 2 moles of periodate to oxidise 1 mole of glucose the percentage of activation is equal to half the moles periodate used over the moles of dextran present times 100.

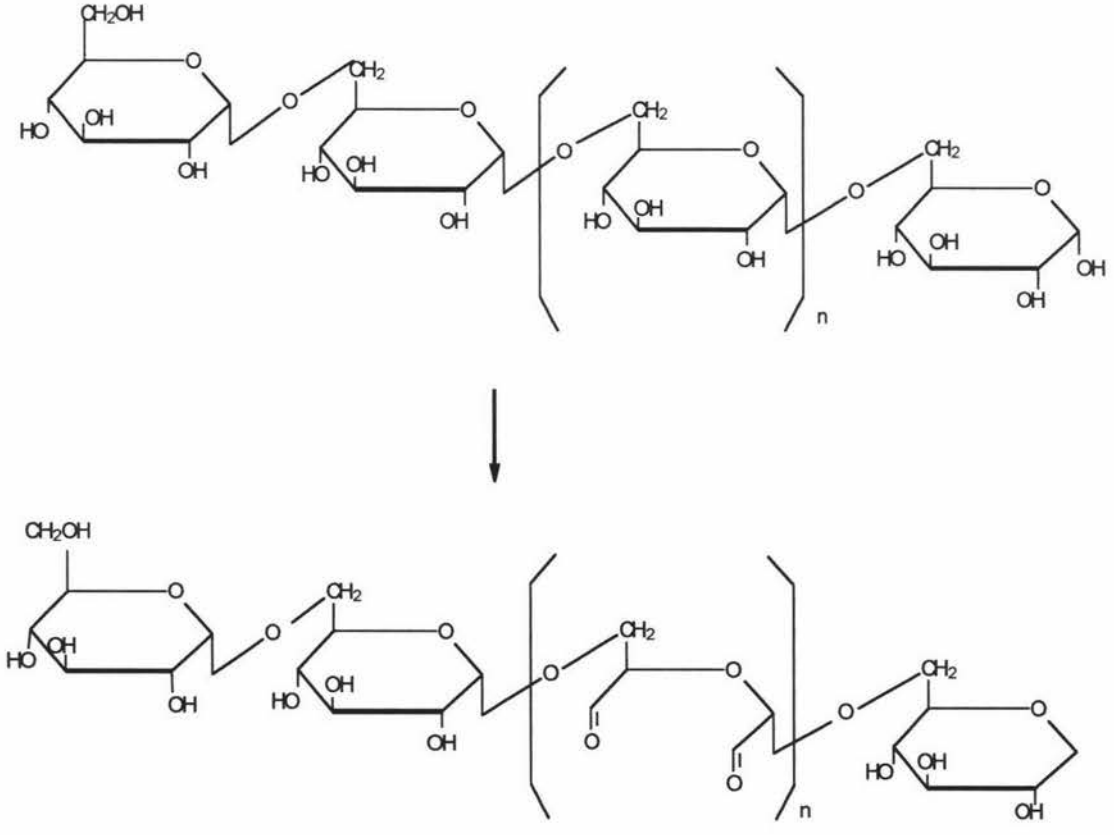


Figure 1.6.3 Overall reaction individual glucose molecule periodate oxidation

Activation of dextran can also be expressed as a percentage of double oxidised residues (%DOR), which is equal to:

$$Ax^2 + Bx + C$$

where x is the percentage oxidation and A, B and C are constants (8.336×10^{-3}, 7.648×10^{-2} and 2.63 respectively).

Kobayashi and Tukatsu (1994) attached trypsin to periodate activated dextran (MW 63 000) at 30°C, pH 8.5. The resultant modified trypsin showed an increase in stability, was not subject to autolysis and was no longer inhibited by soybean trypsin inhibitor.

1.7 Imine Formation

The dialdehyde formed upon reaction of dextran with periodate may react with amino groups in a number of ways. Two of these possibilities, an imine or an oxa-azepine (Kobayashi *et al*, 1990), were investigated with reference to the dialdehyde of cyclodextrin, an α-1,4 linked cyclic glucan. While the oxazepine linkage is stable it caused irreversible inactivation between oxidised dextran and phosphorylase kinase. Reaction conditions dictate to a certain extent the type of product formed. Imine formation is favoured at higher temperatures and pH (Kobayashi and Funane, 1993).

The imine product, or Schiff's base, forms a stable linkage upon reduction in the presence of sodium borohydride or sodium cyanoborohydride. This has been used for immobilisation of several proteins on to periodate oxidised dextran (Battersby *et al*, 1996). Modification by reduction of imine conjugates was seen as a means of avoiding the unstable conjugate. There is now evidence that imine conjugates are more stable than once was thought, and that reduction may not be necessary to obtain a useful product. This was evident in the case of trypsin (Kobayashi and Takatsu, 1994) which after conjugation with dextran was stable enough without reduction to be isolated by SEC.

Variables to be considered upon the binding of a protein to dextran include the MW range of dextran, the type of activation and the amount of protein in the resulting complex (Molteni, 1979). Several agents have been used for the activation of dextran including periodate, azide, cyanogen halides, organic cyanates and epoxyhalopropyl. It is also feasible to dictate coupling via the protein (Mitra *et al*, 1993). For example, bilirubin oxidase had amino groups introduced to allow and enhance its binding to PEG. With purine nucleoside phosphorylase, PNP, additional binding sites for PEG were required. This was achieved by site-directed mutagenesis of some of the arginine

residues to lysine, giving rise to additional binding. The mutant enzyme maintained its catalytic activity and the resulting conjugate was less susceptible to immune responses.

1.8 Complex Formation of Proteins with Dextran

$$RCHO + H_2N\text{-Protein} \leftrightarrow RCH{=}N\text{-Protein}$$

The level of activation of dextran is controllable (Battersby *et al*, 1996). The rate of formation of the complex is proportional to the level of oxidation (Figure 1.8.1), while the rate of release of the protein from dextran is inversely proportional. Therefore a balanced intermediate needs to sought. The degree of oxidation should be chosen so that an acceptable rate of release from the complex is observed. Three levels of periodate activation were initially chosen for the present investigation, 7%, 21% and 56%.

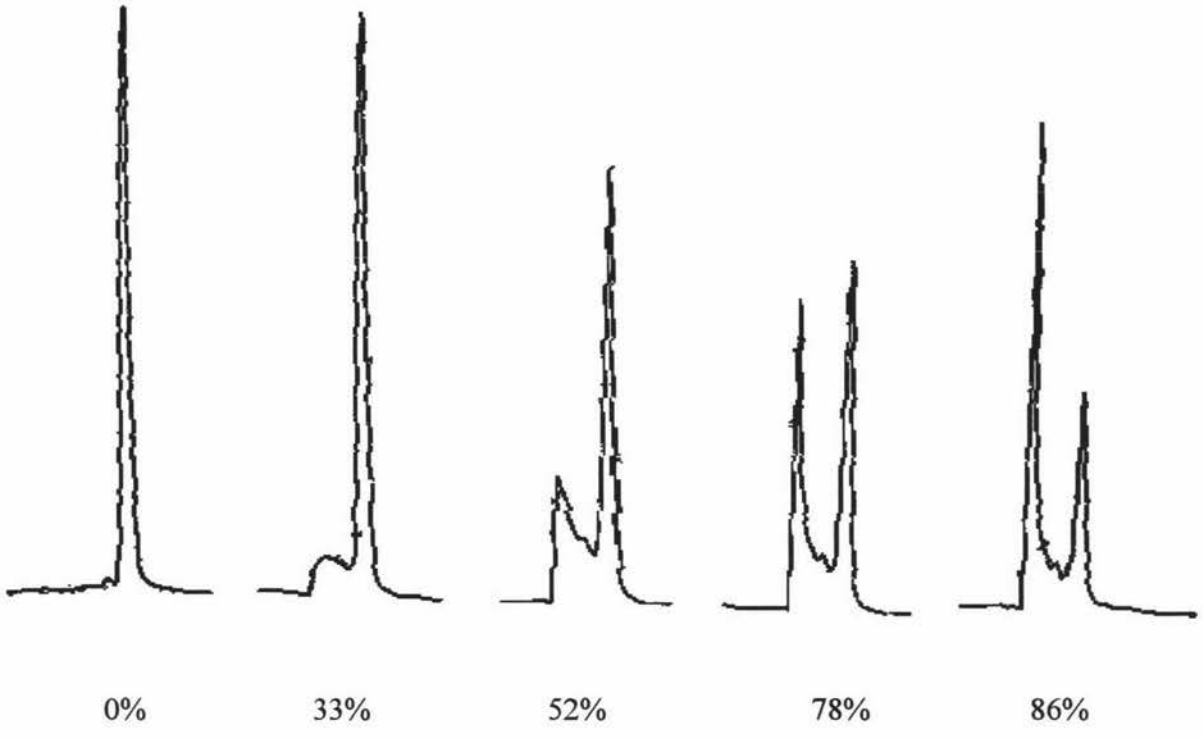


Figure 1.8.1 Extent of complex formation over increasing dextran activation levels for a 24hr period (Puchulu-Campanella, 1991-1993)
met-hGH was incubated with increasing levels of activated dextran for 24hrs. Aliquots were then taken for each level of activation for SEC.

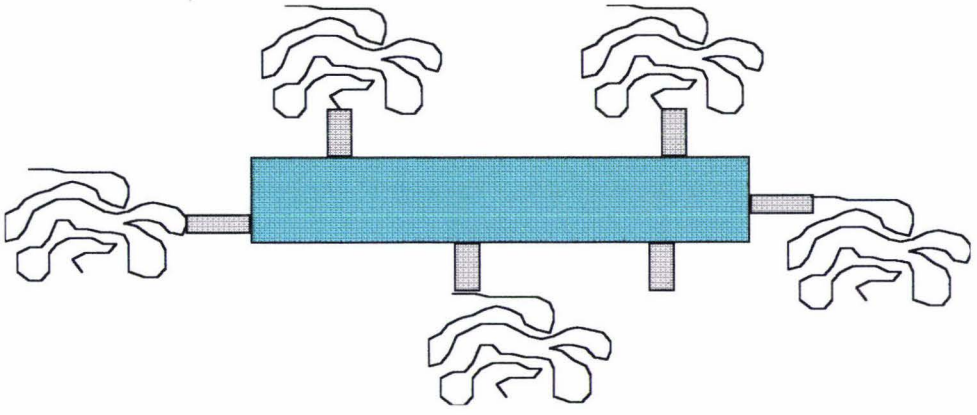
The side effects caused by dextran have been minimised over the last five decades of clinical use (Larsen, 1989). For many years it has been used as a blood expander, to maintain or replace blood volume (Molteni, 1979). Dextran has several properties that

make it a good potential drug carrier. The dextran molecule does not have a selective transport mechanism, but does have a well defined, repetitive chemical structure (Larsen, 1989). It has no limitation on chemical derivation making it a universal carrier, and it is a non-digestible polymer (Berstein *et al*, 1978). Dextran shows excellent physiochemical properties and is physiologically accepted (Larsen, 1989). Dextrans above 55 kDa, that are not cleared via the kidneys, are partially depolymerised by dextranases in the liver and spleen, and then excreted (Molteni, 1979; Larsen 1989). The attachment of ligands may increase or decrease the rate of depolymersation. Rare dextran induced anaphylactoid reactions can be circumvented by preinjection of the monovalent hapten dextran preparation, promiten.

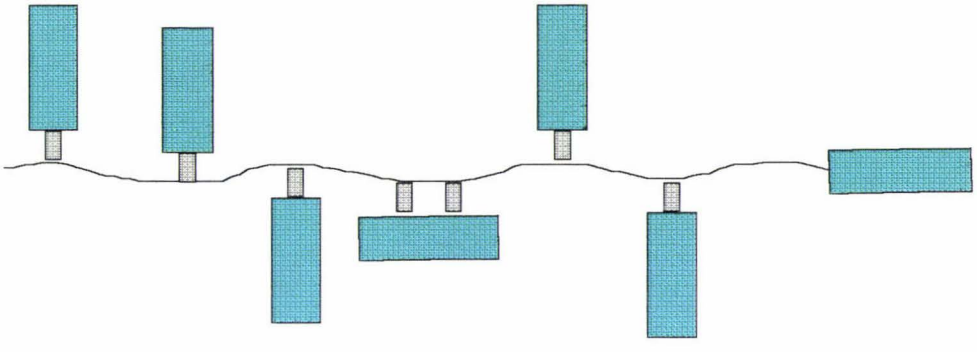
Conjugation of a protein to dextran offers advantages in that it increases its circulatory life time (Marshall, 1978; Mitra *et al* 1993). Clearance of the protein via renal filtration is prevented when the molecular weight of a complex containing it is above 70kDa. Once conjugated there is reduced likelihood of the protein being recognised by proteases, inhibitors and pre-existing antibodies. Conformational stability of the tertiary structure has been suggested to result from the multipoint binding that occurs with dextran conjugates (Mitra *et al*, 1993). Proteins are also protected by the steric hindrance of the complex structure. If the steric hindrance proves to be disadvantageous, spacer arms can be used (Larsen, 1989). This requires careful selection of the spacer arm and spacer-drug bond. It should therefore be possible to choose a spacer-drug link that will be cleaved at the target cells by enzymes secreted locally. Spacer arms can also be used when direct binding between the protein and the polymer is not possible.

The exact final structure of protein-dextran complexes is not known. It is possible that there is one dextran molecule binding to several protein molecules or vice versa (Figure 1.8.2 a and b), but it is probably more likely that the monosaccaride molecules within the dextran bind to several different protein molecules which in turn bind to several different dextran molecules, resulting in intermolecular crosslinking (Figure 1.8.2 c). The situation however may vary depending on the size of the protein and the number of lysine residues present.

Dextran has also been used as a link between drugs and antibody (Ab) carriers that are used to direct the drug to the site of action (Larsen, 1989; Berstein *et al*, 1978; Hurwitz 1983). This technique enables larger amounts of drug to be attached to the Ab (Berstein *et al*, 1978). At high drug concentrations the drug-dextran-Ab carrier conjugates have been shown to be more efficient than the free drug in *in vivo* experiments.



a)



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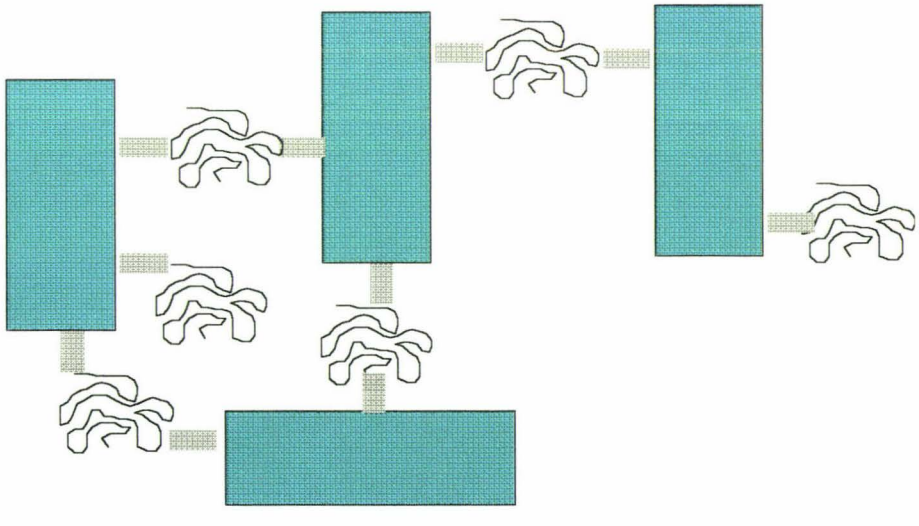


c)

Figure 1.8.2 Possible structure of Protein Dextran Complex

a) Several dextran molecules bound to one protein molecule
b) Several protein molecules bound to one dextran molecule
c) Random crosslinking between dextran and protein molecules

1.9 Protein Modification

Complex formation of proteins to the activated dextran may also provide a means of modifying the protein. Modification of proteins is seen as a way of reinforcing function or obtaining additional or altered functions from the protein. Binding of a protein to dextran itself results in modification of that protein. In nature it has sometimes been found that glycoproteins are more stable than other proteins and this has been attributed to their carbohydrate component (Marshall, 1978). The increased stability of proteins bound to dextran is probably a result of intermolecular crosslinking. It has also been contributed to shells of hydration around the hydrophilic dextran and/or possibly due to additional hydrogen bonding between the dextran and the protein.

Chemical modification of a serine alkaline proteinase, with dialdehyde dextran, resulted in a protein that was less susceptible to surfactants (Yamagata *et al*, 1994). The resulting product showed activity in organic solvents and exhibited increased thermostability. Their protein-dextran complex was used as a model for other proteinases. Evidence suggested that their model could be a useful method of enzyme technology.

Protease-treated gluten, an insoluble wheat protein, was conjugated to dextran through the Maillard reaction resulting in an increase in stability and improved emulsifying properties (Kato *et al*, 1991). This was not achieved by other methods of modification. Gluten is a byproduct from the wheat starch industry. May effort have been made to improve its utilisation. For large scale applications, such as in the food industry, dextran is not the best support for proteins due to its relative cost. In such cases other branched polysaccharides may be used. Initial studies show these may also be effective for improving protein function and stability (Kato *et al*, 1991).

Modification of proteins in the food industry is important due to the desire for food materials with new functionality (Hattori *et al*, 1994). Conjugation of proteins to charged polymers has been investigated as a means of improving biological activity. When carboxylmethyl dextran, an acidic polysaccharide, was conjugate to lysozyme, there was a reduction in the α-helix content of the protein concomitant with a loss of activity of around 40% at 40-60^{0}C. At 4^{0}C and 80^{0}C enzymatic activity was shown to increase and the conjugate became less susceptible to autolysis.

Food preservatives are commonly used to destroy contaminating organisms such as gram-negative bacteria, but because of the fears of toxicity alternatives are being sought

(Nakamura *et al*, 1991). While lysozyme has been used as a food preservative it is limited in that it only acts on gram-positive bacteria. Lysozyme-dextran conjugates formed by the Maillard reaction were shown to have higher emulsifying properties and to act on gram-negative bacteria. The resulting conjugate had greater thermostability and could be used to destroy the outer membrane of gram-negative bacteria. The improved emulsifying properties of the conjugate assist by solubilising the outer membrane, thus exposing the peptidoglycan layer to lytic attack.

1.10 Investigations into Complex Formation of Proteins to Dextran and Subsequent Release

The enzyme study reported in this thesis is aimed at obtaining a model for controlled complex formation and release of bioactive proteins. While this approach may have therapeutic applications it may also provide a means of manipulating proteins to obtain modified activity for other purposes. The enzyme-dextran conjugates were investigated to gain further quantitative knowledge of models of reversible association-dissociation between the macromolecular components. The aim was to show that complex formation between dextran and proteins is not just limited to rhGH which has been extensively studied (Battersby *et al*, 1996).

Proteins were chosen on their availability, relative cost and readily measured activities. It was also deemed desirable to look at proteins with a range of molecular weights. The proteins studied thus ranged from 14 kDa up to ~250 kDa. Proteins were also chosen with differing enzymatic activities to gain an initial impression of the generality of this approach.

Proteins	Molecular weight
Lysozyme	14 300
Trypsin	23 000
Amylase	48 000
Alcohol Dehydrogenase	150 000
Catalase	225 000

Table 1.10.1 Molecular weight range of proteins for dextran complex formation study

The study reported in this thesis (Chapters 2 - 7) has concentrated on the possibility of complex formation of proteins. Activation of dextran was performed by periodate oxidation. Three widely spread activation levels were chosen to find an 'ideal' range. Formation of complexes between the dextran was then followed over time at the varying levels of activation, and the resulting complexes were isolated and analysed for activity and release of the protein. When the protein was released from the complex it was characterised and compared to the original protein used.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Reagents and Equipment

Dextran T-40 was from Pharmacia, Uppsala, Sweden; Lysozyme grade 1, trypsin type 1X (BAEE 14 600), trypsin type XIII (TPCK treated), alcohol dehydrogenase A-7011, catalase type C 10 dimethylsulphoxide (DMSO), *Micrococcus lysodeikticus* and N-α-benzoyl-DL-arginine-ρ-nitrolanilide HCl (BAPNA) were from Sigma Chemical Co., St Louis, MO, USA; Amylase was from Genencor International Inc., Palo Alto, CA, USA and purified by Dr S. Burton, Department of Chemistry, Massey University, New Zealand; rhGH was a gift from Genentech Inc., South San Francisco; Ceralpha substrate was from Deltagen Australia Pty. Ltd., Boronia, VIC, Australia; Sodium acetate, potassium iodide (AR) were from Scientific Supplies Ltd, Auckland, New Zealand; Sodium periodate and sodium azide were from Ajax Chemicals Ltd., Auburn, NSW, Australia; Sodium cyanoborohydride was from Aldrich Chemical Company, Inc., Steinheim, Germany; Convol arsenite, sodium di-hydrogen phosphate, di-sodium hydrogenphosphat-2-hydrat were from Reidel-de Hahn, Selze, Germany; Convol iodine was from May and Baker, Manchester, England. All other reagents were analytical grade wherever possible. Water was MilliQ grade.

Centricons (used for concentration of macromolecular solutions by ultrafiltration through low-adsorption, hydrophilic membranes) MWCO 10 000 were from Amicon®, New Zealand Medical and Scientific Ltd. FPLC and SMART columns were from Pharmacia Biotech, Uppsala Sweden. HPLC columns, Nucleosil C18 300Å 5μm and Vydac C18 250mm x 4.6mm 10μm, were from Alltech, Auckland, New Zealand. Amino acid analyser, FPLC, SMART and Phast systems were all from Pharmacia, Uppsala, Sweden. HPLC system was Waters Associates comprising of a model 680 automated gradient controller, 2 model 510 pumps, a model 441 absorbance detector and a Rheodyne injection system. A Pye Unicam PU 8610 spectrophotometer was used for absorbance measurements. Incubations requiring mixing were shaken on a Ika-Vibrax mix. Centrifugation was carried out using a RC-5B Sorval/Dupont SS-34 rotor angle at 34°C. The gels used with the Phast system were homogenous 7.5, 12.5 and 20% SDS-PAGE. The ultracentrifuge used was a Beckman model E analytical ultracentrifuge with an An-H rotor with Schlieren optics set at 546.1 nm. The laser used was a Spectra Physics 165 argon laser.

2.2 Periodate Oxidation

Dextran T-40 (400mg) was dissolved in 0.2M acetate buffer (50ml) pH 4.5 and added to 0.2M sodium periodate in 0.2M acetate buffer (50ml). Solutions were kept in the dark with constant stirring at 4°C. Oxidation was halted by the addition of 2xw/w ethylene glycol. Oxidised dextran was then purified by ultrafiltration using a Diaflo filter with a 500MW cut off. Purified oxidised dextran was then freeze dried for storage. Level of oxidation was determined by iodometric titration. Large scale oxidation was performed using 4g dextran.

2.3 Iodometric Titration

Saturated bicarbonate solution (10ml), reported to be 6.9g/100ml at 0°C and 16.4g/100ml at 60°C, was added promptly to an aliquot of oxidised dextran (5ml) followed by the addition of arsenite (8ml) and 20% KI solution (1ml). This solution was left in the dark for 15 minutes. Starch (0.4g) was added and the solution was then titrated against iodine. The end point of the titration was taken as the volume of iodine required to give the solution a faint blue-purple colour, stable for 5-10 seconds with stirring.

2.4 Complex Formation

Protein (20/30mg) was dissolved in 100mM sodium phosphate buffer, 0.05% sodium azide, pH 7.5, for all proteins except trypsin where pH 5.5 was used, (2ml) and then added to dextran (20mg) in phosphate buffer (2ml). The mixture was then left in the dark without stirring at room temperature. The complex was isolated on the FPLC using a Superdex 75 (HR 10/30), Superose 12 (HR 10/30) or a Superose 6 column (HR 10/30). Flow rate was 0.4ml/min and the chart speed was 0.4cm/min. Before loading all samples were filtered using 0.2μm millipore membrane in a Swinny filter. Running buffer was 0.05M sodium phosphate, 150mM NaCl, pH 7.0 or 0.2M tris acetate, 0.05% sodium azide, pH 7.5 (Trypsin, pH 5.5). 200μl of complex formation incubation sample was injected per run. All runs on the FPLC were monitored at 280nm. This was chosen because the Tris acetate buffer used in subsequent experiments absorbs at 214nm. At 280nm only tryptophan residues absorb, while at 214nm absorbance results from peptide bonds and carboxylic acid groups.

2.5 Complex Release

Protein dextran complexes were buffer exchanged into 0.2M Tris acetate, 0.05% sodium azide, pH 7.5 (Trypsin, pH 5.5), on the FPLC using a Superdex 75/Superose 6 column and then incubated in the dark at room temperature without stirring. Aliquots were taken and concentrated by ultrafiltration (Centricon 10) and centrifuged for 45-60mins at 3 020g. Retentate was then run on the SMART (50µl) using a Superdex 75 (PC 3.2/30) column at 40µl/min or on the FPLC (200µl) using a Superose 6 column at 0.4ml/min. Released protein species were isolated for further analysis. In some cases several runs were made on the FPLC, and the peaks were pooled and concentrated to obtain enough material for experiments to follow the release of the protein from the complex. This was done for ADH and catalase where there was not an appropriate SMART column, and with trypsin to obtain enough release product for trypsin digest studies. All runs on the FPLC and SMART system were at monitored at 280nm.

2.6 Complex Reduction Studies

Protein-dextran incubations were set up for 24 hours to allow complex formation. Sodium cyanoborohydride, $NaBH_3CN$, was added at 24 and 48 hours to give a final two fold concentration excess (w/w). Upon addition of sodium cyanoborohydride the incubations were kept in the dark with stirring. A control was also set up without the addition of sodium cyanoborohydride. Incubations were then analysed for complex formation using SEC (Superdex 75/Superose 6 column) and the peaks were collected. These complexes were then studied for release by pooling and concentrating the desired fractions and subjecting them to further SEC (SMART 75 column or FPLC Superose 6 column). When the FPLC was used it was necessary to perform several runs and then pool and concentrate the samples. Activity assays were also performed on the complexes. Controls experiments using the protein plus $NaBH_3CN$, without the addition of dextran were also set up for comparison. All runs on the FPLC were monitored at 280nm.

2.7 Lysozyme Lytic Assay

Micrococcus lsodeikicus (20mg) was dissolved in 75mM Tris malate buffer (2ml) by sonication for five minutes. Lysozyme solution (10µl) was added to 40mM Tris malate buffer (2ml). Upon addition of *M. lsodeikticus* (10µl), the solution was shaken and then the absorbance was recorded over time at 450nm. Reagents were kept on ice throughout the reaction. Activity was calculated as the change in absorbance over the

change in time. Glassware and cuvettes were soaked in 20% nitric acid to remove residues of lysozyme (Smales, 1995).

2.8 Laser Light Scattering

NMR tubes were washed and then inverted over a spout above a flask of refluxing acetone (analytical BDH). NMR tubes were then transferred, inverted into a dessicator and left under vacuum overnight Fractions of isolated complex were then filtered through an Acrodisc 0.2μm filter with 0.8μm prefilter into the clean NMR tubes (Harvey, 1995).

Data collection was performed with assistance from Associate Professor D.N. Pinder, Physics Department, Massey University. The laser beam was passed through the centre of the sample at 25°C. After equilibrating for 30 minutes the laser was turned on and the signal was recorded on a computer. The sample was studied at several angles 15°, 50°, 90° and 120° for appropriate time intervals.

2.9 Ultracentrifugation

Samples of lysozyme-dextran complex were prepared and isolated. After freeze drying they were divided into three aliquots of approximately 0.75mg/ml, 1.5mg/ml and 3mg/ml. An aluminium counterpiece 2.5°/12mm was filled with buffer solution and washed several times and left overnight in buffer.

Ultracentrifuge data was achieved with the assistance of Dr J. Lewis, Physics Department, Massey University. An-H rotor with Schlieren optics set at 546.1nm and analyser angle set at 60° was used. The rotor was kept at 25°C throughout the experiments. The experiment was started with the lowest concentration sample and run at 48 000 rpm. Time zero is taken as 2/3 the final speed (32 000 rpm). Progress of each run was monitored visually through a scanner (Harvey, 1995).

2.10 Trypsin BAPNA Assay

1) Trypsin sample (0.3ml) was added to 1M Tris-HCL pH 8 (1.7ml) followed by the addition of 1mg/ml BAPNA in DMSO (1.0ml). Mixture was then incubated at 25/37°C and the absorbance was then taken at 405nm.

2) Enzyme solution (100µl) was added to 100mM Tris-HCl pH 8.0 (350µl), with 400mM $CaCl_2$ and 1mg/ml aqueous BAPNA solution (350µl). Incubations were performed at room temperature. Reactions were halted at various times by the addition of 30% v/v acetic acid aqueous solution (500µl). Absorbance was taken at 410nm (Huckel *et al*, 1996).

2.11 Trypsin Digest of rhGH

rhGH (1mg) was dissolved in trypsin digest buffer (1ml), 10mM Tris base, 1mM $CaCl_2$ pH 8.3. rhGH was then digested by addition of 0.02M trypsin dissolved in 1mM HCL (0.5ml) and incubated at 37°C. After 2 hours a further addition of trypsin (0.5ml) was added. After 4 hours the incubation was halted by the addition of 5% TFA to lower the pH to 2-3.

Tryptic mapping was then followed by HPLC using a Nucleosil C18, 5µm particle, 300Å pore size, 2.1mm x 15cm column. Buffer A was 10% acetonitrile, 0.5% TFA and buffer B was 90% acetonitrile, 0.5% TFA. All samples were filtered through a 0.2µm millipore membrane in a Swinny filter before loading onto the column.

After an isocratic hold at 0% B for five minutes, a linear gradient was run from 0% to 40% B over 133 mins followed by a 15 minute gradient to 60% B. Concentration was held at 60% B for 10 minutes and a short gradient of five minutes was run to return to 0% B.

2.12 Trypsin Digest of Synthetic Peptide

Solid phase peptide synthesis, SPPS, was performed by Ms R. Parshot and Ms J. Cross, Departments of Chemistry and Biochemistry, Massey University, New Zealand. Synthetic peptide (1mg) was dissolved in 0.1M ammonium bicarbonate solution (1ml). The peptide was then digested by the addition of trypsin sample (~0.01mg) in ammonium bicarbonate solution (0.5ml). A second addition of trypsin was given after two hours. Digestion was then either halted after four hours or left over night. Reaction was stopped by the addition of formic acid to lower the pH to 2-3.

Peptides were separated on the HPLC using a reverse phase Vydac C18 column. Buffer A was 100% water, 0.5% TFA and buffer B was 100% acetonitrile, 0.08% TFA. All samples were filtered through a 0.2µm millipore membrane in a swinny filter. A linear gradient was run at 1% per minute up to 40%.

2.13 α-Amylase Activity

p-Nitrophenyl maltoheptaoside (BPNPG-7), Ceralpha substrate, was diluted 4-fold with 50mM Malate buffer, 5mM $CaCl_2$, 0.002% Tween 20, pH 6.7. α-Amylase sample (100µl) was added to the substrate-buffer mixture (700µl) in a cuvette and inverted. The absorbance was then monitored at 410nm (Burton, 1995).

2.14 Alcohol Dehydrogenase Assay

Alcohol dehydrogenase sample (0.2ml) was added to Tris-lysine buffer pH 9.7 (2.4ml) and 0.006M NAD^+ (nicotinamide adenine dinucleotide, 0.2ml) in a cuvette and inverted. Once the absorbance was steady 0.15M ethanol (0.2ml) was added and upon mixing the absorbance was monitored at 340nm for two minutes. The reference solution was Tris-lysine buffer (2.6ml), NAD^+ (0.2ml) and buffer sample (0.2ml) (Cornell and Veech, 1983).

2.15 BCA Protein Concentration Determination

50 parts bicinchoninic acid was added to 1 part copper (II) sulphate pentahydrate 4% solution. 1mg/ml bovine serum albumin was used to make a standard curve of 0 to 100µg protein (0.1ml). Protein determination reagent (2ml) was added to each of the tubes which were then vortexed. Tubes were then incubated for 30 minutes at 37°C. Absorbances were measured at 562nm, using water as a blank. From this, the standard curve of absorbance versus µg protein was produced.

Protein samples (0.1ml) were treated similarly and the amount of protein was determined from the standard curve.

2.16 Amino Acid Analysis Preparation

Samples were prepared for amino analysis by the addition of 6M HCl and 0.1% phenol (1ml per mg sample or 200µl). The sample was then hydrolysed under vacuum at 110°C overnight. Finally the sample was dried down in the dessicator. Amino acid analysis, AAA, was performed by Debbie Frumau, Biochemistry Department, Massey University.

For comparison the amounts of each amino acid present was determined relative to alanine. During the process of AAA Gln is converted to Glu and Asn is converted to

Asp therefore it is not possible to distinguish between Glu and Gln or Asp and Asn. In each case a total amount of the two amino acids is given (Glx and Asx respectively). Tryptophan is lost during hydrolysis prior to AAA. The amounts indicated for Cys and Met are likely to be lower than the actual amounts present due to losses during hydrolysis.

2.17 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophresis, SDS-PAGE, analyses were run on the Pharmacia Phast system. Samples (150ul) were prepared by the addition of 20% SDS (25ul) and bromophenol blue (11ul) and boiling for five minutes. 7.5%, 12.5% or 20% SDS gels were run using separation and silver staining methods in the Phast manual. In the case of lysozyme, native and reverse polarity gels were also performed using the Phast system methods.

CHAPTER THREE

LYSOZYME

3.1 Introduction

The enzyme lysozyme has antibiotic characteristics and is found in a great number of different tissues and secretions in different animals (Jollès, 1960). Hen egg-white lysozyme, a glycosidase, is involved in the breakdown of cell walls of bacteria. It catalyses the hydrolysis of β-1,4 glycosidic bonds in the polysaccharides making up the peptidoglycan layer of cell walls. Lysozyme can also hydrolyse polymers of N-acetyl-D-glucosamine and chitin (Kumagai *et al*, 1993).

Lysozyme has a MW of 14 300Da, and is made up of a single polypeptide chain consisting of 129 amino acids including 6 lysine residues. It is stable at acid pHs which is attributed in part to its disulphide crosslinking (Jollès, 1960). The enzyme has a pKa value of 5.8 (Glazer, 1976), and an isoelectric point of 10.5-10.6 (Keesey, 1987) and is not susceptible to proteolytic attack by trypsin and chymotrypsin (Jollès, 1960). It has been shown that in order to maintain reasonable levels of lytic activity at least three lysine residues must remain free (Smales, 1996).

Lysozyme's six lysine residues and free α-NH_2 terminal group are, in theory, all capable of binding to the aldehyde groups of activated dextran. Because of this and other factors such as its relative purity, availability and low cost lysozyme was an appropriate starting point. It also provided a low MW protein for establishing a ladder of MWs for the subsequent complex formation studies.

Laser light scattering technique, LLST, can be used for the molecular weight determination of polymers (Harvey, 1995; Kato *et al*, 1992). The technique requires only small quantities of samples and is non-perturbing. The principle behind the light scattering technique is that the light beam is scattered when it hits the particle (Figure 3.1.1). The intensity of this scattering is a function of the radius of the particle. As dust and dirt particles also scatter light it is necessary to minimise their presence. The smaller angles used to look at larger particles are more affected by the dust and dirt particles, while larger angles take longer to obtain results.

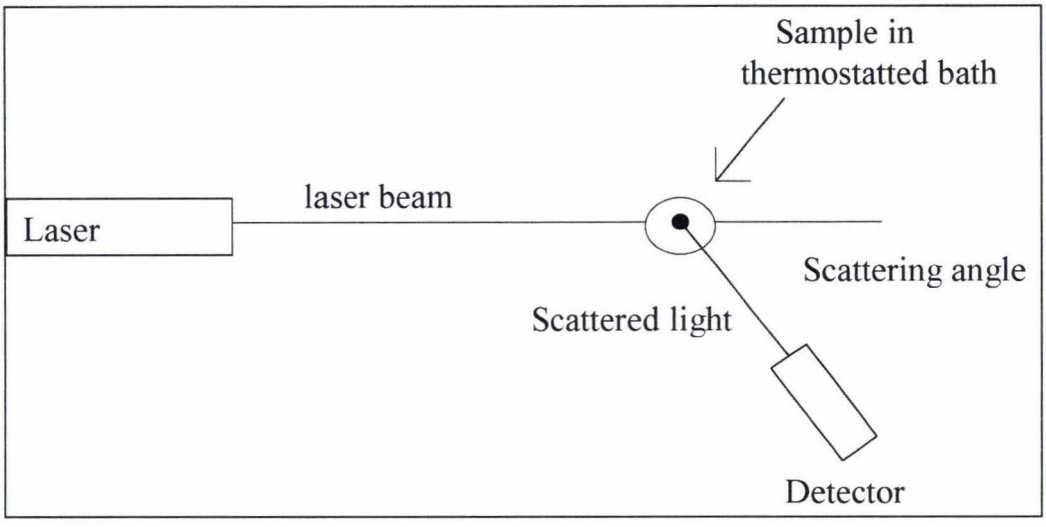


Figure 3.1.1 Laser light scattering apparatus (Harvey, 1995)

Ultracentrifugation can also be used as an analytical technique to determine the molecular weight of a compound (Harvey, 1995). It involves the spinning of a sample at extremely high speeds and monitoring its progress as it sediments. This is monitored by Schlieren optics which can be viewed through the scanner on the ultracentrifuge (Figure 3.1.2). The Schlieren pattern obtained gives a plot of the rate of change in protein concentration with radius. The area under the peak is proportional to the concentration of the protein.

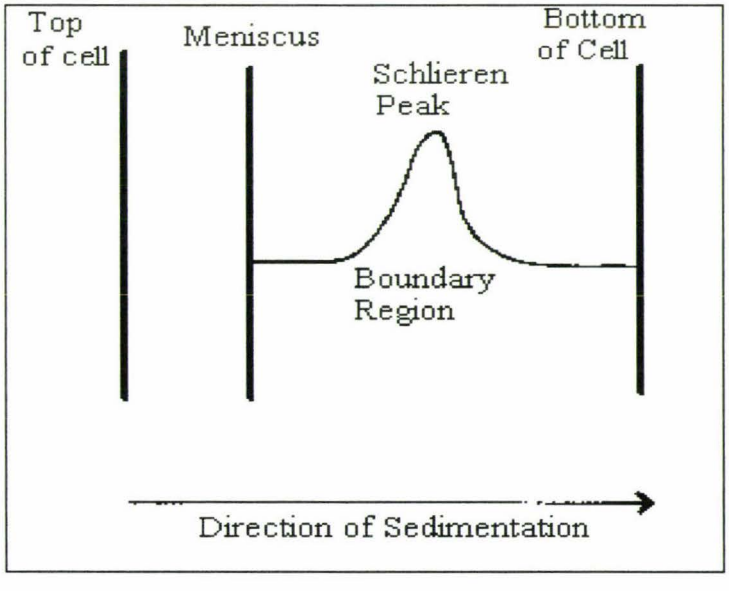


Figure 3.1.2 Diagram of a Schlieren pattern of a homogeneous solution (Harvey, 1995)

3.2 Results and Discussion

Initial studies were begun with the activation level of dextran at 56%. Complex formation occurred rapidly with lysozyme and the 56% activated dextran. FPLC (Superose 12) chromatograms showed that loss of the lysozyme peak during formation was observed in accordance with the formation of two other higher MW species (Figure 3.2.1).

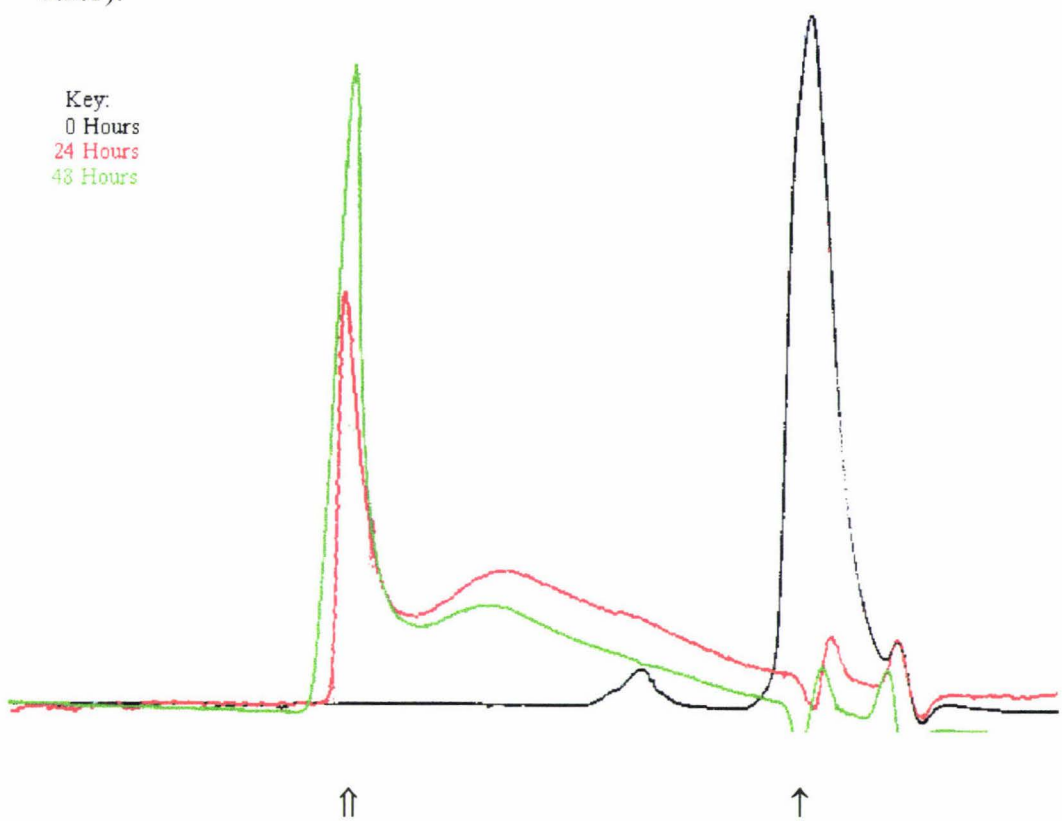


Figure 3.2.1 Complex (⇑) formation over time for lysozyme (↑) and 56% activated dextran

Lysozyme incubated with 56% activated dextran was subjected to SEC (Superose 12, FPLC) at 0hrs, 24hrs and 48hrs in 0.2M sodium phosphate, 150mM $NaCl_2$, pH 7.5.

The absorbance was monitored at 280nm, a wavelength that does not detect non-conjugated dextran. The Superose 12 column, MW range 1000 - 3 x 10^5, was initially chosen on the assumption that it would have the capacity for a complex formed between lysozyme, (MW 14 300Da) and dextran (MW ~ 40 000Da). Within 24 hours of complex formation the peak characteristic of lysozyme had diminished with only a trace remaining. The first of the product peaks was sharp, followed by a broader peak and over time there appeared to be a gradual increase in the first peak associated with a loss of the second peak. This was possibly due to the complex rearranging to the higher MW

species. This could arise from dextran binding to additional amino groups on a single lysozyme molecule. There could also be an increase in crosslinking. The first complex peak was eluted with the void volume, indicating that the complex was equal to or larger than the MW range of the column, 300 000 Da. This is consistent with the results of earlier work done by Puchulu-Campanella, where complex formation studies performed on rhGH with 9% and 18% DOR showed that two higher MW complex bands were observed with SEC profiles (Puchulu-Campanella, 1991-1993). Overtime a preference was also shown for the first of these peaks, eluting with the void volume.

Complex isolated by SEC did not show any bands on 7%, 12.5% and 20% homogenous SDS polyacrylamide gels. Native and reverse polarity native gels were then tried but they gave the same results. It was apparent the sample was not entering the gel. Accordingly it was decided to investigate the apparent extremely high molecular weight complex. This was done using a laser light scattering technique, LLST (Harvey, 1995). This technique has been used before with conjugates formed between globular proteins and randomly coiled polysaccharides (Kato *et al*, 1992). Complexes isolated from the FPLC were prepared for analysis by laser light scattering. Angles of 15°, 50°, 90° and 120° were investigated. Results showed that the complex was a mixture of species covering a range of high molecular weights, in agreement with SEC. LLST results indicated that the radius of the complex was approximately six times larger than that of dextran alone, but the binding of lysozyme to a single dextran molecule, or between multiple dextran molecules, may have restricted the movement of dextran during the LLST. This in turn means that the size of the complex indicated by these experiments could be excessive. Therefore while it appeared to contain approximately 30 dextran molecules it quite possibly contained only about ten. These results tend to indicate that the complex consists of more than one dextran, and therefore is not the result of several protein molecules binding to one dextran molecule. The high MW of the complex also ruled out the likelihood of it being the result of several dextrans binding to a single lysozyme molecule. This would have given a maximum approximate MW of 300 000 Da if all six lysine residues and the α-NH_2 terminal were involved in imine bonds with differing activated dextran molecules. In other words it is likely that the 56% activated complex is an extensive network of interconnected lysozyme and dextran molecules.

The next technique used to examine these complexes was ultracentrifugation (Harvey, 1995). Initial runs were not successful in that there appeared to be nothing in the sample. Runs were repeated using more concentrated samples at which point it was observed that there was a peak on the scanner, that moved across before the ultracentrifuge was up to speed (at less than 10 000rpm). In other words the complex was missed in previous runs because sedimentation had finished by the time the

ultracentrifuge reached maximum speed (48 000rpm). This in turn meant the progress of the Schlieren peak relative to time could not be observed (Figure 3.2.2), and this further supported an emerging picture of a very high molecular weight complex, possibly around 1MDa.

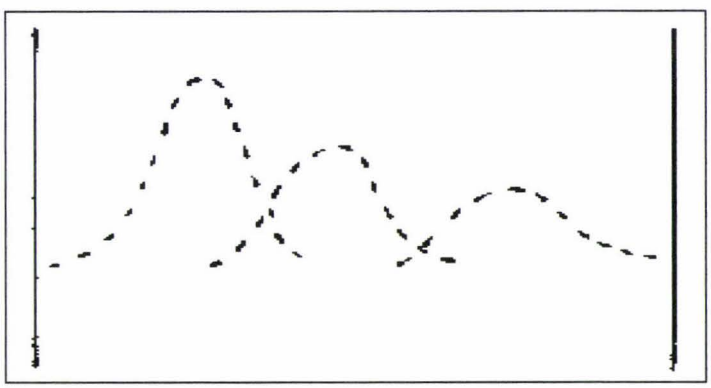


Figure 3.2.2 Expected progress with time of Schlieren peak

Using the standard release conditions, there appeared to be no release of lysozyme or lysozyme fragments from this highly activated complex. Lytic assays performed with the complex also showed no signs of activity. This possibly arises from too many of the lysine residues within the lysozyme molecule being involved in imine bonds with the highly activated dextran. Loss of activity could have also been caused by steric hindrance. That is, the micrococcus might not be accessible to the complexed lysozyme. As there was no indication of release of protein from the complex, and the complex itself did not show any activity no reduction studies were carried out.

Due to the problems encountered with the 56% activated complex, lower levels of activated dextran were then investigated. Activation levels of 7% and 21% were chosen to investigate ideal activation levels required for effective sustained release of active enzyme. Large scale preparations of each oxidation level were performed.

Significantly lower levels of complex were formed with both 7% and 21% activated dextran, and formation was slower compared with the 56% activated dextran. Incubations were subjected to SEC (Superdex 75) and only one broad complex band was observed (Figure 3.2.3 and 3.2.4). Complex formation was faster with the 21% activated dextran which is consistent with the rhGH work by Battersby *et al* (1996). As previously observed, the loss of the lysozyme peak was accompanied by an increase in a peak at higher MW representing the lysozyme dextran complex.

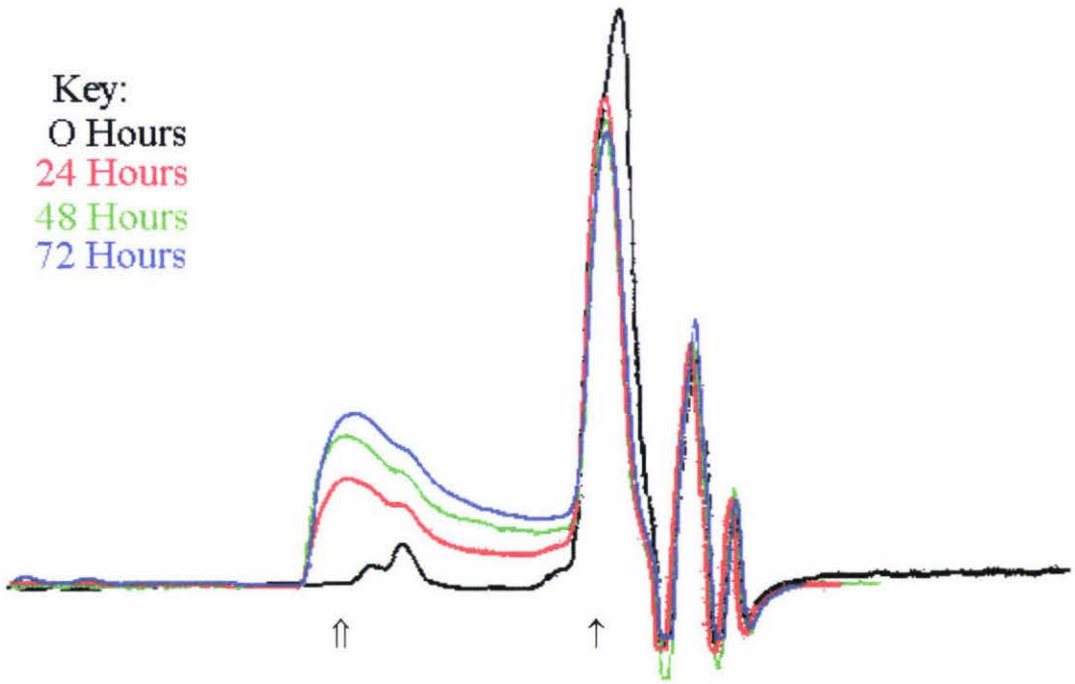


Figure 3.2.3 Complex (⇑) formation over time between lysozyme (↑) and 7% activated dextran

Lysozyme incubated with 7% activated dextran was subjected to SEC (Superdex 75) at 0hrs, 24hrs, 48hrs and 72hrs in 0.2M Tris acetate, 0.05% sodium azide, pH 7.5.

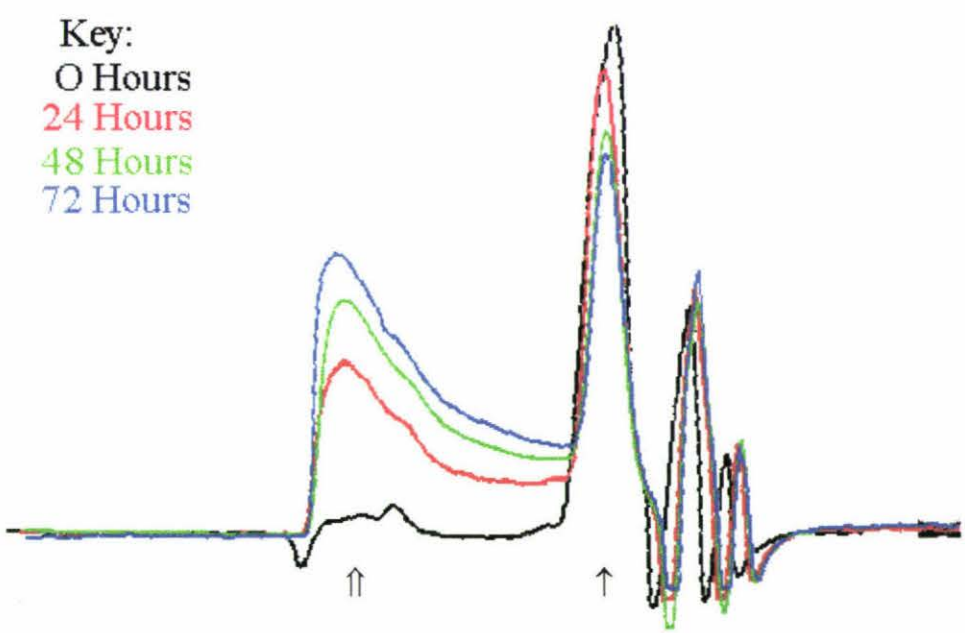


Figure 3.2.4 Complex (⇑) formation over time between lysozyme (↑) and 21% activated dextran

Lysozyme incubated with 21% activated dextran was subjected to SEC (Superdex 75) at 0hrs, 24hrs, 48hrs and 72hrs in 0.2M Tris acetate, 0.05% sodium azide, pH 7.5.

To follow protein release, complexes isolated from the FPLC were concentrated and then after a set period of time analysed by SEC (Superdex 75, SMART system). In each case, a decrease in the peak due to the complex was associated with an increase in a lower MW species with the same retention time as the original lysozyme (Figure 3.2.5).

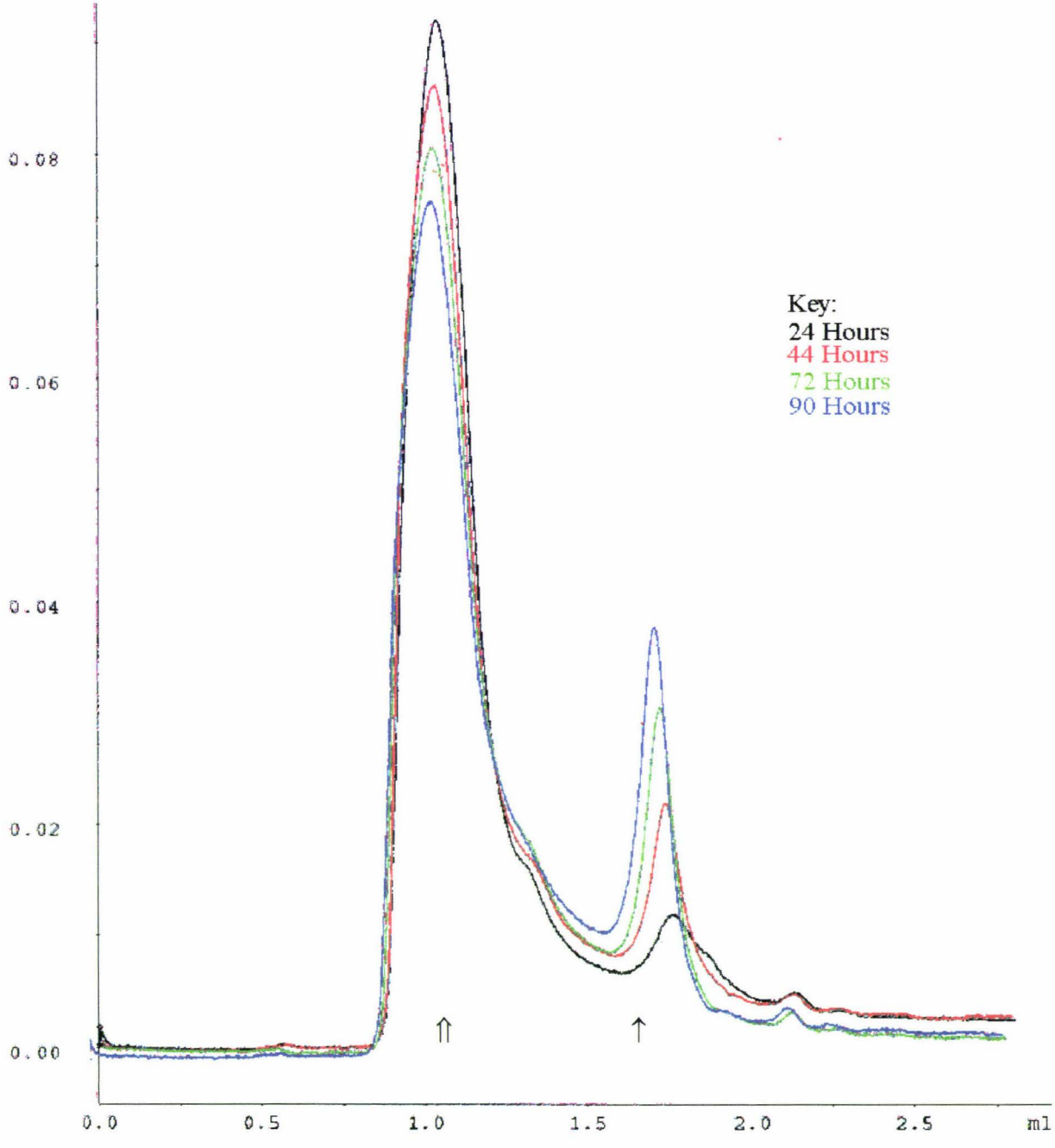


Figure 3.2.5 Release of lysozyme-like (↑) species from complex (⇑) (lysozyme-21% activated dextran) over time

Lysozyme-dextran complex isolated from the FPLC was subjected to SEC (Superdex 75, using the SMART system) at 24hrs, 44hrs, 72hrs and 90hrs in 0.2M Tris acetate, 0.05% sodium azide, pH 7.5.

Both the 7% and 21% activated lysozyme-dextran complexes ran successfully on a 12.5% homogenous SDS polyacrylamide gel, indicating the molecular weight was considerably lower than that of the 56% activated complex (Figure 3.2.6). Lanes 2 and 5 are isolated complexes for the 7% and 21% activated dextrans respectively. The bands due to the complex were smeared, showing that it covers a range of molecular weights. This may result in part from the fact that the dextran has a range of MWs around 40 000Da (Figure 1.6.1), and in part because of the random nature of the complexes formed between the lysozyme and the dextran. In both the complex samples there was also a sharp band present on the gel that ran with the same MW as lysozyme, indicating that release of lysozyme-like material from the complex had occurred after isolation.

Released species isolated by SEC were concentrated and run on a 12.5% SDS polyacrylamide gel (Figure 3.2.6). They gave one clear band running in same place as the lysozyme standard, as shown in lanes 3 and 5 for the 7% and 21% release species respectively.

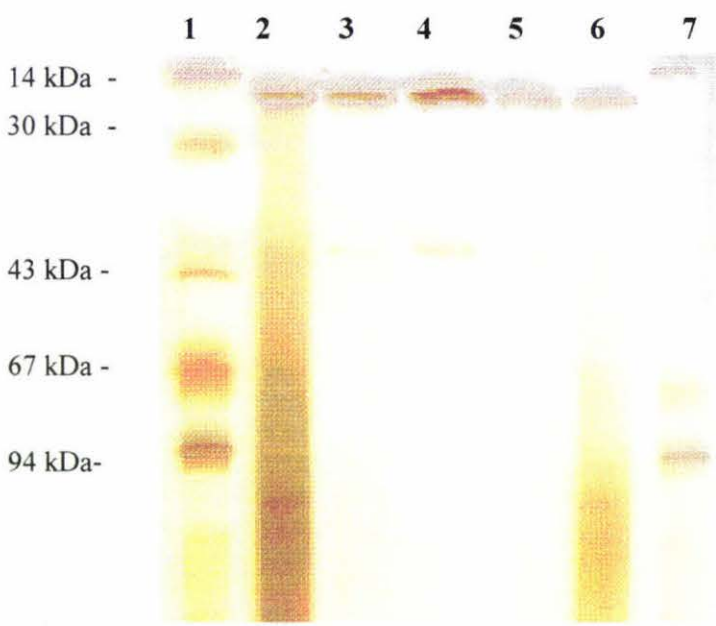


Figure 3.2.6 SDS-Page

Homogenous 12.5 % SDS gel, silver staining development on Phast system.
lane 1 marker: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa)
lane 2 7% activated complex
lane 3 released species from 7% activated complex
lane 4 lysozyme standard
lane 5 released species from 21%; activated complex
lane 6 21% activated complex
lane 7 marker as for lane 1

Activity assays were carried out on the complexes as described previously. They showed lytic activity at both levels of activation. The relative activities for the complex with lysozyme and 7% activated dextran, and the subsequent released lysozyme-like species are shown in Figure 3.2.7. All appropriate aliquots for assaying were isolated by SEC (Superdex 75) and, except for the lysozyme standard, were concentrated approximately 10 fold. Because the release of the protein species appeared to be rapid, it could not be determined whether the observed activity was from the complex itself or from the released protein species. Isolated complex concentrated and re-run straight away on the FPLC indicated that there was release of a lysozyme-like species.

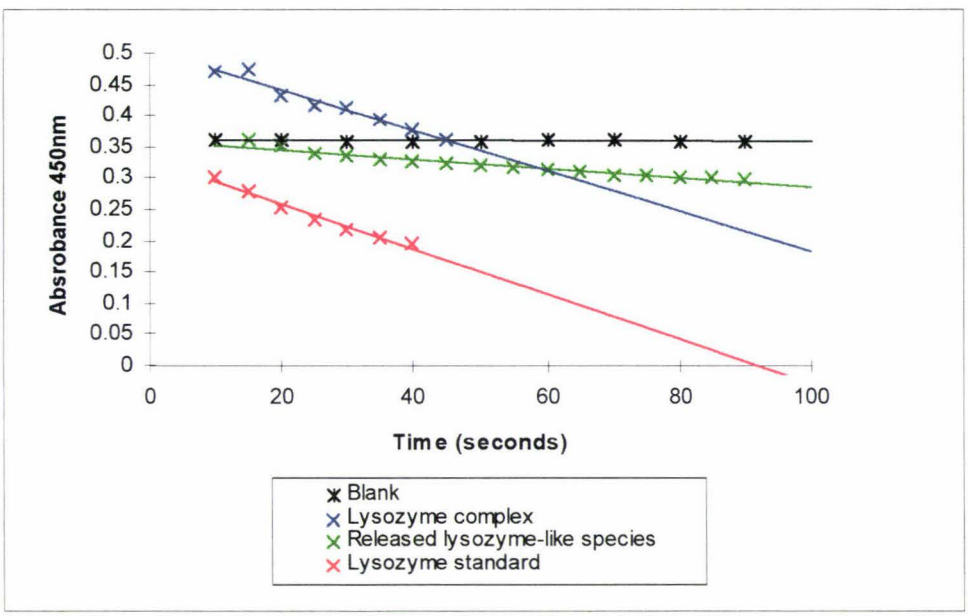


Figure 3.2.7 Lysozyme activity

Lytic assays were performed on the lysozyme-dextran complexes, released lysozyme-like species and on lysozyme standards isolated by SEC (Superdex 75). Complex and release samples were concentrated prior to the assay. Activity was taken as change in absorbance over time at 450nm.

The extent of binding between the lysozyme and dextran is significantly reduced compared with the complex at 56% activation level. This is evident from the results of SDS-PAGE and by the observed lytic activity.

Activity assays were also performed on the isolated complex over three days, as a means of studying the release of protein and to determine the source of the activity. Figure 3.2.8 shows the activity, given as change in absorbance against time, for a three day

period. The increased activity during this period can be attributed to the lysozyme-like species being released from the complex.

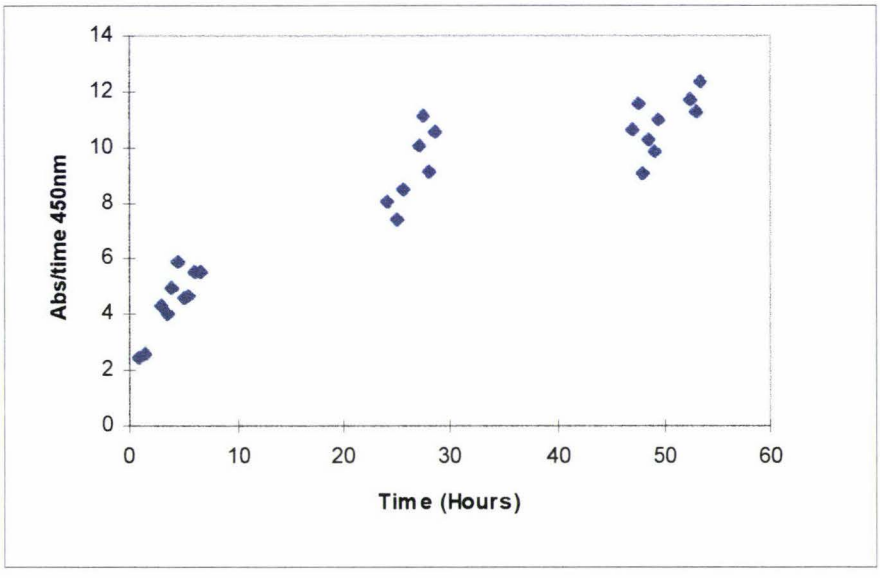
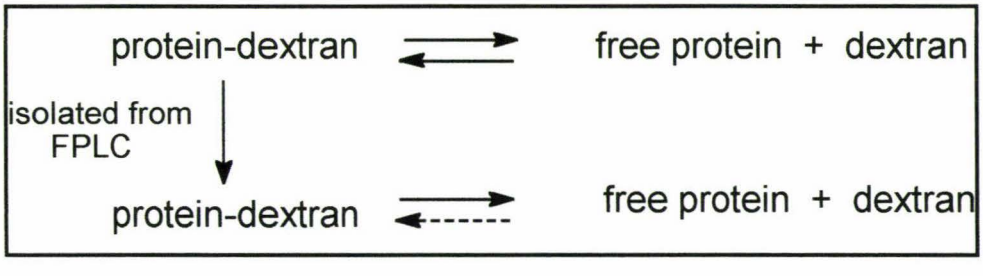


Figure 3.2.8 Activity of Lysozyme Complex with Time

Lysozyme was incubated with 21% activated dextran and the complex formed was isolated by SEC (Superdex 75, FPLC) and concentrated. Complex was then incubated at room temperature, in the dark without stirring. Aliquots were then taken over a three day period and assayed for lytic activity.

The initial activity could either be minimal activity due to complex itself, most likely the lysozyme on the outside of the complex, or from an initial rapid release of lysozyme from the complex. Experiments indicate that release does occur immediately upon isolation of the complex species and the initial increase in activity appears to level off over time. This can probably be accounted for by the fact that the recently isolated complex is re-establishing an equilibrium between the complex and free protein (Scheme 3.2.1).



Scheme 3.2.1 Equilibrium between free protein and dextran

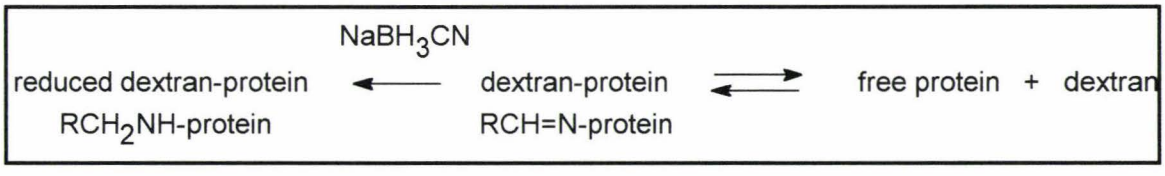
Amino acid analysis was performed on the released species and compared with that of the isolated species from the FPLC and to theoretical data. Relative quantities of each amino acid present was determined with respect to alanine (Table 3.2.1). The amino acid composition of the released species was in good agreement with that of the purified lysozyme. There appears to be no indication of loss of amino acids from either the N-terminal or the C-terminals. Literature data gives the terminals as NH_2- Lys Val Phe Gly Arg.....Arg Gly Cys Arg Leu-COOH (Croft, 1980). There is no value for Cys for the released species but this can be degraded during the hydrolysis carried out before AAA. The biggest variations occurred with Asp, Ser, Tyr and Lys. Only Lys is located near the N-terminal. All of these except for Tyr are shown to be greater for the released species than the purified enzyme indicating the difference is not through loss of amino acids. As Asx actually represents the composition for two amino acids, Asp and Asn, the actual individual difference is possibly less. The released species, with the exception of His does in fact appear to give better correlation with the literature data, differing only in Val and Lys. The value for His is abnormally high and is possibly the result of contamination.

Residue	Total no.	Lit. Seq	Purified	Released
Asx	21	1.8	1.4	1.8
Thr	7	0.6	0.7	0.7
Ser	10	0.8	0.6	1.0
Glx	5	0.4	0.6	0.5
Pro	2	0.2	0.3	0.2
Gly	12	1.0	1.4	1.1
Ala	12	1.0	1.0	1.0
Cys	8	0.7	0.5	-
Val	6	0.5	0.5	0.8
Met	2	0.2	0.2	0.1
Ile	6	0.5	0.5	0.4
Leu	8	0.7	0.6	0.7
Tyr	3	0.3	0.5	0.1
Phe	3	0.3	0.5	0.2
His	1	0.1	0.2	4.7??
Lys	6	0.5	0.4	0.9
Arg	11	0.9	0.9	0.8

Table 3.2.1 Amino acid composition in respect to alanine of the released species in comparison to purified lysozyme and literature sequence

AAA was performed on released lysozyme-like species and lysozyme purified by SEC. Composition of the amino acids present was determined relative to alanine. Theoretical data is also given as a comparison (Croft, 1980).

To further study the complexes, and to establish whether or not they are active, reduction of the complex was carried. Reduction of the complex was performed in the presence of $NaBH_3CN$ which would reduce any imine bonds formed between the lysozyme and the activated dextran. $NaBH_3CN$ was chosen instead of $NaBH_4$ because of its specificity. $NaBH_4$ reduces imine bonds as well as ketones and aldehydes. Normal complex formation was followed by the addition of $NaBH_3CN$. The incubation mixture was then shaken in the dark for 48 hours, and then subjected to SEC on the FPLC. Reduction of the complex should prevent reversal of the imine bond formation preventing the release of lysozyme from the activated dextran (Scheme 3.2.2).



Scheme 3.2.2 Cyanoborohydride reduction of protein-dextran complex

From comparison of the FPLC chromatagraphs of the reduced complex and the control complex (no $NaBH_3CN$ added) it was apparent that reduction of the imine bonds had altered the lysozyme-free dextran ↔ complex equilibrium in favour of complex formation (Figure 3.2.9).

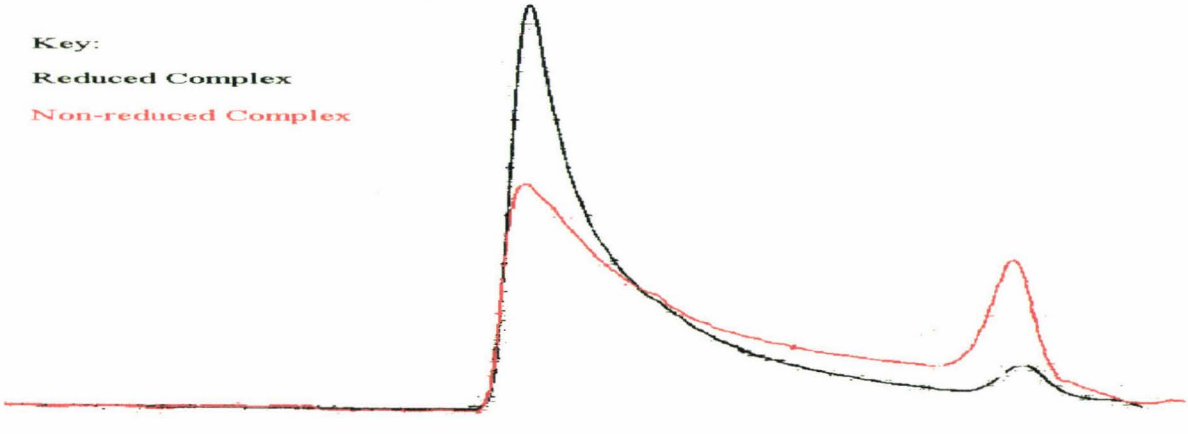


Figure 3.2.9 Complex Formation at 72hrs for reduced and non-reduced complexes
Complex formation incubations were set up with lysozyme and 21% activated dextran. At 24hrs and 48hrs 1x w/w excess of sodium cyanoborohydride was added and the incubations were shaken. At 72hrs aliquots were taken and subjected to SEC (Superdex 75, FPLC) in 0.2M Tris acetate, 0.05% sodium azide, pH7.5.

Activities of the reduced and non-reduced complexes were compared using the lytic assay. Lysozyme incubated with $NaBH_3CN$ in the absence of dextran retained normal lytic activity. This indicated that the presence of $NaBH_3CN$ should not inhibit lytic activity of complexed lysozyme.

While lytic activity was observed with both complexes, it was greater for the non-reduced complex (Figure 3.2.10). Aliquots of the complex were concentrated prior to the activity assay, determination of the protein concentration indicated that the reduced complex contained more protein, approximately 1.65 times more, than the non-reduced complex. As the non-reduced complex had greater activity, yet less protein than the reduced complex it is evident that most of this activity was from released lysozyme-like species.

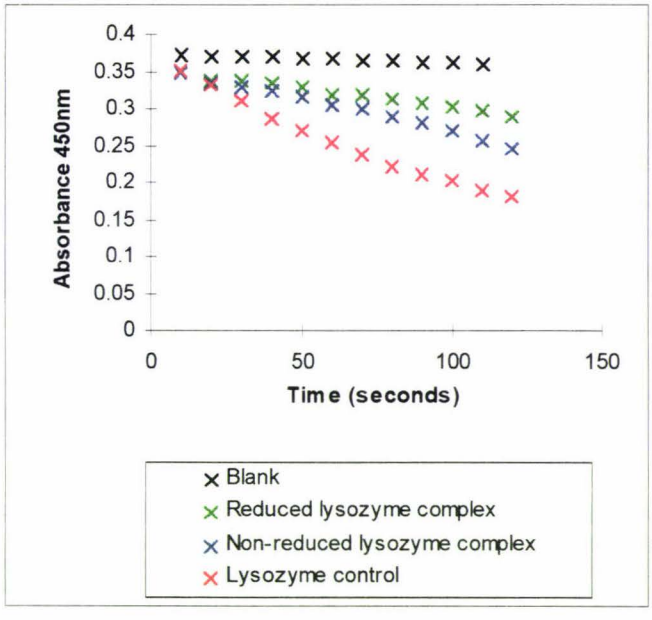


Figure 3.2.10 Lytic activity of reduced and non-reduced complexes
Lytic assays were performed on lysozyme-dextran complexes. Complexes for reduction studies were incubated with $NaBH_3CN$, which reduces the imine bonds preventing release of lysozyme from the complex. Controls were also run on the complexes without $NaBH_3CN$ and on lysozyme plus $NaBH_3CN$. Complexes were isolated by SEC (Superdex 75) and concentrated prior to assaying.

The activity observed with the reduced complex showed that the complexed lysozyme was capable of minimal activity, probably from the lysozymes located on the outer regions of the complex. Any lysozyme 'buried' within the complex is inaccessible to the micrococcus and therefore inactive due to steric hindrance. Adding to this, any

complexed lysozyme bound by more than three lysine residues will also be rendered inactive.

Both the reduced and non-reduced complexes were studied over time for signs of release. After isolating the complexes by SEC (Superdex 75, FPLC) the samples were concentrated and incubated under release conditions. Release of the protein was then monitored by SEC. SMART chromatograms showed only a small release of lysozyme-like species from the non-reduced complex, indicated by the appearance of a peak with the same retention time as lysozyme. For the reduced complex there was no change in the complex peak over time and no indication of lower MW peaks forming. Compared with the earlier standard release experiments, the amount of lysozyme-like species released from the non-reduced complex appears to have been significantly reduced. This is possibly a result of the differing complex formation conditions. For the reduction studies all samples were shaken on the Ika-Vibrax mix. Shaking in the original experiments was not incorporated. This was done to limit adsorption losses during the incubation. This shaking might have possibly allowed for more extensive binding within the complex species. Ultimately this would then reduce the release of lysozyme-like species from the complex.

Kato *et al* (1992) have also investigated the formation of dextran-lysozyme conjugates. In this instance binding was bought about by the Maillard reaction (conjugation by dry heating) that allowed for the binding between the ε-amino groups of the protein and the reducing-end groups of the dextran. From LLST they found that approximately two dextran chains bound to a single lysozyme molecule. A comparison was made with lysozyme conjugates involving cyanogen activated dextran, and it was found that the resulting conjugate consisted of a large number of dextran chains binding to a single lysozyme molecule. Improved emulsifying properties of lysozyme-dextran conjugate over free enzyme were not noted with the CNBr-activated dextran conjugate. Only a partial covering of a protein molecule by dextran chains, as found in the Maillard product, is needed to improve the emulsifying properties.

The lysozyme-dextran study reported in this chapter indicates that, depending on the requirements of the resulting protein-dextran conjugate, the degree of activation by periodate oxidation can be manipulated to determine the extent of dextran binding to the protein. This in turn can be used to establish the properties of the conjugated protein.

In other words the dextran-protein system appears to have a highly desirable range of flexibility. From these initial complex studies it has become apparent that there is a

useful range of activation levels of dextran, activation levels up to at least 21% have resulted in release of lysozyme with relative ease.

The possible roles of lysozyme in cancer therapy are currently being investigated (Sava, 1996). It is hoped that lysozyme could improve the effectiveness of anticancer drugs or assist in immune suppression. Lysozyme has long been considered as a candidate for cancer therapy because of its antibacterial characteristics and its past therapeutical applications.

Lysozyme is also known to have roles in the food industry (Holzapfel *et al*, 1995; Nakamura *et al*, 1991). Applications are limited in part by inactivation caused by endogenous food components or by limited activity. By complexing the lysozyme to a polymer it may be possible to prevent this inactivation. Work by Nakamura *et al* (1991) has already shown that conjugation to dextran extended its enzymatic activity to gram-negative bacteria in addition to gram-positive bacteria (Section 1.9).

3.3 Conclusions

To obtain a useful and viable complex, the oxidation level of dextran needs to be considered. As shown with the 56% activated dextran, complex formation was rapid and it resulted in an extremely high molecular weight complex that then failed to release any protein. The activation level was too high to look at a release mechanism. Nevertheless, for a small basic protein at 56% oxidation the binding was tight and did not need to be reduced for stability. This in turn could be used to trap small proteins or peptides.

At the levels of 21% and 7% activation, complex formation occurred with relative ease and allowed subsequent release of the bound protein. Choosing the level of activation determines the rate at which the complex is formed and this in turn effects the rate of release. Therefore the level of activation can be chosen for the particular end use.

The released species appears to have the same properties as lysozyme. It has lytic activity, elutes at the same time on SEC, has the same MW in SDS-PAGE and has similar amino acid composition.

The complex formed between lysozyme and dextran occurred rapidly and with ease. The spread of molecular weights of the complex observed on SDS-PAGE and SEC probably arises, at least in part, due to random binding of the lysozyme between dextran molecules and is greater than that expected if it resulted only from the varying MW of the dextran (Figure 1.6.1). The complex itself appears to have a limited amount of lytic activity.

These preliminary enzyme studies extend the rhGH study by Battersby *et al* to another species of protein.

Lysozyme is used in the food industry and is being investigated for possible therapeutic uses with cancer therapy. A complex that may have applications in the medicine is one that is easily formed, then promotes sustained and controlled release of one of its components over a definite time course. The food industry may seek additional properties of the enzyme through conjugation. Both areas could benefit from the additional stability of lysozyme in the complexed form.

CHAPTER FOUR

TRYPSIN

4.1 Introduction

Trypsin is a proteolytic enzyme found in the pancreas (Geiger and Fritz, 1984). It is synthesised in the exocrine part of the pancreas as a precursor (zymogen) and stored in the zymogen granules. Activation of the precursor is achieved by removal of a highly negative charged N-terminal peptide. Trypsin is involved in activation of other zymogens in the pancreatic tissue, cleaving proteins and peptides at the carboxamide bond of lysine and arginine residues.

Trypsin is related to several proteolytic enzymes including thrombin, plasmin, kallikren and acrosin, and as such can be used as a model for these important regulatory enzymes (Kasai, 1992).

Trypsin is capable of autolysis, especially in the absence of other proteins. Autolysis can be limited by the presence of Ca^{2+} or low pHs (Green and Neurath, 1953). It was therefore decided to lower the pH of the phosphate incubation buffer and the Tris acetate buffer to 5.5, the previous lysozyme studies were carried out at pH 7.5. $CaCl_2$ is not soluble in phosphate buffers and therefore it was only added to the Tris acetate buffer used in subsequent release studies (Bier and Nord, 1951). The proposed explanation for this effect by Ca^{2+} is that at alkaline pHs the active trypsin is in equilibrium with a reversibly denatured form (Green and Neurath, 1953). Digestion of this inactive form by the active form is thought to be responsible for the loss in activity. The addition of Ca^{2+} shifts the equilibrium towards the active form.

The pocrine trypsin used in this study is made up of 222 amino acids, including ten lysine residues. During the course of this study the original protein used was changed to a TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone) treated trypsin. TPCK is used to inhibit contaminant activity such as chymotrypsin activity. This was of particular importance during the trypsin digest studies.

Proteolytic activity of the trypsin was determined from the BAPNA assay (Figure 4.1.1). This involves trypsin acting on an artificial chromagenic substrate. Therefore the rhGH digest experiments were performed to observe for 'real' activity.

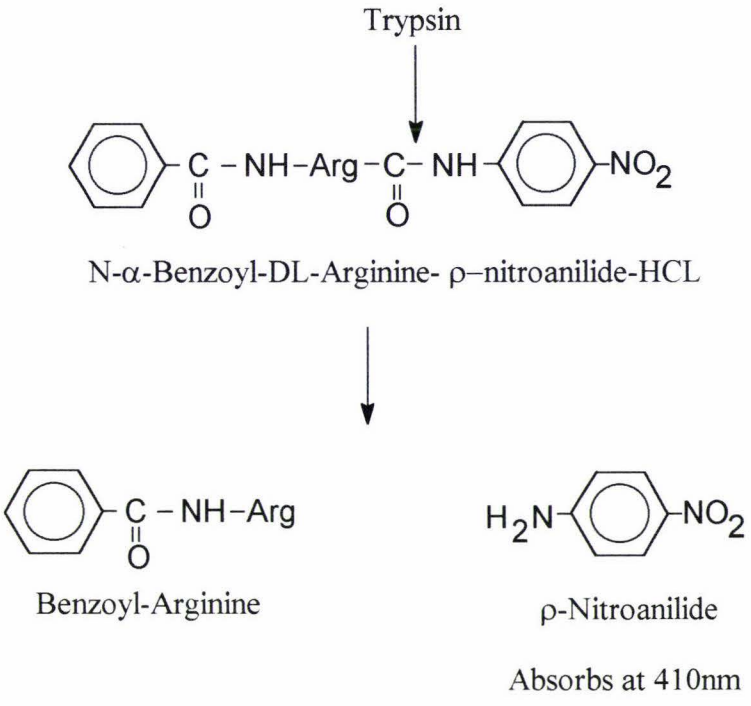


Figure 4.1.1 BAPNA assay for trypsin activity

Trypsin cleaves the carboxamide bond of the arginine producing ρ-nitroanilide, the absorbance of which can be followed at 410nm.

To support the rhGH digest studies when HPLC equipment and buffer difficulties became apparent, digestion was also performed on a synthetic peptide. This was produced by solid phase peptide synthesis (SPPS) by R. Parshot and J. Cross. The peptide was designed as a 24mer with one arginine and two lysine residues. Successful digestion would therefore result in four smaller peptides, each designed to be readily distinguishable by AAA.

4.2 Results and Discussion

Complex formation between trypsin and 56% oxidised dextran was investigated briefly and shown not to be as extensive as in the lysozyme-56% activated dextran case. While the complex formation was rapid and did not produce a manipulable complex, it did run on SDS-PAGE.

From these initial results, and from the lysozyme experiments, it was decided to concentrate the trypsin study on the lower levels of activation. Complex formation was studied over time by SEC at the 7% and 21% activation levels (Figure 4.2.1 and 4.2.2). Formation was shown by the appearance of a broad peak, eluting just after the void volume, in accordance with a loss in the trypsin peak. Complex formation was faster at the 21% activation level. The 7% and 21% complex samples were run with different buffers indicating the differences in chromatographs.

Trypsin has a lower absorbance coefficient at 280 nm compared with lysozyme and therefore greater sensitivity and pooling of samples was required. The absorbance coefficient is determined by the sum of the aromatic residues, in particular Trp, and the carbonyl groups in the protein.

During complex formation some autolysis will occur even at pH 5.5. This was evident from SEC of fresh trypsin standards run immediately compared with that of the incubated standards. In the latter case there was formation of several peaks with longer retention times, therefore smaller MWs, in accordance with a drop in the protein peak. In the presence of dextran, the trypsin should bind in preference to autolysis, and once complexed it should be less susceptible. As mentioned in section 1.6, Kobayashi and Tukatsu (1993) found that trypsin conjugated to periodate activated dextran did not undergo autolysis. Therefore the major concern is to prevent autolysis of any released trypsin-like species. This has been minimised by the addition of $CaCl_2$ to the Tris acetate buffer and by working at pH 5.5.

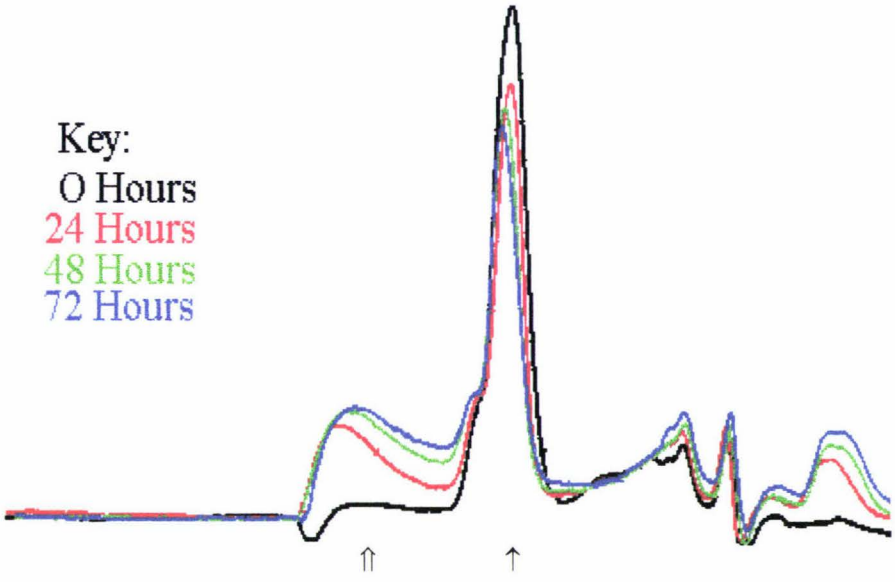


Figure 4.2.1 Complex (⇑) formation over time between trypsin (↑) and 7% activated dextran

Trypsin incubated with 7% activated dextran was subjected to SEC (Superdex 75) at 0hrs, 24hrs, 48hrs and 72hrs in 0.05M sodium phosphate, 150mM NaCl, 0.05% sodium azide, pH 5.5.

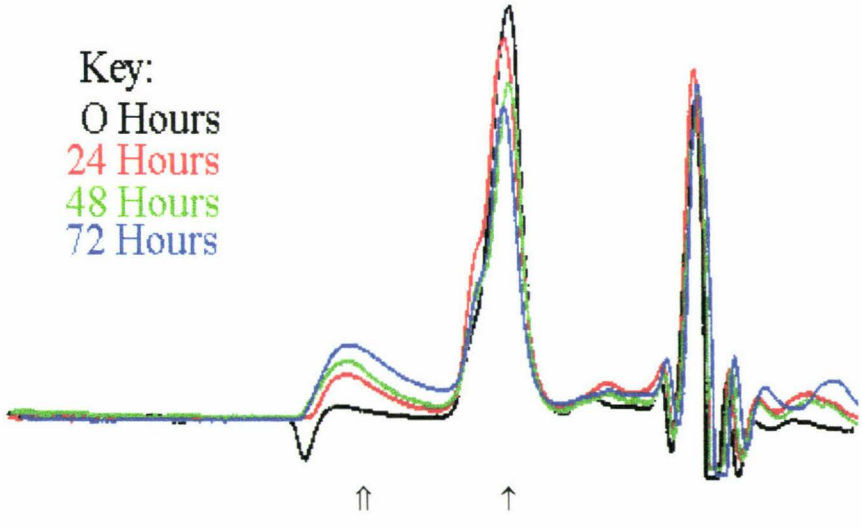


Figure 4.2.2 Complex (⇑) formation over time between trypsin (↑) and 21% activated dextran

Trypsin incubated with 21% activated dextran was subjected to SEC (Superdex 75), at 0hrs, 24hrs, 48hrs and 72hrs in 0.2M Tris acetate, 0.02M $CaCl_2$, 0.05% sodium azide, pH 5.5.

Release from the complex was studied by means of SEC. Complex formation incubations were set up between trypsin and 7% and 21% levels of activated dextran. Upon isolation by SEC (Superdex 75, FPLC) the complexes were incubated under release conditions and aliquots were then subjected to SEC overtime (Figure 4.2.3). At both the 7% and 21% activation levels a decrease in the peak due to the complex was observed in accordance with the formation and increase of a lower molecular weight species with the same retention time as trypsin. For subsequent studies the isolated complex was incubated under release conditions for 72 hours. Aliquots were then subjected to SEC (Superdex 75, FPLC) and released trypsin-like species were pooled and concentrated.

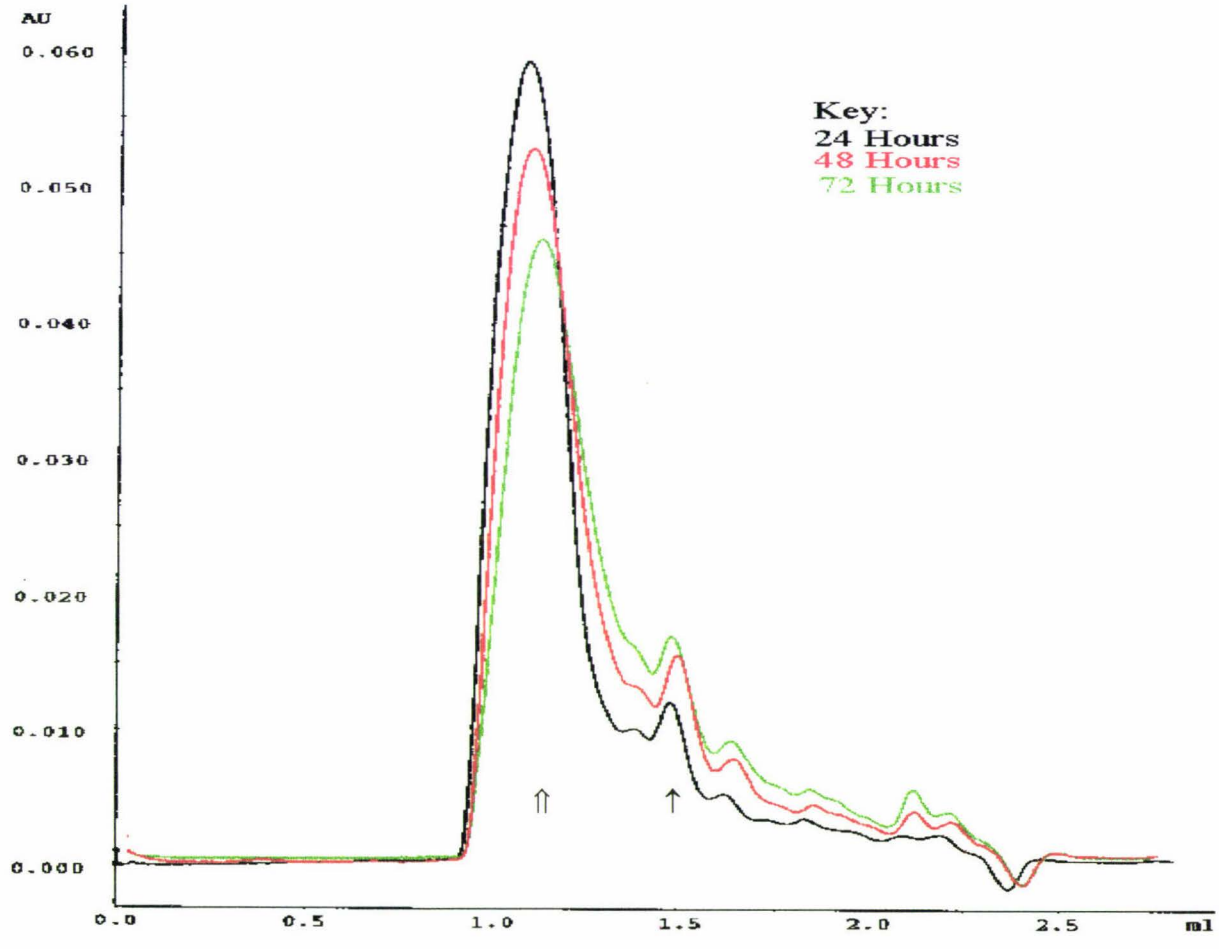


Figure 4.2.3 Release of trypsin-like species (↑) from the complex (⇑)
Trypsin-dextran complexes were isolated off the FPLC and then subjected to SEC (Superdex 75) over time to observe for signs of release in Tris acetate, 0.02M $CaCl_2$, 0.05% sodium azide, pH 5.5.

From the studies performed on the complex it appeared that it maintained only minimal activity at both levels of activation (Figure 4.2.4). Activity was performed by the BAPNA assay previously described. Trypsin has been extensively studied with respect to its immobilisation to a solid support (Huckel *et al*, 1996). Stabilisation of the enzyme was achieved through covalent attachment to the solid support, increasing the duration of enzymatic activity. The applications of this technique range from industrial through to diagnostic. To ensure that severe loss of activity does not occur during covalent coupling, suitable support materials need to be chosen that contain functional groups that can be reacted with the protein under mild conditions.

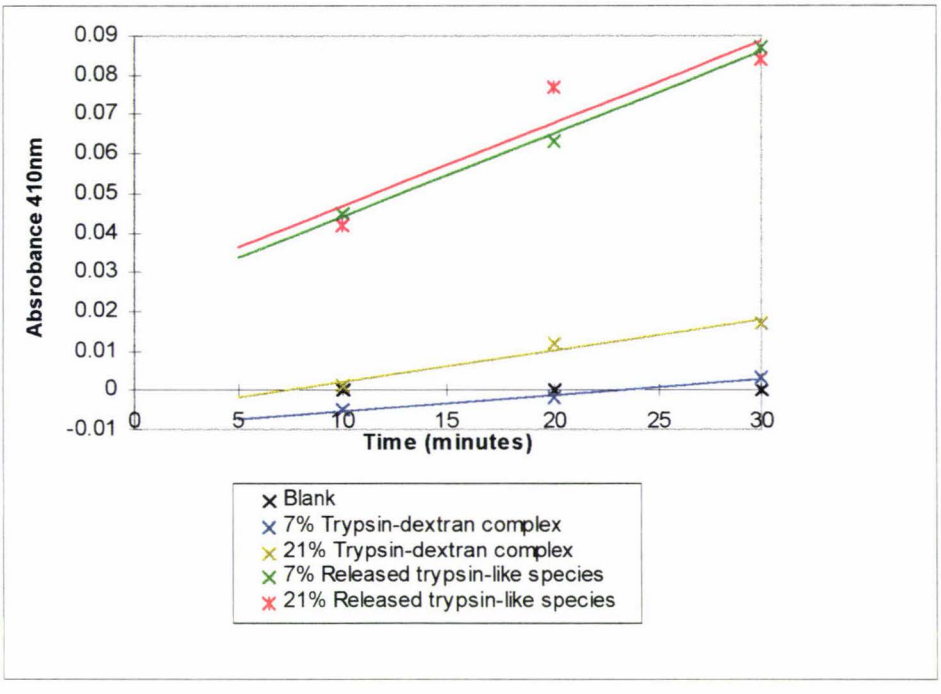


Figure 4.2.4 Trypsin Activity
BAPNA assays were performed on the trypsin complex and released trypsin-like species isolated by SEC and concentrated.

Immobilised trypsin has a lower activity than the free enzyme as a result of distortions to the active site upon binding, but it is more stable (Huckel *et al*, 1996). The activity of free trypsin will decrease more significantly over a period of time in comparison to the immobilised trypsin. Support materials used for the immoblisation of trypsin include zirconia, silica and aminoethylcellulose derivatives which have all achieved varying levels of activity relative to free trypsin. While conjugation with dextran in this investigation appears to give a low relative activity, it is possibly a result of the imine compound being in a dynamic solution situation, compared with the solid support immobilisation case.

Immobilisation techniques allow for the binding of one protein molecule per chemical group, but periodate oxidation of dextran allows for multiple binding between the protein and the dextran. This additional crosslinking may further limit the activity due to steric hindrance. Reduction studies were required, as with the lysozyme studies, to determine if this minimal activity is from the complex itself or from trypsin released straight away.

Activity of the complexed trypsin is possibly affected by the pH used. Experiments were performed at pH 5.5 to help minimise autolysis of the protein, but studies with immobilised trypsin have shown it to be more active around pH 9 (Huckel *et al*, 1996). Therefore there is the possibility for future work to investigate the effect of altering the pH after isolation of the complex. This would be more feasible if the complexed trypsin is protected from autolysis. Presence of $CaCl_2$ should minimise subsequent autolysis of the released trypsin.

Specific activity values indicated that the released protein is far more active than the complexed trypsin (Table 4.2.1). This indicates that the observed activity of the complex is probably due to releasing trypsin.

	Specific Activity $\mu mol\ min^{-1}\ mg^{-1}$
7% Complex	0.0037
21% Complex	0.0056
7% Release	0.0259
21% Release	0.0194

Table 4.2.1 Specific activity for the trypsin complex samples and the release trypsin-like species
Specific activity is expressed in μmoles p-nitroanilide produced per min per mg protein.

Activity assays were also performed over time on the trypsin-dextran complex isolated by SEC (Figure 4.2.5). Levels of activity were very low so it is not apparent how significant the changes in activity where. Indications from the BAPNA assays performed, are that the activity appears to drop initially but then increase again.

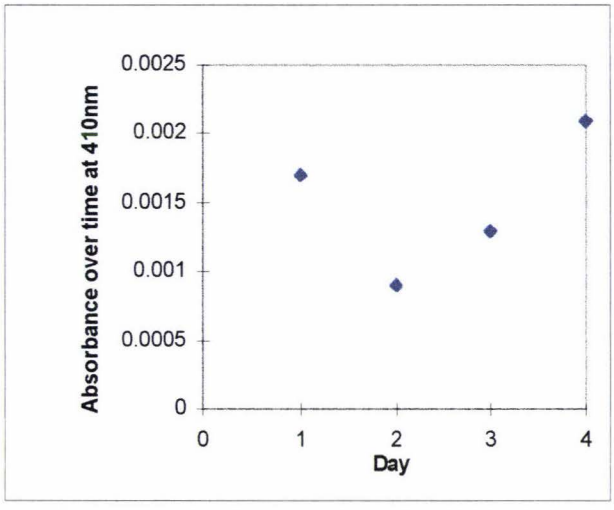


Figure 4.2.5 Activity of trypsin-dextran complex over time

Trypsin was incubated with dextran and after 72 hours the complex was isolated by SEC (Superdex 75). Activity of the complex was then followed over four days using the BAPNA assay.

Amino acid analysis was performed on the released species and compared with that of trypsin purified by SEC, and theoretical data (Croft, 1980). There is very good agreement between the amino acid composition for the released species and the purified trypsin (Table 4.2.2). The biggest differences occurs with tyrosine, proline and histidine. The sequence for trypsin is NH_2-Ile Val Gly Gly Tyr.....Gln Gln Thr Ile Ala (Ala Asn)-CH_2 (Croft, 1980). For this situation it is unlikely that loss from the N-terminal will occur as it is 'protected' within the active site. There appears to be no loss of amino acids from either terminal (Desnvelle, 1960).

Residue	Total no.	Lit. seq	Purified	Released
Asx	23-24	1.6	1.2	1.1
Thr	10	0.6	0.7	0.8
Ser	27	1.9	1.0	1.3
Glx	16	1.2	1.0	1.1
Pro	9	0.6	0.8	0.5
Gly	25	1.8	1.7	1.9
Ala	14-15	1.0	1	1.0
Cys	12	0.9	0.5	0.4
Val	16-17	1.1	0.8	0.7
Met	2	0.1	0.2	0.1
Ile	15-16	1.1	0.7	0.7
Leu	16	1.1	0.8	0.9
Tyr	8	0.5	0.7	0.2
Phe	4	0.4	0.4	0.2
His	4	0.3	0.3	0.9
Lys	10	0.7	0.5	0.7
Arg	5	0.3	0.5	0.2

Table 4.2.2 Amino acid composition with respect to Alanine of the released species in comparison to purified trypsin and the literature sequence

AAA was performed on released trypsin-like species and trypsin isolated by SEC. Amount of each amino acid present was made relative to alanine. Theoretical data was also referred to (Croft, 1980).

Analytical HPLC runs were performed courtesy of J. Battersby on the released trypsin-like species and the original starting material. (Figure 4.2.6). Although there are only small quantities of the released material it did appear to be trypsin like, eluting close to the original trypsin peak. This was just a one off experiment.

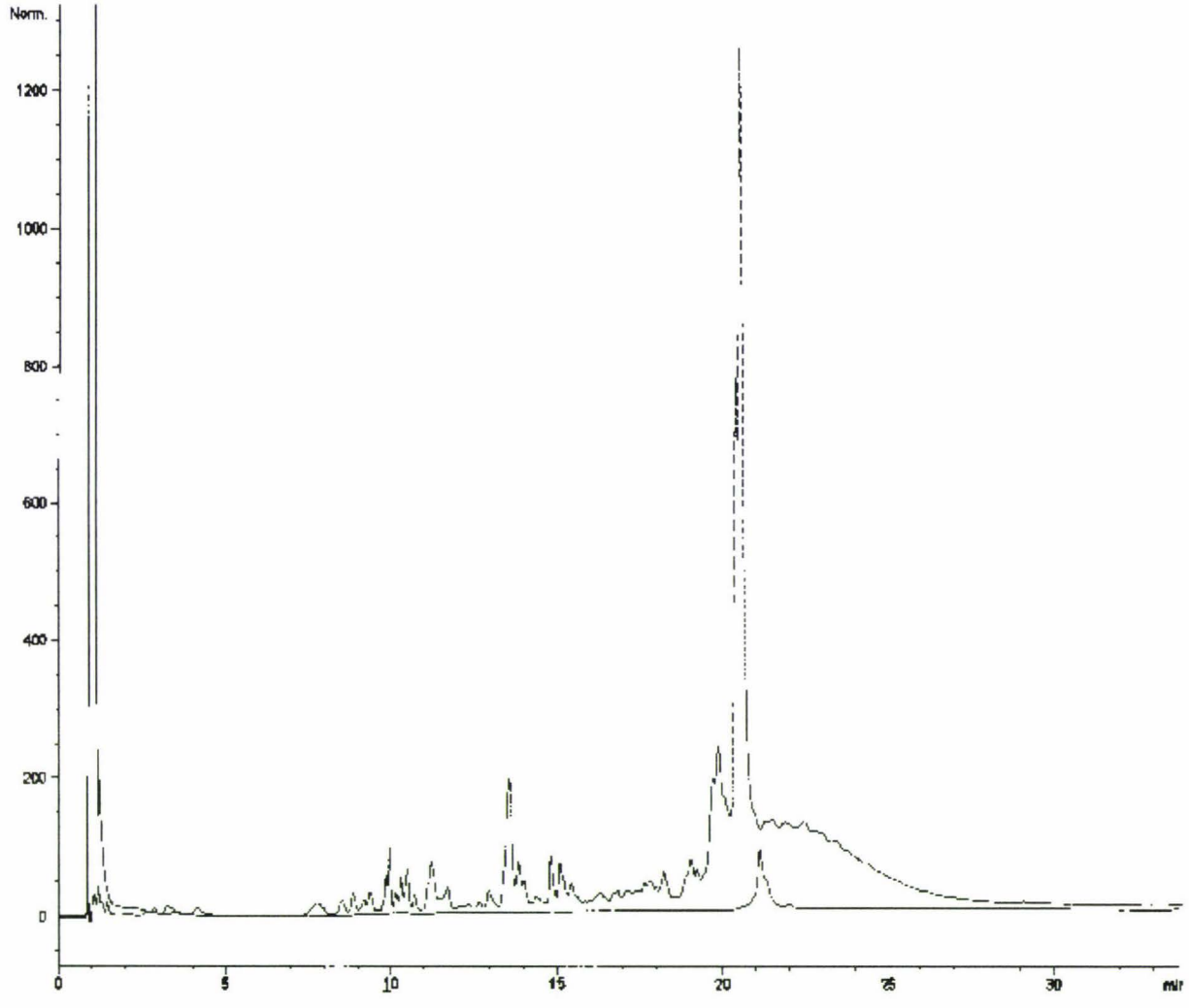


Figure 4.2.6. Analytical reverse phase chromatography of the released trypsin-like species and the original trypsin (Courtesy J. Battersby).
The top line represents the original trypsin and the bottom line is the released-trypsin-like species, present in significantly lower concentrations.

Both the released species and the complex were run on SDS-PAGE (Figure 4.2.7). The Complex gave rise to a smeared band covering a range of MWs (lane 2 and 6). The release of trypsin from the complex subsequent to isolation is indicated by the band running with the same MW as trypsin (lane 6). The released trypsin isolated by SEC (lane 3) was also shown to be the same MW as the original trypsin. The additional lower MW material present is possibly a result of autolysis.

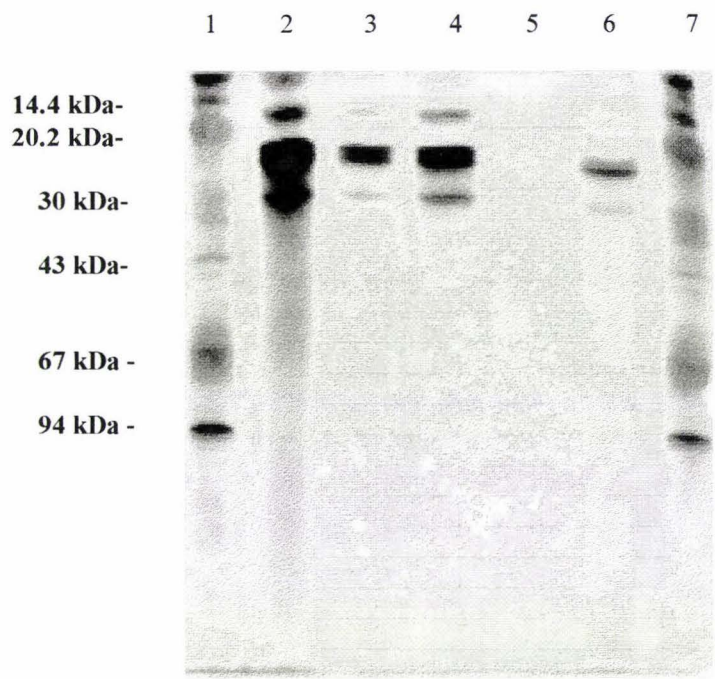


Figure 4.2.7 SDS-PAGE analysis

Homogenous 12.5% SDS gel using the silver staining development on the Phast system

lane 1 marker: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.2 kDa), α-lactalbumin (14.4 kDa)
lane 2 complex formation incubation mixture
lane 3 released trypsin-like species
lane 4 trypsin standard
lane 5 filtrate
lane 6 7% trypsin-dextran complex
lane 7 marker as per lane 1

Reduction studies were performed on trypsin as for lysozyme (section 3.2). $NaBH_3CN$ was shown not to inhibit the activity of trypsin (Figure 4.2.8). Aliquots of the various complexes were assayed after concentrating. No activity was observed at all with the reduced complexes, while the non-reduced complexes were active. As trypsin is not inhibited by the presence of $NaBH_3CN$ this implies that the activity for the non-reduced

complex was due to release of the trypsin from the complex. For the non-reduced controls there was less release of trypsin-like species over time compared with normal release studies. This could have resulted from the shaking conditions required for the reduction studies, and is consistent with the lysozyme study (section 3.2).

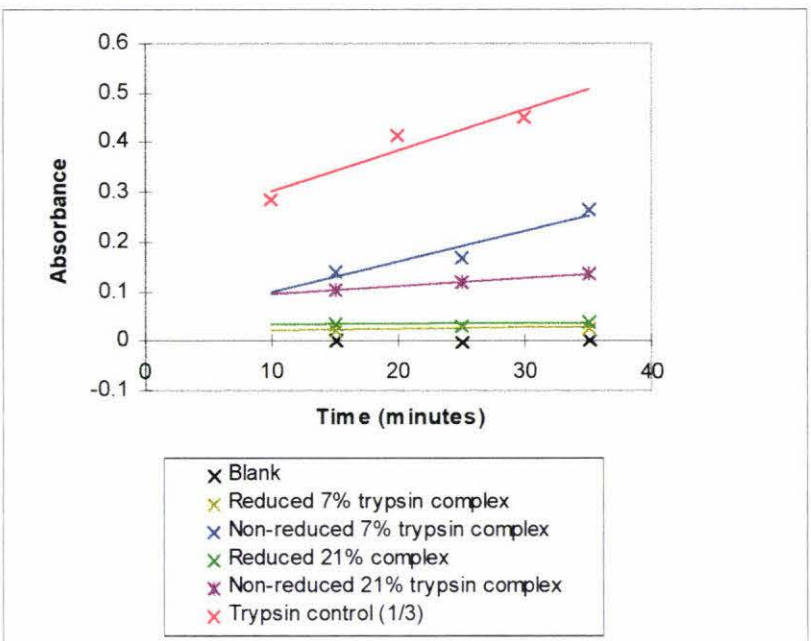


Figure 4.2.8 Activity Studies on reduced and Non-reduced complexes

BAPNA assays were performed on the trypsin-dextran complexes. Complexes for reduction studies were incubated with $NaBH_3CN$, reducing the imine bonds and preventing release of trypsin from the complex. Controls were also run with complexes in the absence of $NaBH_3CN$, and on trypsin incubated with $NaBH_3CN$ alone.

Activity studies showed that the complex had lower specific activity than the release trypsin (Table 4.2.1). Reduction studies hence showed all the activity in the complex sample was from the released protein. Therefore the lower specific activity value for the complex was because there was both conjugated and released trypsin like-species present (increasing the amount of protein) but only the released species was active.

To further confirm the presence of trypsin's proteolytic activity a digest with the released trypsin was performed on rhGH. The peptide map obtained from reverse phase chromatography (C18) was then compared with a tryptic map produced from the action of the original trypsin. While the map from the released trypsin-like species did indicate that considerable digestion had taken place, it did not produce an identical map. It is possible that only partial digestion had taken place. This could occur if lower amounts of

the released trypsin-like species were used compared with the original trypsin. Possible means of clarifying this would include analysing the peaks obtained in each digest, running the digest for longer periods or by obtaining greater amounts of the released trypsin-like species. In the rhGH digest studies relative amounts of the released trypsin were lower than the recommended protein to trypsin ratio (Battersby *et al*, 1995).

In an attempt to simplify the situation a synthetic peptide was chosen for the digestion studies. The peptide was designed to have two lysines and one arginine present. The synthetic 24mer would then produce four equivalently sized fragments upon digestion. Compared with the rhGH these individual peptides would be easier to identify. Unique amino acids were positioned in each fragment to allow for ease of detection by AAA (Scheme 4.2.1). Trypsin-dextran incubations were carried out in order to obtain greater amounts of released product.

P1 P2 P3

Gly[1] - Ala - **Met** - Leu - Ala - Lys - Gly - **Glu** - Val - Ala - Leu - Arg - Gly - **Pro** - **Phe** -

↑ ↑

P4

Val - Ala - Lys - Gly - **Asp** - **Ser** - Ala - Gly - Ala[24]

↑

Scheme 4.2.1 Sequence of the 24mer, synthetic peptide

↑ expected cleavage sites by trypsinolysis of 24mer. Amino acids unique to each theoretical peptide are shown in bold. (P = peptide) P1 to P4 represents the expected peptides after trypsin digestion.

The synthetic peptide produced was purified and then shown to give a single peak with reverse phase chromatography (Figure 4.2.9). AAA was performed on the synthetic peptide to confirm that the desired monomer had been produced (Table 4.2.3). Amounts of each amino acid are given relative to glycine. The AAA indicated that there was one less alanine present than expected. It is possible that one was deleted during the SPPS. The low value for Met is not unexpected as it is partially destroyed in the hydrolysis process. Although the values were not in absolute agreement, presence of the 'marker' amino acids appeared to be efficient enough for the trypsin digest. Having established the identity of the synthetic peptide trypsin digests were then performed with both the original trypsin and the released trypsin-like species. At 4 hours and 15 hours aliquots from each digest were taken and subjected to reverse phase chromatography (Figures 4.2.10 and 4.2.11). Fractions were isolated and then analysed by AAA.

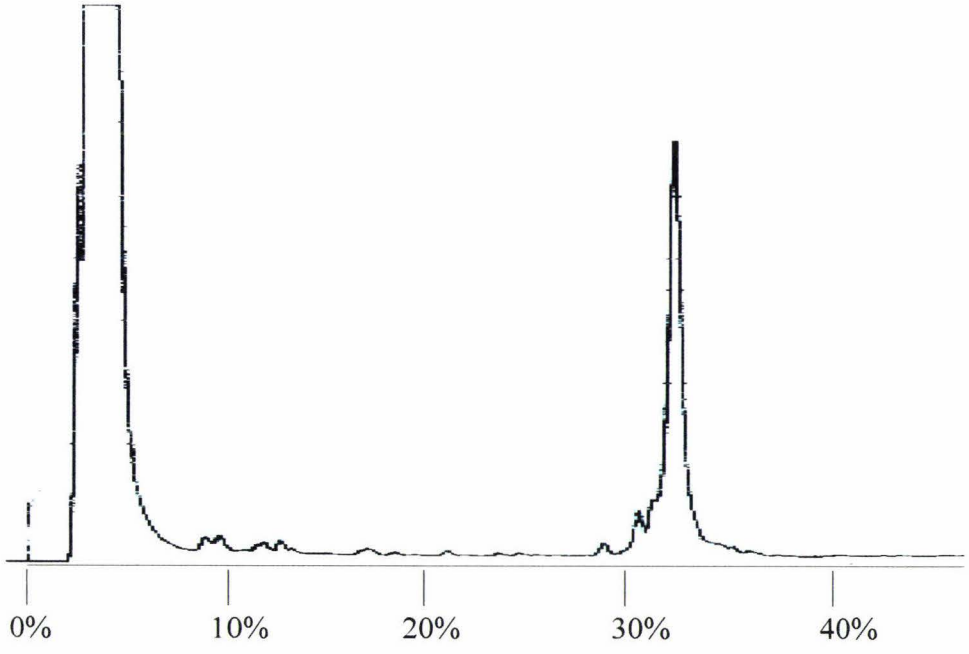


Figure 4.2.9 Reverse-phase analytical run of the synthetic peptide
The synthetic peptide was produced and purified by R. Parshot and J. Cross. The peptide was then analysed by reverse phase chromatography. Scaling is given with respect to the percentage of buffer B.

	Number expected	Amount present
Gly	5	5.0
Ala	6	5.0
Met	1	0.3
Leu	2	1.6
Lys	2	1.9
Glu	1	0.8
Val	2	2.1
Arg	1	1.1
Pro	1	1.5
Phe	1	1.3
Asp	1	1.5
Ser	1	1.0

Table 4.2.3 AAA of the synthetic peptide
A 24mer synthetic peptide was designed to produce four fragments upon proteolytic attack. The synthetic peptide was produced by SPPS. AAA was performed on the purified fragment and the amino acid composition was determined with respect to glycine.

Comparisons of the 4 hour digests showed four similar peaks at 8.3%, 16.8%, 17.8% and 18.5% (percentage given relative to buffer B) for both digests (Figures 4.2.10 and 4.2.11). For the released trypsin-like species there is also an additional peak at 36%, shown by AAA to contain no protein material. The peak at 8.3% occurs as a doublet, the earlier eluting of the two was shown to contain no amino acid material. The equivalent peaks for the two digests were designated 1-4 as indicated in Figures 4.2.10 and 4.2.11. AAA comparisons with the four predicted peptides were carried out (Tables 4.2.4). P4, the most polar of the peptides, appears to be eluting first.

P1 of the synthetic peptide elutes at 16.8% B. The two digests show similar amino acid compositions. Met is slightly lower for the original trypsin but this amino acid is susceptible to hydrolysis prior to amino acid analysis. The amount of Met indicated here is higher than relative amount present in the intact synthetic peptide of 0.3. For P2 the original trypsin showed that there was good agreement with the expected amino acid composition. The valine value was lower than expected for the released trypsin-like digest. The presence of arginine was unable to be determined from the AAA because the trace went off the scale well before the expected elution time for arginine. The reasons for this are unknown, possibly contamination of the sample occurred between isolation from the HPLC and performing of the AAA. A similar pattern was seen when the AAA was first performed on the intact synthetic peptide. Repetition of the AAA indicated no contamination. Analyse of the peak at 18.5% B, P3, showed good agreement between the two digests. The proline value did appear to be a bit low. For P4 (8.3%), the released trypsin-like digest showed better amino acid composition than the original digest which indicated high amounts of aspartate and low amounts of serine. Composition of alanine for both digests pointed to the fact that only one residue was present in the peptide, although two were predicted. Looking back at the amino acid composition of the intact synthetic peptide there was also evidence of the loss of an alanine residue (Table 4.2.3). This could have arisen due to an amino acid deletion during the SPPS at -Ser-Ala-Gly- or due to a failed deprotection of the FMOC amide resin such that the FMOC-Gly was the first amino acid to couple, therefore the peptide produced may have only been 23 residues and not 24.

In each case there were no signs of any intact synthetic peptide (31% B). It appears that the tryptic digest has gone to completion. The overnight digest (15 hours) gave the same patterns of the four peaks representing the four peptides. The chromatograph of the released trypsin-like species still showed an additional peak at 36% while the original trypsin had an additional peak at 6.2%. AAA of the latter showed that it contained no peptidic material.

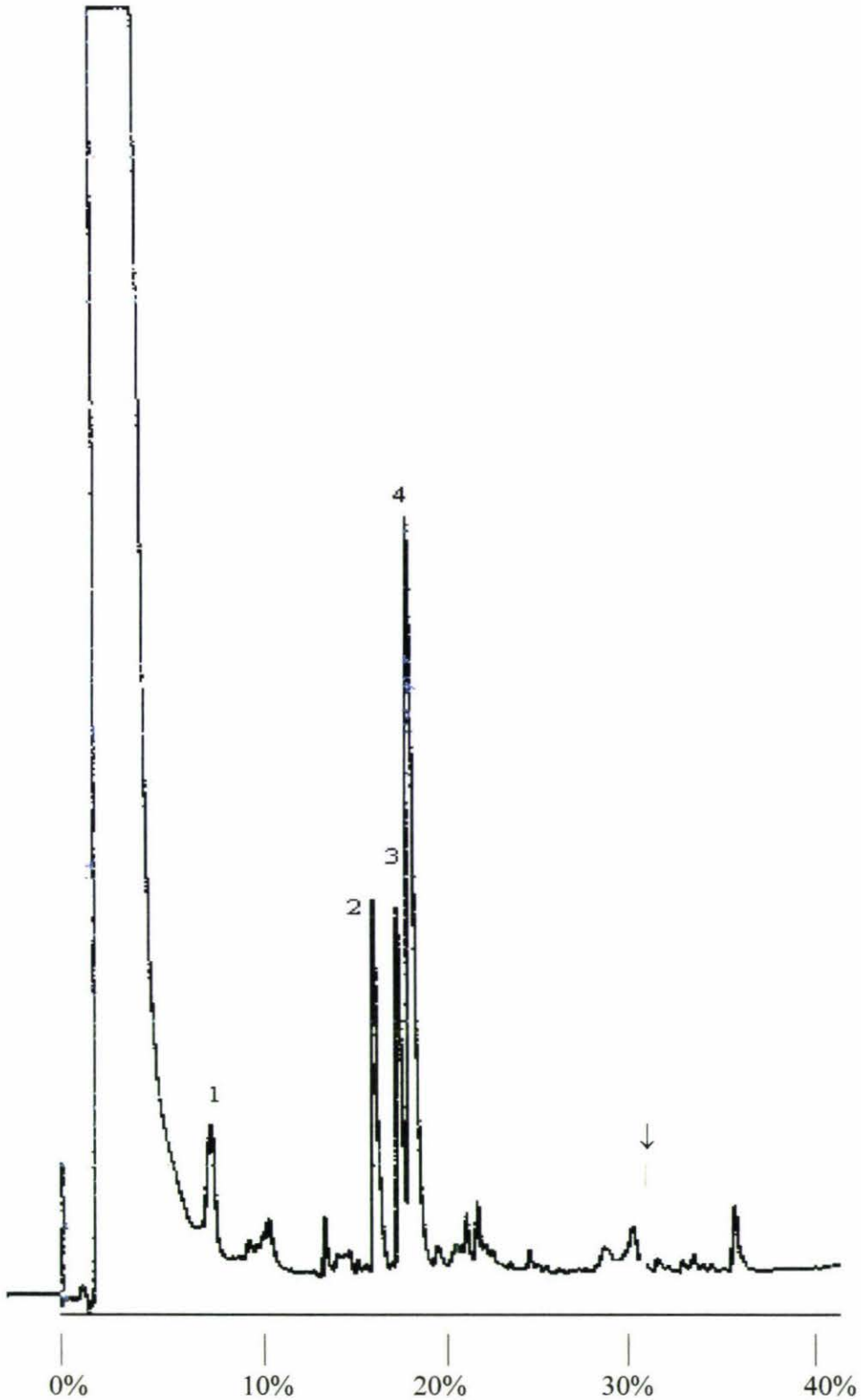


Figure 4.2.10 HPLC chromatograph of trypsin digest on the 24mer by the original trypsin.

Synthetic peptide (1mg/ml) in 0.1M ammonium carbonate was incubated at 37^{0}C. Trypsin sample was added at 0 hours and at 2 hours (final peptide:trypsin of 50:1). At four hours the reaction was halted by the addition of formic acid to lower the pH to 2-3. ↓ indicates elution time of the intact synthetic peptide.

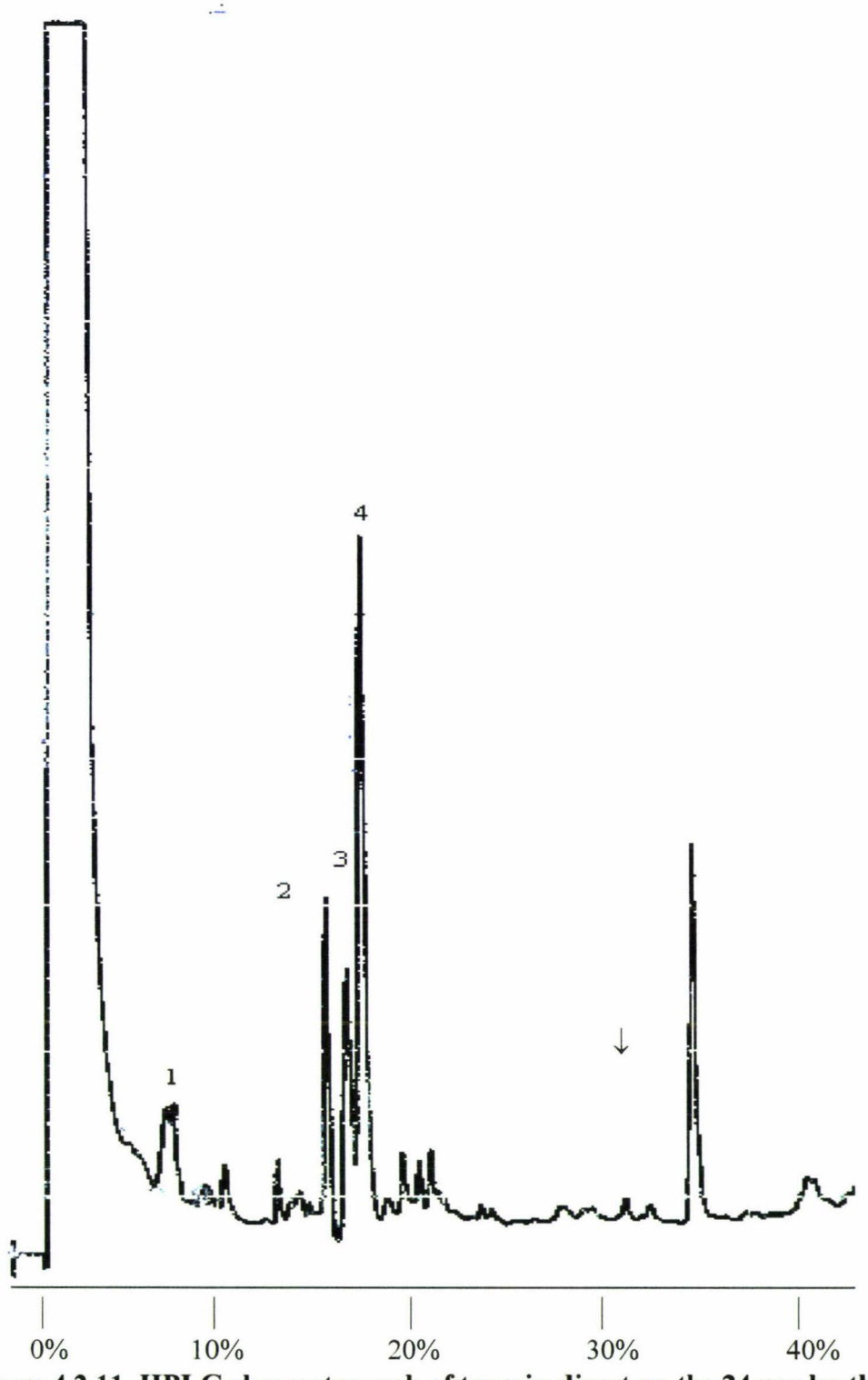


Figure 4.2.11 HPLC chromatograph of trypsin digest on the 24mer by the released trypsin-like species.

Synthetic peptide (1mg/ml) in 0.1M ammonium carbonate was incubated at 37^{0}C. Trypsin sample was added at 0 hours and at 2 hours (final peptide:trypsin of 50:1). At four hours the reaction was halted by the addition of formic acid to lower the pH to 2-3. ↓ indicates elution time of the intact synthetic peptide.

a) Peptide 1 Gly-Ala-Met-Leu-Ala-Lys

	Number AA present	Original Trypsin	Released trypsin-like
Gly	1	1.4	1.2
Ala	2	2	2.0
Met	1	0.6	0.9
Leu	1	1.0	1.1
Lys	1	1.1	1.1

b) Peptide 2 Gly-Glu-Val-Ala-Leu-Arg

	Number AA present	Original Trypsin	Released trypsin-like
Gly	1	1.1	0.8
Ala	1	1.1	1.2
Val	1	0.8	0.4
Ala	1	1.0	1.0
Leu	1	1.0	0.9
Arg	1	1.0	-

c) Peptide 3 Gly-Pro-Phe-Val-Ala-Lys

	Number AA present	Original Trypsin	Released trypsin-like
Gly	1	1.1	1.3
Pro	1	0.8	0.6
Phe	1	1.0	0.9
Val	1	0.9	0.9
Ala	1	1.0	1.0
Lys	1	1.0	1.2

d) Peptide 4 Gly-Asp-Ser-Ala-Gly-Ala

	Number AA present	Original Trypsin	Released trypsin-like
Gly	2	2.0	2.5
Ala	2	0.9	1.2
Ser	1	0.5	0.9
Asp	1	3.2	2.0

Table 4.2.4 AAA composition of peptides from trypsin digest

a) Peptide 1, P1
b) Peptide 2, P2
c) Peptide 3, P3
d) Peptide 4, P4

Tryptic digests were performed for 4 hours on the synthetic peptide by the original trypsin and the released trypsin-like species. The digests were subjected to reverse phase chromatography and the subsequent peaks were then analysed by AAA. Composition was then determined relative to alanine (except for P4, were compositions were made relative to glycine).

Reverse phase chromatographs indicated that the released trypsin-like species performed an equivalent digest on the synthetic peptide as the original trypsin. Each peptide was identified by AAA.

	Peak	% B
P1	2	16.8
P2	3	17.8
P3	4	18.5
P4	1	8.3

Table 4.2.5 Summary of synthetic peptide digestion

Therapeutic applications of trypsin include being used as a digestive aid (cystic fibrosis), in the treatment of bruising and fractures along with chymotrypsin, and it has been used for cleaning wounds (Wiseman, 1975). Trypsin has also found industrial uses including preparation of leather, treatment of raw silk and in the preparation of protein hydrolysates.

4.3 Conclusions

Initial studies with 56% activated dextran and trypsin indicated similar difficulties to those noted during the lysozyme study. Therefore this level of activation was not investigated further.

Trypsin proposed more problems than the lysozyme due in part to its ability to undergo autolysis. Once this was minimised trypsin was shown to form complexes with dextran with relative ease. Release from the complex appeared to be quite slow.

BAPNA assays performed on the reduced complex indicated that it had no activity. Observed activity with the complex samples was therefore probably due to the released trypsin-like species. Loss of activity is most likely a result of alterations to the active site upon binding. Steric hindrance may also play a role here, but if this was the case minimal activity would be expected from the protein on the outer surface of the complex. The released species did appear to be trypsin like. It gave a positive BAPNA assay, eluted at the same time for SEC, and was shown to have the same MW by SDS-PAGE.

Digestion of a synthetic peptide by the released trypsin-like species was performed in order to confirm the indications from the rhGH digest studies that the release product had 'real' trypsin activity. The released species produced a peptide map from the synthetic peptide that compared well with that of the original trypsin. AAA was also supportive, although troublesome. "Variations in AAA values can arise from slight impurities in the sample preparation, due to incomplete hydrolysis and the fact that some of the amino acids can get destroyed during the hydrolysis process" (Moore, 1994). The fact that the digest maps showed none of the remaining intact peptide, and gave rise to only four peaks with peptide material indicates that the digestion had gone to completion. Apart from Met, amino acids that can be destroyed were avoided in this peptide. The main concerns were the inability to detect the arginine in P2 for the released trypsin-like species as well as the non-consistent values for Met and the high Asp value in P4 for the original trypsin digest. The presence of the marker amino acids did identify each of the expected peptides.

Not only are there possible therapeutic and industrial benefits for a slow release system involving trypsin, but also other proteolytic enzymes. Trypsin can be used as a model for proteolytic enzymes. Investigations with trypsin indicate that other proteolytic enzymes should also be able to bind to activated dextran and subsequently be released over time.

CHAPTER FIVE

α-AMYLASE

5.1 Introduction

α-Amylase, α-1,4 glucan 4-glucanohydrolase, is widely distributed in micro-organisms, mammalian tissues and plants (Nakajima *et al*, 1986). It hydrolyses the α-1,4 linkages of amylose, a component of starch, giving rise to a mixture of glucose and maltose (Chang *et al*, 1992; Lehninger, 1977) but it is unable to hydrolyse α-1,6 links. In mammals it is secreted by the salivary glands and the pancreas.

α-Amylase is stable in the pH range of 4.8-8.5, with a preference for pH 5-7 (Burton, 1995). The MW range of α-amylase from different sources is 45-51 kDa. The α-amylase used in this study is a recombinant variety (courtesy of Genencor International) from *Bacillus licheriformis*, MW 48 000 Da.

α-Amylase is a major industrial enzyme used in brewing, bread making, and in the paper and textile industries (Yamane, 1989; Debabov, 1982). The starch industry uses α-amylase for production of glucose and high fructose sugar from starch (Yamane, 1989). In the textile industry starch is added to prevent breaching of threads. α-Amylase is then used to remove the starch and starch derivatives.

The assay for α-amylase activity uses blocked ρ-nitrophenyl maltoheptaoside, BPNPG7, as a substrate. The presence of α-amylase will break down BPNPG7 to blocked maltosaccharide and p-nitrophenyl maltosaccharride (Figure 5.1.1). The latter is then further broken down to p-nitrophenol by glucoamylase and alpha-glucosidase. Free p-nitrophenol forms a yellow colour upon addition of a Trizma base that can be followed at 410nm.

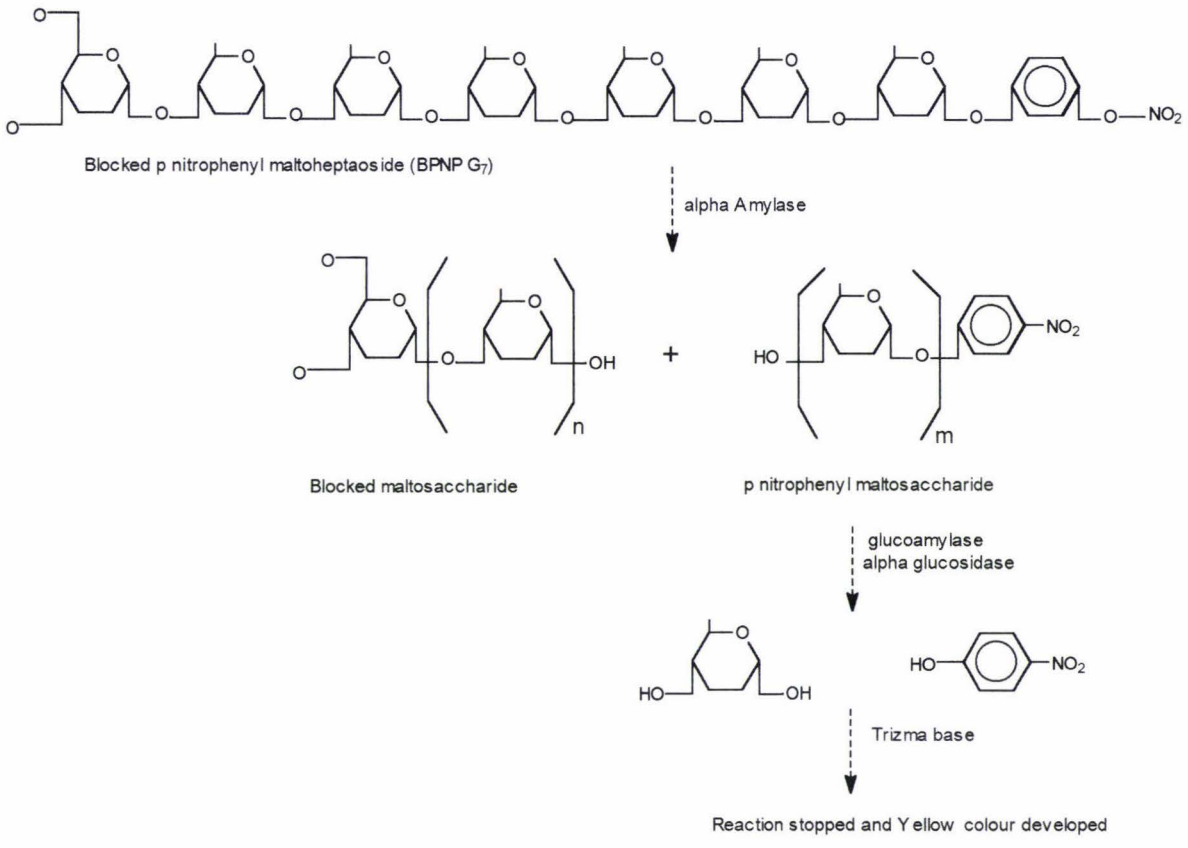


Figure 5.1.1 Theoretical basis of α-amylase assay procedure (Megazyme)
Yellow colour observed is from the production of phenolate

5.2 Results and Discussion

Both the 7% and 21% levels of activated dextran were investigated for complex formation with α-amylase. Due to the difficulties encountered with the 56% activated dextran and the lower MW proteins, lysozyme and trypsin, this level of activation was not investigated for α-amylase.

Complex formation was studied by SEC (Superdex 75). At both levels of activation, significant amounts of complex formation were observed within the first 24 hours of incubation, which then levelled off (Figure 5.2.1 and 5.2.2). The complex covered a range of MWs, and formation was greater with the 21% activated dextran, consistent with the lysozyme and trypsin experiments (Sections 3.2 and 4.2). The MW of the complex formed started just above that of the α-amylase protein, therefore the fractionation between the free protein and the complexed protein was less distinct than for the smaller proteins (Figures 3.2.3, 3.2.4 and 4.2.1, 4.2.2). When isolating the complex, a cut-off was selected to ensure that none of the free amylase protein was included (indicated on Figures 5.2.1 and 5.2.2).

Release of the protein from the complex was followed by SEC (Figure 5.2.3). Over time there was a drop in the peak due to the complex in accordance with an increase in a peak with the expected retention time of α-amylase. The most significant amount of release appears to occur within 24 hours, consistent with the reforming of an equilibrium between the complex and the free protein (Scheme 3.2.1). For *in vivo* situations, such an equilibrium would be severely altered. In this case the released protein would be removed from the vicinity of the complex, pushing the equilibrium to the left and increasing the overall rate of release.

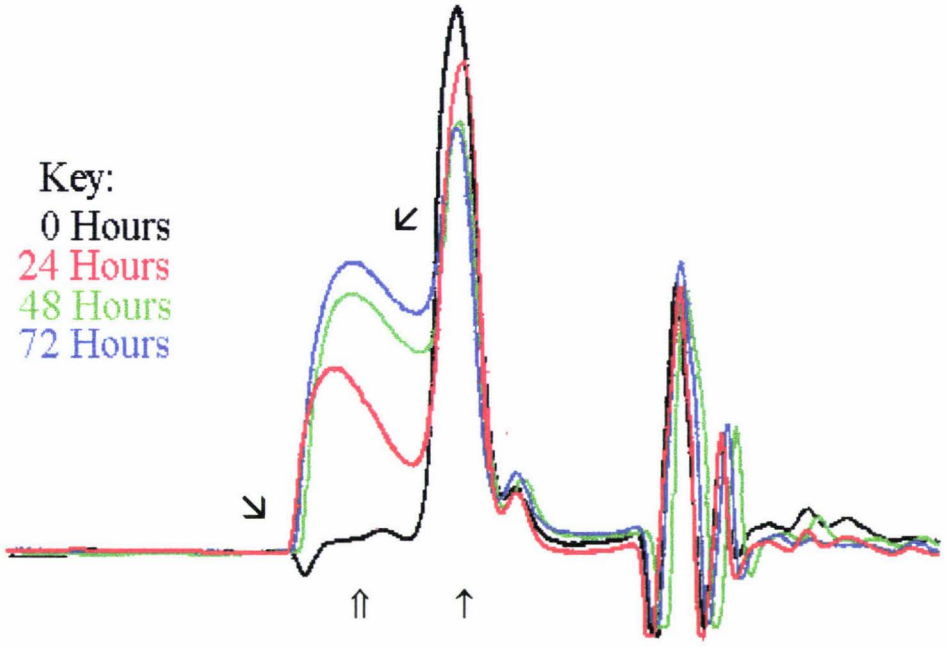


Figure 5.2.1 Complex (⇑) formation over time between α-amylase (↑) and 7% activated dextran

Amylase incubated with 7% activated dextran was subjected to SEC (Superdex 75) at 0hrs, 24hrs, 48hrs and 72hrs in 0.2M Tris acetate, 0.05% sodium azide, pH 7.5. Cut off marks to ensure isolation of the complex without free protein are indicated by the arrows.

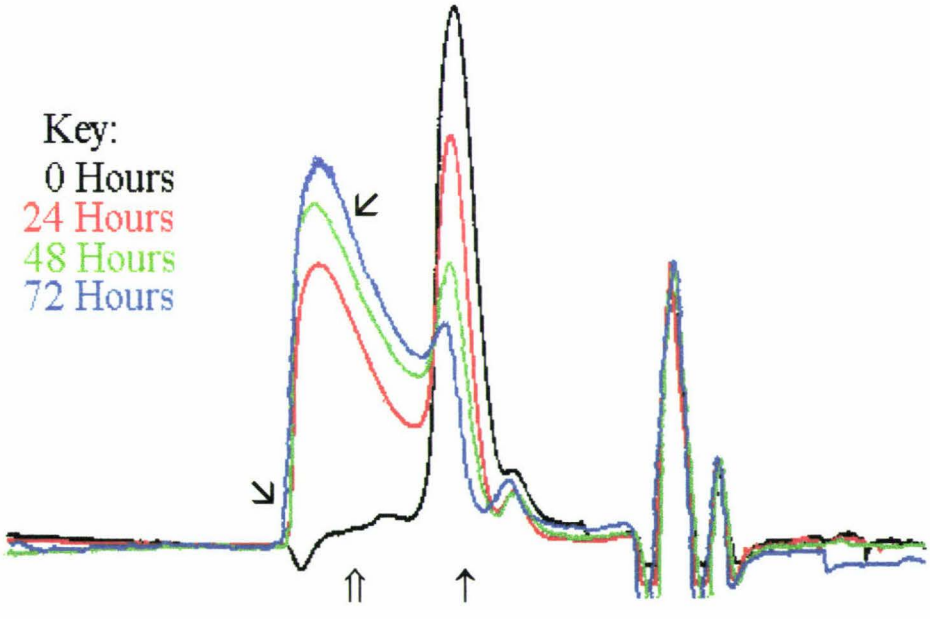


Figure 5.2.2 Complex (⇑) formation over time between α-amylase (↑) and 21% activated dextran

Amylase incubated with 21% activated dextran was subjected to SEC (Superdex 75) at 0hrs, 24hrs, 48hrs and 72hrs in 0.2M Tris acetate, sodium azide, pH 7.5. Cut off marks to ensure isolation of the complex without free protein are indicated by the arrows.

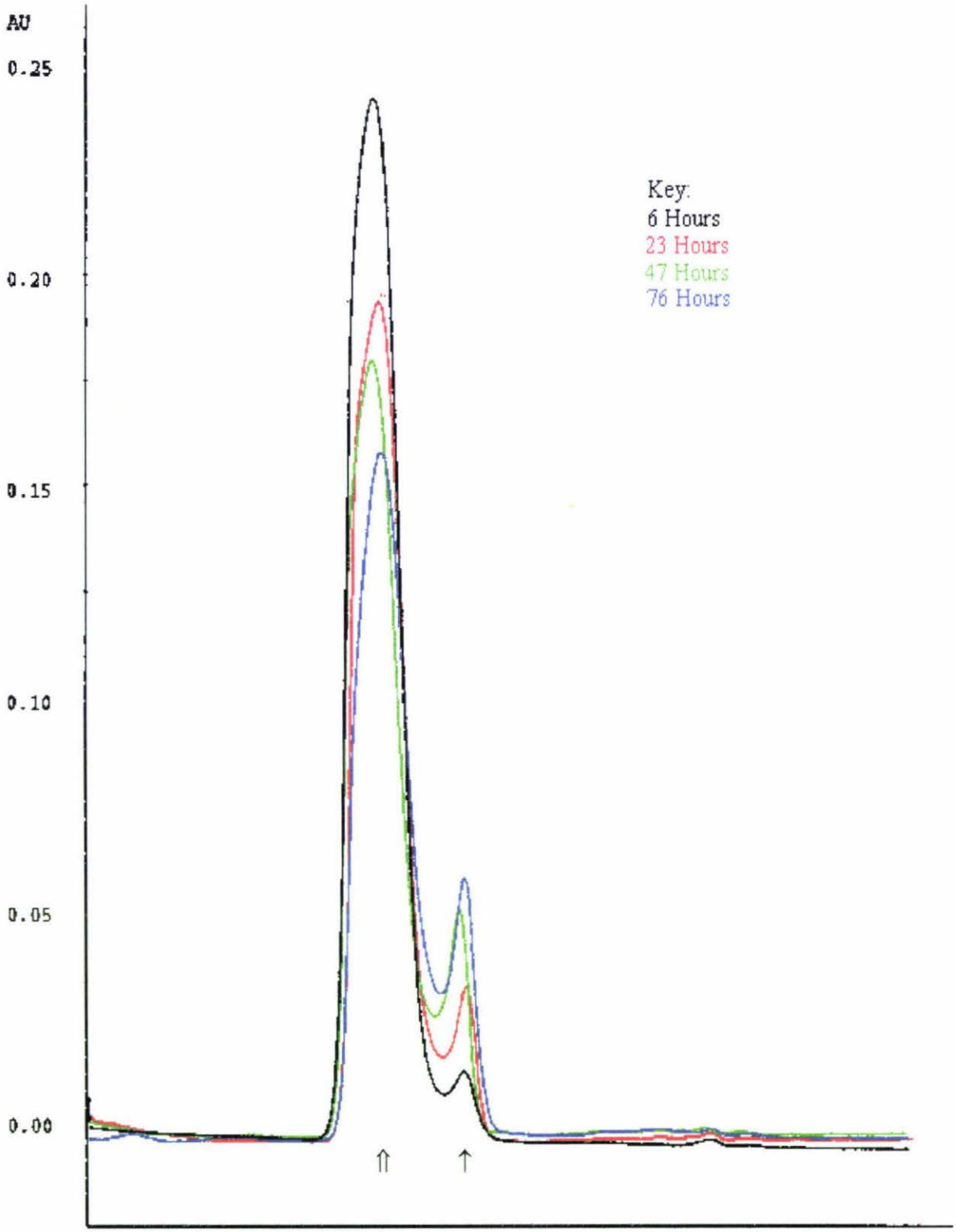


Figure 5.2.3 Release of α-amylase-like species (↑) from the dextran complex (⇑) over time

Amylase-dextran complex was isolated from the FPLC and then subjected to further SEC (Superdex 75) over time in 0.2M Tris acetate, 0.05% sodium azide, pH 7.5. Drop in the complex peak was observed in accordance with the formation of peak with equivalent retention time to α-amylase.

α-Amylase activity was determined, as previously described, and is shown by an increase in the absorbance at 410nm over time. While the complex isolated by SEC did show activity, it could not be determined whether the activity was due to complex itself, or if it was a result of protein being released from the complex (Figure 5.2.4). The α-amylase standard had a higher protein concentration, hence was significantly more active.

The α-amylase-dextran complex was formed and isolated, and then incubated under release conditions. The released α-amylase-like species was then isolated using SEC (Superdex 75, FPLC), instead of the SMART system, in order to obtain larger quantities for subsequent analysis. The released protein species appeared to have retained α-amylase activity (Figure 5.2.4).

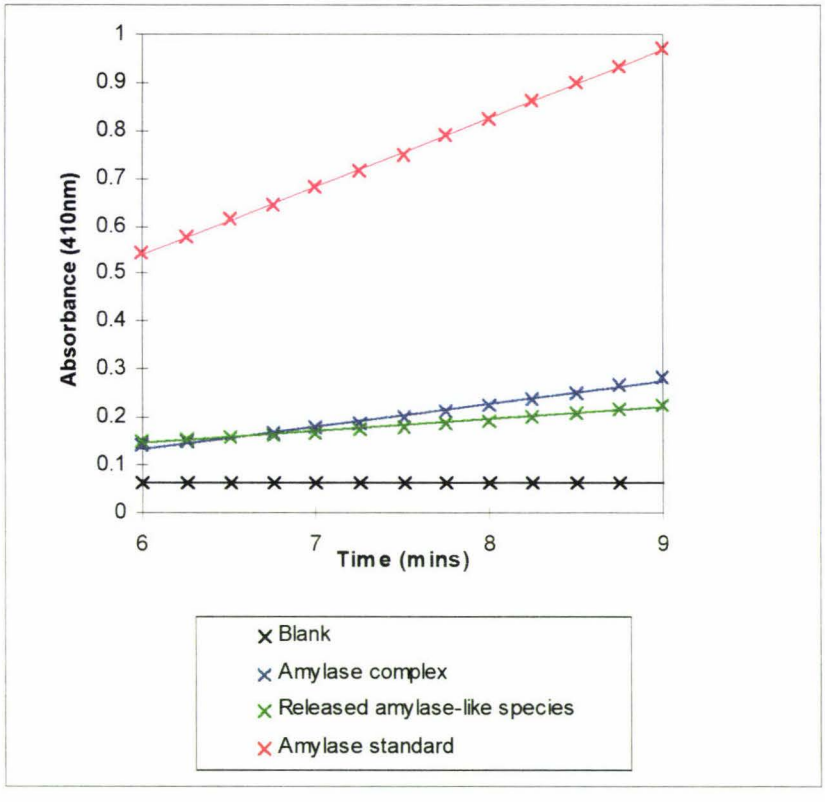


Figure 5.2.4 Activity of α-amylase

The complex and release samples where isolated from the FPLC and concentrated. Aliquots were than taken to assay for activity. α-Amylase standard was isolated from the FPLC and used immediately. α-Amylase standard had higher protein content.

The released α-amylase-like protein species (lane 4) had the same MW as α-amylase on SDS-PAGE (Figure 5.2.5). Analysis of the complex on SDS-PAGE showed a smeared

band consistent with a wide range of MWs (lane 2). Release of α-amylase-like species from the complex is evident from the band running at the same place as amylase. This indicated that initial release had occurred straight away, in the time taken to concentrate the isolated fraction.

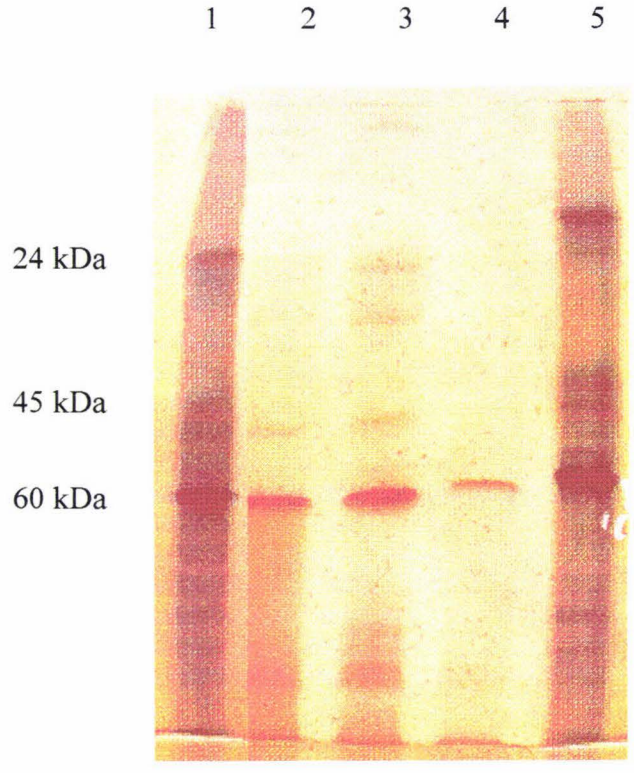


Figure 5.2.5 SDS Homogenous gel

lane 1 marker: trypsinogen (24kDa), albumin egg (45 kDa), albumin bovine (60 kDa)
lane 2 amylase-7% activated dextran
lane 3 amylase standard
lane 4 7% released amylase-like species
lane 5 marker as per lane 1

Activities were also performed on the α-amylase-dextran complexes over time (Figure 5.2.6). Complexes at both levels of activation were assayed and showed no significant changes in activity. Studies by SEC had already shown that release takes place within the first 24 hours (Figure 5.2.3). For the lysozyme-dextran case (Section 3.2) there was an increase in activity over time relating to the release of lysozyme-like species. This was not the case for α-amylase where the enzyme-dextran complex had a similar activity to the free enzyme, as shown in Table 5.2.1. The standard exhibited only a slightly higher specific activity.

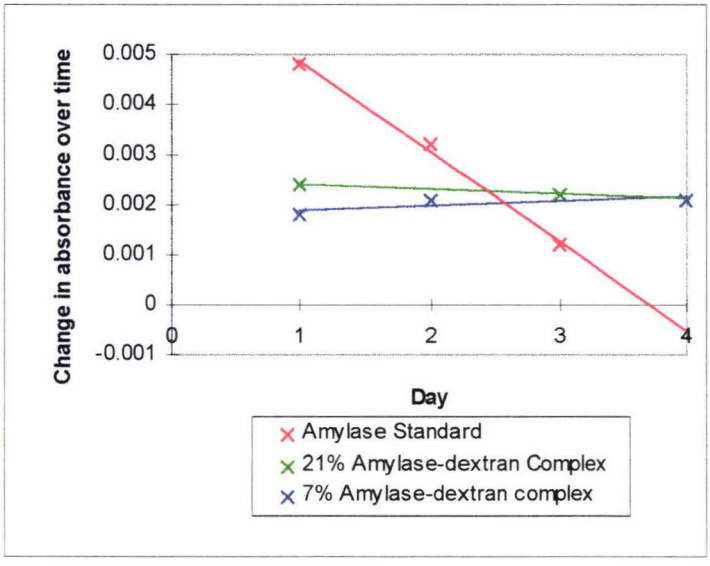


Figure 5.2.6 Activity of amylase complex over time

Complex formation incubations were set up with amylase and 7% and 21% activated dextran. Complexes formed were then isolated by SEC (Superdex 75). Aliquots were then taken over four days to determine activity.

	Specific Activity (μmoles min^{-1} mg^{-1})
Released α-amylase	3.9×10^{-3}
Complex	3.9×10^{-3}
α-Amylase standard	4.4×10^{-3}

Table 5.2.1 Specific activity

Specific activity was determined for both the released α-amylase-like species and the complexed α-amylase in μmoles of p-phenolate produced min^{-1} mg^{-1} of protein (from activity in Figure 5.2.4 and protein determinations).

The activity of the α-amylase standard, purified by SEC (Superdex 75, FPLC) was also analysed and showed that there was a significant drop in activity over the four days (Figure 5.2.6). As this decrease in activity was not observed for the α-amylase-dextran complexes, there is an increase in the stability of α-amylase conjugated to dextran.

AAA was performed on the released species (Table 5.2.2) and showed similarity in the compositions of the purified amylase and the released species. The main differences occur with Thr, Gly, and Glx (Gln and Glu) of which only the Gln residue is located near the C-terminal. The N- and C-terminal sequences for amylase are NH_2-Met Lys Gln Gln Lys Arg.....Val Ser Ile Tyr Val Gln Arg-COOH (Swiss-Prot, 1992). The composition for the terminal amino acids of the released α-amylase species is consistent with that of the purified α-amylase, indicating that no loss has occurred.

Reduction studies were performed to confirm whether or not the α-amylase-dextran complex was active. Both the reduced and non-reduced complexes were isolated from the FPLC and concentrated. Aliquots of each were taken and assayed for α-amylase activity. The remainder of the complex was incubated as for normal release experiments and then subjected to SEC (Superdex 75) over time. No release was observed from the reduced complexes. The non-reduced complex showed a significantly decreased rate of release compared with complexes formed under normal conditions. This is consistent with the lysozyme and trypsin studies (Section 3.2 and 4.2).

Only slightly greater complex formation was observed with the reduced complexes compared with the non-reduced. In the lysozyme study this difference was quite significant (Figure 3.2.9). It is possible that the binding of α-amylase was already at maximum capacity. That is, even though the equilibrium had been altered in favour of complex formation steric hindrance prevented further binding between α-amylase and dextran.

The α-amylase control, incubated with $NaBH_3CN$, retained its activity showing that the presence of the cyanoborohydride did not inhibit it. Both the reduced and non-reduced complexes were active (Figure 5.2.7). This further supported the evidence that the α-amylase is active in the conjugated form.

Residue	Total No.	Theoretical	Purified	Released
Asx	62	1.5	1.3	1.6
Thr	28	0.7	0.8	1.6
Ser	27	0.7	0.7	0.9
Glx	47	1.1	1.0	0.6
Pro	16	0.4	0.5	0.4
Gly	45	1.1	1.2	1.7
Ala	41	1.0	1.0	1.0
Cys	0	0.0	0.0	0.2
Val	32	0.8	0.6	1.0
Met	8	0.2	0.1	0.2
Iso	21	0.5	0.5	0.6
Leu	36	0.9	0.8	0.9
Tyr	31	0.8	0.8	0.7
Phe	22	0.5	0.6	0.4
His	25	0.6	0.6	0.9
Lys	30	0.7	0.6	0.8
Arg	24	0.6	0.6	0.7

Table 5.2.2 Amino acid composition of α-amylase and release species

AAA was performed on the released α-amylase species and purified α-amylase isolated by SEC. Composition of amino acids was determined relative to alanine. Composition was also compared to the theoretical data for sequence α-amylase (Swiss-Prot, 1992).

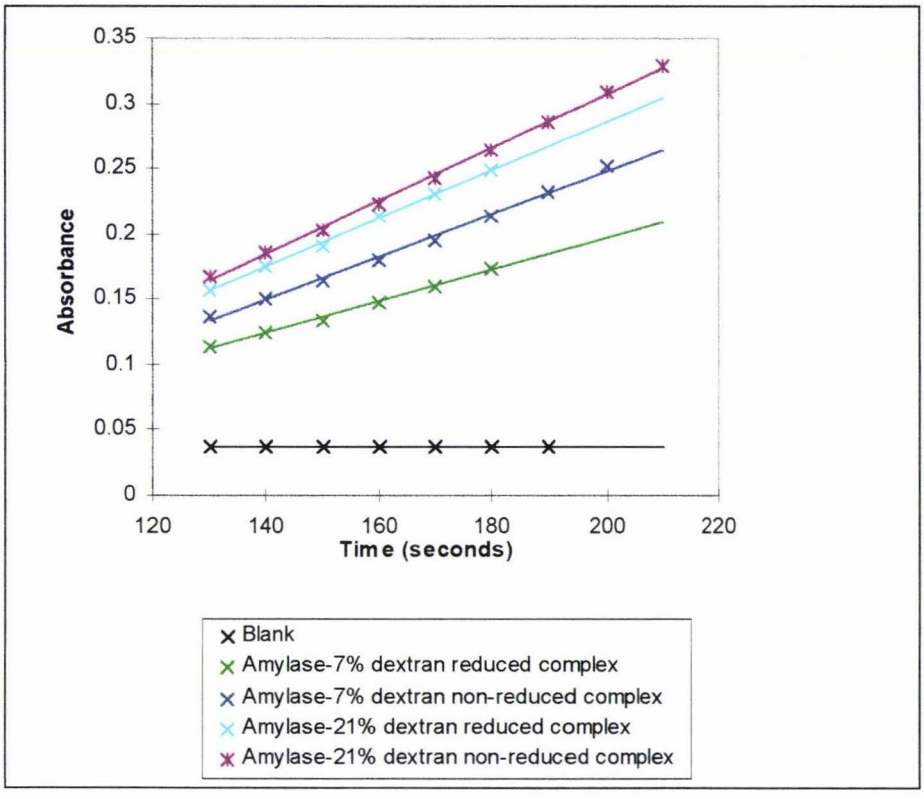


Figure 5.2.7 Comparison of activities for reduced and non-reduced complexes
Assays were performed on α-amylase-dextran complexes. Complexes were reduced in the presence of $NaBH_3CN$, which reduces the imine bonds and prevents release of the α-amylase from the complex. Controls were also run on the complexes in the absent of $NaBH_3CN$ and on α-amylase plus $NaBH_3CN$. Complexes were isolated from the FPLC and concentrated prior to assaying.

In the lysozyme study the complexed-protein exhibited only minimal activity (Section 3.2), however for α-amylase the activity was the same for both the complexed and free states. This could be accounted for by the fact that α-amylase is a bigger enzyme and therefore less likely to be 'buried deeper' within the complex because of steric hindrance. Hence all enzyme molecules would be located near or on the outside of the polymer and would be accessible to substrates. Also, the site of binding to dextran is an important factor. In the case of α-amylase it would appear that the active site is not disturbed upon conjugation to dextran.

These findings are in agreement with a previous study on α-amylase conjugation by Srivastava (1991), that showed no alteration to the active site occurred upon conjugation of α-amylase to dextran. When complexed with dextran the α-amylase was less susceptible to heat and denaturing agents, and its thermostability was increased. Conformation of the active site was not thought to have been affected because there was no difference in pH optima between the conjugated amylase and the native enzyme. This in turn implies that the functionality at the active site had not been affected.

5.3 Conclusions

The α-amylase protein was a much larger molecule than the two proteins already studied, but complex formation still occurred with ease. Fewer difficulties were encountered with the complex formation and release compared to trypsin. This is due to a greater absorbance at 280nm and probably the fact that α-amylase is a non-proteolytic protein.

At both levels of activated dextran, complex formation and release were shown to occur rapidly and with ease. The released species was found to be α-amylase-like due to its equivalent retention times with by SEC and by the MW obtained from SDS-PAGE. The released species also indicates good amylase activity and showed similar amino acid composition.

The complex formed between α-amylase and dextran retained a reasonable level of α-amylase activity, indicating that it was unaffected by binding to dextran. Therefore conjugation did not appear to produce structural distortions that may have adversely affected the active site. It is possible that the size of amylase prevents it from being surrounded extensively by dextran, reducing its access to substrates. That is, there is less extensive crosslinking between dextran and α-amylase compared to the lysozyme. It is possible that the resulting structure is moving away from the random crosslinking (Figure 1.8.2c) and towards a situation of dextran molecules binding to an α-amylase molecule (Figure 1.8.2a).

Although complex formation at the 56% activation level was not investigated here, its usefulness can not be negated. Larger proteins may in fact crosslink with the higher activated dextran to produce manipulable complexes. The increased size of the protein may prevent the extensive crosslinking that can give rise to loss of activity, as in the case of lysozyme and trypsin. It appears that in the case of α-amylase the active site is unaffected by binding, and therefore it could possibly be advantageous to investigate a complex at higher activation levels.

α-Amylase remains significantly active after conjugation to dextran. The complexed α-amylase exhibits greater stability compared with the free protein. The predominant use of α-amylase is as an industrial enzyme, involved in brewing, baking through to the paper industry. These processes often require high temperature (e.g. up to 80°C in the brewing industry). Therefore conjugation, reduced or non-reduced, to a polymer such as dextran could be advantageous for maintaining or increasing stability.

CHAPTER SIX

ALCOHOL DEHYDROGENASE AND CATALASE

6.1 Introduction

6.1.1 Alcohol Dehydrogenase

The alcohol dehydrogenase (ADH) used in this study came from Bakers yeast and is 150 kDa (Brändén *et al*, 1975). Yeast ADH enzyme differs from the mammalian ADH in that it exhibits a more specific substrate specificity. The yeast enzyme is primarily involved with alcohol fermentation in the glycolytic pathway, and is more specific for ethanol and acetaldehyde. The enzyme is stable over a wide pH range but it shows most stability around pH 8.5 - 9.5. Yeast ADH enzyme is tetrameric, comprising of four identical polypeptide chains (Reid and Fewson, 1994), while the mammalian ADH enzyme is a dimer. The enzyme does not readily dissociate into dimers and monomers (Kitson, 1997) but certain reagents including urea, maleylation, SDS, sulphydryl reagents and thiol oxidation and chelating agents will cause the protein to dissociate into its subunits (Brändén *et al*, 1975). The enzyme is not active in its monomeric forms although it might exhibit some activity in the dimeric form (Kitson, 1997), which is the active form of the mammalian ADH (Reid and Fewson, 1994)

Activity of ADH was determined from the production of NADH which has a strong absorbance at 340nm. The assay was based on the ethanol assay method described by Cornell and Veech (1983). Alcohol dehydrogenase catalyses the reaction:

$$\text{ethanol} + NAD^+ \rightarrow \text{acetaldehyde} + NADH + H^+$$

Regeneration of NAD^+ during fermentation is required for other oxidation and energy yielding metabolic processes. This in turn is also important in the industrial production of alcohol and solvents (Reid and Fewson, 1994). ADH is used to assay for alcohols, but it uses NAD^+ which is expensive (Wiseman, 1975).

6.1.2 Catalase

Catalase is a high MW protein, 220 to 350kDa, involved in the removal of free radicals (Hass and Brehm, 1993). The protein itself has both a catalytic and a peroxide activity. The catalytic activity involves the decomposition of hydrogen peroxide to water and

oxygen. This is required in living systems to prevent the formation of OH^- radicals (Halliwell and Gutteridge, 1985). Catalase's second activity involves the oxidation of H donors such as methanol, ethanol, formic acid and phenols. Related to peroxidase, catalase differs in that it can use H_2O_2 as an electron acceptor and an electron donor (Sharma *et al*, 1989). It also has the ability to act on aliphatic alcohols while other peroxidatic enzymes are unable to utilise such substrates (Halliwell and Gutteridge, 1985).

Catalytic Activity:

$$H_2O_2 \rightarrow H_2O + O_2$$

Peroxide Activity:

$$ROOH + AH_2 \rightarrow H_2O + ROH + A$$

It has been proposed that, because of the dual function of catalase, the function may vary from tissue to tissue. The microenviroment of the cells will determine the role catalase takes on (Halliwell and Gutteridge, 1985).

The protein consists of four identical subunits (60 kDa) that will dissociate at extreme pH (<3, >10), in the presence of detergents and upon storage and freeze drying (Halliwell and Gutteridge, 1985; Percy, 1983). Stability of catalase is dependant on the maintenance of the oligomeric structure (Burton, 1995).

Industrial applications of catalase include the removal of H_2O_2 from textiles after bleaching, from food and to remove spilt H_2O_2 from the skin (Wiseman, 1975). The ability of catalase to liberate oxygen is used in several applications including sterilisation, microbial growth and in the production of a wide variety of products from porous cement to baked goods.

6.1.3 Higher MW Proteins

Catalase and alcohol dehydrogenase were selected to extend this imine study to higher MW proteins, as well as to broaden the range of enzymatic activities investigated. These proteins where thought to be more likely to incur difficulties with binding due to steric hindrance, limiting the extent of binding between dextran and the proteins. Studies on these two enzymes were not as extensive as for the previous enzymes.

6.2 Results and Discussion

6.2.1 ADH Complex Formation

Complex formation was investigated at the levels of 7% and 21% activated dextran (Figure 6.2.1.1 and 6.2.1.2). Complex formation is indicated by a drop in the ADH peak in accordance with the formation of a higher MW peak that merges with the ADH peak. At both levels of activation most of the complex formation appears to have occurred within 48 hours. After this time there is no significant amount of change in the complex band formed. Complex formation is faster at the 21% level of activation and there is a greater amount of higher MW species formed (maximum absorption occurs at shorter retention time). In the case of ADH incubated with 21% activated dextran there was just one broad peak visible by 72 hours. This is due to the extent of overlap between the ADH peak and the ADH-dextran complex peak.

6.2.2 Catalase Complex Formation

For catalase, complex formation appears to have reached equilibrium within the first 24 hours. This was evident at both 7% and 21% levels of activated dextran (Figure 6.2.2.1 and 6.2.2.2). The beginning of the complex band comes out with the void volume indicating that the MW of the complex is greater than the capacity of the column, (5 x 10^6 Da). Formation of two peaks was apparent as a result. This is similar to the pattern obtained with the lysozyme-56% dextran complex which eluted with the void volume (Figure 3.2.1). Complex formation is greater at the 21% activation level. The complex band at the 21% activation level is more distinct from the catalase peak showing less overlap (Figure 6.2.2.2).

6.2.3 Complex Formation

Both catalase and ADH indicated that the complex formation with the 21% activated dextran produced higher MW complex species compared with the 7% level. These differences were not apparent with the lower MW proteins previously studied. While the lower MW proteins showed greater amount of complex formation with the 21% activated dextran, they still covered a similar MW range as the 7% activation level. These differences were apparent between the 21% and 56% activated dextran conjugated to lysozyme.

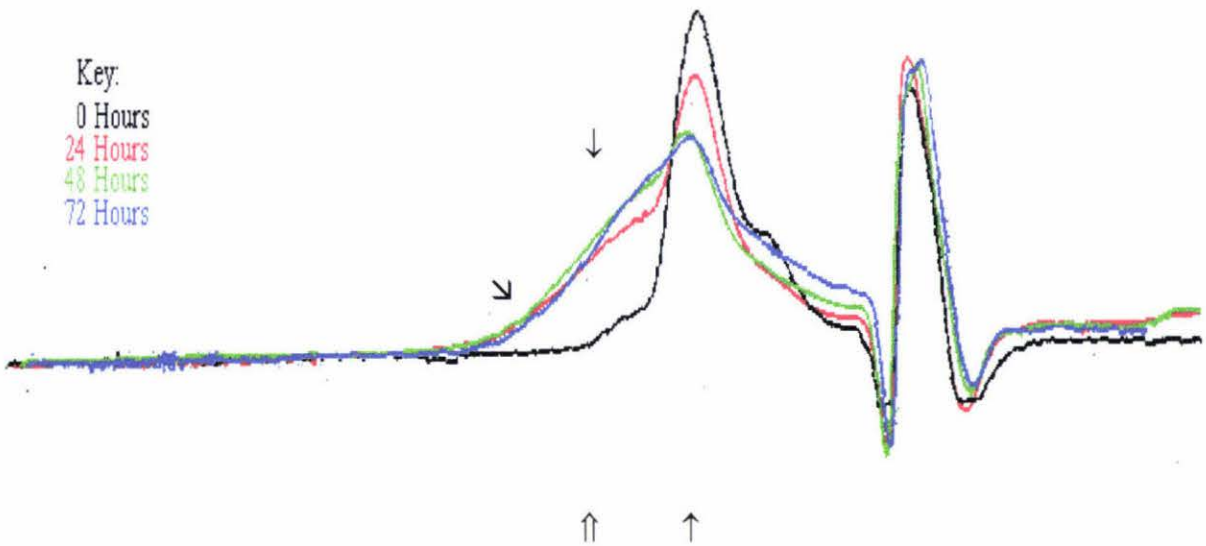


Figure 6.2.1.1 Complex (⇑) formation over time for ADH (↑) and 7% activated dextran

ADH was incubated with 7% activated dextran. Aliquots were taken and analysed by SEC (Superose 6) at 0hrs, 24hrs, 48hrs and 72hrs in 0.2M Tris acetate, 0.05% sodium azide, pH 7.5. The arrows indicate the cut off marks when isolating the complex.

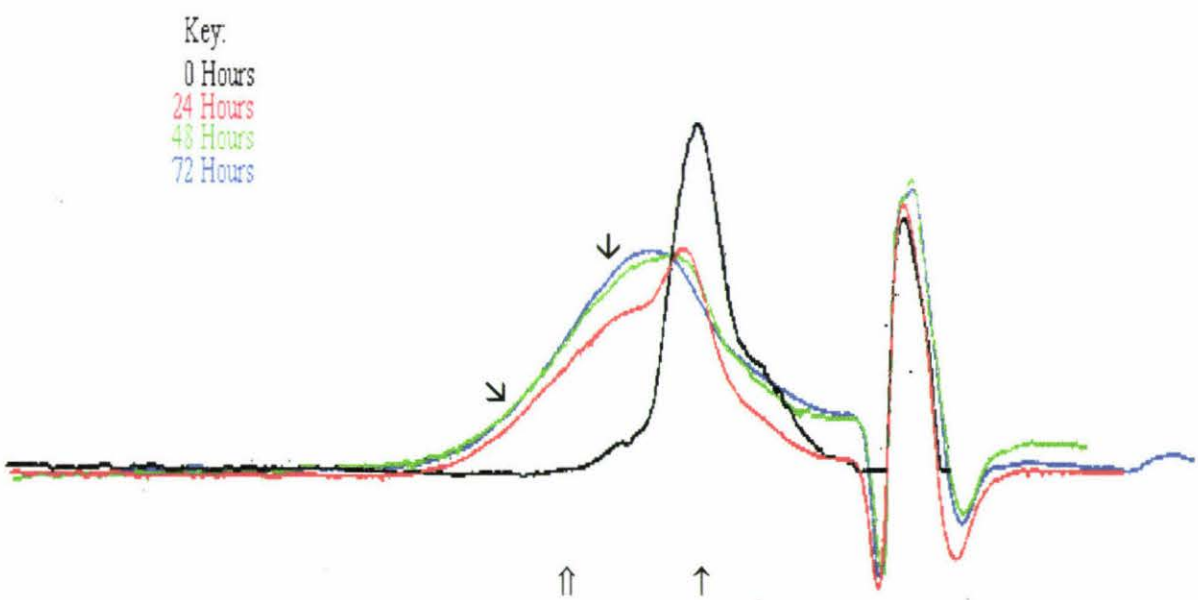


Figure 6.2.1.2 Complex (⇑) formation over time for ADH (↑) and 21% activated dextran

ADH was incubated with 21% activated dextran. Aliquots were taken and analysed by SEC (Superose 6) at 0hrs, 24hrs, 48hrs and 72hrs in 0.2M Tris acetate, 0.05% sodium azide, pH 7.5. The arrows indicate the cut off marks when isolating the complex.

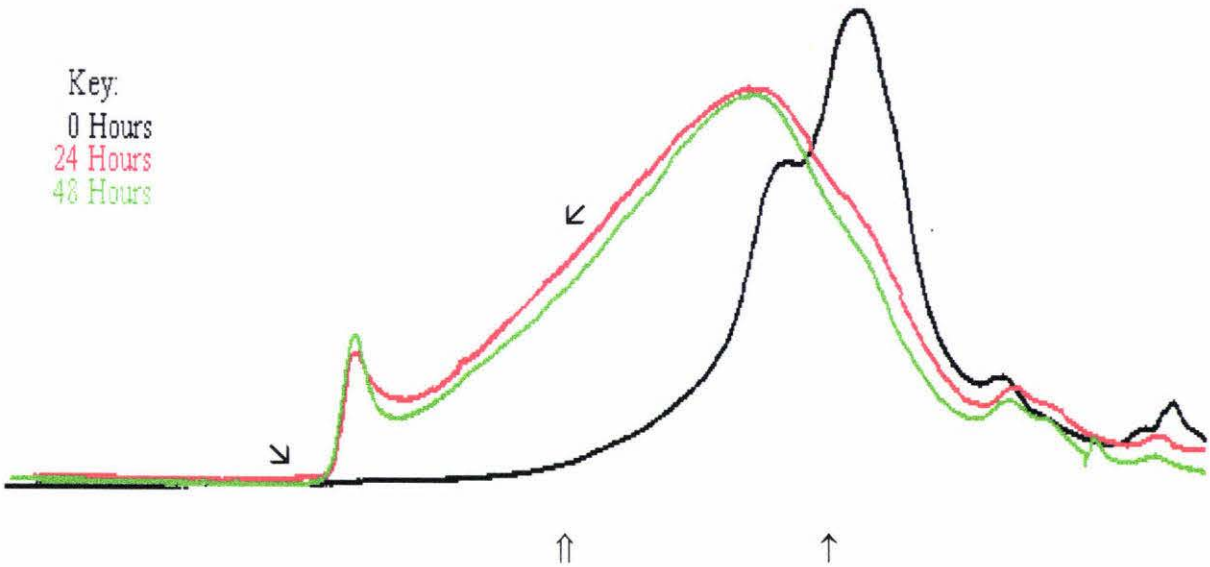


Figure 6.2.2.1 Complex (⇑) formation over time for catalase (↑) and 7% activated dextran

Catalase was incubated with 7% activated dextran. Aliquots were taken and analysed by SEC (Superose 6) at 0hrs, 24hrs and 48hrs in was 0.2M Tris acetate, 0.05% sodium azide, pH 7.5. The arrows indicate cut-off marks when isolating the complex.

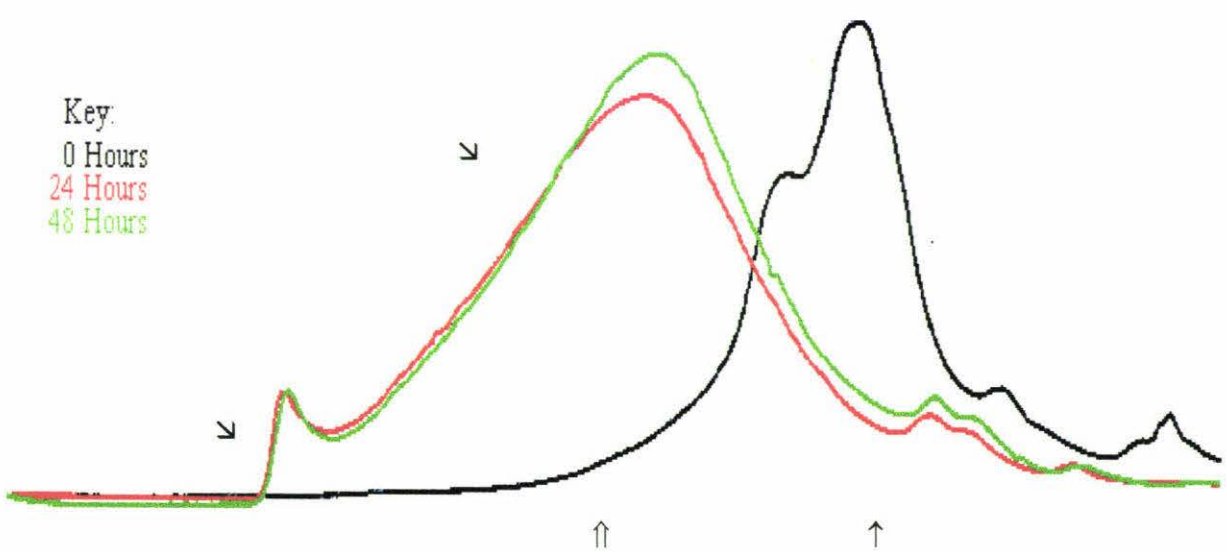


Figure 6.2.2.2 Complex (⇑) formation over time for Catalase (↑) and 21% activated dextran

Catalase was incubated with 21% activated dextran. Aliquots were taken and analysed by SEC (Superose 6) at 0hrs, 24hrs, 48hrs and 72hrs in was 0.2M Tris acetate, 0.05% sodium azide, pH 7.5. The arrows indicate the cut off marks when isolating the complex.

In each case of complex formation the resulting complex peak merged with the original protein peak. This in turn made it more difficult to isolate the complex for subsequent experiments. In order to ensure that no free protein was incorporated into the complex, clear cut-off marks had to be chosen. This in turn caused a bias as only the higher molecular weight complexes were investigated. If the molecular weight range of the complex was a result of random binding between the proteins and the dextran, then isolation of this higher MW complex would not only result in a re-establishing of the free protein equilibrium, it would also re-establish an equilibrium with the lower MW complex species.

6.2.4 ADH-dextran Complex and Release Investigations

A complex formation incubation was set up between ADH and 7% activated dextran. After 48 hours an aliquot was subjected to SEC (Superose 6). Fractions were isolated over the range of complex and free protein peak. (Figure 6.2.4.1).

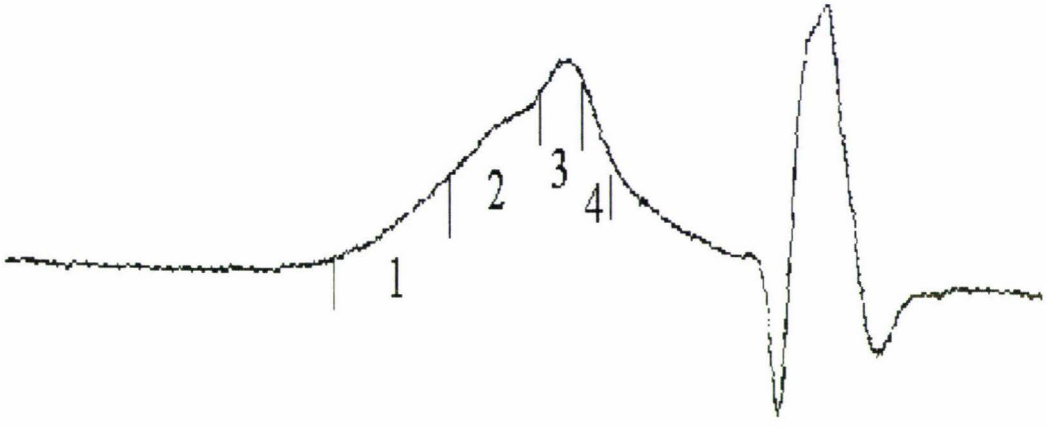


Figure 6.2.4.1 Complex Formation at 48 Hours between ADH and 7% Activated Dextran

ADH was incubated with 7% activated dextran. At 48 hours an aliquot was subjected to SEC (Superose 6). Fractions (1-4) were isolated over the range of the overlapping complex-free ADH protein peaks.

ADH activity was then determined, as previously described, for each fraction (Figure 6.2.4.2). All fractions indicated activity. This was significantly greater for fractions 2 and 3. Fraction 3 should consist mostly of free protein. Fraction 2 is an overlap between the complex peak and the protein peak, so from this fraction it can not be determined if any of the observed activity is from the complexed ADH or solely from free protein.

Fraction 1 should have contained no free ADH at the time of isolation. Therefore activity in this instance was either from the complex itself or from ADH being released.

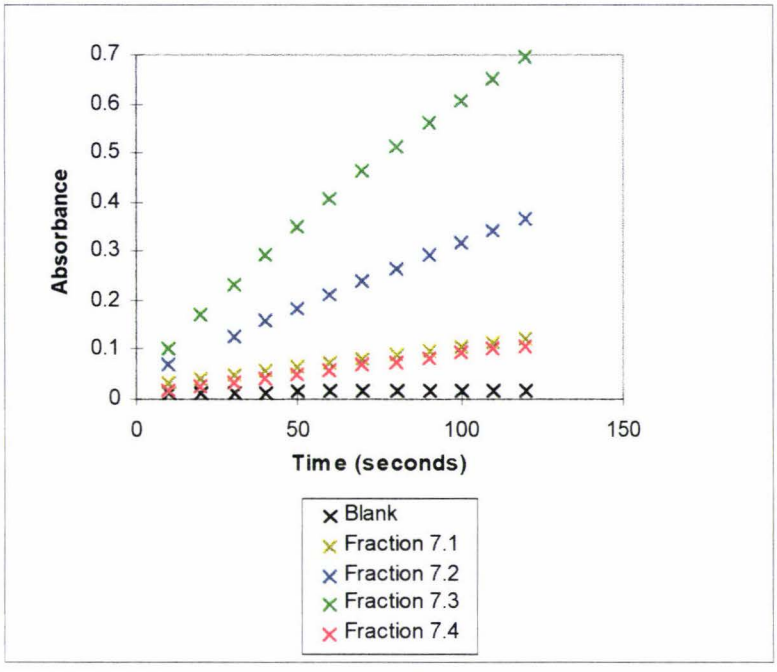


Figure 6.2.4.2 Activity assays performed on isolated fractions from ADH-7% dextran complex from figure 6.2.4.1

Complex formation incubation was set up between ADH and 7% activated dextran. At 48 hours an aliquot was subjected to SEC (Superose 6). Fractions were collected over the complex-free protein peak in 0.2M Tris acetate, 0.05% sodium azide, pH 7.5. The activity was then followed at 340nm.

Release experiments were attempted by SEC on the SMART system, Superose 12. This was found to be limited due to the capacity of the column. A drop in the complex peak was observed over time but there was no evidence of the formation of a release peak. The expected retention time for ADH is on the edge of the complex peak, therefore a Superose 6 column was required for optimal separation. There did appear to be a shift towards the lower MW complex species.

The release experiments were repeated using the FPLC. Incubations were set up with ADH and 7% activated dextran. Aliquots were then subjected to SEC (Superose 6) and the initial eluting complex was collected. Samples were pooled, concentrated and incubated under release conditions. Aliquots were then analysed over time by SEC (Figure 6.2.4.3). There was a definite shift of the complex species to lower MW forms. Over time there was also the formation and increase in size of peaks at longer retention

times (smaller MW). These were break down products of the ADH protein, possibly into dimers and monomers, upon release from the complex.

The final eluting fraction of the complex peak could be due to either released ADH-like species or lower MW forms of the protein-dextran complex.

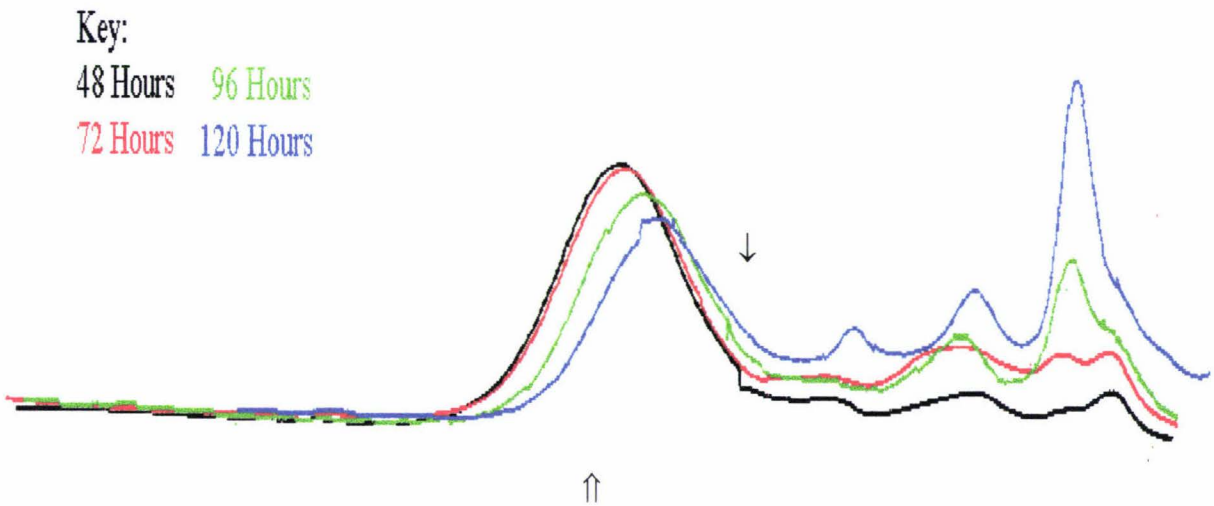


Figure 6.2.4.3 Release from ADH-7% complex (⇑) over time
Complex formation incubations were set up between ADH and 7% activated dextran. At 70 hours the complex was run on the FPLC (Superose 6). Fractions were pooled over several runs and concentrated. The isolated complex was then incubated under release conditions. Aliquots were taken at 48hrs, 72hrs, 96hrs and 120hrs and analysed by SEC (Superose 6) in 0.2M Tris acetate, 0.05% sodium azide, pH 7.5. ↓ expected retention time of ADH.

High and low MW samples were obtained from complex peak. Activities were performed on each indicating that the complex does have activity (Figure 6.2.4.4). Activities were also performed on the break down product with the greatest absorbance, while this was shown to consist of proteinaceous material from protein determination and AAA, it showed no signs of activity. The break down products were not further analysed. It is possible that this peak with the greatest absorbance was a monomer and therefore predicted to be inactive. If one of the earlier peaks was a dimer it may have exhibited activity.

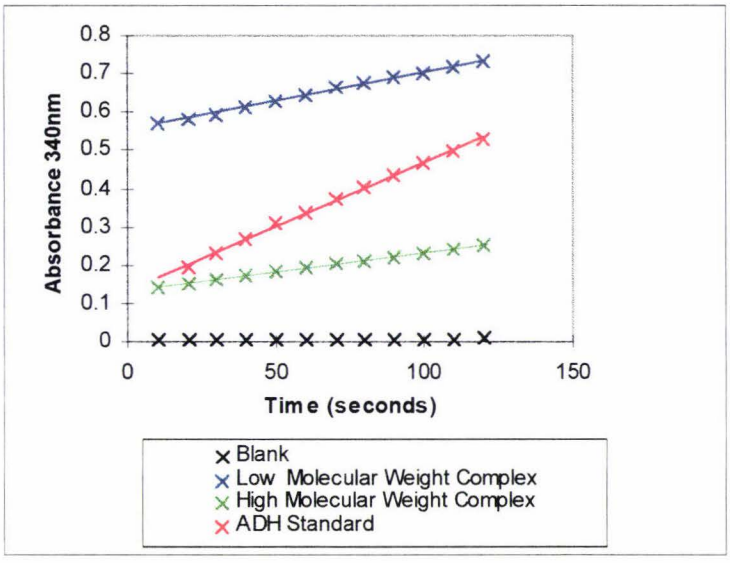


Figure 6.2.4.4 ADH Activity
Complex formation was set up between ADH and 7% activated dextran. An aliquot was run on the FPLC, Superose 6. The high MW complex (complex only) was isolated along with lower MW complex (possible released protein)

From the activities (Figure 6.2.4.4) and protein determinations the specific activities were determined for the lower and higher MW complexes (Table 6.2.4.1). There is a significantly higher specific activity value for the lower MW complex. This could mean that there was in fact released ADH-like species in this fraction eluting on the edge of the ADH-dextran complex, or alternatively the ADH might be more active in the lower MW complex species due to less steric hindrance.

	Specific Activity $\mu moles\ min^{-1} mg^{-1}$
Higher MW Complex	0.52
Lower MW Complex	3.2

Table 6.2.4.1 Specific activity comparison for ADH-dextran complex
Specific activity was determined for the high and low MW complexes isolated from the FPLC. Specific activity is given as μmoles NADH produced per min per mg protein.

Samples of the possible release product were also obtained from the lower MW end of the complex, for AAA (Table 6.2.4.2). There was no significant difference between the 7% and 21% ADH proposed release species except for Thr, higher for the 7% complex

Amino Acid	Purified	21%	7%
Asp	0.9	1.2	1.1
Thr	0.5	0.6	1.2
Ser	0.6	0.9	1.0
Glu	0.9	1.3	0.9
Pro	0.4	0.5	0.6
Gly	1.3	1.8	1.8
Ala	1.0	1.0	1.0
Cys	0.1	0.8	0.8
Val	1.0	1.2	1.3
Met	0.1	0.2	0.1
Ile	0.6	0.6	0.5
Leu	0.8	1.0	0.9
Tyr	0.3	0.1	0.1
Phe	0.2	0.4	0.4
His	0.3	0.3	0.2
Lys	0.7	0.7	0.7
Arg	0.3	0.4	0.4

Table 6.2.4.2 Amino acid compositions with respect to alanine

Lower MW complex (possible released ADH) was isolated from the FPLC for both ADH-7% and 21% complexes for AAA. Composition of amino acids was determined with respect to alanine and compared to ADH purified by SEC (Superose 6)

release sample. There is also reasonable agreement with the ADH purified by SEC. In each case higher quantities were indicated for Gly, Ser and Cys. This of course does not show whether it is in the bound form or not. Aliquots of the suspected release ADH-like species were then prepared for SDS-PAGE. If the protein is present then it should run as a single band on the SDS polyacrylamide gel. Complexed protein will run as a higher MW smeared band.

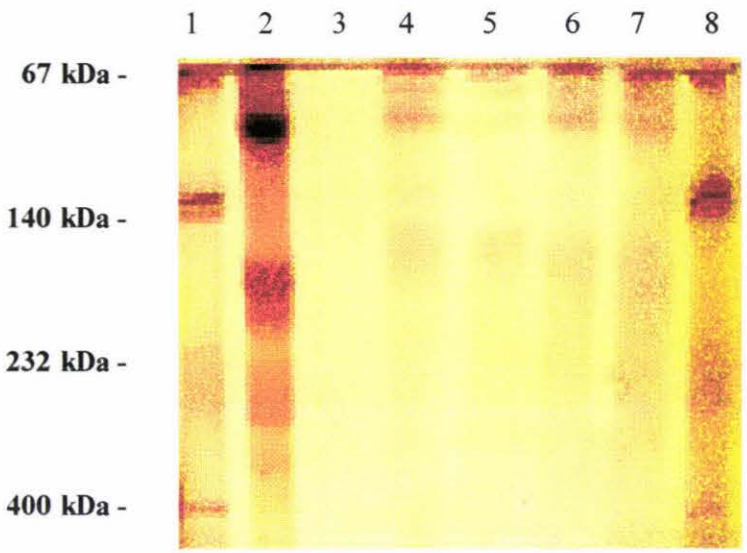


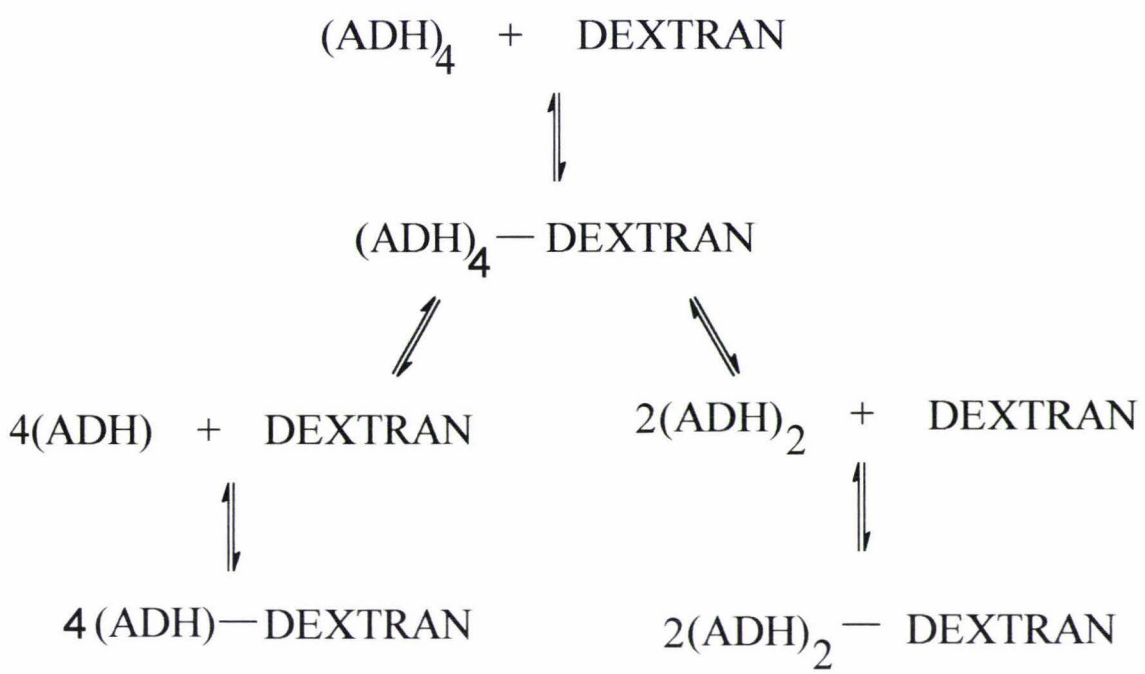
Figure 6.2.4.5 SDS- PAGE analysis

lane 1 marker: ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), bovine serum albumin (67 kDa).
lane 2 ADH standard
lane 3 Lower MW complex
lane 4 Lower MW complex
lane 5 Higher MW complex
lane 6 Higher MW complex
lane 7 marker as per lane 1

For the ADH standard two main peaks are present, one at 150kDa and the other ~75kDa, possibly a dimer (Figure 6.2.4.5). Lane 3 was one of the lower MW breakdown products which appears to have run off the gel. The lower MW fractions of the complex (lanes 4 and 5) exhibited MW products of 75kDa and below. They also show a smeared band around 150 kDa. This does not distinguish them from lower MW complex or free protein. Lanes 6 and 7 are high MW complex which show smeared complex band and released products, 75kDa and below.

One possibility is that released ADH elutes with the edge of the complex peak during SEC. ADH in the uncomplexed form may not be stable under the conditions used in

these experiments and therefore dissociates into its monomers as it is released from the complex. It may also be possible that the ADH monomers or dimers could be binding to the complex (Scheme 6.2.4.1).



Scheme 6.2.4.1 Possible reactions occurring with ADH-dextran incubations
$(ADH)_4$ = tetramer, $(ADH)_2$ = dimer, (ADH) = monomer.

If the monomers of ADH bind to dextran they will not be active, but the dimeric forms might be. As there were indications of a MW species around 75kDa from SDS-PAGE, there definitely appears to the possibility that ADH was binding as a dimer to the activated dextran.

There appears to be two main possibilities. The ADH could be binding solely in the tetrameric form, only breaking down into monomers and dimers upon release. The second option is that ADH is initially bound to the dextran in the tetrameric form, but this can then rearrange to the dimer and monomer forms as a result of releasing and rebinding (Scheme 6.2.4.1).

Reduction studies were also performed on the ADH complex. Due to problems associated with the column (Superose 6) low sensitivity and peak spreading was a noted. Acitivty studies performed (Figure 6.2.4.6) on ADH indicated that it was not inhibited by

the presence of $NaBH_3CN$ (control ADH standard plus $NaBH_3CN$). Non-reduced complexes indicated ADH activity. Only minimal activity was observed for the reduced complex. It did indicate that the complexed ADH had some activity. The relative amounts of protein present were not equivalent.

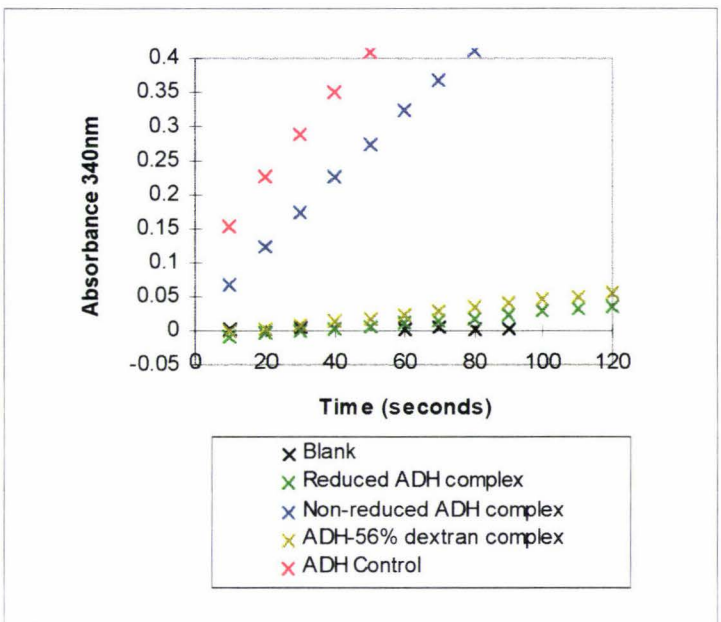


Figure 6.2.4.6 ADH Reduction studies

Assays were performed on ADH-dextran complexes. Complexes were reduced in the presence of $NaBH_3CN$, which reduces the imine bonds preventing release of ADH from the complex. Controls were also run on complexes in the absence of $NaBH_3CN$ and on ADH plus $NaBH_3CN$ (no dextran). Complexes were isolated off the FPLC.

There was also a bias noted here in that the complex-ADH free protein equilibrium was shifted in the direction of complex formation (Scheme 3.2.1) for the reduced complex. Therefore maybe the higher MW species assayed for activity, is less active due to an increase in the extent of binding. The additional activity from the non-reduced complex either comes from released ADH from the complex before it dissociates, or possibly as a result of the reduced extent of binding.

A brief study was also set up to investigate the interaction between ADH and 56% activated dextran. Initial studies showed that significant complex formation was seen after only 12 hours. This was assayed for activity at the same time as the reduced complexes (Figure 6.2.4.6) and showed only minimal activity. At this level of activated dextran there is probably more crosslinking between the dextran and the protein. While

this may support the possibility that the increased binding had limited the activity of the reduced ADH complex compared with the non-reduced complex, the minimal activity may also result from the slower release rate at 56% level of activation.

6.2.5 Catalase Release

Initial release studies with catalase showed a drop in the peak due to the complex but no signs of catalase-like release (Figure 6.2.5.1). There is formation of several lower MW species indicated by bands with longer retention times. This suggests that the catalase is dissociating into dimers and monomers upon release from the complex. Catalase is known to readily dissociate into these inactive forms (Halliwell and Gutteridge, 1985). Initially the most obvious change is the decrease in MW of the complex. This is followed by a drop in the complex peak and the appearance of the lower MW fragments.

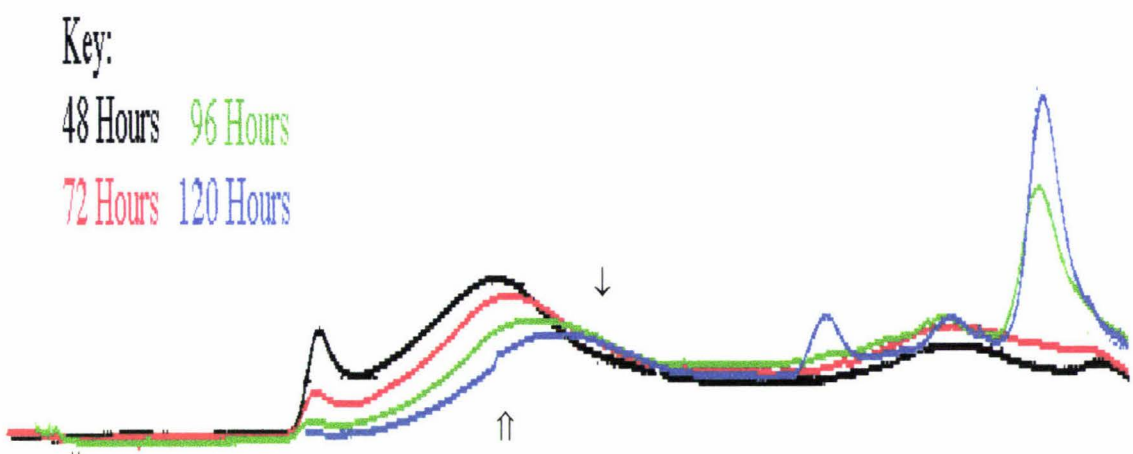


Figure 6.2.5.1 Release studies for catalase-21% activated dextran complex (⇑)
Complex formation incubations were set up between catalase and 21% activated dextran. The complex was then isolated by SEC (Superose 6, FPLC). Fractions were pooled over several runs and concentrated. Isolated complex was then incubated under release conditions. Aliquots were taken at 48hrs, 72hs, 96hrs and 120hrs and then subjected to SEC (Superose 6) in 0.2M Tris acetate, 0.05% sodium azide, pH 7.5. ↓ expected retention time for catalase.

6.3 Conclusions

The inability to obtain release of the intact active protein was a major problem encountered with ADH and catalase. The previous proteins studied were all monomers and were relatively stable. Possibly for these larger proteins, which dissociate to their dimer and monomer forms, it would be more efficient to use the reduced form of the complex. This way the dextran carrier is being used principally as a means of stabilising the respective proteins. For therapeutic use of high MW proteins such as ADH and catalase, conjugation would not necessarily be required to reduce renal clearance as they are already above 70kDa. Nevertheless slow release or covalent bonding may be useful for transport of the protein and extending the lifetime within the body. It may offer protection from proteolytic enzymes and other degradation agents, as well as increasing biological stability.

As ADH shows minimal activity in the reduced form, then maybe this form of the complex could be used as a stabilising factor. Although further investigations are required the evidence points to the possibility of ADH binding to the dextran as a dimer. In this form the enzyme could still exhibit activity. This statement is supported by the lower MW fractions observed by SEC and SDS-PAGE during release experiments. Releasing dimers could then be further dissociating into monomers.

The extensive overlap between the complex and ADH or catalase indicates the MW of the complex is not significantly higher. Therefore if the protein is in the tetrameric form then only a limited number of protein molecules are binding to each dextran. This indicates that the possible structure of the complex is changing with the increasing MWs of the proteins. Lysozyme investigations indicated that the resulting complex was an intricate mixture of crosslinking between different lysozyme and dextran molecules (Figure 1.8.2c). Extent of this binding increases with an increase in activation levels of dextran. Evidence from the ADH and catalase studies indicates that the amount of crosslinking between the protein and dextran was decreasing as the protein size increased, and moved towards a structure of dextran molecules binding to a single protein molecule. In other words the situation depicted in Figure 1.8.2c has shifted towards that shown in Figure 1.8.2a.

If the proteins are also binding to dextran in the dimer and monomer forms, then this would affect the resulting structure. Given the MW of the complex, this would indicate that more extensive crosslinking with the dextran is taking place. This clearly indicates

that further studies are required to help establish the way in which the protein is binding, which in turn dictates the final structure of the complex.

It is possible that higher levels of activated dextran could be useful for higher MW proteins. The extent of conjugation between ADH/catalase and dextran may be limited by steric hindrance and the number and location of activated glucose molecules. Therefore higher percentage activation would make more glucose molecules within the dextran available for conjugation, meaning steric hindrance was the main limiting factor. This is supported by the results of complex formation obtained for catalase and ADH (Figure 6.2.1.1 - 6.2.2.2). There was a distinctive shift in retention times between the 21% and 7% complexes in each case. At the 21% level of activation there is a shorter retention time (therefore higher MW) for the maximum absorption of the complex peak.

These preliminary studies into ADH and Catalase are not extensive but they do indicate the possibilities of binding larger MW proteins to dextran.

CHAPTER SEVEN

CONCLUSION AND FUTURE WORK

7.1 Conclusions

The study reported in Chapters 2-6 investigated the conjugation of proteins to dextran as a potential model for obtaining a slow release mechanism. Sustained release of proteins was primarily considered for therapeutic use, but there are also possibilities for commercial applications. In biomedicine sustained release of a therapeutic protein or drug from a carrier-conjugate confers several advantages over existing methods of drug administration. Most notably it reduces frequent or multiple doses of drugs for the patient. If the slow release device can be directed to the site of action then it could be possible to limit the presence of the protein or drug in the body, and hence the amount of metabolites. This also means it is possible to have more of the therapeutic agent available at the desired site of action. Protein conjugation to dextran was also expected to confer stability.

Dextran was chosen as a carrier because of the numerous possibilities for chemical derivatisation. It is appropriate for therapeutic use because the side effects have been well minimised (Section 1.8). That is it has a good track record over the last five decades (Larsen, 1989). The method of derivatisation chosen for this study was oxidation followed by imine formation with a selected protein.

The proteins chosen covered a MW range of 14 kDa to 220 kDa. They were investigated for their ability to bind to dextran. The smaller proteins, lysozyme, trypsin and amylase, all conjugated to dextran with relative ease. In the case of lysozyme and trypsin the extent of binding to 56% activated dextran resulted in a complex that did not release protein under the conditions studied. This non-viable slow release situation was overcome by using lower levels of dextran activation for these two enzymes. Conjugation to dextran of these smaller proteins was not limited by steric hindrance. The amount of activated glucose molecules available limited the extent of binding. The reverse situation was seen to occur for the larger proteins. Upon going from 7% to 21% activated dextran, the ADH and catalase showed a distinct increase in the average MW of the complex species, as might be expected. Steric hindrance of the larger proteins possibly limited the conjugation with dextran. Higher percentages of activation would mean that there are more available sites for the proteins to bind to. Therefore while the higher level of activation offered little promise for slow release of the smaller proteins, it is possible that larger proteins may benefit. α-Amylase might also be useful at higher activation levels as the active site was not affected by binding to the dextran.

Lysozyme and α-amylase proved to be good models for slow release. Facile complex formation occurred and release of a protein species was readily observed. While the dextran-lysozyme imine complex exhibited only minimal activity, the conjugated α-amylase retained normal activity. The reduced activity observed for lysozyme could result from too many of the lysine residues being involved in conjugation with dextran. In addition, the resulting structure of the lysozyme-dextran complex may result in some of the lysozyme molecules being inaccessible to the micrococcus used for the lytic assay. That is, only the protein molecules on the outer surface of the complex would be able to exhibit cell lysis. This would be less likely to occur for α-amylase because the increased size of the protein should reduce the extent of binding. The availability of the α-NH_2 groups and lysines would vary from protein to protein.

Trypsin is a lower MW protein than α-amylase but there were no signs of it being active in the complex. Possibly the conditions used were not ideal for the protein. Optimum pH for trypsin activity is 8 but the complex investigations were carried out at pH 5 in order to minimise autolysis. There is also the possibility that the active site is distorted during conjugation. Immobilisation studies with trypsin bound to zirconia and silica have shown a decreased relative activity because binding had effected the active site (Huckel *et al*, 1996). For future experiments the possibility of increasing the pH to 8 after complex formation could be investigated. This would increase the autolysis of the releasing trypsin like-species, but the addition of $CaCl_2$ should help minimise this. Autolysis is reduced in the presence of other proteins and peptides therefore *in situ* this should be less of a problem.

The larger MW proteins investigated are both oligomers, consisting of four subunits each. ADH and catalase appeared to exhibit less stability and readily dissociated into dimers and monomers. Complex formation appeared to reach equilibrium faster than the lower MW proteins. This was thought to be because at the activation levels used steric hindrance would probably limit the extent of binding. Difficulties were encountered during release experiments. Only the higher MW range of the complex species were investigated for release. These protein-dextran complexes did appear to re-establish an equilibrium with lower MW range. Protein-like species were being released from the complex, but it became apparent that they were no longer stable and had dissociated, as evidenced by the formation of lower MW moieties observed with SEC and SDS-PAGE. Dissociation may be occurring earlier, and the dimer and monomer forms may still bind to dextran. The usefulness of the released product in this situation would depend on how long it maintained its oligomeric structure in an *in vivo* situation if at all. More work is required on ADH and catalase before these speculations can be confirmed. Catalase requires maintenance of its oligomeric structure for activity (Burton, 1995). Possibly the main advantage for conjugation of ADH and catalase at these levels of activated dextran is the stability conferred on the protein while in the complex.

It has become apparent that the percentage activation of dextran plays an important role in the properties of the protein-dextran complex. For individual proteins the level of activation can be chosen for a particular desired effect. Properties of the complexed protein change with varying activation levels. In general it appears that lower levels of dextran activation are preferable for smaller proteins. This will lead to a more viable complex. Very preliminary evidence suggests that higher levels of activation might be suitable for larger proteins such as ADH and catalase.

7.2 Future work

Investigations to date have indicated a potential benefit for the sustained release of proteins from dextran. It is apparent that the slow release from dextran noted with rhGH (Battersby *et al*, 1996), can be extended to other bioactive proteins. This is of particular interest in biomedicine where therapeutic use of proteins is often limited. Binding of proteins to the dextran polymer would not only increase the stability of the protein *in vivo*, but it would reduce its clearance rate from the body, and possibly reduce the chances of immunological responses. Attack by proteolytic enzymes could also be reduced.

The results from this investigation clearly indicate that the level of activated dextran used can be varied to suit the protein in question and the desired use for the complex. While varying the level of activation plays an important role, it would also be beneficial to investigated the varying ranges of dextran size. This investigation centred on the use of Dextran-T40, but the usefulness of larger and smaller MW dextrans (such as 70 kDa and 10 kDa) could be investigated. For therapeutic applications release experiments would need to be studied at physiological conditions. Relative amounts of protein to dextran could also be studied.

Small activated dextrans may provide a means of trapping unwanted proteins and peptides and removing them from the body. This is envisioned from the lysozyme-56% dextran complex where extensive conjugation resulted in an inactive complex without signs of release. Although theoretically this is a possibility, other proteins not designated for removal could also bind to the dextran. This proposed scavenging system would perhaps be more suited if the targeted protein or peptide was in higher concentrations and involved in a life threatening situation.

Taking the sustained release model of proteins a step further there is the possibility of binding two proteins to the dextran. Different methods of chemical modification could be used to

obtain binding of each protein, otherwise there would probably be preference for one protein over the other. One approach therefore would be to covalently bind the first protein for the purpose of targeting, and then bind the second through periodate oxidation to obtain slow release of the therapeutic protein at the targeted site. This approach could use two, one or no proteins in a double drug therapy situation.

For *in vivo* use the equilibrium of protein release from the complex would be altered. The protein would be moved away from the complex site pushing the equilibrium in the direction of release. Therefore *in vitro* it could be advantageous to use competition experiments to investigate the rate of release. That is, upon release of the protein a competitor would bind in its place, preventing it from re-associating with the complex. This could also have commercial applications where it is necessary to increase or control the rate of release, for example where the released species are used as catalysts to increase the rate of reaction.

Another approach to further study the rate of release of the proteins from the dextran complex could be through ultrafiltration or a hollow fibre apparatus. An isolated protein-dextran complex could be placed in an ultrafiltration unit. Using an appropriate MW cut off membrane, any of the released protein species could be isolated through the membrane and assayed. Such studies would require refinement of the MW range of dextran, and the MW of both the dextran and the protein studied would have to be taken into account.

Binding of proteins to dextran in this study could also be seen as a method of altering and improving the properties and function. This is particularly evident from the possible use of lysozyme in the food industry. Studies have shown that conjugation can increase emulsifying properties as well extending enzymatic action to gram negative bacteria (Nakamura *et al*, 1991). Different levels of dextran activation will affect these modifications.

For the larger MW proteins more extensive investigation is required, not only to find an ideal activation level in each case, but also to establish the binding modes of the protein. That is whether they are also binding as dimers and monomers and the implications of this phenomenon. Further investigations into reduced and non-reduced complexes would be beneficial for characterising the complex and the release mechanism. The periodate oxidation step is relatively inexpensive ($NaIO_4$, US$0.39 per g, 500g price Aldrich). If the reduction was thought to be a useful possibility, scale up would probably necessitate using the cheaper sodium borohydride ($NaBH_4$, US$0.16 per g, 2kg price; $NaBH_3CN$ US$ 0.72 per g, 1kg price, Aldrich)

For large scale production of enzymes complexed to dextran the cost would need to be considered. This is of particular importance for commercial applications such as the food industry. In certain cases it could be beneficial to use a cheaper polymer then dextran. The method of activation of the dextran or another polymer would also be chosen on a cost basis.

Application of dextran conjugates is not just limited to protein molecules but can also be extended to small drug molecules. These preliminary investigations into sustained release of proteins from periodate activated dextran have shown that conjugation can be extended to five more bioactive proteins of varying size and biological activity.

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