

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**DEXTRAN ENZYME IMINE COMPLEXES:
A PRELIMINARY STUDY**

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

Louisa Jane Fisher
1997

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

First and foremost I would like to thank my supervisor Associate Professor David R.K. Harding for his time, input and encouragement over the last two years.

I would also like to acknowledge Debbie Frumau for running my amino acid analysis samples, and Dick Poll for his constant help with the FPLC and SMART systems. Thanks are also due to Associate Professor D.N. Pinder and Dr J. Lewis for their time and help with the LLST and Ultracentrifugation experiments respectively.

Special thanks and appreciation to Rekha Parshot and Jenny Cross for the SPPS and purification.

Thank you also to J. Battersby, Genentech Inc., South San Francisco, for assistance and suggestions with the tryptic digest studies, and for running the HPLC of the trypsin as well as for the gifted rhGH and the rough sketch that lead to Figure 1.8.2.

I would also like to thank the Departments of Biochemistry and Chemistry for their assistance along the way, especially the members of the Centre for Separation Science and Gill Norris's lab.

Finally I would like to thank my parents and friends, in particular Suzette, Ruth, Kimberley and Morris for putting up with me especially through the last stages of my thesis.

TABLE OF CONTENTS

Abstract	ii
Acknowledgements	iv
Table of Contents	v
List of Figures	viii
List of Tables and Schemes	x
List of Abbreviations	xi
CHAPTER ONE INTRODUCTION	1
1.1 Drug Delivery	1
1.2 Controlled Release of Drugs.....	2
1.3 Encapsulation.....	3
1.4 Non-reversible Covalent Bonding.....	4
1.5 Sustained Release of rhGH from Dextran	5
1.6 Periodate Oxidation of Dextran.....	6
1.7 Imine Formation.....	9
1.8 Complex Formation of Proteins with Dextran.....	10
1.9 Protein Modification	13
1.10 Investigations into Complex Formation of Proteins to Dextran and Subsequent Release	14
CHAPTER TWO MATERIALS AND METHODS	16
2.1 Reagents and Equipment	16
2.2 Periodate Oxidation.....	17
2.3 Iodometric Titration.....	17
2.4 Complex Formation.....	17
2.5 Complex Release.....	18
2.6 Complex Reduction Studies	18
2.7 Lysozyme Lytic Assay.....	18
2.8 Laser Light Scattering	19
2.9 Ultracentrifugation	19
2.10 Trypsin BAPNA Assay.....	19
2.11 Trypsin Digest of rhGH.....	20
2.12 Trypsin Digest of Synthetic Peptide.....	20
2.13 α -Amylase Activity	21

2.14	Alcohol Dehydrogenase Assay	21
2.15	BCA Protein Concentration Determination.....	21
2.16	Amino Acid Analysis Preparation	21
2.17	SDS-polyacrylamide gel electrophoresis	22
 CHAPTER THREE LYSOZYME		23
3.1	Introduction.....	23
3.2	Results and Discussion	25
3.3	Conclusions.....	39
 CHAPTER FOUR TRYPSIN		40
4.1	Introduction.....	40
4.2	Results and Discussion.....	42
4.3	Conclusions	56
 CHAPTER FIVE α-AMYLASE		60
5.1	Introduction.....	60
5.2	Results and Discussion	62
5.3	Conclusions.....	71
 CHAPTER SIX ALCOHOL DEHYDROGENASE AND CATALASE		72
6.1	Introduction	72
6.1.1	Alcohol Dehydrogenase	72
6.1.2	Catalase	72
6.1.3	Higher MW Proteins.....	73
6.2	Results and Discussion	74
6.2.1	ADH Complex Formation.....	74
6.2.2	Catalase Complex Formation	74
6.2.3	Complex Formation	74
6.2.4	ADH-dextran Complex and Release Investigations.....	77
6.2.5	Catalase Release	85
6.3	Conclusions.....	86

CHAPTER SEVEN	88
CONCLUSION AND FUTURE WORK.....	88
7.1 Conclusions.....	88
7.2 Future work	90
REFERENCES	93

LIST OF FIGURES

Figure 1.6.1	Molecular weight distribution by gel filtration of Dextran T-40	6
Figure 1.6.2	Periodate oxidation of Dextran	7
Figure 1.6.3	Overall reaction individual glucose molecule periodate oxidation	8
Figure 1.8.1	Extent of complex formation over increasing dextran activation levels for 24hr period	10
Figure 1.8.2	Possible structure of protein dextran complex	12
Figure 3.1.1	Laser light scattering apparatus	24
Figure 3.1.2	Diagram of a Schlieren pattern of a homogeneous solution	24
Figure 3.2.1	Complex ($\uparrow\uparrow$) formation over time for lysozyme (\uparrow) and 56% activated dextran	25
Figure 3.2.2	Expected progress with time of Schlieren peak	27
Figure 3.2.3	Complex ($\uparrow\uparrow$) formation over time between lysozyme (\uparrow) and 7% activated dextran	28
Figure 3.2.4	Complex ($\uparrow\uparrow$) formation over time between lysozyme (\uparrow) and 21% activated dextran	28
Figure 3.2.5	Release of lysozyme-like (\uparrow) species from complex ($\uparrow\uparrow$) (lysozyme-21% activated dextran) over time	29
Figure 3.2.6	SDS-Page	30
Figure 3.2.7	Lysozyme activity	31
Figure 3.2.8	Activity of lysozyme complex with time	32
Figure 3.2.9	Complex formation at 72hrs for reduced and non-reduced complexes	35
Figure 3.2.10	Lytic activity of reduced and non-reduced complexes	36
Figure 4.1.1	BAPNA assay for trypsin activity	41
Figure 4.2.1	Complex ($\uparrow\uparrow$) formation over time between trypsin (\uparrow) and 7% activated dextran	43
Figure 4.2.2	Complex ($\uparrow\uparrow$) formation over time between trypsin (\uparrow) and 21% activated dextran	43
Figure 4.2.3	Release of trypsin-like species (\uparrow) from the complex ($\uparrow\uparrow$)	44
Figure 4.2.4	Trypsin activity	45
Figure 4.2.5	Activity of trypsin-dextran complex over time	47
Figure 4.2.6	Analytical reverse phase chromatography of the released trypsin-like species and the original trypsin	49
Figure 4.2.7	SDS-PAGE analysis	50
Figure 4.2.8	Activity studies on reduced and non-reduced complexes	51
Figure 4.2.9	Reverse-phase analytical run of the synthetic peptide	53

Figure 4.2.10	HPLC chromatograph of trypsin digest on the 24mer by the original trypsin.	55
Figure 4.2.11	HPLC chromatograph of trypsin digest on the 24mer by the released trypsin-like species.	56
Figure 5.1.1	Theoretical basis of α -amylase assay procedure	61
Figure 5.2.1	Complex (\uparrow) formation over time between α -amylase (\uparrow) and 7% activated dextran	63
Figure 5.2.2	Complex (\uparrow) formation over time between α -amylase (\uparrow) and 21% activated dextran	63
Figure 5.2.3	Release of α -amylase-like species (\uparrow) from the dextran complex (\uparrow) over time	64
Figure 5.2.4	Activity of α -amylase	65
Figure 5.2.5	SDS Homogenous gel	66
Figure 5.2.6	Activity of amylase complex over time	67
Figure 5.2.7	Comparison of activities for reduced and non-reduced complexes	70
Figure 6.2.1.1	Complex (\uparrow) formation over time for ADH (\uparrow) and 7% activated dextran	75
Figure 6.2.1.2	Complex (\uparrow) formation over time for ADH (\uparrow) and 21% activated dextran	75
Figure 6.2.2.1	Complex (\uparrow) formation over time for catalase (\uparrow) and 7% activated dextran	76
Figure 6.2.2.2	Complex (\uparrow) formation over time for Catalase (\uparrow) and 21% activated dextran	76
Figure 6.2.4.1	Complex Formation at 48 Hours between ADH and 7% Activated Dextran	77
Figure 6.2.4.2	Activity assays performed on isolated fractions from ADH-7% dextran complex from figure 6.2.4.1	78
Figure 6.2.4.3	Release from ADH-7% complex (\uparrow) over time	79
Figure 6.2.4.4	ADH Activity	80
Figure 6.2.4.5	SDS- PAGE analysis	82
Figure 6.2.4.6	ADH Reduction studies	84
Figure 6.2.5.1	Release studies for catalase-21% activated dextran complex (\uparrow)	85

LIST OF TABLES AND SCHEMES

Table 1.10.1	Molecular weight range of proteins for dextran complex formation study	14
Scheme 3.2.1	Equilibrium between free protein and dextran	32
Table 3.2.1	Amino acid composition in respect to alanine of the released species in comparison to purified lysozyme and literature sequence	34
Scheme 3.2.2	Cyanoborohydride reduction of protein-dextran complex	35
Table 4.2.1	Specific activity for the trypsin complex samples and the release trypsin-like species	46
Table 4.2.2	Amino acid composition with respect to alanine of the released species in comparison to purified trypsin and the literature sequence	48
Scheme 4.2.1	Sequence of the 24mer, synthetic peptide	52
Table 4.2.3	AAA of the synthetic peptide	53
Table 4.2.4	AAA composition of peptides from trypsin digest	58
Table 4.2.5	Summary of synthetic peptide digestion	58
Table 5.2.1	Specific activity	67
Table 5.2.2	Amino acid composition of α -amylase and release species	69
Table 6.2.4.1	Specific activity comparison for ADH-dextran complex	80
Table 6.2.4.2	Amino acid compositions with respect to alanine	81
Scheme 6.2.4.1	Possible reactions occurring with ADH-dextran incubations	83

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 ₃ CN	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 electrophoresis
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