THE BIOLOGICAL PROPERTIES OF SHIGA-LIKE TOXIN I

Submitted for the degree of

Philosophical Doctorate

Beverley Jane Burgess (BSc. Hons.)

University of Warwick

March 1993

To Graeme, Mummy, Daddy and Lindsay.

CONTENTS page

page

SECTION 5 APPENDICES

SECTION 6 REFERENCES

ACKNOWLEDGEMENT

Over the last three years of study I have received help from many different people, without their help and encouragement I may never have reached this final stage. For this reason I would like to say 'thank you' to them.

Firstly I would like to thank Dr Lynne Roberts, under whose expert guidance my research project developed, for providing me with the opportunity to further my education and for her invaluable expertise, enthusiasm and support throughout.

For the provision of additional financial and technical support throughout and for his interest, I would like to thank my external supervisor Professor Tony Atkinson (PHLS, CAMR, Porton Down).

Professor Mike Lord and Dr Martin Hartley have also shown a constant interest and enthusiasm for my studies and have both proven to be a great source of specialist advice for which I would like thank them. I would also very much like to thank Dr George Salmond, Dr Ben Swoboda, Dr Liz Jones and Marie Ryd (Stockholm, Sweden) all of whom have been generous with their valuable time in the sharing of their expert knowledge and experience.

With regard to the practial aspect of my research there are many people to whom I owe a great many thanks. I am indebted to Dr Howard Tranter, Helen King and Roger Rhind-Tutt at Porton Down, whose hard work and specialist technical support provided me with some of the much appreciated raw material for my research.

The greatest source of practical 'on the spot' help in the laboratory has to have been from the inhabitants of PBL II! Thanks to everyone in PBL II for all the helpful hints and the occasional reagent, but mainly for making the lab. a friendly and team spirited environment to work in.

For their support and encouragement through all my years of study, I would like to thank my parents and my sister, Lindsay.

Finally to my husband Graeme - my greatest 'result'!. What can I say, except that without him, I would never have got this far?

FIGURE INDEX

DECLARATION

Research presented in this thesis was obtained by the author unless specifically indicated in the text. The research presented in this thesis has not been submitted for any previous degree. All sources of information used in the preparation of this thesis are indicated by reference.

ABBREVIATIONS

AMP	ampicillin
ATP	adenosine triphosphate
BSA	bovine serum albumen
CAP	m7G(5')ppp(5')G
CD_{100}	100% cytotoxic dose
CIP	calf intestine phosphatase
CP	creatine phosphate
CT	cholera toxin
DMEM	Dulbecco modified Eagles medium
ds	double stranded
DT	diphtheria toxin
DTT	dithiothreitol
EDTA	ethylene diaminotetraacetic acid
EGTA	ethylene glycol-(βaminoethyl ether)-N,N,N,N',N'-tetraacetic acid
Gb_3	globotriosylceramide
g	gramme
IC_{50}	50% cytotoxic dose
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
KDa	1000 Daltons
LB	Luria broth
LT	Escherichia coli heat labile toxin
mRNA	messenger RNA

u	micro (10^{-6})
m	milli (10^{-3})
M	Molar
n	nanno
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	*Pseudomonas* exotoxin A
p	pico (10^{-9})
PMSF	phenyl methyl sulphonyl fluoride
RA	ricin A subunit
RNAse	ribonuclease
rRA	recombinant ricin A subunit
rRNA	ribosomal RNA
rSLT IA	recombinant Shiga like toxin I A subunit
SDS	sodium dodecyl sulphate
ss	single stranded
SLT I	Shiga-like toxin I
SLT II	Shiga-like toxin II
SLT IA	Shiga-like toxin I A subunit
SLT IB	Shiga-like toxin I B subunit
ST	Shiga toxin
STA	Shiga toxin A subunit
tRNA	transfer RNA
TCA	trichloroacetic acid
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone (treated trypsin)

v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolylβ-D-galactopyranoside

SECTION 1 **INTRODUCTION**

SUMMARY

Shiga toxin and the Escherichia coli Shiga-like toxins (SLTs) are type 2 ribosome inactivating proteins (RIPs), exhibiting a specific RNA N-glycosidase activity comparable to that of the plant toxin ricin (Endo et al. 1988). Not only do they possess the same enzymatic activity as a plant toxin, but on examination at the molecular level, they may be seen to be classic bipartite toxins. The present study examines in detail the RNA N-glycosidase activity of Shiga-like toxin I (SLT I) ie, the susceptibility of a range of different eukaryotic ribosomes to the toxin, the exact site of SLT I-catalysed depurination of yeast ribosomal RNA and the kinetics of such depurination. The cytotoxic effect of SLT I on Vero cells is also examined. In addition to its characterisation and subsequent comparison to ricin, an attempt has been made to correlate certain structure - function aspects of SLT I. By structural comparison with other bacterial toxins it has been proposed that proteolytic cleavage within an arginine rich, trypsin sensitive, disulphide-bonded loop structure is essential for expression of cytotoxicity. Examination of the enzymatic activity and cytotoxicity of a 'protease insensitive' mutant SLT I A subunit (SLT IA) generated in the present study has led to the conclusion that proteolytic processing within this disulphide-bonded loop is not essential, but that processing may occur at alternative sites within SLT IA to compensate for loss cleavage at the proposed target sites.

SECTION 1.1 **History and disease.**

It was Kiyoshi Shiga, a Japanese microbiologist, who in 1898 first described a bacterium to be the causative agent of a type of dysentery distinct to that of amoebic dysentery. He called the bacterium Bacillus dysenteriae which was later renamed Shigella dysenteriae and the disease shigellosis. It was the continued work of Shiga and his colleagues on bacterial extracts that led eventually to the discovery by Conradi, in 1903, of the associated, highly potent cytotoxin - Shiga toxin (ST). However, although work by Shiga and his colleagues had shown that crude preparations of the toxin when given parenterally to mice and rabbits caused hind limb paralysis and eventual death, the role of Shiga toxin in pathogenesis was not understood for many years and remained a matter of conjecture. Indeed, for many years Shiga toxin was regarded to be a neurotoxin until improvements in purification and toxin yield allowed a closer examination of its cytotoxic effects. These studies demonstrated that, the neurological manifestations were a secondary effect of the toxin with direct damage to vascular endothelial cells in the central nervous system (CNS) being the primary effect.

Konawalchuk et al. (1977) first described an E.coli toxin which was cytotoxic to Vero cells and distinct from other E.coli toxins such as the heat labile and heat stable toxins. O'Brien et al. (1982), later screened E.coli isolates known to cause

infantile diarrhoea which were also known to be cytotoxic for Vero and HeLa cells. They screened for neutralisation of cytotoxic activity using rabbit antisera raised against ST. Neutralisation of the cytotoxic effect confirmed their hypothesis that certain strains of E.coli, which were non invasive and which produced neither heat stable nor heat labile toxin, produced a Shiga - like toxin (SLT). On closer examination, it was found that two types of SLT were produced; one which could be neutralised with anti ST antisera and one which could not. The former was designated SLT I and the latter antigenically distinct version, SLT II (Strockbine et al. 1986).

Human infection with a Shiga-like toxin (SLT)-producing strain of E.coli, defined by Levine et al. (1987) as an enterohaemorrhagic E.coli (EHEC), may manifest itself in a range of different disease symptoms (Hunt et al. 1989). These range from an asymptomatic infection to a mild non bloody form of diarrhoea to the more complex and in some cases life threatening haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombocytopenic purpura (Karmali, 1989).

Not only is there a spectrum of disease as a result of EHEC infection, but it would also appear to be the case that the type of people susceptible to infection is indefinable. EHEC have been associated with the above mentioned diseases in

infants, children and adults in both developed and developing countries (Riley, 1987).

A variety of EHEC serotypes are also involved. Riley *et al.* (1983) first implicated *E.coli* 0157 in an outbreak of haemorrhagic colitis with patients suffering severe abdominal pain and an afebrile diarrhoea which progressed to bloody diarrhoea. In Chile, studies revealed three different EHEC serotypes to be most often involved in causing HUS in children (Cordovez *et al.* 1992); whilst an Italian study revealed serotype 0157 to be the most prevalent in paediatric HUS (Capriolini *et al.* 1992). In cases of infantile diarrhoea serotype 026 was isolated with the most frequency (Scotland *et al.* 1990).

The actual role of ST and SLTs in pathogenesis remains unclear. Work was initially focused on dysentery resulting from infection with *S.dysenteriae* type 1 after the finding that patients with this organism suffered a more severe form of dysentery than did those infected with other *Shigella* species. Indeed it was also found to be the case that *S.dysenteriae* type 1 associated HUS was of a more severe nature than that as a result of EHEC infection (O'Brien and Holmes, 1987).

Investigations into the role ST plays in dysentery have involved the use of mutant strains of *S.dysenteriae* type 1. Levine *et al.* (1973) found that volunteers fed with a low toxin

producing mutant strain of S.dysenteriae type 1 suffered a less severe form of the disease than did the volunteers fed the highly toxigenic non mutant strain. ST appeared in some way to exacerbate the effects of the infection. In support of this, Fontaine et al. (1988) noted significant differences in disease manifestations when they infected macaque monkeys with a tox^- strain of S.dysenteriae type 1 or with the wild type toxin producing strain. Less bloody stools and less inflammation and ulceration of the colonic mucosa were observed following infection with the tox^- strain. With regard to the role played by SLT in HC, Pai et al. (1986), induced diarrhoea in infant rabbits, found that a non toxin producing strain did not result in diarrhoea, a low toxin producer resulted in some of the rabbits developing diarrhoea, whilst all of those infected with 0157:H7 displayed colon damage and diarrhoea symptoms. Thus results strongly implicate ST and SLT in the pathogenesis of dysentery and HC respectively.

Donohue-Rolfe et al. (1986) were able to detect free toxin in the faeces of patients infected with S.dysenteriae type 1. It is suggested that free toxin in the gut may bind to and directly damage colonic epithelial cells. The resultant ulceration of the colonic mucosa may then allow leakage of blood , inflammatory elements and alteration of the homeostatic capacity of the colon (Hale, 1991). Sekizaki et al. (1987), later demonstrated that, both ST and SLT were indeed cytotoxic to human colon epithelial cells.

The mechanisms by which Shigella spp. and EHEC deliver ST or SLT to the target cells are very different, since Shigella spp. are invasive and E.coli are not. Shigella spp. penetrate the colonic epithelial cells, multiplying intracellularly and spreading to adjacent cells and tissues. However, whilst ST plays little or no role in proliferation and survival of the bacterium (Brunton, 1990) it is delivered to the cells in highly concentrated doses (Tesh and O'Brien 1991), as is SLT by non-invasive adherence of EHEC.

Karch et al. (1987) were able to detect attachment in vitro of serotype 0157, the serotype most commomly associated with HC. Further to this, Scotland et al. (1990), found that 13 out of the 14 isolates tested by Karch et al. (1987) were capable of attachment to a line of human intestinal cells (Intestine 407 cells). Tiporizi et al. (1987, 1989), found complete degeneration of the mucosal surface of the colon to be associated with the formation of attaching, effacing lesions by 0157:H7 in studies on gnotobiotic piglets with 0157:H7 induced diarrhoea. SLT production was not, however, a prerequisite for formation of these lesions, since a tox^- mutant of 0157:H7 still caused lesions, demonstrating that SLT is not the only factor involved in mediating damage to the colon.

Levine et al. (1987), on examination of 0157:H7 serotypes from HC and HUS patients observed that the E.coli possessed fimbriae. Possession of such fimbriae correlated with the

possession of a 60MDa plasmid, the loss of which resulted in the loss of capacity of 0157 isolates to attach to Intestine 407 cells. More recently, the *eae* gene responsible for attaching and effacing by EHEC has been identified and cloned (Yu and Kaper, 1992). In comparison with the *eae* gene of enteropathogenic *E.coli* (EPEC), which are also capable of forming attaching and effacing lesions on epithelial cells, the *eae* gene of EHEC was found to have a high degree of homology at both nucleotide and amino acid levels. It has been proposed that the *eae* gene encodes a 94KDa protein adhesin (Yu and Kaper *et al*. 1992).

The role of ST and SLT in dysentery and HC remains to be clarified, although strong evidence suggests that the toxins in some way serve to exacerbate the disease manifestations.

Haemolytic uremic syndrome (HUS) is a further and more complex manifestation of EHEC or *Shigella* infection. Once again the role played by ST and SLT in pathogenesis remains unclear. Indeed it is thought that the disease involves multiple factors (Karmali, 1989).

HUS is of highest incidence in children, causing haemolytic anaemia, thrombocytopenia and acute renal failure (Karmali, 1989). There exists no animal model for HUS, thus links between EHEC infection and HUS are mainly on an epidemiological basis (Brunton, 1990). However, other evidence does suggest a link

between the two. Studies by Lopez *et al.* (1989) on Argentine children, suggest that of those children infected with EHEC, those with bloody diarrhoea are more likely to develop HUS than those who do not display bloody diarrhoea. Patients are often found to display symptoms similar to those of HC prior to development of HUS (Karmali, 1989). It has been suggested that this may allow leakage of free ST or SLT into the bloodstream with the result of direct damage to vascular endothelial cells (Karmali *et al*, 1985). Levine *et al.* (1992) propose that if this is the case, then toxin must be liberated in quantities large enough to exert their biological effect but too small to ellicit an immune response, since on examining the sera of 18 Thai patients in both acute and convalescent phases of infection with *S.dysenteriae* type 1, only a minority had antibody to ST. Such behaviour is similar to that of tetanus toxin.

Glomerular endothelial cells in particular are thought to be a target for direct damage by ST and SLT. Histopathological studies on the kidneys of HUS patients are found to have a large degree of structural damage to their glomeruli (Tesh and O'Brien, 1991). In support of this Obrig *et al.* (1988), have shown the cytotoxic effect of ST on human umbilical vein endothelial cells (HUVEC). However, other factors such as bacterial lipopolysaccharide (LPS) have been implicated in augmenting damage to the kidney by ST and SLT (Koster, 1984). Louise and Obrig (1992), report the synergystic cytotoxic

effect of ST plus LPS on HUVEC suggesting a role for LPS in development of HUS with regard to human vascular endothelial cell damage. The role of LPS in HUS is, however, unclear. Tesh et al. (1991), report that LPS does not augment the cytotoxic effect of ST on HUVEC. They do not, however, rule out the involvement of LPS in HUS noting that glomerular endothelial cells and vascular endothelial cells are very different both morphologically and functionally.

The type of SLT produced by the bacterium is also seen to be a factor in the development of EHEC-induced HUS. SLT II production would seem to be the predominant factor in development of renal disease. Ostroff et al. (1988), found that patients infected with EHEC producing both SLT I and SLT II or SLT II alone developed renal problems, whilst those infected with an SLT I only producer did not.

It would appear that the multifactorial nature of HUS does not currently allow clear definition of the role played by ST and SLT in the development of the disease.

ST and the SLTs have not only been implicated in the diseases described above, but it has also been suggested that ST may play a role in the neurological disorders experienced by some sufferers of shigellosis. Recent work by Ashkenazi et al. (1990), has shown that other toxic products of Shigella spp. may be responsible for the neurological manifestations of

shigellosis, since the toxin isolated from infected patients' stools was not neutralised by antisera to purified ST. The toxin was not however, found to be SLT I or SLT II. In addition to this, Shigella spp. isolated from such patients did not carry the structural genes for ST. The cytotoxin isolated was suggested to be a protein of approximately 100 - 125KDa, crude preparations of which, when injected into mice resulted in neurological symptoms similar to those seen during shigellosis. Ashkenazi et al. (1990), conclude that ST production is not necessary for the neurological manifestations of shigellosis.

ST and SLT are implicated in a range of disease manifestations the specific nature of their involvement remains to be clarified.

SECTION 1.2 **Structural comparison of ST and the SLTs.**

Classically ST and the SLTs, of which ST is considered the type member, were classified according to their biological properties. This encompassed their ability to cause fluid accumulation in ligated rabbit ileal loops, cytotoxicity to Vero and HeLa cells and their ability to cause hind limb paralysis and eventual death to mice and rabbits when injected

intraparentally. This latter property initially led ST to be considered as a neurotoxin. However, until the toxins could be purified to homogeneity, it was unclear whether such multiple activities were attributable to a single toxin or to multiple contaminating toxins (Eiklid and Olsnes, 1983).

More recently, advances in molecular technology have allowed classification of ST and the SLTs with regard to their immunological, genetic and biochemical properties.

Extensive immunological and biochemical studies have revealed that SLT I is essentially identical to ST (O'Brien and LaVeck 1982); whilst SLT II, although sharing 55% homology at the nucleotide level with ST and SLT I (Jackson *et al.* 1987), is antigenically distinct (Strockbine *et al.* 1986). SLT I, in contrast, is completely neutralised by ST antisera (O'Brien and LaVeck, 1982, Marques *et al.* 1986).

Cloning and sequencing of the genes encoding ST and SLT I (Calderwood *et al.* 1987, DeGrandis *et al.* 1987, Kurazono *et al.* 1987, Strockbine *et al.* 1988) has further facilitated the comparison between ST and SLT I (FIG 1.2.1).

Existing in a single copy located in the *trp* - *pyrF* region of the *Shigella* chromosome, ST is encoded by a bicistronic operon (Strockbine *et al.* 1988) encoding both the A and B subunits of the toxin.

FIG 1.2.1 **Nucleotide sequence of the genes encoding the A and B subunits of Shiga toxin and Shiga-like toxin I** (Stockbine *et al*. 1988).

Nucleotides are numbered above the sequence. Differences in the nucleotide sequence of Shiga-like toxin I (SLT I) compared to that of Shiga toxin (ST) are indicated above the sequence (nucleotide 223 = **G** (SLT I), **T** (ST), nucleotide 359 = **T** (SLT I), **A** (ST), nucleotide 850 = **C** (SLT I), **T** (ST)). Nucleotide substitution at position 359 results in an amino acid substitution from **threonine** in ST to **serine** in SLT I. This is indicated below the nucleotide sequence. **ATG** indicates START codons. Horizontal lines above the sequence indicate putative ribosome binding sites. In addition **-10** and **-35** above the sequence indicates promoter recognition sites.

```
                                                                  50
TGACCAGATATGTTAAGGTTCGAGCTCTCTTTGAATATGATTATCATTTTCATTACGTTATTG
                                                             100
TTACGTTTATCCGGTGCGCCGTAAAACGCCCGTCCTTCAGGGCGTGGAGGATGTCAAGAATATA
                    150
GTTATCGTATGGTGCTCAAGGAGTATTGTGTAAT ATG AAA ATA ATT TTT AGA GTG
                                200                            G
CTA ACT TTT TTC TTT GTT ATC TTT TCA GTT AAT GTG GTT GCG AAG GAA
                                    250
TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT TCG CTG AAT
                                        300
GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT TCA TCA GGA
                                    350                T
GGT ACG TCT TTA CTG ATG ATT GAT AGT GGC ACA GGG GAT AAT TTG TTT
                                        400          Serine
GCA GTT GAT GTC AGA GGG ATA GAT CCA GAG GAA GGG CGG TTT AAT AAT
                                    450
CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA GGA TTT GTT
                                            500
AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT TCA CAT GTT
                                                550
ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC AGT AGC TAT
                                                    600
ACC ACG TTA CAG CGT GTT GCA GGG ATC AGT CGT ACG GGG ATG CAG ATA
                                                650
AAT CGC CAT TCG TTG ACT ACT TCT TAT CTG GAT TTA ATG TCG CAT AGT
                                                    700
GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG TTA CGG TTT GTT
                                                        750
ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG AGG GGA TTT
                                                            800
CGT ACA ACA CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA ATG ACT GCT
                                                          C
GAA GAT GTT GAT CTT ACA TTG AAC TGG GGA AGG TTG AGT AGT GTC CTG
                                                                900
CCT GAC TAT CAT GGA CAA GAC TCT GTT CGT GTA GGA AGA ATT TCT TTT
                                                                    950
GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA ATA CTG AAT TGT
                                                                   1000
CAT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT GAG TTT CCT

TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG CAC AAT AAA
 1050
ATA TTG TGG GAT TCA TCC ACT CTG GGG GCA ATT CTG ATG CGC AGA ACT
     1100
ATT AGC AGT TGA GGG GGT AAA ATG AAA AAA ACA TTA TTA ATA GCT GCA
         1150
TCG CTT TCA TTT TTT TCA GCA AGT GCG CTG GCG ACG CCT GAT TGT GTA
             1200
ACT GGA AAG GTG GAG TAT ACA AAA TAT AAT GAT GAC GAT ACC TTT ACA
                 1200
GTT AAA GTG GGT GAT AAA GAA TTA TTT ACC AAC AGA TGG AAT CTT CAG
                     1300
TCT CTT CTT CTC AGT GCG CAA ATT ACG GGG ATG ACT GTA ACC ATT AAA
                         1350
ACT AAT GCC TGT CAT AAT GGA GGG GGA TTC AGC GAA GTT ATT TTT CGT
                     1400
TGA CTCAGAATAGCTCAGTGAAAATAGCAGGCGGAGATTCATAAATGTTAAATACATCTCAA
    1450                                                      1500
TTCAGTCAGTTGTTGCCGGTTCTGATAATAGATGTGTTAGAAAATTTCTGCATG
```

In the case of SLT I a short intergenic region of 12 base pairs exists between the two regions encoding SLT IA and SLT IB (Calderwood et al. 1987). SLTs are, however, encoded by temperate, lambda-like bacteriophages (Williams-Smith et al. 1983, Newland et al. 1985, Strockbine et al. 1986, Kurazono et al. 1987, Kozlov et al. 1988). Expression of the genes are controlled by the binding of an iron-Fur corepressor complex to the promoter of the gene (Calderwood and Mekalanos, 1987, Weinstein et al. 1988). Thus expression is controlled at the level of transcription.

Nucleotide and amino acid sequence analysis have revealed that the ST B subunit (ST B) and SLT I B subunit (SLT IB) are identical in their amino acid sequence (Calderwood et al. 1987, DeGrandis et al. 1987) (FIG 1.2.2). Most groups (Calderwood et al. 1987, DeGrandis et al. 1987, Strockbine et al. 1988) report the only difference between the ST A subunit (ST A) and the SLT IA subunit (SLT IA) is the substitution at position 45 of the amino acid threonine in ST A for a serine residue in SLT IA (FIG 1.2.3). Takao et al. (1988) report their own finding that residue 45 of SLT IA is also a threonine residue and not serine. From this they suggest that the structural gene encoding ST in S.dysenteriae type 1 has been transferred in an identical form to certain strains of E.coli. This has led them (Takao et al. 1988) and others (O'Brien and LaVeck, 1982, Strockbine et al. 1988) to conclude that ST and SLT I are essentially the same.

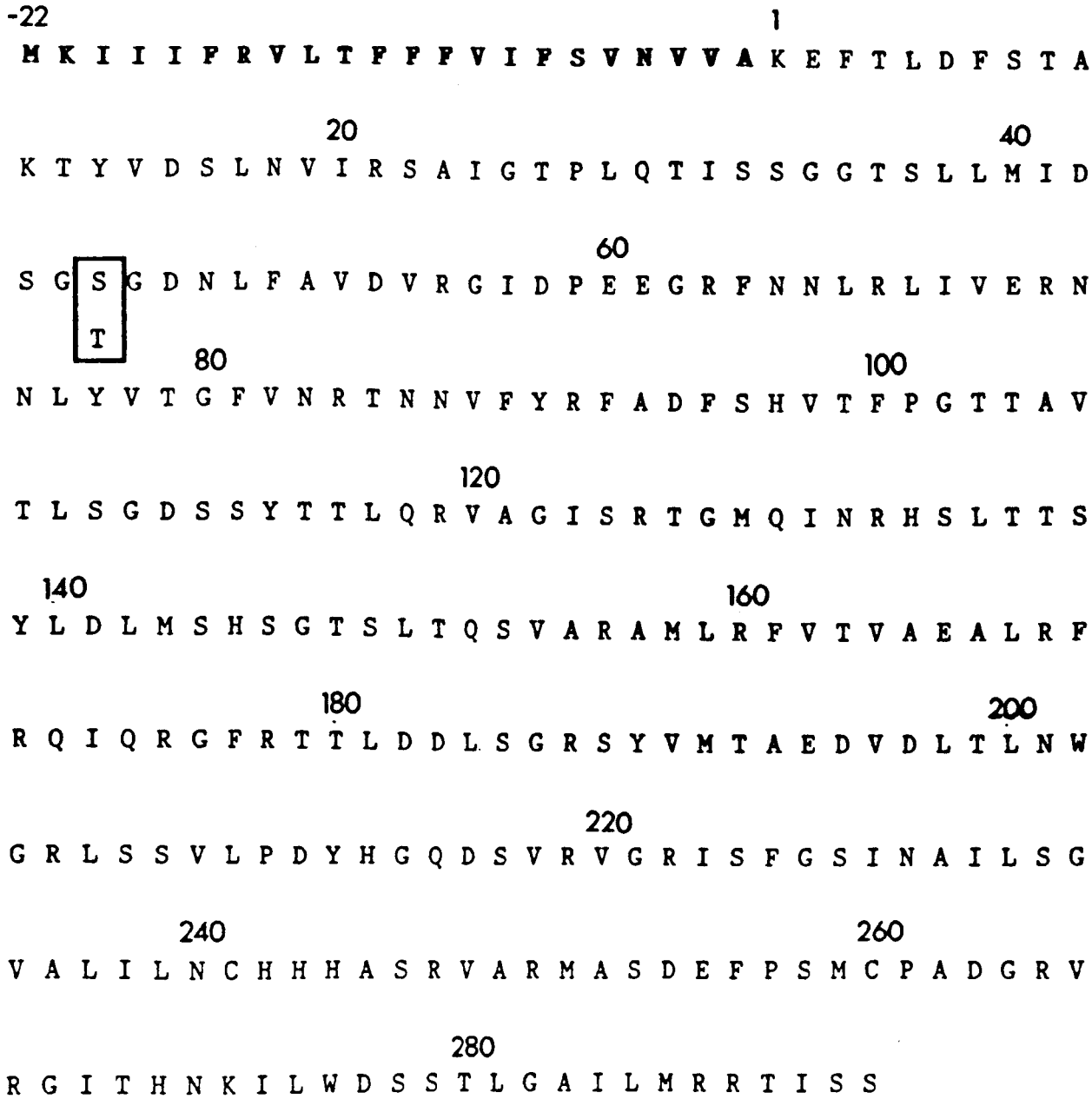
FIG 1.2.2 **Amino acid sequence of the B subunit of Shiga toxin / Shiga-like toxin*I*** (Stockbine et al. 1988).

```
-20                                                 1
M K K T L L I A A S L S F F S A S A L A T P D C V T G K V E Y T

                  20                                                40
K Y N D D D T F T V K V G D K E L F T N R W N L Q S L L L S A Q

                                    60
I T G M T V T I K T N A C H N G G G F S E V I F R
```

Bold type indicates proposed signal peptide

Residues are numbered above the sequence

FIG 1.2.3 **Amino acid sequence of the A subunit of Shiga toxin / Shiga-like toxin1**(Stockbine et al. 1988).



☐ indicates differences between SLT I A and ST A

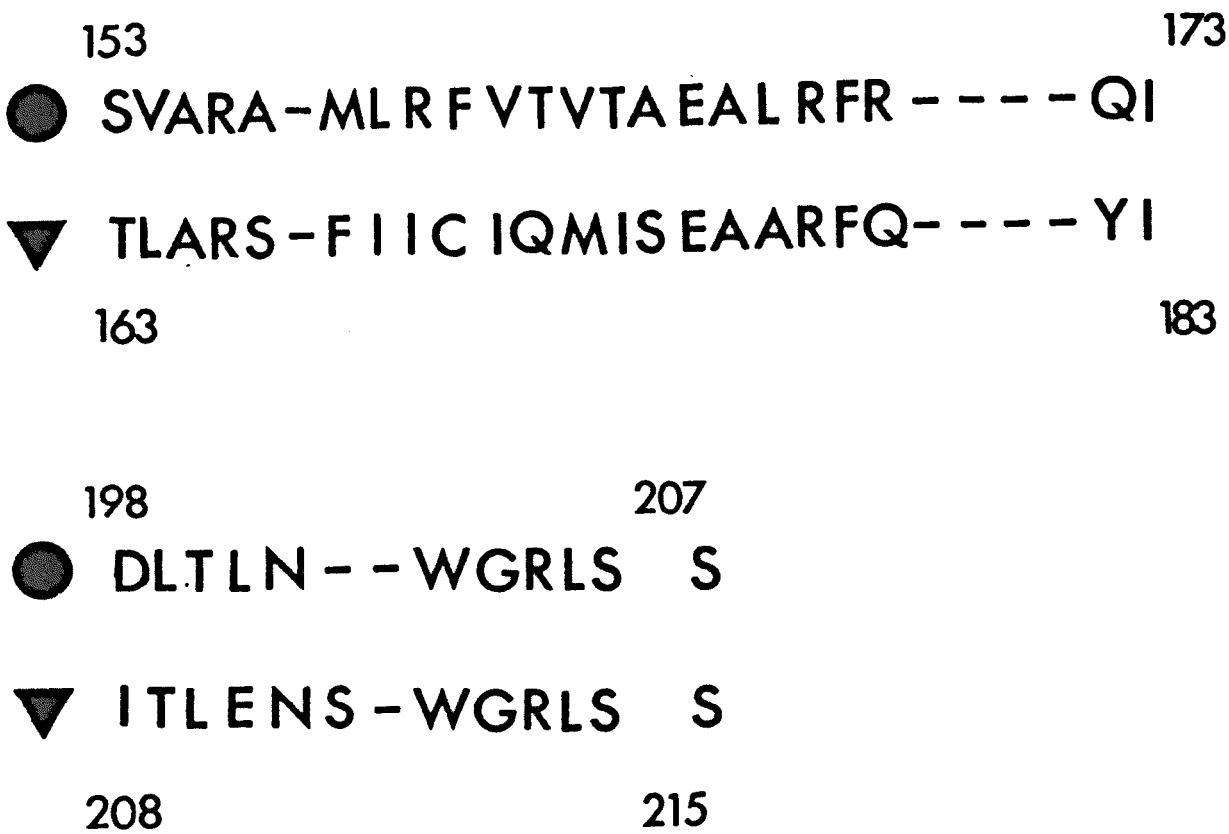
bold type indicates proposed SIGNAL PEPTIDE

Nucleotide sequence analysis of the SLT I operon predicts the mature SLT IA subunit to have 293 amino acids with a molecular weight of 34,804; whilst the mature B subunit comprises 69 amino acids and a molecular weight of 7,692 (Calderwood et al. 1987). In both cases the SLT I subunits are synthesised as precursor forms, each possessing a signal peptide of 22 amino acids in the case of SLT IA and 20 amino acids in the case of SLT IB. The separate A and B subunits are then translocated to the periplasm for holotoxin assembly as is the case with Escherichia coli heat labile toxin (LT) and cholera toxin (Hirst et al 1984a, Hofstra and Witholt, 1984). Treatment of the A subunit with protease and reduction has demonstrated that the A subunit comprises two peptides; A_1 and A_2, of approximately 27 - 28KDa and 4 - 5KDa respectively (O'Brien and LaVeck, 1983). Following treatment with protease, these remain linked together by a disulphide bond. Calderwood et al. (1987) propose that fragments of the sizes observed by O'Brien and LaVeck (1983) ie 27 - 28KDa and 4 - 5KDa would result following proteolytic cleavage of SLT IA within the region flanked by cysteine residues 242 and 261 of SLT IA: residues capable of forming a disulphide bridge.

SECTION 1.3 **Comparison of the putative active site of ST / SLT I with that of ricin**

Endo et al. (1988) demonstrated that ST and SLT II were single site RNA-specific N-glycosidases with the capacity to depurinate 28S or 26S rRNA and irreversibly inactivate eukaryotic ribosomes in a manner analogous to that of the plant toxin, ricin. Indeed the degree of amino acid homology observed between SLT IA and ricin A subunit (RA), shown by Calderwood et al. (1987) and DeGrandis et al. (1987), has allowed the putative active site of SLT IA to be defined based on current knowledge of the putative active site of RA (Montfort et al. 1987, Katzin et al. 1991). Alignment of the putative active sites of RA and SLT IA revealed 32% of residues to be identical (Calderwood et al. 1987) (FIG 1.3.1). On this basis the relative importance of amino acid residues within the 'active site' of SLT IA has been investigated with regard to those of RA. This has involved mutation of residues seen to be conserved between the two; working on the basis that residues of enzymatic importance are probably those which appear to have been conserved. Hovde et al. (1988), mutated glutamic acid (Glu) residue 167 of SLT I A subunit to aspartic acid (Asp) based on knowledge from the crystallographic structure of RA which shows glutamic acid residue 177 to lie within the active site cleft. Mutation of this residue in SLT IA resulted in a 1000 fold decrease in in vitro RNA N-glycosidase activity of SLT IA. By comparison, conversion of this residue in RA to Asp

FIG 1.3.1 Homology comparison of the putative active sites of Shiga-like toxin I A subunit (SLT IA) and ricin A subunit (RA)



Shiga-like toxin I A subunit ●

ricin A subunit ▼

Numbers relate to amino acid sequence in each case

resulted in an 80 fold decrease in activity (Schlossman *et al*. 1988), whilst its conversion to glutamine or alanine resulted in a 1000 fold and 20 fold decrease in enzymatic activity respectively (Ready *et al*. 1991 and Schlossman *et al*. 1989 respectively). It is also interesting to note that glutamic acid is also to be found at the active sites of the bacterial toxins *Pseudomonas* exotoxin A (Caroll and Collier, 1987), pertussis toxin (Cortina and Barbieri, 1989), diphtheria toxin (Tweten *et al*. 1985, Wilson *et al*. 1990) and SLT II. Mutation of the 'active site' glutamic acid residues of these various bacterial toxins has shown them to play an important role in enzymatic activity. Conversion of Glu 553 in *Pseudomonas* exotoxin A to Asp reduced the *in vitro* enzymatic activity by 10,000 fold (Douglas and Collier, 1987). Tweten *et al*. (1985) reported that conversion of Glu 148 to Asp in diphtheria toxin resulted in a mutant with only 0.6% the activity of the wild type; whilst Jackson *et al*. (1990), demonstrate a 100 fold reduction in activity of SLT II on conversion of Glu 166 to Asp.

More recently Frankel *et al*. (1989), have suggested that two regions of sequence homology between SLT IA and RA appear to be involved in RNA N-glycosidase activity of the RA and SLT IA subunits. With regard to this, when mutated to serine (Ser), tyrosine (Tyr) residue 77 in SLT I A has been found to cause a 1000 fold reduction in activity (Deresiewics *et al*. 1992). This residue may be seen to be conserved across the RNA N-

glycosidases of which ricin is the type member (Ready et al. 1988). Mutation of the corresponding tyrosine residue (Tyr 80) in RA to phenylalanine (Phe) resulted in a 15 fold reduction in activity (Ready et al. 1991). Such a reduction in activity compares favourably with the observed reduction in activity (10 -20 fold) of SLT IA on conversion of Tyr 77 to Phe (Deresiewics et al. 1992). Deresiewics et al. (1992) feel that Tyr 77 plays an important role in the enzymatic activity of SLT IA. They do not however, eliminate a change in conformation resulting from mutation of Tyr 77 as a reason for the observed reduction in activity of SLT IA. Molecular modelling reveals that the phenolic ring of Tyr 77 is one of three in a cluster close to the proposed active site cleft of SLT IA. Molecular modelling of RA has revealed that the corresponding Tyr 80 residue is also one of three aromatic residues in the putative active site cleft of RA.

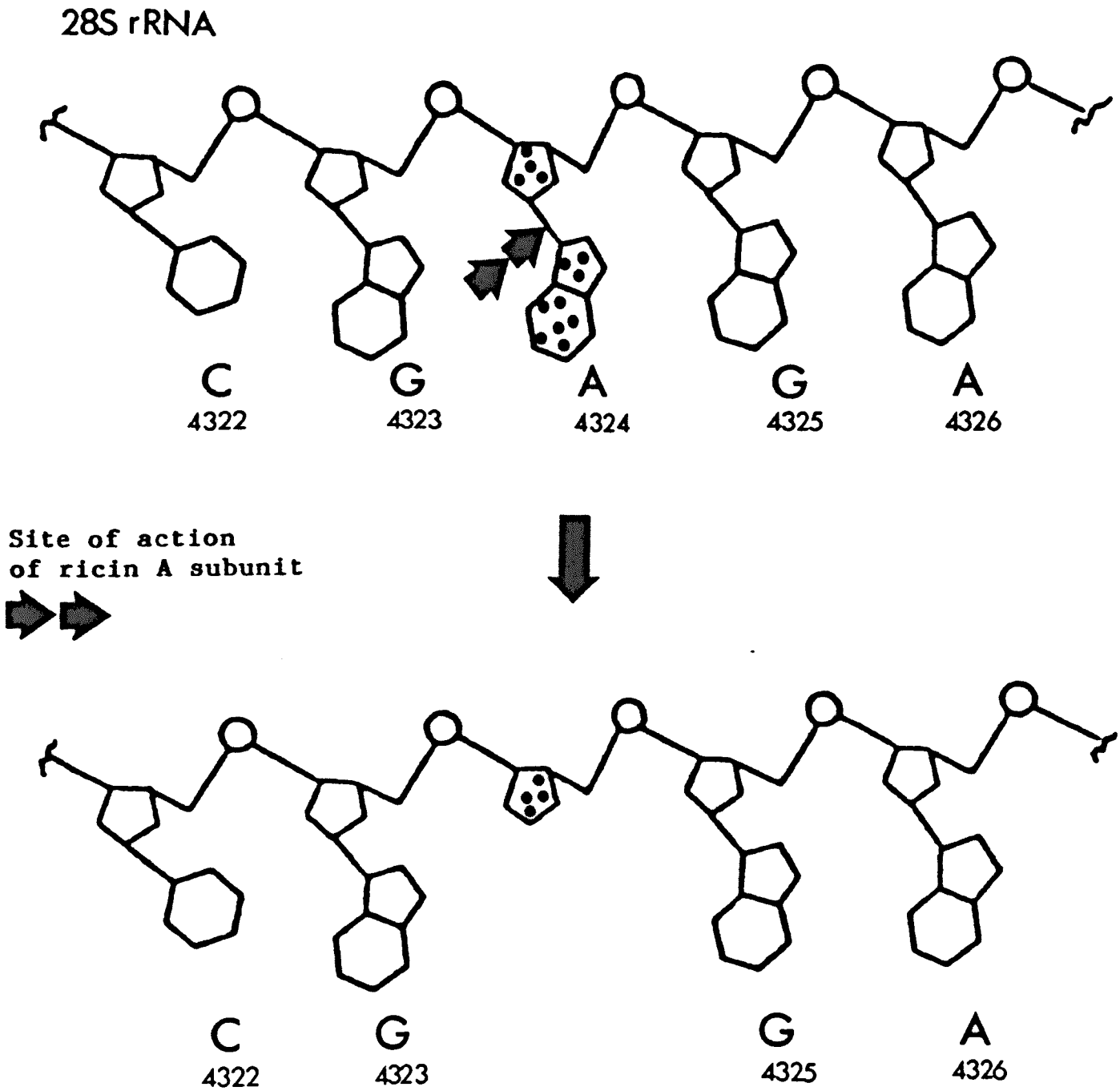
SECTION 1.4 **RNA N-glycosidase activity of ricin and ST / SLT I**

Of particular interest to this study is the RNA N-glycosidase activity of ST A subunit, which has been shown to be comparable to that of the plant toxin ricin (Endo et al. 1987, Endo and Tsurugi, 1987, Endo et al. 1988), leading to an irreversible inhibition of protein synthesis in eukaryotic cells and eventual cell death.

Reisbig et al. (1981), had previously demonstrated that the A_1 fragment of ST was capable of inactivating salt washed rabbit reticulocyte and salt washed rat liver ribosomes at a rate of 40 ribosomes per minute by selectively inactivating the 60S ribosomal subunits. Endo et al. 1988 went on to examine this inactivation at the molecular level on the basis of his own observations with regard to the action of ricin on eukaryotic ribosomes. Nucleotide sequence analysis of the 3' terminal fragment released from rat liver 28S rRNA following modification by ST and SLT II and aniline cleavage revealed that both specifically removed adenine residue 4324 (A_{4324}) of rat liver 28S rRNA. This was achieved by cleavage of the N-glycosidic bond between the adenine residue at position 4324 and the connecting ribose sugar of the 28S rRNA. The observed depurination of the 28S rRNA of rat liver ribosomes at (A_{4324}) by ST and SLT II was exactly the same as that previously observed to be the case with ricin (Endo et al. 1987, Endo and Tsurugi, 1987) (FIG 1.4.1).

Further comparisons between ST, SLT II and ricin were made by Saxena et al. (1989). Ricin, ST and SLT II were all microinjected into Xenopus oocytes in order to study their effects in living cells. Nucleotide sequencing of the rRNA extracted from the modified oocyte ribosomes revealed that in each case the same adenine residue - 3732 had been removed from the 28S rRNA, thus augmenting the fact that the RNA N-glycosidase activity demonstrated by ricin, ST and SLT II is

FIG 1.4.1 Site of depurination of rat liver 28S ribosomal RNA (rRNA) by the action of ricin A subunit



highly specific.

Since these studies reveal that ST and SLT II have an identical action on rRNA then it is reasonable to assume that they ought to have an identical effect on the partial reactions of protein synthesis.

ST had been shown to inactivate eukaryotic protein synthesis both *in vivo* (Brown *et al*. 1980) and in *in vitro* systems (Brown *et al*. 1980, Olsnes *et al*. 1981) by inhibiting peptide elongation (Brown *et al*. 1980). Obrig *et al*. (1987) then went on to examine the mechanism by which ST inhibited the process of peptide elongation demonstrating that the action of ST on ribosomes directly inhibits elongation factor 1 (EF1)-dependant transfer of aminoacyl-tRNA. It is suggested from their findings that the action of ST on the ribosome actually causes some structural changes to the ribosome in the region of the ribosome normally concerned with binding of elongation factor 1. Igarashi *et al*. (1987) confirmed that this was also the case with SLT I from their studies on the ability of hybrid (ribosomes in which either the 60S or 40S ribosomal subunits had been treated with SLT I) to synthesise polyphenylalanine; (polyPhe) finding that only those ribosomes containing a SLT I treated 60S subunit gave a significant decrease in polypeptide synthesis. They concluded that SLT I behaved in a manner identical to that observed with ST inactivation of eukaryotic ribosomes. More recently Furutani *et al*. (1992) compared the

mechanisms by which ricin and SLT II inhibited protein synthesis. Their overall conclusion was that SLT II and ricin both inhibit protein synthesis in exactly the same way - by interfering with the process of binding of the aminoacyl-tRNA to the ribosomes. Results revealed that both toxins inhibited EF1- and GTP-dependant aminoacyl-tRNA binding to ribosomes and in addition, were found to inhibit the EF1-dependant GTPase activity during binding of the above mentioned aminoacyl-tRNAs to the ribosomes.

In most cases however, these studies were performed using non physiological systems involving salt washed ribosomes and the addition of elongation factors and translation of polyPhe RNA (a transcript which cannot undergo physiological initiation). In addition, problems which may be encountered in the use of such systems include cross contamination of elongation factors and failure to add factors to saturation.

Originally RA was thought to affect both EF1 and EF2-dependent stages of protein synthesis. However, recent studies by Osborn and Hartley (1990) to examine the effect of RA on the partial reactions of translation of globin messenger RNA (mRNA) in a reticulocyte lysate system (a physiological translation system), have shown the primary block by RA to be the EF2-dependent stage. It is presumed therefore, that this is also the case with inhibition of protein synthesis by ST , SLT I and SLT II. Osborn and Hartley (1990) clearly demonstrated that RA

modification of the 60S subunit reduced the rate of association of that subunit with the 40S preinitiation complex by a factor of six and that the EF2-dependant translocation step was completely blocked. This confirms the earlier view of Nilsson and Nygard (1986) but is inconsistent with the findings of Igarashi et al. (1987) whose work on ST and SLT I as described above, revealed that ST and SLT I modification of ribosomes inhibited the binding of aminoacyl-tRNA to ribosomes but did not affect the translocation step.

Recent studies have examined the kinetics of RA-catalysed depurination of eukaryotic ribosomes. Depending upon the source of ribosomes and the assay conditions RA has been found to have K_m values of 0.1 - 2.6uM and k_{cat} values of 201 - 1500min^{-1} (Endo and Tsurugi, 1988, Osborn, 1990, Chaddock, 1991, Ready et al. 1991). Little kinetic investigation into ST / SLT I / SLT II-catalysed depurination of ribosomes has been carried out. Reisbig et al. (1981) do however, report the A_1 fragment of the ST A subunit to catalyse the depurination of 40 ribosomes min^{-1}.

It may be seen that most comparisons have been made between the activities of ST and ricin. Since SLT I is regarded to be virtually identical to ST (O'Brien and LaVeck, 1982), and thus information relating to the activity of SLT I has been derived from knowledge of the activity of ST. This present study

directly addresses a comparison between the catalytic activity of ricin and SLT I.

SECTION 1.5 **Structural comparison of ST / SLT I with other bacterial toxins**

Further to this study are the structural comparisons which may be made by comparing SLT I with other bacterial toxins such as cholera toxin (CT) and E.coli heat labile toxin (LT) in addition to DT and PE. Such comparisons allow the delegation of specific roles to each domain of SLT I.

The bipartite nature of ST and SLT I is typical of many bacterial toxins, comprising an enzymatically active A domain and a cell binding B domain (Olsnes et al. 1981) (FIG 1.5.1). The holotoxin has a molecular mass of approximately 70KDa. It comprises a single A subunit (SLT IA) of molecular weight 32KDa non covalently associated with multiple copies of a B subunit. Each B subunit is of approximately 7.7KDa in size, as deduced from the nucleotide sequences of stxA and stxB respectively (Kozlov et al. 1988, Strockbine et al. 1988). The B subunits are thought to associate with the single A subunit as a pentameric structure (Olsnes et al. 1981, Donohue -Rolfe et al. 1984, O'Brien and Holmes 1987, Stein et al. 1992) comparable to that of CT (Gill, 1976) and LT (Sixma et al 1991). From examination of the crystal structure of the B subunit (Stein et

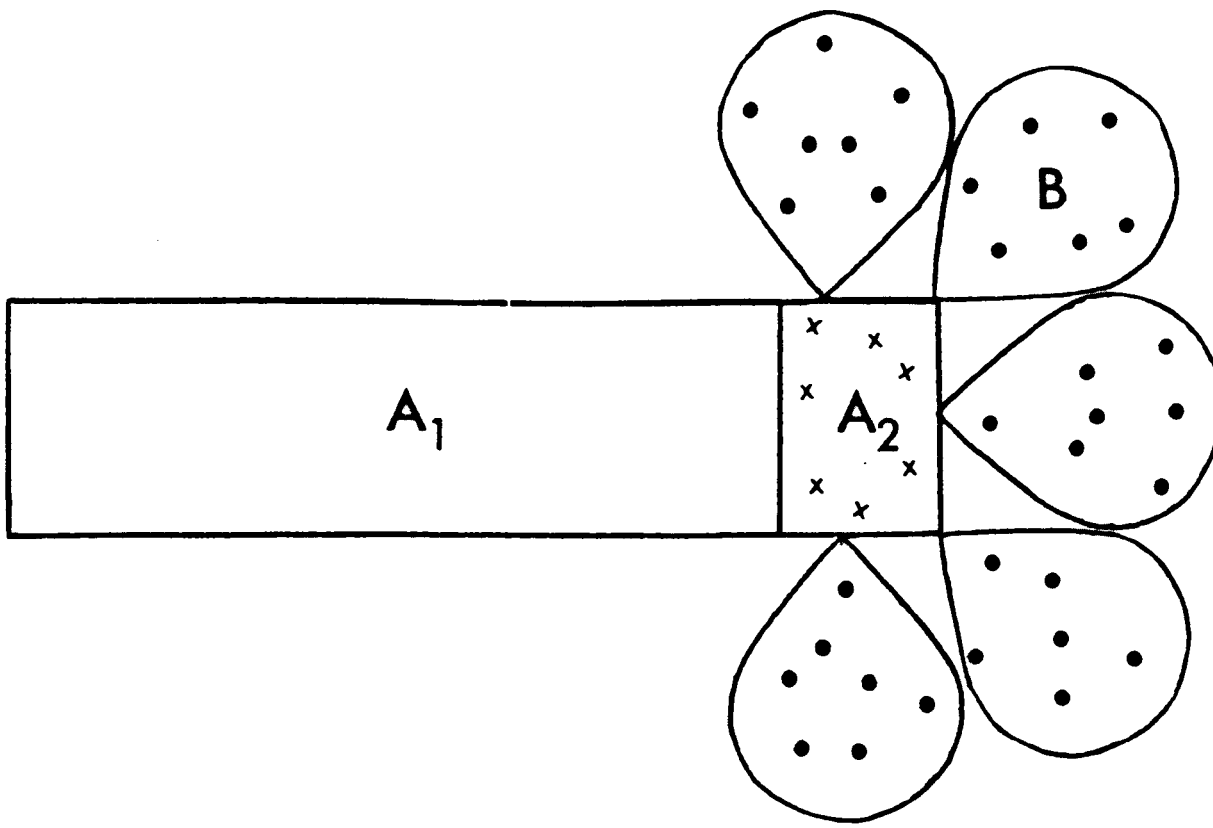


FIG 1.5.1 **Proposed subunit arrangement of Shiga-like toxin I**

al. 1992) suggest that this conveys five potential binding sites per molecule of holotoxin. Alternatively, it is suggested by Hart et al. (1991) from high resolution X-ray analysis of crystallised SLT IB that the B subunits associate with the SLT IA subunit in the form of a tetramer.

Examination of the crystal structure of the SLT I B subunit by Stein et al. (1992) revealed that although there is no primary sequence homology between SLT IB and the B subunit of LT, both subunits adopt a similar structural fold, if allowance is made for the larger size of LT B (103 amino acids) compared to SLT IB (69 amino acids) (Spangler, 1992) (FIG 1.5.2). The similarities in three-dimensional structure led Stein et al. (1992) to suggest that the B subunits may have diverged from a common ancestoral pentameric protein. This protein they suggest, may not have originated from another toxin, instead it may originally have been involved in the recognition of cell surface carbohydrate moieties. From their results Stein et al. (1992) conclude that a distant evolutionary relationship may exist between the Shiga toxin and cholera toxin families.

Considerable structural similarities are seen to exist between CT, LT and ST / SLT I. All are comprised of a single enzymatically active A subunit with the multiple B subunits responsible for cell binding being non covalently associated with the A_2 peptide (Gill, 1976, Sixma et al. 1991 and Olsnes et al. 1981 respectively). It is with regard to these apparent

FIG 1.5.2 **Amino acid sequences of Shiga-like toxin I B subunit and Escherichia coli heat labile toxin (human isolate).**

Residues are numbered above sequence

Amino acid sequence SLT IB (Strockbine et al. 1988)

```
-20                                                 1
M K K T L L I A A S L S F F S A S A L A T P D C V T G K V E Y T

                20                                                  40
K Y N D D D T F T V K V G D K E L F T N R W N L Q S L L L S A Q

                                                60
I T G M T V T I K T N A C H N G G G F S E V I F R
```

Amino acid sequence LT (human strain) (Spangler, 1992)

```
1                                                   20
A P Q S I T E L C S E Y H N T Q I Y T I N D K I L S Y T E S M A

                40                                                  60
G K R E M V I I T I K S G A T F Q V E V P G S Q H I D S Q K K A

                                                80
I E R M K D T L R I T Y L T E T K I D K L C V W N N K T P N S I

      100
A A I S M E N
```

similarities that we may speculate on the role of the A_2 region of ST / SLT I.

On closer examination both CT and LT may be regarded as tripartite molecules (Gill *et al.* 1981 and Sixma *et al.* 1991 respectively) each clearly comprising a single enzymatically active A_1 peptide, a pentamer of B subunits responsible for binding of the toxin to the cell surface receptor ganglioside GM_1, plus an A_2 peptide which has been proposed to play an important role in membrane translocation of the A subunit (Gill *et al.* 1981). In addition, it is with the A_2 region that the B subunit pentamer associates non covalently (Gill, 1976, and Sixma *et al.* 1991). Recent studies on the A_2 regions of CT (Jobling and Holmes, 1992) and LT (Streatfield *et al.* 1992) have strongly implicated the A_2 region in the stable association of the relative B subunit pentamers with the A subunit to form holotoxin.

Keusch (1981) has proposed that ST / SLT I should also be regarded as a tripartite molecule. He has suggested that the A_2 peptide may be responsible for translocation of the enzymatically active A_1 peptide to its intracellular target, following the successful binding and endocytic uptake of the holotoxin. However, it should be stated that even as yet, there is no direct evidence for A_2 playing a role in protein translocation. In addition and far more likely, the A_2 peptide may also be considered to play an important structural role

with regard to its interaction with the multiple B subunits . In this sense, it would be similar to the equivalent portion of CT and LT.

SECTION 1.6 **Binding and entry of ST / SLT I**

As is the case with other bacterial toxins for example PE and DT, it is necessary for the A subunit of ST and SLT I to enter the cell in order to gain access to its cytosolically located substrate; in this case, the ribosome. It has been suggested (Middlebrook and Dorland, 1984) that intracellularly acting bacterial toxins such as ST and SLT I may in fact utilise the cellular glycolipid receptors which would normally facilitate the binding of glycoprotein hormones.

Entry of ST and SLT I is mediated by binding of the B subunit to a cell-borne glycolipid receptor - globotriosylceramide (Gb_3) ie a Galα 1-4Galβ 1-4Glu trisaccharide linked to a ceramide (Cohen *et al*. 1987, Lindberg *et al*. 1987, Lingwood *et al*. 1987), facilitating receptor mediated endocytosis (RME). Binding of the SLT IB to the Gb_3 receptor is highly specific requiring that at least the terminal disaccharide Gal-α1-4Galβ be available (Tesh and O'Brien, 1991). Lindberg *et al*. (1987) found that the insensitivity of certain cell lines was afforded by a lack of Gal-α1-4Galβterminating glycolipid receptors required for the high specificity binding of ST.

Once bound to the cell, the next requirement is for entry of the enzymatically active A subunit into the cell cytosol. Keusch (1981), proposed receptor mediated endocytosis as the mechanism by which the ST A subunit accesses the cytosol. Sandvig and Brown (1987) and Sandvig et al. (1989) have demonstrated entry of ST to be via endocytosis through clathrin coated pits. Sandvig et al. (1991) then presented evidence to suggest that a proportion of internalised ST was transported to the trans Golgi network (TGN) as had previously been seen to be the case with ricin (Van Deurs et al. 1986). Sandvig et al. (1992) have recently visualised ST within the lumen of the endoplasmic reticulum (ER) and nuclear envelope of chemically stimulated A431 cells. It has therefore been suggested that the early Golgi or even ER itself, may be the compartments physiologically important for the subsequent membrane translocation step which precedes ribosome inactivation. This hypothesis has been proposed by Pelham et al. (1992).

SECTION 1.7 **Intracellular processing and translocation of ST / SLT I compared with that of other bacterial toxins**

In common with many other bacterial toxins is the presumed requirement for proteolytic processing of endocytosed ST and SLT I in order to generate an enzymatically active particle. It remains unclear as to where such processing occurs within the target cell, but such processing is presumed to be obligatory (Olsnes et al. 1981, Reisbig et al. 1981).

Many bacterial toxins are secreted as inactive precursor forms requiring a certain degree of proteolytic processing in order to 'activate' them. Such a requirement is seen in not only ST (Olsnes et al. 1981) and SLT I but also in PE (Leppla et al. 1978, Ogata et al. 1990, Fryling et al. 1992), DT (Collier, 1975, Sandvig and Olsnes, 1981, Moskaug et al. 1989), CT (Gill, 1976), tetanus toxin (Helting and Zwisler, 1974), E.coli heat labile toxin (Spicer and Noble, 1982, Yamamoto et al. 1987) and pertussis toxin (Burns, 1988). In ST and SLTs the processing site is found within the A subunit.

The enzymatically active A subunit of ST and SLT I is comprised of two regions, designated A_1 and A_2. The two are separated by a protease sensitive linker region; treatment with trypsin yielding a 'nicked' A subunit analogous to that seen in DT, CT and LT. However, only under reducing conditions are the constituent A_1 (27.5KDa) and A_2 (4.5KDa) peptides completely

separated, since they are bridged by a single disulphide bond formed between cysteine (Cys) residues 242 and 261, which flank the arginine (Arg)-rich protease-sensitive linker region (FIG 1.7.1). Proteolytic cleavage within an Arg rich region flanked by Cys residues is also evident in DT (Collier, 1975, Moskaug et al. 1989) and PE (Ogata et al. 1990). Possession of a protease sensitive region is commonly found amongst the bacterial toxins; examples include tetanus toxin (Helting and Zwisler, 1974), DT (Collier, 1975, Moskaug et al. 1989, Choe et al. 1992) CT (Gill, 1976, Gill et al. 1981), LT (Gill et al. 1981, Spicer and Noble, 1982, Yamamoto et al. 1984) and PE (Ogata et al. 1990) (FIG 1.7.2).

DT, secreted by β lysogenised strains of Corynebacterium diphtheriae as a single polypeptide proenzyme of 58KDa, yields an enzymatic A subunit of 21KDa and a B subunit of 37KDa on treatment with trypsin and reducing agent. The B subunit is responsible for cell binding and translocation (Caroll et al. 1988). Cleavage occurs within a 14 residue loop structure (residues 187 - 200) (Choe et al. 1992) (FIG 1.7.3). This region may be seen to be arginine-rich. Indeed, cleavage is thought to occur at arginine residues 190, 192 or 193 (Moskaug et al. 1989). After cleavage (nicking) the A and B subunits remain covalently linked by a single disulphide bond between cysteine residues 186 and 201 (Choe et al. 1992). It is thought that reduction of this disulphide bond liberating the free A and B subunits, occurs on exposure of the nicked toxin to the

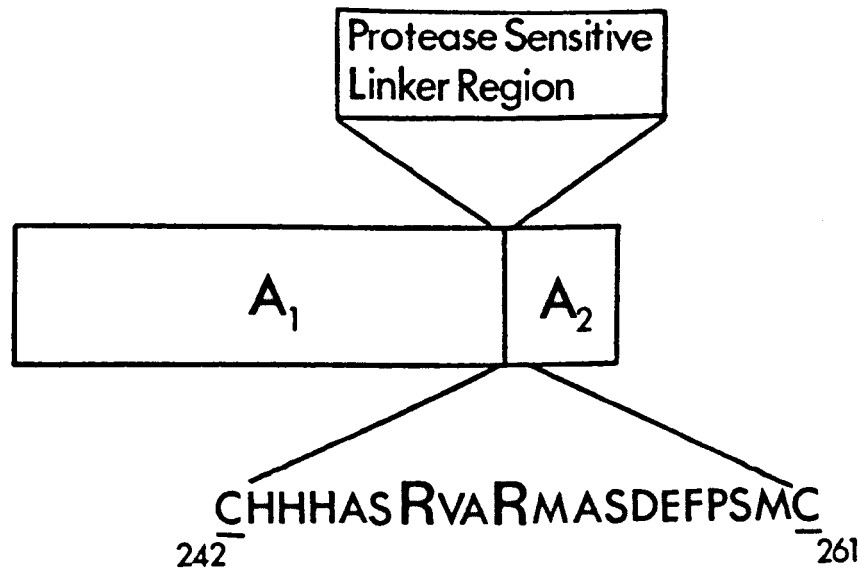


FIG 1.7.1 **The proposed Shiga-like toxin I A subunit (SLT IA) protease sensitive region.**

The amino acid sequence for this region is detailed with the cysteine residues which form a disulphide bridge at this region denoted C.

FIG 1.7.2 **Amino acid sequence at proposed sites of proteolytic processing for diphtheria toxin (DT), Shiga-like toxin I (SLT I) and Escherichia coli heat labile toxin (LT) (human isolate)**

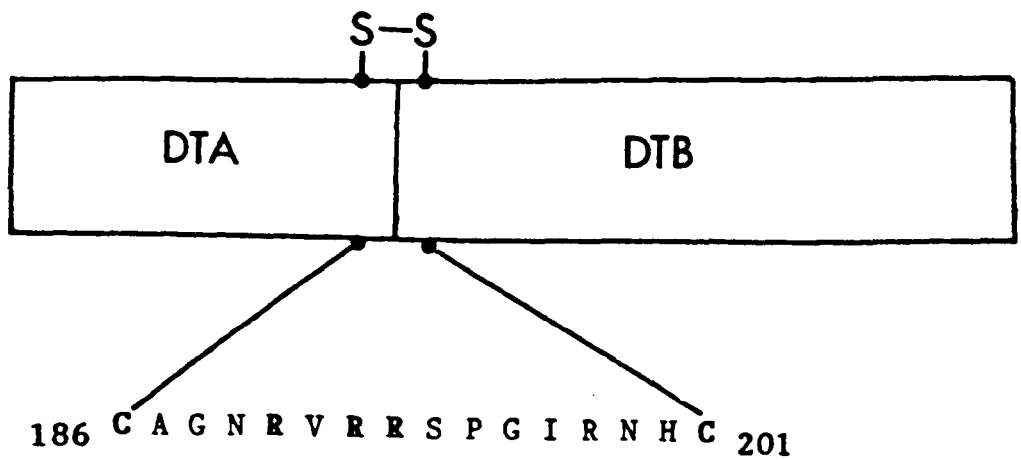
186 **C A G N R V R R S P G I R N H C** 201 **DT**

242 **C H H H A S R V A R M A S D E F P S M C** 261 **SLT I**

187 **C G N S S R T I T G D T C** 198 **LT**

Numbers relate to amino acid sequence in each case

FIG 1.7.3 **Proposed site of proteolytic cleavage of diphtheria toxin (DT)**



S-S denotes a disulphide bond.

DTA and DTB denote diphtheria toxin A and B fragments respectively.

reducing environment of the cytosol during membrane translocation (Sandvig and Olsnes, 1981).

Proteolytic cleavage and reduction of PE, a 66KDa polypeptide, releases a 37KDa enzymatically active fragment (Jinno et al. 1989, Ogata et al. 1990, Fryling et al. 1992). Again proteolytic cleavage may be seen to take place at an arginine-rich region flanked by cysteine residues (residues 265 and 287) within domain II of PE (residues 252 - 364). The importance of proteolytic processing at this site was demonstrated by Jinno et al. (1989). On mutation of Arg 276 and Arg 279 whilst the mutant molecule demonstrated unchanged enzymatic and cell binding properties, its cytotoxicity was greatly reduced. Using radiolabelled PE Ogata et al. (1990) followed the intracellular processing of PE and identified the site of proteolytic cleavage within domain II as being close to Arg 279 within the disulphide loop region bounded by cysteine residues 265 and 287.

Again, a requirement for proteolytic processing has been demonstrated with CT and the closely related E.coli heat labile toxin (LT). Synthesised as a single polypeptide precursor (Gill, 1976), proteolysis generates an A_1 peptide of approximately 21KDa which Gill et al. (1981), have reported as displaying the highest in vitro activity and an A_2 peptide of approximately 5KDa. During secretion CT becomes nicked by haemagglutinin/protease (Booth et al. 1984). In contrast LT is

not secreted from E.coli but remains in an unnicked state in the periplasm (Hirst et al. 1984a). Following secretion the CTA fragments remain linked by a disulphide bond between Cys residues 187 and 198 of CT A subunit. As previously discussed with regard to DT (Sandvig and Olsnes, 1981) the fragments remain linked in this unreduced and essentially inactive state until the toxin enters a cell (Spangler, 1992). With regard to generation of an enzymatically active A_1 peptide in vivo; Janicot et al. (1991) put forward the suggestion, based on their findings, that processing of the CT A subunit takes place within an acidic endosomal compartment. It is then from this compartment that the enzymatically active A_1 peptide is released. This location would be compatible therefore, with the findings of Ogata et al. (1990) and Fryling et al. (1992) that the PE processing enzyme is enriched in endosomal fractions. Location of a protease known to be responsible for the intracellular processing of PE (Fryling et al. 1992) suggests that the same protease may be responsible for the processing of other toxins. Its endosomal location has not however, been convincingly demonstrated. The presence of such a protease in all eukaryotic cells is also an unknown factor.

DTA has also been shown to be released from an endosomal compartment (Papini, 1987, Moskaug et al. 1991). Following binding of the DT B subunit (DTB) to a cell-borne receptor, the toxin is endocytosed to endosomes where the toxin undergoes conformational changes. Such conformational changes are thought

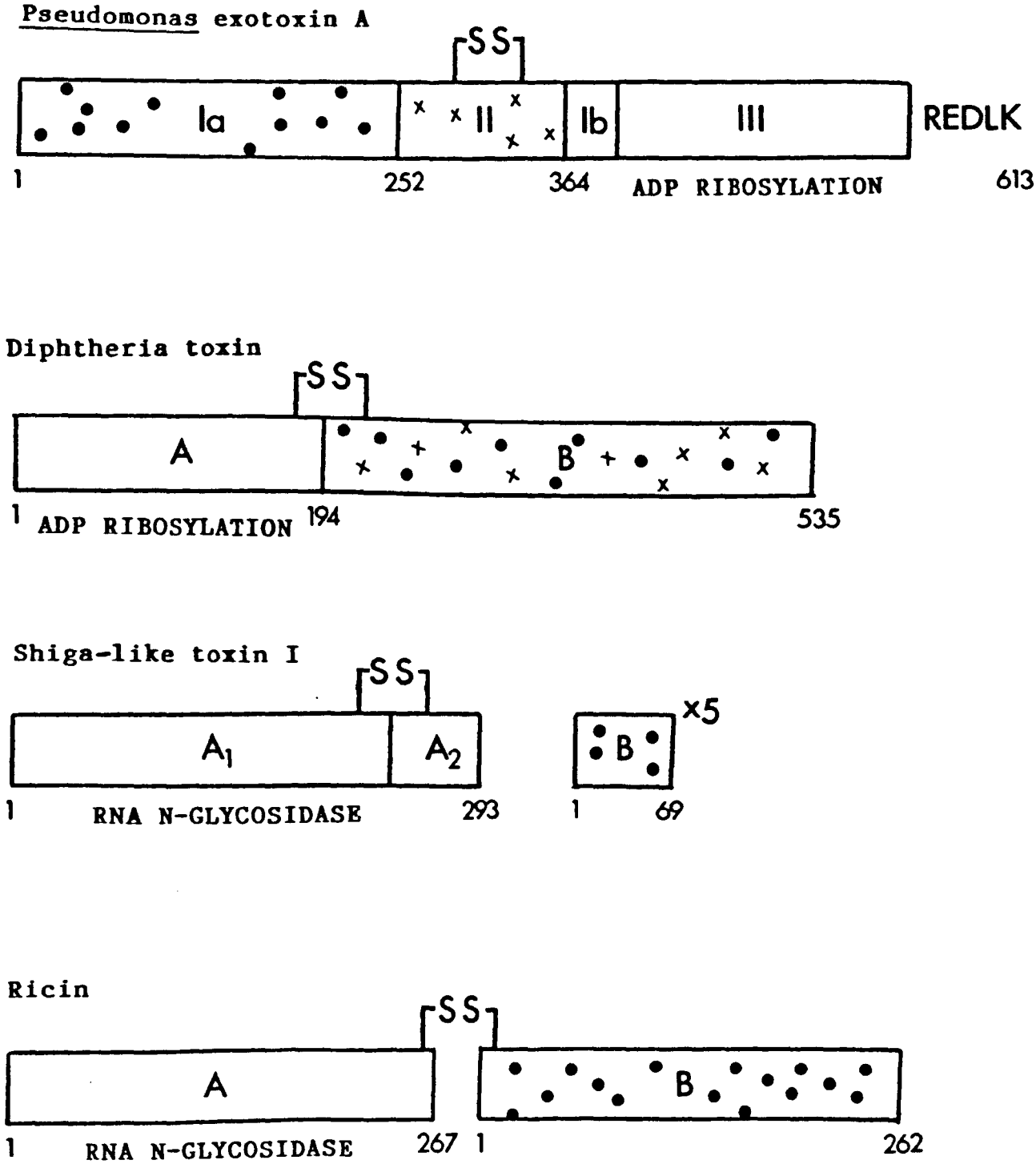
to promote insertion and formation of an ion-selective channel in the endosomal membrane allowing translocation of DTA into the cytosol (Papini, 1987, Moskaug et al. 1991). It is thought, although as yet unknown, that the low pH environment of the endosome may trigger conformational changes in the DT molecule leading to its insertion into the endosomal membrane (Choe et al. 1992).

The contribution of the A_2 peptide of ST and SLT I to the enzymatic activity remains unclear, since Olsnes et al. (1981) and Reisbig et al. (1981), demonstrate that in the case of ST, the A_1 peptide alone, is approximately six times more active in vitro than the unnicked, non reduced ST A subunit. A further report (unpublished) suggests that A_2 is not necessary for activity of the A subunit (Paige Anderson, Boston MA); whilst Kongmuang et al. (1988) have demonstrated that there is no difference in the cytotoxic nature of either nicked or unnicked SLT I. However, the necessity for proteolysis and reduction in order to release an enzymatically active subunit, suitable for efficient membrane translocation has been demonstrated in other intracellularly acting bacterial toxins such as PE and DT. It may be rationalised that if the structural arrangement of SLT I is as shown in FIG 1.5.1, then proteolysis in the hypersensitive region and reductive cleavage would afford a means of releasing a catalytically active A_1 fragment from the rest of the toxin. This would make the release of A_1 analogous to that of the release of the A

fragment from the B fragment of DT (Sandvig and Olsnes, 1981, Moskaug et al. 1989) and domain III from the N terminal portion of PE (Ogata et al. 1990, Fryling et al. 1992) (FIG 1.7.4), ie the active enzyme can be released from the cell binding portion of the toxin to cross the membrane and effect ribosome inactivation. Alternatively, it may be necessary to cleave the A subunit in order to allow full expression of A_1 catalytic activity, and have nothing to do with allowing effective membrane translocation to access the cytosol in the absence of the cell binding domain.

In order to examine the proposed contribution of the SLT I A_2 peptide to translocation of the enzymatically active A_1 subunit experiments similar to those carried out to define the proposed translocation domain of PE could be undertaken. Of the three functional domains constituting PE, it is domain II (residues 253 - 364) which has been shown to be an essential requirement for translocation of the enzymatically active domain III (Hwang et al. 1987) across the cell membrane (Jinno et al. 1989). Perhaps the most conclusive evidence for the translocation role of domain II of PE comes from the work of Prior et al. (1992) who by construction of a fusion protein comprising PE domain II and barnase (the extracellular ribonuclease of Bacillus amyloliquefaciens) successfully demonstrated the translocation of a foreign protein to the cell cytosol. They concluded that domain II, essentially independent of other PE domains, was

FIG 1.7.4 Schematic representation of Pseudomonas exotoxin A (PE), diphtheria toxin (DT), Shiga-like toxin I (SLT I) and ricin.



sufficient for translocation of a soluble enzyme to the cell cytosol.

The contribution of the ST / SLT I A_2 peptide to the enzymatic activity of the toxin is also pertinent to this study. Comparison of SLT I to other bacterial toxins may suggest what this role may be, but as yet this remains unclear. The need for proteolytic processing of SLT IA into A_1 and A_2 will therefore be investigated by studying the effects of a non cleavable, recombinant SLT IA on ribosomes *in vitro* and on cultured cells. Comparisons will be made with a recombinant wild type SLT IA. It is hoped that a clearer picture will emerge as to the contributions (advantageous, neutral or deleterious) of A_2 towards the *in vitro* enzymatic activity of SLT IA. The cytotoxic effects of a holotoxin containing such a non cleavable A subunit will also address the issue of whether proteolytic processing is essential for cell killing.

SECTION 1.8 **Project Aims**

1. Production of biochemically pure SLT I in order to allow a direct comparison to be made between the RNA N-glycosidase activity of SLT I and ricin.

2. Production, by oligonucleotide site directed mutagenesis, of SLT IA subunit mutants in order to examine any contributory role of the A_2 peptide in RNA N-glycosidase activity of SLT IA and to examine the necessity of the proteolytic processing step for the expression of cytoxicity.

SECTION 2 MATERIALS AND METHODS

SECTION 2.1 MATERIALS

The majority of analytical grade chemicals used were obtained from either BDH Chemicals Ltd. or FSA/Fisons where available. The sources of other specific reagents are detailed below.

Amersham International PLC, Amersham, Buckinghamshire.

Amplify^{TM}
[α^{35}S] dATP (10mCi/ml, >1200Ci/mmol)
L-[^{35}S] methionine (15mCi/ml, >1000Ci/mmol)

Biocarb AB, S-223 70 Lund, Sweden.

Globotriose-Fractogel TSK gel matrix

Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire.

Bio-Rad Assay protein determination kit, low range SDS PAGE molecular weight standards

Boehringer Mannheim (UK) Ltd., Lewes, East Sussex.

calf intestinal phosphatase, creatine kinase, creatine phosphate, deoxyribonucleotides, dideoxyribonucleotides

DIFCO Laboratories, Basingstoke, Hampshire.

Bacto-agar, Bacto-peptone, Bacto-tryptone, yeast extract

FSA/Fisons, FSA Laboratory Supplies, Loughborough, Leicestershire.

acetic acid (glacial), acids and organic solvents, 2-mercaptoethanol, urea

GIBCO/BRL, Life Technologies Ltd, Paisley, Renfrewshire, Scotland.

T4 DNA ligase, SP6 RNA polymerase, T7 RNA polymerase, ultra-pure RNAse free sucrose

Kodak Ltd., Hemel Hempstead, Hertfordshire.

N,N'-methylenebisacrylamide (electrophoresis grade)

Life Sciences Inc., St Petersburg, Florida, USA.

AMV reverse transcriptase

LKB-Produkter AB, Bromma, Sweden.

Ultropac TSK DEAE-5PW HPLC column

MERK/BDH BDH Chemicals Ltd., Poole, Dorset.

acrylamide (Electran^{TM}), aniline, bromophenol blue, Duolite MB6113 mixed resin, sodium dodecyl sulphate, polyethylene glycol 6000, triton X-100

Northumbria Biologicals Ltd., Cramlington, Northumberland.

restriction endonuclease Ssp I

Pharmacia (UK) Ltd., Milton Keynes, Buckinghamshire.

m7G(5')ppp(5')G (CAP), restriction endonucleases

Promega Biotechnology, P&S Biochemical Ltd., Liverpool, Merseyside.

RNasin, rabbit reticulocyte lysate

Sigma Chemical Company Ltd., Poole, Dorset.

ampicillin, bovine serum albumen (BSA), Coomassie brilliant blue, dithiothreitol (DTT), ethidium bromide, L-amino acids, phenylmethylsulphonyl fluoride (PMSF), polymyxin B sulphate, sephadex G100-120, TPCK trypsin

United States Bioscience, Cambridge Bioscience, Cambridge.

Sequenase^{TM} DNA sequencing kit, T7-GEN^{TM} <u>in vitro</u> mutagenesis kit, T4 polynucleotide kinase

Whatman International Ltd., Maidstone, Kent.

3MM chromatography paper

Recombinant ricin A subunit used in this study was obtained from ICI, Alderley Edge, Cheshire. The non-glycosylated, recombinant protein is identical in primary amino acid sequence to native, plant purified ricin A subunit. It does however, contain an N-terminal methionine prior to the native N-terminal isoleucine residue.

SECTION 2.2 GROWTH AND MAINTENANCE OF BIOLOGICAL MATERIALS AND BIOCHEMICALS.

2.2.1 Bacterial cultures.

All bacterial strains used in the following study are listed in Appendix 1.

(i) Maintenance.

Stock laboratory cultures were maintained at 4°C on minimal media/glucose plates: 1xM9 salts (10x M9 salts is 60g/L Na_2HPO_4, 30g/L KH_2PO_4, 10g/L NH_4Cl, 5g/L NaCl) 1mM $MgSO_4$,1mM thiamine HCl, 0.1mM $CaCl_2$, 0.2% w/v glucose, 1.5% Bacto-agar. In this way bacterial cultures could be stored for up to one month prior to replating.

(ii) Growth and short term storage on solid media.

L-broth (1% w/v Bacto-tryptone, 0.5% w/v yeast extract, 1% w/v NaCl) solidified with 1.5% w/v Bacto-agar (L-agar) provided short term maintenance of bacterial strains. Supplemented with ampicillin to a final concentration of 100ug/ml this provided selection for cells transformed with plasmid DNA

carrying antibiotic resistance to ampicillin. Plates were then incubated inverted at 37°C overnight. Transformant colonies were then streaked on to fresh L-ampicillin plates, where following growth at 37°C they were maintained at 4°C for up to 2 weeks.

(iii) Growth in liquid media.

10mls of L-broth inoculated with a single colony was incubated at 37°C in an orbital shaker (200rpm) overnight, resulting in an overnight culture. Addition of ampicillin to a final concentration of 100ug/ml again providing a means of selection for growth of transformant colonies.

2.2.2 Yeast cultures

ABYS 1, a vacuolar protease deficient strain of yeast (Achstetter et al., 1984) was used for the production of yeast ribosomes. Liquid cultures were grown at 30°C in an orbital shaker at 200rpm in YPD (1% w/v yeast extract, 2% w/v Bacto-peptone, 2% w/v glucose) supplemented with 1% v/v uracil and 1% v/v adenine each originally a 1mg/ml stock. Cells were maintained on YPD plates (YPD solidified with 2% w/v Bacto-agar) at 4°C for up to 4 weeks. Growth and

storage of yeast cultures was as described by Sherman et al. (1986).

2.2.3 Storage of Ribosome Inactivating Proteins.

Shiga-like toxin I was stored at -20°C in PBS (8g/L NaCl, 0.2g/L KCl, 1.44g/L Na_2HPO_4, 0.24g/L KH_2PO_4, pH7.2). Ricin and recombinant ricin A chain (rRA) (ICI) were stored at 4°C.

SECTION 2.3 PREPARATION OF RIBOSOMES.

(i) Yeast ribosomes.

A 500ml overnight culture of yeast ABYS 1 was grown as detailed in section 2.2.2 to an OD of 2 at 600nm. This was then harvested at 5,000 x g, 4°C for 5mins. The resultant cell pellet was washed twice by resuspending in sterile distilled water (SDW). The washed pellet was then resuspended in 3 pellet volumes of lysis buffer (100mM KOAc, 2mM MgOAc, 20mM HEPES-KOH pH7.4, 20% v/v glycerol with the addition of 0.005 volumes 20mg/ml PMSF prior to use). 0.5 volumes of acid washed glass beads (40 mesh) were

then added and the mixture vortexed at 4°C until >50% cell lysis could be seen on microscopic inspection. The glass beads and cell debris were then pelleted and the ribosome containing supernatant centrifuged at 15,000 x g, 4°C for 20mins in order to pellet the mitochondria. The resultant supernatant then underwent ultracentrifugation at 50,000 x g, 4°C for 3 hours which yielded a pellet of ribosomes. The ribosome pellet was washed in 500ul Endo buffer (25mM Tris HCl pH7.6, 25mM KCl, 5mM $MgCl_2$) and gently resuspended in 500ul of the same buffer using an acid washed glass rod. The ribosome concentration was calculated using the formula that 1mg/ml of ribosomes is equivalent to 12.5 OD units at 260nm.

(ii) Salt washed wheatgerm ribosomes.

20g of high grade, milled wheatgerm was ground thoroughly in 100mls grinding buffer (200mM Tris HCl pH9, 200mM KCl, 35mM $MgCl_2$, 25mM EGTA, 200mM RNase-free sucrose) plus 0.5 volumes sterile, crushed glass at 4°C using a pre-chilled, alkali-washed pestle and mortar. The debris was pelleted by centrifugation at 5,000 x g, 4°C, 10mins. The resultant supernatant (excluding the surface layer of fat) was then centrifuged again (15,000 x g, 4°C, 15mins) in order to pellet unwanted nuclei and mitochondria. The

ribosome-containing supernatant was layered over a 20ml sucrose pad (1M RNase-free sucrose, 40mM Tris HCl pH9, 200mM KCl, 30mM $MgCl_2$, 5mM EGTA) and centrifuged at 50,000 x g, 4°C, for 3 hours. The resulting ribosome pellet was washed and resuspended in 500ul Endo buffer as in section 2.3(i).

(iii) Rabbit liver ribosomes.

Rabbit liver ribosomes were prepared using a modification of the methods described by Martin et al. (1969) and Moldave and Sadnik (1979).

10g fresh rabbit liver was homogenised in 23mls Medium A (50mM Tris HCl, 12.5mM $MgCl_2$, 80mM KCl, pH7 plus 0.25M sucrose) Martin et al. (1969). Cell debris was removed by centrifugation of the homogenate at 10,000 x g, 4°C for 15mins followed by filtering the supernatant through cheesecloth. The filtrate then underwent ultracentrifugation (50,000 x g, 4°C for 1.5 hours) in order to produce a crude ribosome pellet which was washed and resuspended in 2ml Medium A. This was then spun through a 1M sucrose cushion (1M sucrose in Medium A) at 40,000 x g, 10°C for 4 hours. The resultant ribosome pellet was resuspended in 500ul Endo buffer as previously described (2.3(i)).

Ribosomes isolated from tobacco leaf, E.coli, and rabbit reticulocytes were kind gifts from Dr Martin Hartley and Andrea Massiah (Warwick).

SECTION 2.4 ISOLATION OF NUCLEIC ACIDS.

2.4.1 Extraction of RNA from ribosomes and cell free translation systems.

Extraction of total RNA from such reaction mixes was achieved by the addition of 0.1 volumes 10% (w/v) sodium dodecylsulphate (SDS) and an equal volume of TE (10mM Tris HCl pH 8, 1mM EDTA) equilibrated phenol chloroform (1:1 v/v). The further addition of 0.1 volumes of 2M NaOAc/acetic acid, pH 6 and 2.5 volumes 100% ethanol to the resultant top aqueous layer followed by incubation at -20°C for from 2 hours to overnight, allowed precipitation of the RNA. The RNA was then pelleted by centrifugation at 11,000 x g at R° in a microcentrifuge and the resultant pellet washed with 70% v/v ethanol and dried in vacuo. The pellet was then redissolved in 10 - 20ul of sterile distilled water and stored at -70°C. The ribosomal RNA (rRNA) concentration was determined using the assumption that at 260nm the optical density (OD) of

a 1mg/ml solution of RNA is 25 (Maniatis et al., 1982).

2.4.2 Preparation of plasmid DNA

(i) Small scale preparation - 'mini-preps'.

The alkaline lysis method as described by Maniatis et al. (1989) provided a rapid means of preparing plasmid DNA for the screening of transformant colonies.

(ii) Large scale preparation.

A modified version of the method described by Holmes and Quigley (1981), was used. 400mls of SOB (2% w/v Bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 10mM $MgCl_2$, 2.5% KCl) with ampicillin added to a final concentration of 100ug/ml, was inoculated with an overnight culture of the plasmid bearing transformant of interest. The culture was incubated overnight at 37C as previously described in section 2.2.1(iii).

Cells were then pelleted at 5,000 x g and 4°C for 10mins before being resuspended in 7.2mls of SET (25mM Tris-HCl pH8, 10mM EDTA, 15% w/v sucrose). 7.2 mls of a 4mg/ml solution of lysozyme in SET was then

added and incubated for 5mins at room temperature prior to the further addition to the suspension of 12mls of 10% w/v Triton X-100. The solution was then briefly boiled over a bunsen flame until it became gelatinous. The flask containing the gelatinous mixture was then plunged first into a boiling water bath and then into icy water. The flask contents were then subjected to centrifugation at 20,000 x g for 30mins at 4°C. In order to precipitate unwanted proteins 0.5 volumes of 7.5M ammonium acetate was then added to the resultant supernatant and the mixture left on ice for 20mins. A further centrifugation - 15,000 x g for 10mins at 4°C pelleted the protein precipitate which was then discarded. Precipitation of the nucleic acids was achieved by the addition to the supernatant of 0.7 volumes of isopropanol and incubation at -20°C for 10mins followed by centrifugation at 15,000 x g , 4°C for 10mins. The nucleic acid pellet was then drained and resuspended in 4mls of low TE (10mM Tris HCl pH8, 0.1mM EDTA).

A caesium chloride gradient allowed purification of the required supercoiled plasmid DNA from the remaining intrinsic nucleic acids present at this stage. To the 4mls of nucleic acid was added 4.3g caesium chloride and 0.5ml (5mg/ml in water) ethidium

bromide. Sealing of the solution in a Beckman 4.5ml 'quick seal' ultracentrifuge tube allowed centrifugation in a Beckman Vti 65 rotor at 50,000 x g at 20°C for 6 hours. The resultant lower band of supercoiled plasmid DNA was removed from the ultracentrifuge tube by syringe and the ethidium bromide extracted from the DNA with isopropanol equilibrated with caesium chloride-saturated TE. Precipitation of the plasmid DNA was by the addition of 4 volumes of Low TE to the ethidium free aqueous phase, the addition of 0.1 volume 3M sodium acetate and addition of 2 volumes of 100% ethanol followed by precipitation at -20°C for 2 hours to overnight.

The precipitated DNA was pelleted at 15,000 x g, 4°C for 10mins, washed with 70% ethanol and dried <u>in vacuo</u> and resuspended in 400ul TE. Storage of such DNA was at -20°C.

(iii) Preparation of bacteriophage M13 replicative form (RF) DNA.

50ul of an overnight culture of TG2 cells plus a single M13 plaque were used to inoculate 5mls of 2YT broth (Bacto Tryptone 1.6% w/v, yeast extract 1% w/v, NaCl 1% w/v) and incubated at 37°C, 200rpm overnight. This was then used to inoculate a further 200mls 2YT

broth with incubation and shaking as before until the culture reached an OD of 0.5 at 550nm. The culture was then shaken at 300rpm, 37°C for a further 4 hours. Preparation of M13 RF DNA was as described for large scale preparation of plasmid DNA.

(iv) Preparation of bacteriophage M13 single-stranded (ss) DNA.

The method of Schreier and Cortese (1979) describes the way in which the positive (+) ss form of M13 (Messing, 1986) vector and M13 derived cloning constructs were prepared. Briefly, a single plaque was used to inoculate 1.5ml of 2YT media containing 100ul exponential phase E. coli JM101. The culture was then incubated with shaking at 37°C for 5 - 6 hours. Centrifugation of the culture (11,000 x g at 4°C for 10min) resulted in a single-stranded phage-containing supernatant. The addition of 0.25 supernatant volumes of 20% polyethylene glycol 800 in 3.5M Na acetate allowed precipitation of the ss DNA by incubation at room temperature for 30min. This was then centrifuged once again (11,000 x g at R° for 10min) in order to pellet the ss DNA. Following thorough removal of the resultant supernatant, the ss DNA pellet was extracted and precipitated (2.4.3) and the final pellet resuspended in 20 - 40ul TE.

2.4.3 Extraction and precipitation of DNA.

Following restriction with restriction endonucleases, DNA was extracted with an equal volume of TE equilibrated phenol chloroform (1:1 v/v) as described by Maniatis et al. (1989). DNA was then precipitated by the addition of 0.1 volumes 3M NaOAc/acetic acid pH 6 plus 2 volumes 100% ethanol as described by Maniatis et al. (1989). DNA was then pelleted by centrifugation , washed , dried and resuspended as described by Maniatis et al. (1989).

2.4.4 Isolation of DNA following gel electrophoresis.

Following gel electrophoresis of restriction digested DNA on an Agarose - TBE gel (see below) required fragments were isolated by elution on to dialysis membrane. Long wave UV light allowed visualisation of the required DNA band and an incision to be made directly ahead of it. Into this cut was inserted a piece of Whatman 3MM paper with a dialysis membrane backing soaked in 1 x TBE buffer. Electrophoresis of the gel at 60mA was then continued this time with the gel unsubmerged. This was continued until the required band had migrated into the paper - prevented from leaving it by the dialysis membrane.

Both were then placed in a 1.5ml Eppendorf tube with a hole pierced in the bottom. This was then placed in a 2ml Eppendorf tube and centrifuged for 1min in a microcentrifuge. 100ul of TE was added to the smaller tube and the centrifugation repeated. Addition of TE and centrifugation was repeated four times in total. The resultant eluate collected in the larger of the two tubes was then centrifuged for a further 5mins in a microcentrifuge in order to pellet any debris. DNA was then precipitated from the supernatant as previously descibed (2.4.3) and then redissolved in an appropriate volume of 1 x TE (10 - 20ul).

2.4.5 Spectophotometric quantitation of DNA.

The concentration of DNA was estimated by measuring the absorbance at 260nm and calculated using the formula that 1 OD unit at 260nm is equivalent to 40ug/ml of double stranded DNA or 30ug/ml single stranded DNA.

SECTION 2.5 MODIFICATION AND MANIPULATION OF NUCLEIC ACIDS.

2.5.1 Treatment of modified rRNA with aniline reagent.

Endo et al. (1987), first described the cleavage of ricin A chain modified rRNA. This method was however, modified by May et al. (1989) and was the method used in this study. 20ul aniline reagent (1M aniline-acetic acid pH 4.5) was added to 2-4ug of modified rRNA contained in a volume of 2-4ul. This was then incubated at 60°C for 2 minutes. Precipitation of the rRNA was achieved by the addition of 0.1 volumes 7M NH_4OAc and 2.5 volumes 100% ethanol followed by incubation on dry ice in the dark for 20mins. The rRNA was pelleted in a microcentrifuge at 11,000 x g for 15mins at 4°C and the pellet washed with 70% v/v ethanol prior to being dried in vacuo.The pellet was then resuspended in 20ul of 60% v/v formamide in 0.1 x Endo buffer (3.6mM Tris, 3mM NaH_2PO_4, 0.2mM EDTA).

2.5.2 Preparation of aniline reagent.

Aniline was distilled twice and stored at -20°C in the dark until required. 1ml of this aniline was then

mixed with 7ml H_2O and 0.5ml glacial acetic acid in a glass universal previously acid washed in order to remove RNase. The pH of the aniline reagent was then made 4.5 with glacial acetic acid and made up to a final volume of 11ml. It could then be stored in the dark at 4°C for up to two months.

2.5.3 Restriction endonuclease digestion of DNA.

Restriction endonuclease digestion of plasmid and M13 RF DNA was carried out using restriction enzymes and buffers supplied by Pharmacia. All digests were incubated for 1.5-2 hours at 37°C except for those involving Sma I, which were incubated at 30°C.

2.5.4 Dephosphorylation of plasmid vector DNA.

Removal of 5' phosphate groups from DNA in order to minimise self ligation of vector DNA was achieved by incubating DNA with calf intestinal phosphatase as in the method described by Maniatis et al. (1989).

2.5.5 Ligation of DNA.

Ligation of DNA was achieved using T4 DNA ligase fromBRL Life Technologies Inc. using conditions recommended by the manufacturer.

2.5.6 Phosphorylation of mutagenic oligonucleotides.

Oligonucleotides used in site directed mutagenesis were phosphorylated exactly as described by the United States Biochemical (USB) T7-GEN^{TM} *in vitro* mutagenesis system manual using T4 polynucleotide kinase.

2.5.7 Site directed mutagenesis.

The USB T7-GEN^{TM} *in vitro* mutagenesis system is based on the work of Vandeyar *et al.* (1988) and serves to increase the percentage mutant progeny by eliminating non mutant parental template at an early stage in the procedure. The mutant strand is synthesised in the presence of 5-methyl-dCTP resulting in a methylated mutant strand. Selection for the mutant strand may then be accomplished by nicking of the parental non mutant strand with an enzyme - *Msp* I which nicks only non methylated DNA and, therefore, not the methylated mutant strand. The nicked strand is then removed by

the action of Exonuclease III, leaving only the mutant strand to act as template. Transformation of this methylated mutant strand into a host E.coli strain lacking the methylation dependant restriction systems mcrA and mcrB, which are ordinarily involved in the restriction of methylated DNA propagates the synthesis of methylated mutant strand DNA. Use of this method greatly enhances the efficiency of the mutagenesis process.

Site directed mutagenesis of SLT IA subunit was achieved using the mutagenic oligonucleotides in FIG 3.3.3 exactly as detailed by the USB T7-GEN™ in vitro mutagenesis system protocol.

2.5.8 Dideoxy-sequencing of DNA.

Dideoxy-sequencing of DNA was carried out using SEQUENASE™ (a modified form of T_7 DNA polymerase) exactly as detailed in the United States Biochemical (USB) SEQUENASE™ manual.

(i) Dideoxy-sequencing of M13 ssDNA.

This was carried out as detailed in the United States Biochemical (USB) SEQUENASE™ manual.

0.5pmoles of primer and 1ug of template DNA were mixed and incubated with 40mM Tris HCl pH7.5, 10mM $MgCl_2$, 50mM NaCl for at 65°C, 2min in a final volume of 10ul. This was then cooled slowly to a temperature below 30°C. 1ul DTT, 2ul labelling mix (1.5uM dGTP, 1.5uM dCTP, 1.5uM dTTP), 0.5ul [α -^{35}S]-dATP (10uCi/ul) and 2ul Sequenase™ (diluted 1:8 in TE) were then added to the annealed template/primer reaction mix and incubated for 5min at room temperature. 3.5ul of this was then transferred to 2.5ul of each of the termination mixes (which had been pre-warmed to 37°C) and incubated at 37°C for a further 5min. To each termination reaction was added 4ul of 95% v/v formamide, 20mM EDTA, 0.05% w/v bromophenol blue, 0.05% w/v xylene cyanol. Prior to gel electrophoresis the reaction mixes were heated to 80°C for 2min. Typically 3ul of each reaction mix was loaded on to a sequencing gel (2.6.3).

(ii) Dideoxy-sequencing of plasmid DNA.

Prior to carrying out dideoxy-sequencing as detailed in the USB SEQUENASE manual, denaturation of the plasmid DNA was necessary. This involved the incubation at room temperature of 1ug DNA in 8ul H_2O with 2ul of 2M NaOH for 10mins. The DNA was then ethanol precipitated as previously detailed and

resuspended in an amount of SDW relevant to the annealing of primer/template reaction as described by the USB SEQUENASE manual.

2.5.9 Primer extension.

The modified method of Moazed *et al.* (1986) as described by May *et al.* (1989) was used in the annealing of oligonucleotide primer to ribosomal RNA and its subsequent extension using reverse transcriptase in order to closely investigate the site of depurination of 28S rRNA by SLT IA. The procedure used was as described by May *et al.* (1989) except that the depurinated rRNA was extracted from yeast ribosomes following their exposure to toxin, that is, either recombinant ricin A subunit (RA) or Shiga like toxin I A subunit (SLT IA).

SECTION 2.6 RESOLUTION OF NUCLEIC ACIDS BY GEL ELECTROPHORESIS.

2.6.1 Formamide - Agarose gels.

Resolution of aniline-treated ribosomal RNA was achieved using 1.2% w/v agarose in 0.1 x TPE buffer

(3.6mM Tris, 3mM NaH_2PO_4, 0.2mM EDTA),50% formamide - 100ml in a 15 x 15cm gel former. 10ul aniline-treated rRNA was heated to 65°C for 5 mins in a denaturation step and cooled briefly on ice. 3ul sample buffer (50% v/v glycerol, 0.1% w/v bromophenol blue) was then added and the rRNA sample loaded on to the gel. Electrophoresis of the gel in 0.1 x TPE buffer, unsubmerged, at 20mA for 2-3 hours was then followed by staining of the gel in 2ug/ml ethidium bromide for 10-20 mins and destaining in distilled water for a further 30 mins. Visualisation was by UV illumination of the gel. The gel was then photographed using a polaroid instant camera and polaroid 667 or 665 film.

2.6.2 Agarose - TBE gels.

Restricted DNA was fractionated on 1% w/v agarose in 1 x TBE (10 x TBE comprises 108g/L Tris HCl, 55g/L Boric acid, 9.3g/L EDTA) as described by Maniatis et al. (1989). Gels were electrophoresed submerged in 1 x TBE buffer containing 0.5ug/ml ethidium bromide at 60mA for 2-3 hours. Visualisation of the resolved DNA was by UV illumination.

2.6.3 Polyacrylamide gels.

Linear 6% w/v acrylamide gels as described by Maniatis *et al.* (1989), were used to resolve the DNA products of dideoxy-sequencing. For a gel of dimensions 20cm x 40cm 21g of urea was dissolved in 5ml 10xTBE with the addition of 7.5ml 40% acrylamide (38g w/v acrylamide, 2g w/v N,N'-methylenebisacrylamide, deionised with Duolite monobed resin MB11) and diluted with SDW to a final volume of 50mls. The gel mix was polymerised using 250ul of 10% w/v ammonium persulphate and 25ul TEMED and poured immediately. Prior to loading samples were denatured at 80°C for 2mins in loading dye (95% formamide, 20mM EDTA, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol). The gel was run at 40W constant power in 1 x TBE for 2 - 3 hours. The gel was fixed in 10% v/v methanol, 10% v/v acetic acid for 15 mins prior to being dried on Whatman 3MM paper at 80°C *in vacuo*. Autoradiography was carried out using Fuji X-ray film overnight.

Products of primer extension were also resolved using this method. The gel was, however, 8% w/v polyacrylamide.

SECTION 2.7 TRANSFORMATION OF BACTERIA.

2.7.1 Preparation of competent cells.

An overnight culture of E. coli was used to inoculate fresh L-broth and the culture incubated with shaking at 37°C until it reached exponential phase. 50ml cells were then pelleted at 5,000 x g for 5mins at 4°C before being resuspended in 0.5 volumes of 0.1M $MgCl_2$. The cells were then repelleted as before and resuspended in 2mls 0.1M $CaCl_2$. Cells were then left on ice for a minimum of 2 hours before use.

2.7.2 Transformation of E.coli with plasmid DNA.

Transformation of E.coli with DNA was achieved by mixing 100ul of competent cells and approximately 10ng of DNA, for 45min at 4°C on ice followed by heat shock at 42°C for 45secs. The mixture was then returned to ice for a further 45 mins. Cells were then spread on L-agar plates containing the required antibiotic for selection of the plasmid DNA and the plates incubated at 37°C overnight.

2.7.3 Transformation of E.coli with M13 DNA.

1ng of M13 DNA was incubated on ice with 100ul competent cells (TG2) as above. Following the second incubation on ice for 45mins, the mixture of cells and DNA were added to 3ml molten H-top agar containing 40ul isopropyl-β-D-thiogalactopyranoside (IPTG) (20mg/ml), 40ul 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (20mg/ml in dimethylsulphonide) and 200ul of exponentially growing E.coli. This mixture was poured on to glucose minimal media plates and incubated at 37°C overnight.

SECTION 2.8 IN VITRO EXPRESSION OF RECOMBINANT SLT IA SUBUNITS.

2.8.1 in vitro transcription.

Recombinant Shiga-like toxin I A subunits (rSLT IAs) resulting from site directed mutagenesis (SDM) were each cloned into plasmid vector pGEM2 (FIG 3.3.9) and orientated such that transcription was driven by the viral promoter T7. (A control vector used in subsequent in vitro experiments was under

transcriptional control of viral promoter SP6). The resultant plasmid was then linearised in each case with Eco RI and the linear DNA extracted with phenol-chloroform, ethanol precipitated and resuspended in TE to a final concentration of 1ug/ul and used in the following reaction:

Transcription reaction

Linearised DNA (1ug/ul)	2.0ul
H_2O	2.5ul
Transcription Premix	12.0ul
RNasin	0.5ul
5mM M-G(5')ppp(5')G (CAP)	1.0ul
T7/SP6 polymerase	2.0ul
TOTAL REACTION VOLUME	20.0ul

Transcription Premix

10 x salts (20mM spermidine, 400mM HEPES KOH pH7.5, 60mM magnesium acetate)	1000ul
10mg/ml BSA	100ul
500mM DTT	100ul
50mM rUTP, rATP, rCTP	100ul
5mM rGTP	200ul
H_2O	4500ul
TOTAL VOLUME	6mls

Stored at -70°C.

Transcription was carried out at 40°C for 1 hour with the addition of 1ul 8mM rGTP in 20mM HEPES KOH pH7.6 after 30 minutes. The resultant transcripts were then stored at -70°C.

2.8.2 *in vitro* translation.

(i) Wheatgerm lysate.

Wheatgerm lysate was a kind gift from Dr R. Wales (Warwick) and was used in the following reaction to translate *in vitro* transcripts previously synthesised. Translation reactions were assembled on ice and the components added in the following order:

Wheatgerm translation reaction

Wheatgerm Premix	2.35ul
H_2O	4.40ul
mRNA transcript (1ug)	1.00ul
^{35}S-Methionine (15uCi)	1.00ul
Wheatgerm lysate	3.75ul
TOTAL REACTION VOLUME	12.00ul

NB Wheatgerm lysate was defrosted, centrifuged and used immediately.

Wheatgerm Premix

1M HEPES KOH pH7.6	350ul
0.1M ATP	250ul
0.4M Creatine Phosphate (CP)	500ul
10mg/ml CP Kinase	100ul
1.5mg/ml spermine pH7.0	500ul
0.1M DTT	425ul
2mM GTP (40mM GTP/magnesium acetate stock pH7)	250ul
5mM each amino acid mix	125ul
1M potassium acetate	2100ul
H_2O	100ul
TOTAL VOLUME	4.7ml

Translation was carried out at 30°C for 60mins. The products were then analysed by SDS polyacrylamide gel electrophoresis (SDS PAGE) (2.10.3) and visualised by fluorography (2.10.3(iii).

(ii) Rabbit reticulocyte lysate

Rabbit reticulocyte lysate was purchased from Promega and used as detailed in the instructions supplied by them. However, in most cases only half the prescribed reaction volumes were used, using 15uCi ^{35}S-methionine and 1ug *in vitro* transcribed mRNA per translation reaction. Translation was carried out at 30°C as before and the products analysd by SDS PAGE followed by fluorography to visualise them.

SECTION 2.9 *IN VIVO* EXPRESSION OF RECOMBINANT SHIGA-LIKE TOXIN I A SUBUNITS.

Plasmid pSC25 (FIG 3.3.5) expressing the SLT IA subunit under control of the lacZ promoter of pUC19, was kindly donated by Dr.S.B. Calderwood (Boston MA, USA). It was used to transform *E.coli* strain JM101 as previously described and grown exactly as described by Hovde *et al*. (1988). IPTG induced expression of SLT IA was also achieved as described by Hovde *et al*. (1988).

2.9.1 Preparation of periplasmic extracts.

Typically a 1L culture of E.coli strain JM101 expressing SLT IA was grown under the conditions described by Hovde et al. (1988). The cells were harvested by centrifugation at 2500 x g and 4°C for 30mins. The cell pellet was then gently resuspended in 20 mls Tris/Sucrose (1M Tris pH8, 0.5M EDTA pH8, 1M $MgCl_2$, 200g/L Sucrose) and the mixture incubated at room temperature for 10mins before a further centrifugation step 7500 x g, 4°C for 5mins. The resultant supernatant fluid was drained from the pellet and the pellet resuspended in 10mls 1mM Tris pH7.5. Incubation of the cells on ice for 10mins was followed by a centrifugation step 19,000 x g, 4°C for 15mins. The resultant supernatant in this case was the required SLT IA-containing periplasmic fraction.

SECTION 2.10 ANALYSIS OF PROTEINS.

2.10.1 Determination of protein concentration.

Protein concentrations were determined using a commercially available Bio-Rad Assay. The assay is based on the Bradford dye-binding procedure

(Bradford, 1976) providing a simple colorimetric assay. Protein concentrations were determined by comparison to a standard curve using Bovine Serum Albumin (BSA) as the standard. The procedure was exactly as described by Bio-Rad.

2.10.2 Treatment of protein products with trypsin.

The trypsin sensitivity of *in vitro* translated, recombinant SLT IA subunits was determined by their incubation with increasing concentrations of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated (TPCK) trypsin (Sigma) (0 - 50ug/ml in PBS) at 37°C for 15mins. The resultant protein products were analysed by SDS-PAGE.

2.10.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Proteins were analysed using 12% or 15% SDS polyacrylamide gels as described by Laemmli (1970) under reducing and non reducing conditions the latter being achieved by the omission of 2-mercaptoethanol (0.75M) from the sample loading buffer. Protein bands could be visualised by two methods of staining:

(i) Coomassie Blue staining.

Following electrophoresis, gels were immersed in a stain consisting of 0.25% w/v Coomassie Brilliant Blue R, 50% v/v methanol, 10% v/v acetic acid for 1 hour prior to several washes in destain (40% v/v methanol and 10% v/v acetic acid) in order to visualise the protein bands.

(ii) Silver staining.

Gels were silver stained as described by Merril et al. (1981).

(iii) Visualisation of radioactively labelled proteins following SDS-PAGE.

Gels were firstly fixed in destain (above) for 30 mins and then subjected to fluorography by immersion in 'Amplify' (Amersham) for 15 mins. Gels were then dried by vacuum dessication at 80°C on to 3MM paper and exposed to Fuji RX X-Ray film in a light proof cassette at -70°C.

SECTION 2.11 DETERMINATION OF THE BIOLOGICAL ACTIVITIES OF SLT I HOLOTOXIN AND RECOMBINANT SLT IA SUBUNITS.

2.11.1 RNA N-glycosidase activity.

(i) Incubation with susceptible eukaryotic ribosomes.

Potentially toxic extracts were incubated with 30ug of susceptible eukaryotic ribosomes - typically yeast ribosomes were used. Prior to incubation with susceptible ribosomes, SLT IA containing extracts were first incubated with 2-mercaptoethanol to a final concentration of 6mM at 30°C for 30min. Incubation with ribosomes at 30°C for 20mins in 1 x Endo buffer (25mM Tris HCl pH7.6, 25mM KCl, 5mM $MgCl_2$) plus 2-mercaptoethanol to a final concentration of 6mM then followed. The ribosomal RNA was then extracted and subjected to aniline treatment, as described previously in SECTION 2.5.

(ii) Extraction and aniline treatment of ribosomal RNA following in vitro expression of recombinant SLT IA subunits.

Following translation of potentially toxic mRNAs in a reticulocyte lysate cell free system exactly as detailed in the manual supplied by Promega, ribosomal RNA was extracted from the ribosomes involved in the translation and subjected to aniline cleavage as previously described in section 2.5.

(iii) Examination of the ability of reticulocyte ribosomes to translate a 2^{o} transcript.

Translation of mRNA in a reticulocyte lysate cell free system was carried out exactly as described in the manual supplied by Promega. Following the initial translation of potentially toxic transcripts at 30°C for 1 hour, 0.5-1ug of a second transcript encoding a non toxic product was added to the reaction mix and incubated for a further 1 hour at 30°C. Translation products were analysed by SDS-PAGE and visualised by autoradiography.

2.11.2 Cytotoxic activity of SLT I on Vero cells.

Vero cells were plated out in 96 well flat bottomed tissue culture plates at a concentration of $1.5x10^4$cells per well in Dulbecco Modified Eagle's Medium (DMEM) (DME plus 5% heat inactivated foetal calf serum, 2mM glutamine, 50IU/ml penicillin, 300ug/ml steptomycin). Incubation at 37°C overnight in a 5% CO_2 incubator resulted in the formation of a confluent monolayer of cells. The DMEM was removed from the wells and the monolayers washed once with PBS. Toxin diluted in 25ul of PBS was then added to each well and the cells incubated for a further 1 hour at 37°C, 5% CO_2. 100ul DMEM was then added to the wells and intoxication of the cells left to proceed for 20-24 hours at 37°C, 5% CO_2. Toxin containing supernatant was removed at the end of this period and the cell monolayers washed once again with PBS. This was followed by the addition of 1uCi ^{35}S-methionine contained in 50ul PBS to each well and a further incubation at 37°C, in 5% CO_2 for 2 hours. The supernatant was removed from the cells and the cell monolayers washed x3 with 5% trichloroacetic acid (TCA) and once with PBS. The addition of 0.5M NaOH to each monolayer led to cell lysis over a period of 45mins at room temperature allowing the contents of each well to be mixed with liquid scintillant and the

incorporation of the isotope into cell proteins to be counted using a scintillation counter. Thus IC_{50} values for SLT I could be determined.

SECTION 2.12 PURIFICATION OF SHIGA-LIKE TOXIN I (SLT I).

Shiga-like toxin I (SLT I) only producing E.coli serotype 026:H11, strain E3787 was purchased from the National Type Culture Collection on the advice of Dr S M Scotland (Public Health Laboratory Service, Collingdale). This was then grown in iron-depleted syncase medium (1.17g/L NH_4Cl, 5g/L KH_2PO_4, 5g/L Na_2HPO_4, 0.2% glucose, 0.002% nicotinic acid, 0.04% tryptophan, 1ml/L trace salts [5% $MgSO_4$, 0.5% $MnCl_2$in 0.001M H_2SO_4,pH8.0]) (O'Brien et al. 1982) as 2 x 10L cultures in A Braun Biostat E glass fermenter for 18 - 20 hours at 37°C with aeration 5L/min. This was carried out under Category II containment at PHLS, CAMR, Porton Down. Purification of SLT I followed the method described by Ryd et al. (1989) for purification of ST, with slight modification. Typically 4g cell paste was resuspended in 20mls PBS and sonicated using an MSE Soniprep 150 sonicator 10 x 20sec bursts at amplitude setting 22. Centrifugation (4°C, 10,000 x g, 45 min) allowed

separation of the cell debris from the supernatant which was then precipitated with ammonium sulphate to 60% saturation Petric et al. (1987) (1 hour, 4°C with stirring). Centrifugation (4°C, 10,000 x g, 10min) allowed separation of the resultant ammonium sulphate precipitated proteins. These were then resuspended in 20ml PBS and dialysed against 3L PBS overnight at 4°C before being filter sterilised (Ryd et al. 1989) and applied directly to a 1ml globotriose (Gb_3)-Fractogel column (Biocarb) (equilibrated with PBS) at a constant temperature of 4°C. The column was then washed with approximately 40 column volumes ie 40mls PBS in order to remove proteins which failed to bind. Elution of the affinity-bound proteins was as described by Ryd et al. (1989) using 6M guanidine HCl pH6.7 at 4°C. 6 x 1ml fractions were collected and dialysed immediately at 4°C against 3L PBS which was replaced at intervals of 1, 3 and 12 hours. Fractions were analysed by SDS polyacrylamide gel electrophoresis (2.10.3), Bio-Rad assay (2.10.1) and for RNA N-glycosidase activity (2.11.1(i)).

2.12.1 Storage and preservation of globotriose-Fractogel column.

Following use the column was cleared of residual SLT I by applying approximately 10 column volumes of 6M guanidine HCl pH6.7 through the column followed by approximately 40 column volumes of PBS. Microbial growth was prevented by storage of the column matrix in PBS containing 0.02% v/v sodium azide. The column was then stored at 4°C.

Prior to use of the column in the purification of recombinant Shiga-like toxin I A subunit containing holotoxins it was analysed for the existence of residual SLT I or SLT IB by elution with 6M guanidine HCl pH6.7. 7 - 8 x 1ml fractions were collected in each case and dialysed against PBS as previously described. Fractions were then analysed by SDS polyacrylamide gel electrophoresis (2.10.3) and for RNA N-glycosidase activity on yeast ribosomes (2.11.1(i)).

SECTION 2.13 PURIFICATION OF SLT IB SUBUNIT.

Plasmid pSBC32 (FIG 3.4.1) contained in E.coli strain JM105 was kindly supplied by Dr S B Calderwood

(Boston, USA). It was grown and expression of SLT IB induced as described by Calderwood et al. (1990). Following this step, the protocol was then modified. Firstly the preparation of periplasmic extract was achieved using method (2.9.1). Periplasmic extracts were then applied directly to the 1ml globotriose-Fractogel column (Biocarb) as described (2.12). Elution and dialysis of SLT IB from this column was then achieved exactly as described for elution and dialysis of ST described by Ryd et al. (1989).

SECTION 3 RESULTS AND DISCUSSION

CHAPTER 1

SECTION 3.1 Purification of Shiga-like toxin I (SLT I).

Introduction

Previously, many groups have attempted to purify Shiga toxin (ST), and Shiga-like toxins I and II (SLT I and SLT II) to homogeneity with varying degrees of success. This may be attributed to the low level production of the toxins, which constitute less than 0.1% of the total cell proteins (Donohue-Rolfe et al. 1991).

In order to be able to compare the activity of SLT I with that of ricin, it was necessary firstly to purify the toxin. Initial attempts to purify SLT I were based on a combination of protocols developed and used successfully by others in the purification of both ST and SLT I.

Purification of ST has been achieved by several groups (Brown et al. 1980, O'Brien et al. 1980, Olsnes and Eiklid, 1980, Kongmuang et al. 1987) using a variety of methods including size fractionation, isoelectric focussing (IEF), ion exchange chromatography and anti-ST affinity chromatography. Toxin yields were however, relatively low. For example, Kongmuang et al. (1987) reported a yield of only 0.32 - 0.75mg of purified toxin from a 5L culture. More recently Donohue-Rolfe et al. (1991) have presented a purification procedure yielding 1mg of

ST from 4L of culture. In addition to the the use of Cibacron Blue, chromatofocussing and size fractionation chromatography, they have employed the high specificity binding of the ST B subunit to the disaccharide Galα1-4Galβ1, which forms part of the terminal trisaccharide of glycoprotein P_1, in their purification procedure.

The production of many bacterial toxins may be seen to be regulated by iron. This is also seen to be the case with ST (O'Brien and LaVeck, 1982) and SLT (O'Brien et al. 1982, Calderwood et al. 1987, Weinstein et al. 1988). Weinstein et al. (1988) examined the effects of iron on production of SLT I and ST. The presence of iron in the growth medium was found to suppress production of SLT I. Under conditions of iron deprivation, large quantities of ST were detected extracellularly ie 79.5% of the toxin produced was detected extracellularly under conditions of iron deprivation compared to 29.5% in the presence of iron . However, iron deprivation did not result in a massive increase in the release of SLT I from Escherichia coli. Weinstein et al. (1988) suggest that iron deprivation may in some way affect the integrity of the outer membrane of Shigella dysenteriae resulting in release of ST to the medium; an effect which is not so marked in the SLT I producing E.coli.

The role of iron in the regulation of SLT I expression was investigated by Calderwood and Mekalanos (1987). The expression

of certain E.coli genes, for example those involved in biosynthesis of iron-binding ligands, were known to be repressed by the presence of iron. A control gene, fur, the protein product of which acts as a negative regulator of transcription, was also implicated in repression of such genes in the presence of iron. Neilands (1982) proposed that this was achieved by binding of iron as a corerepressor in conjunction with the fur gene product repressor protein, to the operator sites of iron-regulated genes. Studies by Calderwood and Mekalanos (1987) revealed that in cases where the fur gene product was absent expression of SLT I was constitutively high even in the presence of iron. This observation led them to suggest that the SLT I promoter was regulated by the fur gene product. In addition, comparison of the nucleotide sequence of the SLT I promoter region with those of other iron regulated promoters also revealed significant sequence homology. Calderwood and Mekalanos (1987) suggested that this may represent a binding site for iron and the fur gene product and thus a mechanism for iron regulation of SLT I.

Iron starvation has also been seen to enhance release of the periplasmically located (Hirst et al. 1984a) E.coli heat labile toxin (LT) (Hunt and Hardy, 1991). Thus yields of ST and SLT I have been improved in many purification protocols by the use of an iron depleted growth medium (O'Brien et al. 1982, O'Brien and LaVeck, 1983, Ryd et al. 1989, Donohue-Rolfe et al. 1991).

Methods to improve the release of ST and SLTs have also been investigated. Incubation of cells with polymyxin B sulphate was found to be a successful means of releasing the periplasmically located ST (Donohue-Rolfe and Keusch, 1983, Griffin and Gemski, 1983). Indeed, Donohue-Rolfe and Keusch observed 80 - 90% of the ST produced to be released following treatment of cells with polymyxin B. Incubation of cells with polymyxin B was also incorporated into the successful purification protocol of SLT I by Petric et al. (1987).

The SLT I purification protocol developed by Petric et al. (1987) was used as a starting point in the purification of SLT I in this study.

Results and Discussion

E.coli 026:H11 strain E3787 was purchased from the National Type Culture Collection (NTCC) on the advice of Dr S M Scotland (PHLS Collingdale). This strain in particular was chosen, since it produces SLT I alone.

A 1L culture of E.coli strain 026:H11 was grown in Penassay broth (Difco) at 37°C with shaking (200rpm) for 5 hours as described by Petric et al. (1987). Following harvesting of the cells by centrifugation (5,000 x g, 10 min) the cell pellet was resuspended in a mixture of 50ug/ml phenylmethylsulphonyl fluoride (PMSF) (Sigma) in 250ml phosphate buffered saline

(PBS) containing 0.2mg/ml polymyxin B sulphate (Sigma) and further incubated for 1 hour as described by Petric et al. (1987) prior to a centrifugation step (10,000 x g, 10min). The resultant supernatant underwent ammonium sulphate precipitation as detailed by Petric et al. (1987). At this stage the precipitated proteins were tested for the presence of active SLT I by the ability of the preparation to depurinate susceptible eukaryotic ribosomes (2.11.1), in this case rabbit reticulocyte ribosomes. With the addition of vanadyl ribonucleoside complex (VRC) to the crude protein preparation in order to inhibit the action of non-specific ribonucleases, the crude extract was incubated with the ribosomes. The ribosomal RNA (rRNA) was then extracted (2.4.1) and subjected to aniline treatment (2.5.1) prior to gel electrophoresis (2.6.1). This assay is based on the fact that depurinated rRNA is susceptible to aniline catalysed hydrolysis. Treatment of depurinated rRNA with acetic-aniline results in the release of a fragment of approximately 390 bases from the 3' end of the large subunit rRNA. FIG 3.1.1 reveals that the crude protein preparation did indeed contain active SLT I showing the release of a 390 base fragment diagnostic of toxin catalysed depurination of rRNA (lane 4). The rRNA fragment is comparable in size to that resulting from the depurination of rRNA by ricin A subunit (RA) (lane 2).

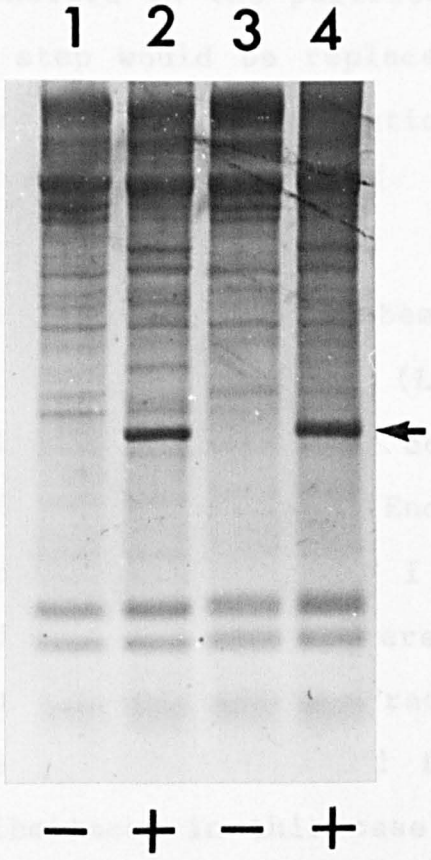
A size fractionation step was then introduced in order to continue the purification process. The crude preparation was

FIG **3.1.1** **Examination of the use of polymyxin B sulphate in the extraction of biologically active Shiga-like toxin I.**

2ul of periplasmic extract produced by incubation of Shiga-like toxin producing Escherichia coli with polymyxin B sulphate as described by Petric et al (1983), was incubated with 30ug rabbit reticulocyte ribosomes for 20min at 30°C (2.11.1(i)). Vanadyl ribonucleoside complex (VRC) was also included at this stage (2mM final concentration) in order to reduce non-specific degradation of the ribosomal RNA (rRNA). The resultant rRNA was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). Fractionation and visualisation of the resultant rRNA was then carried out (2.6.1).

Lane 2 = positive control: resultant rRNA following incubation with recombinant ricin A subunit (rRA).
Lane 4 shows resultant rRNA following incubation with periplasmic extract.

+ indicates treatment of extracted rRNA with aniline reagent (lanes 2 and 4).
- indicates non treatment of extracted rRNA with aniline reagent (lanes 1 and 3).
➡indicates the characteristic fragment released following aniline cleavage of toxin-modified rRNA.


1 2 3 4
− + − +

applied to a 1.5 x 80cm Sephadex G100 -120 (Sigma) column. The proteins were eluted using 1 x Endo buffer (2.3(i)) and the resultant fractions tested for SLT I activity by the ability to depurinate rabbit reticulocyte ribosomes (2.11.1(i). No such activity was detected. Following several repeated attempts using size fractionation in the purification protocol, it was decided that this step would be replaced by high performance liquid chromatography (HPLC) resolution using a DEAE ion exchange column.

1 x Endo buffer pH 7.6 (2.3(i)) had been used to equilibrate ULtropac TSK DEAE - 5PW HPLC column (LKB) and also to elute proteins which failed to bind to the column matrix. A linear gradient of KCl (50mM to 0.5M) in 1 x Endo buffer was then used to elute the bound proteins (pI SLT I = 6.72 (Petric et al. (1987)). 24 fractions each of 3ml were collected. FIG 3.1.2 shows the resultant elution profile. Fractions 4, 8 and 12 from each of the three peaks were tested for SLT I activity by incubation with ribosomes, in this case yeast ribosomes. Each peak was found to contain active toxin. FIG 3.1.3. Lanes 4, 5 and 6, representing fractions 4, 8 and 12 respectively, each display the characteristic rRNA fragment released following aniline cleavage of modified rRNA. This is an indication of the presence of biologically active SLT IA. The fragment sizes are directly comparable to that seen in lane 2 following modification of yeast rRNA by ricin A subunit.

FIG **3.1.2 High performance liquid chromatography (HPLC) elution profile.**

Crude Shiga-like toxin containing extract was applied to an Ultropac TSK DEAE - 5PW (LKB) HPLC column and eluted using a linear gradient of KCl (50mM - 0.5M) in 1 x Endo buffer (25mM Tris HCl pH7.6, 25mM KCl, 5mM $MgCl_2$). 24 x 3ml fractions were collected.

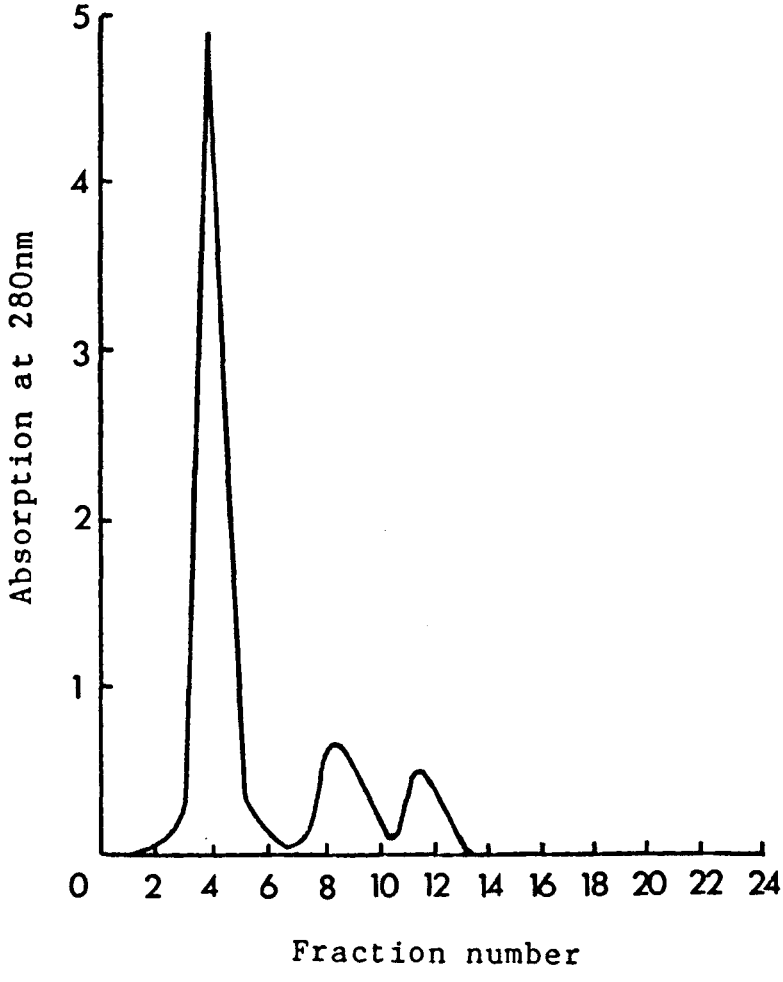

Absorption at 280nm
5
4
3
2
1
0
2
4
6
8
10
12
14
16
18
20
22
24
Fraction number

FIG 3.1.3 **Examination of high performance liquid chromatography (HPLC) fractions for RNA N-glycosidase activity.**

10ul of the 3ml fractions 4, 8 and 12 (FIG 3.1.2) collected following purification of crude periplasmic extract by high performance liquid chromatography using an Ultropac TSK DEAE - 5PW HPLC column (LKB), was incubated with 30ug yeast ribosomes for 20min at 30°C (2.11.1(i)). In order to reduce non-specific degradation of ribosomal RNA (rRNA) vanadyl ribonucleoside complex (VRC) was also included at this stage (2mM final concentration). Prior to incubation with the ribosomes, each of the fractions had been preincubated with 2-mercaptoethanol to a final concentration of 6mM to ensure maximum activity of any SLT I present. The resultant rRNA was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). The resultant rRNA was then fractionated and visualised (2.6.1).

Lane **2** = positive control: rRNA resulting from incubation with recombinant ricin A subunit (rRA).
Lanes **4, 5 and 6**: rRNA following incubation with HPLC fractions **4, 8 and 12** respectively.
+ indicates treatment of extracted rRNA with aniline reagent.
- indicates non treatment of extracted rRNA with aniline reagent.
➡ indicates the characteristic fragment released following aniline cleavage of toxin-modified rRNA.

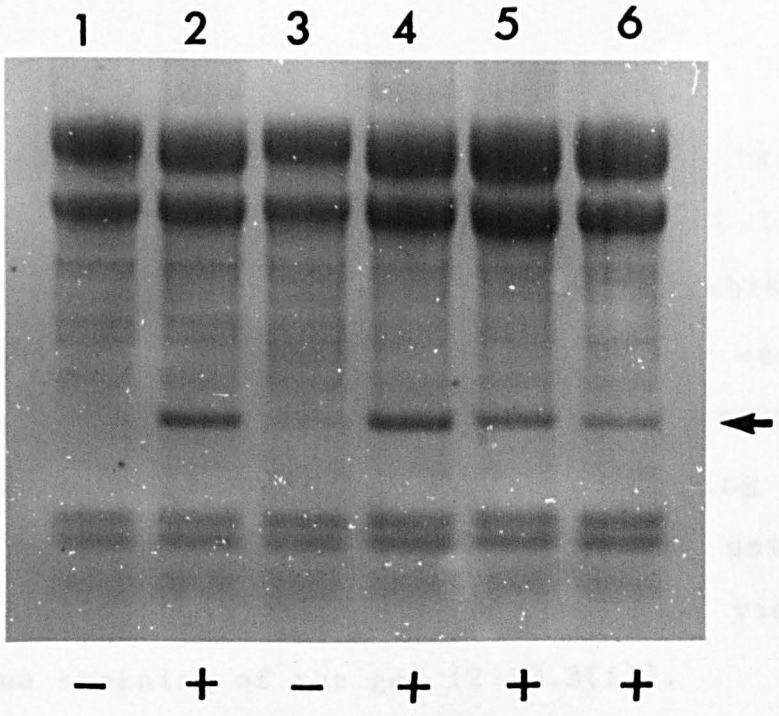
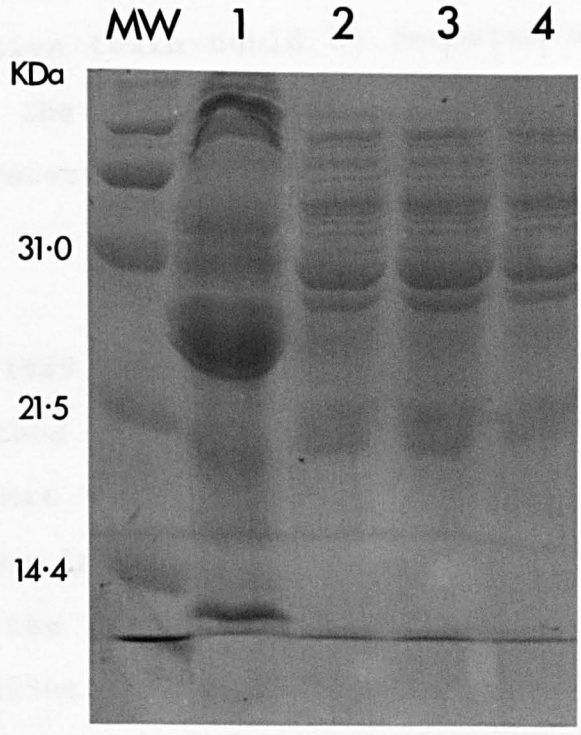

1
2
3
4
5
6
−
+
−
+
+
+

FIG 3.1.4 Analysis of high performance liquid chromatography (HPLC) fractions by SDS polyacrylamide gel electrophoresis (SDS PAGE).

The elution profile for high performance liquid chromatography (HPLC) purification of Shiga-like toxin I (SLT I) using an Ultropac TSK DEAE - 5PW HPLC column (LKB) exhibited three distinct peaks FIG 3.1.2. The relevant fractions were analysed by SDS polyacrylamide gel electrophoresis (SDS PAGE) (2.10.3) in comparison to pre column, crude SLT I containing polymyxin B periplasmic extract (Petric et al. 1981) using a 15% polyacrylamide gel. The resolved proteins were visualised by coomassie blue staining of the gel (2.10.3(i)).

Lane 1 = crude SLT I containing pre column proteins.
Lanes 2 - 4 represent fractions 4, 8, and 12 (FIG 3.1.2) respectively.
MW = molecular weight markers.


MW
1
2
3
4
KDa
31·0
21·5
14·4

Analysis of the active fractions 4, 8 and 12 by SDS polyacrylamide gel electrophoresis (SDS PAGE) FIG 3.1.4 however, revealed that in each of the fractions there remained a high degree of contamination with other proteins. Thus active fractions 4, 8 and 12 were pooled and applied to the Sephadex G100 - 120 (Sigma) column mentioned previously and eluted as above. No active toxin could be detected and at this stage it was decided that purification of SLT I would follow the successful protocol described by Ryd et al. (1989) to purify ST (2.12).

Ryd et al. (1989) reported the purification of ST by a simple and rapid method exploiting the high specificity binding of the SLT I B subunit (SLT IB) to globotriose (Gb_3), a trisaccharide possessing the important terminal disaccharide Galα1-4Galβ1. Coupling of the trisaccharide to a gel matrix, Fractogel TSK AF-Amino 32-63um, allowed single step purification of ST with 87% recovery of biologically active material. Elution of the holotoxin from the gel matrix required the use of a strong denaturant, 6M guanidine HCl pH6.7 in order to overcome the tight binding of ST to the gel matrix.

Successful purification of SLT I from E.coli 026:H11 was achieved as described by Ryd et al. (1989) with only slight modification (2.12). 2 x 10L cultures of E.coli 026:H11 were grown in a Braun Biostat E 10L glass fermenter for 18 - 20 hours, at 37°C, with aeration of 5L/min (no pH control) under

Category II containment at Public Health Laboratory Service (PHLS), Centre for Applied Microbiology Research (CAMR), Porton Down, Salisbury. As described by Ryd *et al.* (1989) in the production of ST, *E.coli* 026:H11 was grown in iron-depleted syncase media described by O'Brien *et al.* (1982). The iron concentration of the medium prior to inoculation was found to be 0.32 parts per million (ppm).

The total yield from 20L of culture was 120g of wet cell paste. Typically 4g of cell paste was resuspended in 20ml PBS and sonicated (10 x 20sec) using a MSE Soniprep 150 sonicator, amplitude 22. The whole cell sonicate was then centrifuged (4°C, 10,000 x g, 45min).

A 60% ammonium sulphate precipitation step was added as a pre-affinity column step to eliminate non salt precipitable material which previously led to blockage of the mini-column. This additional step was found to effectively eliminate the viscous nature of the cell sonicate allowing it to pass through the column matrix more freely and extending the number of times that it could be used to purify SLT I. Therefore the resultant supernatant was subjected to ammonium sulphate precipitation (60% saturation) for 1 hour at 4°C with stirring (Petric *et al.* 1987) at this stage of the purification. The precipitate was removed by centrifugation (4°C, 10,000 x g, 10min) and resuspended in 20ml PBS pH7.2 and dialysed overnight against PBS pH7.2 at 4°C. This was then filter sterilised as described

by Ryd et al. (1989) prior to being applied to the Gb_3-Fractogel column matrix which had been equilibrated with PBS pH7.2. The purification was carried out at 4C.

Proteins which had failed to bind were removed from the matrix by washing with approximately 40 column volumes, ie 40ml of PBS pH7.2 Bound products were eluted using 6M guanidine HCl pH6.7. Six 1ml fractions were collected. The resultant elution profile is shown in FIG 3.1.5. As each fraction was collected it was dialysed immediately against 3L PBS pH7.2 which was replaced at intervals of 1, 3 and 12 hours.

FIG 3.1.6 shows the purification scheme used to purify of SLT I.

SDS PAGE analysis of each stage in the purification of SLT I (FIG 3.1.7), compares the crude whole cell sonicate (lane 1), 60% ammonium sulphate precipitated proteins (lane 2) and affinity purified SLT I (lane 3). In lane 3, a protein band of approximately 32KDa (presumably SLT I A subunit) and a single band running just below the dye front are clearly visible, the latter of which are assumed to represent the SLT I B subunits. In addition two faint bands are evident. The first, at approximately 27 - 28KDa, indicates the existence of proteolytically cleaved and reduced SLT IA, since it may be seen to be of the same size as the SLT I A_1 fragment (27 - 28KDa). The second faint band, approximately 22 - 23KDa in size

FIG **3.1.5 Absorbance profile showing the elution of affinity bound material from a 1ml globotriosylceramide (Gb_3) - Fractogel column.**

20mls of a 60% ammonium sulphate precipitated preparation of 026:H11 whole cell sonicate proteins (typically from 4g cell paste) was applied to the 1ml Gb_3 - Fractogel column (Biocarb). Proteins which failed to bind to the matrix were washed from the column using 40 column volumes ie 40ml PBS. Bound proteins were eluted using 6M guanidine HCl pH6.7 as described by Ryd *et al*. (1989). Six 1ml fractions were eluted and dialysed immediately against PBS pH7.2.

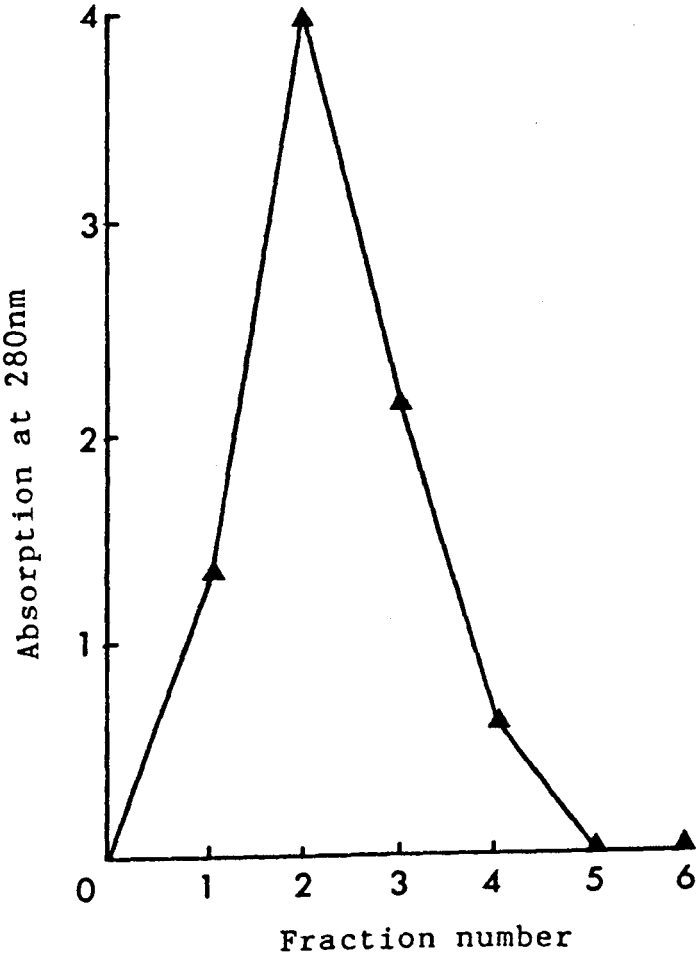

Absorption at 280nm
4
3
2
1
0
1
2
3
4
5
6
Fraction number

FIG 3.1.6 Purification scheme used in the production of biochemically pure Shiga-like toxin I (SLT I).

Escherichia coli serotype **O26:H11**
grown in **iron-depleted syncase media** (O'Brien *et al.* (1982))
2 x 10L, 18 - 20 hours, 37°C, with aeration 5L/min
Braun Biostat E 10L glass fermenter
(PUBLIC HEALTH LABORATORY SERVICE, PORTON DOWN)

↓

total yield = **120g cell paste**

↓

4g cell paste resuspended in 20mls PBS pH7.2
Sonication 10 x 20sec, amplitude 22
MSE Soniprep 150 sonicator

↓

centrifugation (4°C, 10,000 x g, 45min)

↓

supernatant (15ml) 60% $(NH_4)_2SO_4$ precipitation
1 hour, 4°C with stirring

↓

centrifugation (4°C, 10,000 x g, 10min)

↓

precipitate resuspended in 20ml PBS pH7.2
dialysis overnight against PBS pH7.2 (4°C)

↓

Filter sterilisation (0.45um Millipore filter)

↓

application to **1ml Gb_3-Fractogel column** (Biocarb)
equilibrated with PBS pH7.2 (4°C)

↓

unbound proteins washed from column
40 column volumes PBS pH7.2

↓

affinity bound proteins eluted
6M guanidine HCl pH6.7 (4°C)

↓

6 x 1ml fractions collected
dialysed IMMEDIATELY against PBS pH7.2 (4°C, 18 hours)

↓

1. Bio-Rad determination of protein concentration
2. SDS PAGE analysis
3. Determination of biological activity (aniline assay)

↓

yield = 25ug SLT I/g cell paste

FIG **3.1.7 Purification of Shiga-like toxin I (SLT I).**

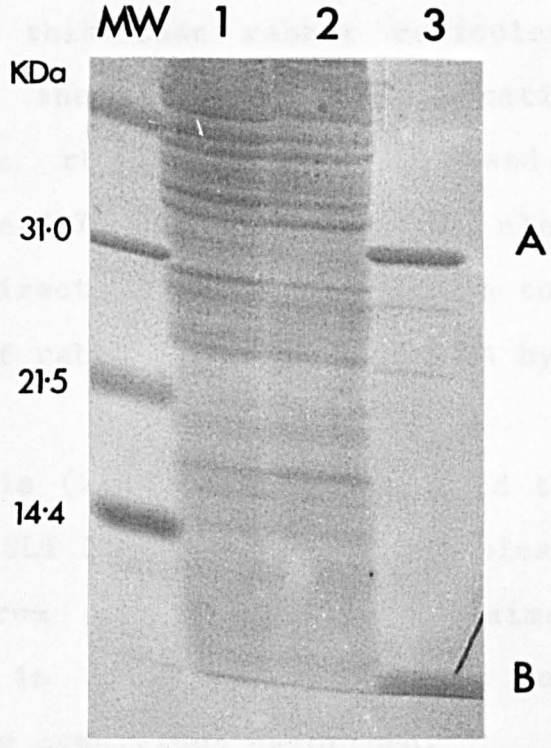
Production and purification of SLT I from SLT I-only producing E.coli strain 026:H11 was achieved using a slightly modified method of Ryd et al. (1989). Analysis of the elution products following overnight dialysis (2.12) in comparison to whole cell sonicate and 60% ammonium sulphate precipitation products was by SDS polyacrylamide gel electrophoresis (SDS PAGE) on a 15% gel (2.10.3) followed by Coomassie staining (2.10.3(iii)).

1: E.coli 026:H11 whole cell sonicate.

2: 60% ammonium sulphate precipitated proteins.

3: Affinity purified Shiga-like toxin I - Gb_3 column fraction.

MW: molecular weight markers.


MW
1
2
3
KDa
31·0
21·5
14·4
A
B

may be a degradation product of SLT IA resulting from non specific cleavage by small amounts of contaminating proteases.

The resultant purified product was shown to be biologically active by its ability to depurinate susceptible eukaryotic ribosomes, in this case rabbit reticulocyte ribosomes. FIG 3.1.8 shows the resultant fractionated rRNA. The characterisitic rRNA fragment released following aniline cleavage of the SLT IA modified rRNA is clearly distinguishable (lane 2) and directly comparable in size to that resulting from depurination of rabbit reticulocyte rRNA by ricin A subunit.

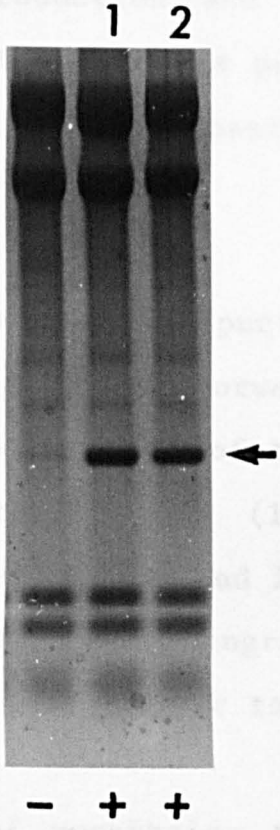
Bio-Rad analysis (2.10.1) of toxin yield typically indicated a yield of 25ug SLT I / g cell paste. Typically 1g of cell paste is derived from a culture of approximately 160mls E.coli 026:H11 grown in iron-depleted syncase media (O'Brien et al. 1982) under the conditions described.

FIG 3.1.8 **Examination of the activity of pure Shiga-like toxin I A subunit on rabbit reticulocyte ribosomes.**

0.4ug of affinity purified Shiga-like toxin I (SLT I) was incubated with 30ug rabbit reticulocyte ribosomes for 20min at 30C (2.11.1(i)). In order to ensure maximum possible RNA N-glycosidase activity of the toxin prior to incubation with the ribosomes the toxin was preincubated with 2-mercaptoethanol to a final concentration of 6mM. Following incubation with toxin the ribosomal RNA (rRNA) was extracted (2.4.1) and subjected to aniline cleavage (2.5.1). Fractionation and visualisation of the rRNA was then carried out (2.6.1).

Lane 1 = positive control: rRNA extracted from rabbit reticulocyte ribosomes incubated with recombinant ricin A subunit.
Lane 2 shows rRNA following incubation with pure SLT I.

+ indicates treatment of extracted rRNA with aniline reagent.
- indicates non treatment of extracted rRNA with aniline reagent.
→ indicates the characteristic fragment released following aniline cleavage of toxin-modified rRNA.


1
2
−
+
+

Conclusion Chapter 1

After many unsuccessful attempts to purify SLT I to homogeneity by a variety of different methods, the slightly modified method developed by Ryd et al. (1989) finally proved to be a successful means of production and purification of SLT I. Highly pure toxin results from this purification strategy, the yield from the starting wet cell paste is however, relatively low (0.0025%).

At first glance this method of purification appears to be relatively simple and straight forward. However, the first problem to be encountered is that of the iron-depleted syncase media described by O'Brien et al. (1982). Production of the media is extremely time consuming and laborious, since not only does it consist of numerous 'ingredients' but also the requirement for iron depletion prior to sterilisation.

The considerable cost of purchasing the Gb_3-Fractogel column matrix (Biocarb) presents the second problem, as does its size of 1ml. It was found that prior to the addition of an ammonium sulphate precipitation step to the purification procedure, the viscous nature of the whole cell sonicate supernation resulted in 'clogging' of the column. This severely reduced the number of times the column could be used. Even after addition of the ammonium sulphate precipitation step to the procedure, as a

direct result of its small size the column still became 'clogged' with material after only limited usage.

The low toxin yield (0.0025%) compounded the problem of the limited life span of the 1ml column matrix. The requirement for toxin in the present study, as will be seen in subsequent chapters, was not extensive. However, a constant supply of biochemically pure SLT I was needed. Extended use of the column matrix was therefore a necessity. For this reason careful regeneration of the column matrix was carried out after each purification.

Ultimately pure toxin was generated in sufficient amounts to fulfill the requirements of the present study.

CHAPTER 2

A comparison of the biological activities of Shiga-like toxin I and ricin.

Introduction

The RNA N-glycosidase activity of ricin A subunit (RA) has been investigated extensively. By contrast, current knowledge of the RNA N-glycosidase activity of Shiga-like toxin I (SLT I) has, in the main, been derived from investigation into the RNA N-glycosidase activity of Shiga toxin (ST). For example, Olsnes et al. (1981) and Reisbig et al. (1981) reported the inhibition of protein synthesis in rabbit reticulocyte lysate cell free translation systems by ST, whilst Endo et al. (1988) examined the mechanism by which Shiga toxin (ST) and SLT II were capable of modifying rat liver ribosomes. The latter study identified the specific site of modification by ST and SLT II to be adenine residue 4324 (A_{4324}) of the 28S ribosomal RNA (rRNA) of rat liver ribosomes as had also been found to be the case with RA modification of rat liver ribosomes (Endo et al. 1987).

Since ST and SLT I were considered to be identical (Strockbine et al. 1988) it was assumed that SLT I would possess a biological activity identical to that of ST. Thus most studies compare the RNA N-glycosidase activities of ST and ricin.

To date few direct comparisons have been made between the RNA N-glycosidase activities of ricin and SLT I. In this chapter a direct comparison between the biological activities of SLT I and ricin is made. Where appropriate, comments are made on the comparable activities of SLT I and ST.

SECTION 3.2.1 Comparison of the cytotoxicity of Shiga-like toxin I with that of ricin on Vero cells.

Introduction

Both ricin and Shiga-like toxin I (SLT I) cause an irreversible inhibition of protein synthesis in eukaryotic cells, a process which leads to eventual cell death.

With regard to Shiga toxin (ST) and thus SLT I, it has been found that some cell lines are resistant to the effects of the toxin. These include human melanoma cells, chinese hamster ovary cells (CHO) and foetal hamster kidney cells (BHK) (Olsnes and Eiklid, 1980). ^{125}I labelled ST allowed Eiklid and Olsnes (1980) to examine whether or not toxin actually bound to these insensitive cell lines. Their findings were, that toxin bound to some but not all of the insensitive cell lines. They concluded that the existence of the cellular receptor is necessary but not sufficient for ST to be able to cause death of that cell line. More recently however, on identification of the glycolipid receptor for ST, Lindberg *et al.* (1987) observed that cells naturally resistant to ST were those which did not possess glycolipids with the terminal disaccharide, Galα1-4Galβ required for the binding of ST.

The cytosolic target for action of ST and SLT I has since been identified as being the ribosome, as is the case with ricin and thus the requirement for internalisation has also been realised.

In addition to identifying certain cell lines as being insensitive to ST, Olsnes and Eiklid (1980) found both Vero and HeLa S_3 cell lines to be sensitive to ST. Whereas no cytotoxic effect could be detected with CHO and BHK cell lines at toxin concentrations of up to 10ug/ml, 50% cell death of HeLa S_3 cells was achieved with toxin concentrations of only 0.1ng/ml.

Later investigations by Sandvig and Brown (1987) into the ionic requirements for the entry of ST into cells revealed that the sensitivity of Vero and HeLa cells to ST was dependent upon the presence of certain ions. For instance, in the presence of Co^{2+} which blocks calcium transport across the cell membrane, both Vero and HeLa cells were found to be insensitive to ST. Thus it was suggested that entry of ST into the cytosol requires calcium transport. Incubation of Vero and HeLa cells in buffer containing high a concentration of K^+ also resulted in insensitivity of the cells to ST. Under the same conditions however, Vero cells were found to retain their sensitivity to ricin. Protection of Vero and HeLa cells by high K^+ concentrations against ST is suggested to be as a consequence of a reduction in membrane potential, which in turn may affect not only the membrane itself, but also the interaction between

the membrane and the bound ligand. Blockage of Cl^- transport by anions such as SO^{4-} and SCN^- and also inhibitors of anion transport such as 4,4-diisothiocyano-stilbene-2,2-disulphonic acid were found to protect both Vero and HeLa cells against ST, whilst the entry of ricin into the cytosol is unaffected by the presence of Cl^- or SO^{4-}. From these observations regarding ST and ricin in addition to those regarding other toxins, Sandvig and Brown (1987) concluded that despite sharing many common properties the toxins studied appear to employ different mechanisms to cross the cell membrane and to enter the cytosol. Sandvig and Brown (1987) identified an ID_{50} value for ST on Vero cells of 0.6ng/ml.

Olsnes et al. (1981), again examining the cytotoxic effect of ST on HeLa S_3 cells, identified a requirement of only 50 - 100 molecules of ST to be added per cell for 50% cell death to occur overnight. This was compared to a figure of 1000 molecules of ricin or abrin bound per cell required in order to achieve 50% cell death overnight (Sandvig et al. 1978). By comparison of the number of receptor sites per HeLa cell for ST and ricin (10^6 and 3×10^7 (Sandvig et al. 1976) respectively) it is suggested that the significant difference in the number of molecules required must be related to a difference in association constant K_a between the toxins and their receptors. The K_a values of ST and ricin for their receptors are $10^{10}M^{-1}$ and $2 \times 10^7 M^{-1}$ (Sandvig et al. 1978) respectively. However, there are now known to exist many other factors which may

account for the 10 fold difference in the number of ST and ricin molecules required to cause 50% cell death overnight observed by Olsnes et al. (1981).

In order to reach their intracellular target - the ribosome, both ST and ricin must bind to their respective surface receptors and be effectively endocytosed. Ricin is capable of binding opportunistically to a number of different galactose-terminating glycolipids and glycoproteins (Sandvig et al. 1991). In many cases such binding may not lead to adsorptive endocytosis of the ricin molecule. The binding of ST to Galα1-4Galβ-terminating glycolipids however, is highly specific (Cohen et al. 1987, Lindberg et al. 1987, Lingwood et al. 1987). Therefore it is likely that in each case the highly specific binding of ST to a receptor molecule will lead to receptor mediated endocytosis. Recent studies by (Stein et al. 1992) of the crystal structure of the SLT I B subunit pentamer suggest that each pentamer has five potential receptor binding sites. This may account for the high binding affinity of the SLT IB subunit for its cellular receptor globotriosylceramide (Gb_3).

The entire process of binding, uptake and translocation is probably very complex. For this reason it is unlikely that the observed differences in the cytotoxicity of ricin and ST of Olsnes et al. (1981) are simply as a result of a difference in

the binding affinities of the toxins for their respective receptors.

O'Brien and LaVeck (1983) and Kongmuang et al. (1988) have examined the cytotoxic effect of SLT I. In the latter a comparison has been made between the cytotoxic effect of SLT I on Vero cells in both its nicked (ie proteolytically cleaved SLT IA) and unnicked forms. A resultant 100% cytotoxic dose (CD_{100}) value of 0.1ng/ml SLT I for both nicked and unnicked toxin led Kongmuang et al. (1988) to suggest that nicking did not alter the cytotoxic nature of SLT I as had also been found to be the case with ST (Olsnes et al. 1981). Studies on binding of nicked and unnicked SLT I also suggests that nicking of the toxin has no effect on the time taken to bind to susceptible cells (Kongmuang et al. 1988).

Again it may be seen that much of that which is known about the activity, in this case cytotoxicity, of SLT I has been deduced from studies with ST. Here a direct examination of the cytotoxic effect of SLT I on Vero cells is undertaken and the result compared to that of ricin on Vero cells.

Results and Discussion

Using the procedure described (2.11.2), the cytotoxic nature of SLT I on Vero cells was examined. Vero cells were incubated

(2.11.2) with 0 - 100ng/ml pure SLT I for 24 hours. FIG 3.2.1 shows the result of this incubation on the protein synthesis of intoxicated Vero cells by measuring the incorporation of ^{35}S-methionine into cell protein. The results shown are representative of results obtained from repeating the experiment four times.

The resultant plot (FIG 3.2.1) allowed the 50% cytotoxic dose (IC_{50}) value ie the concentration required for 50% inhibition of protein synthesis, to be determined for the action of SLT I on Vero cells, a value which was then compared directly to the IC_{50} value for the action of ricin on Vero cells.

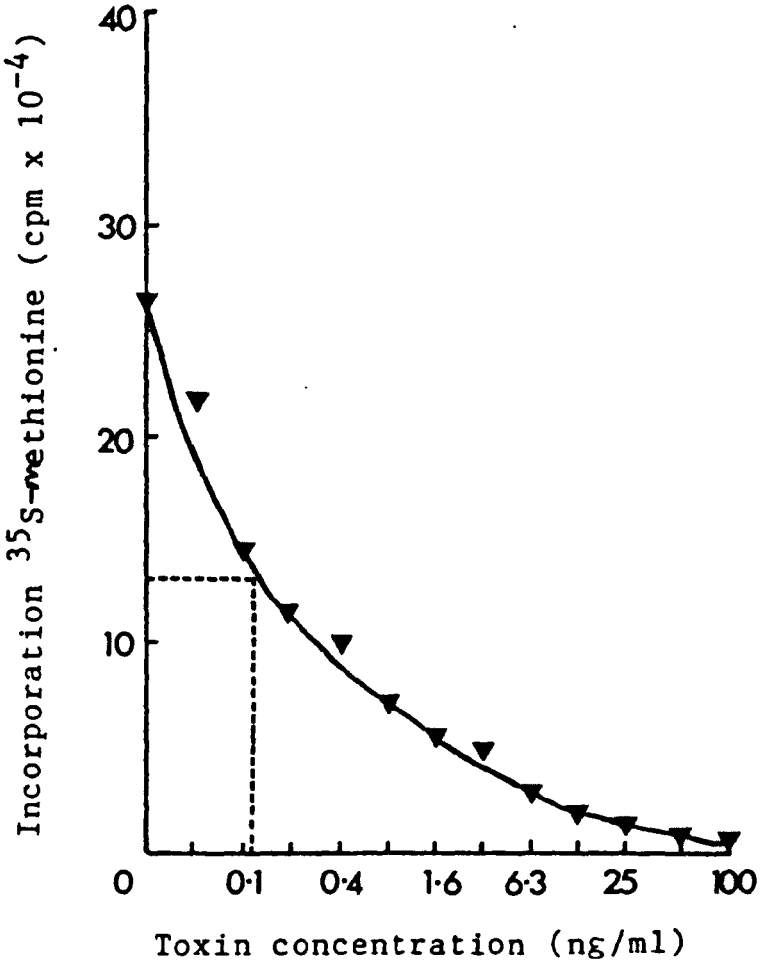
The IC_{50} value for SLT I on Vero cells was found to be 0.1ng/ml, a value which is 10 fold lower than that of ricin on Vero cells (IC_{50} = 1 - 3ng/ml) (O'Hare et al. 1987, 1990, Yoshida et al. 1991).

As previously discussed, there are a number of factors which may account for the apparent difference in cytotoxicity of SLT I compared with those published for ricin. These include the cellular distribution of receptors, differences in binding affinities, plus the relative efficiencies of endocytosis and membrane translocation.

The advantage afforded by the high specificity binding of ST and therefore SLT I, to glycolipids possessing the terminal

FIG **3.2.1 Examination of the cytotoxic effect of Shiga-like toxin I (SLT I) on Vero cells.**

Concentrations of SLT I from 0 - 100ng/ml were incubated in quadruplicate with 1.2 - 1.5 x 10^4 cells per well for 24 hours. The addition of 1uCi ^{35}S-methionine to each well following this incubation period allowed a measure of protein synthesis in order to indicate the extent of cell killing at each concentration of toxin. This was achieved by measuring the incorporation of isotope into cell protein in comparison to that of non toxin treated controls. Hence the 50% cytotoxic dose (IC_{50}) value could be determined for SLT I on Vero cells.


Incorporation ^{35}S-methionine (cpm x 10^{-4})
40
30
20
10
0
0·1
0·4
1·6
6·3
25
100
Toxin concentration (ng/ml)

disaccharide Galα1-4Galβ resulting in efficient endocytosis of the ST / SLT I has been discussed in comparison to the opportunistic binding of ricin to many different galactose-terminating molecules, not all of which may result in endocytosis of the toxin. The events following endocytosis remain unclear in both cases.

With regard to ST / SLT I there is evidence of its accumulation within the endoplasmic reticulum (ER) following endocytosis (Sandvig et al. (1992). This suggests that ST is either transported by glycolipids to the ER, or that as is thought to be the case with ricin, routing of the toxin to the ER occurs as a result of receptor exchange by the toxin in the trans-Golgi network (TGN) and interaction with proteins containing a KDEL-like sequence.

Reduction, processing and translocation of ST / SLT I and ricin remains an area of uncertainty which may represent a major rate limiting step in the cytotoxicity of the toxins.

Finally, the observed difference in cytotoxicity of ricin and SLT I may be a reflection of a difference in the catalytic activities of the toxins; a question which is addressed in SECTION 3.2.4.

It may be seen that there are many factors, any number of which may account for the difference in cytotoxicity of SLT I on Vero

cells observed in this present study (IC_{50} = 0.1ng/ml) in comparison to that of ricin (IC_{50} = 1 - 3ng/ml (O'Hare et al. 1987, 1990, Yoshida et al. 1991). In addition it is important to note that differences in cytotoxicity between SLT I (this study) and those published for ricin may have arisen as a result of slight variations in the experimental methods used. The IC_{50} values obtained for the action of ricin on Vero cells (O'Hare et al. 1987, 1990, Yoshida et al. 1991) of 1 - 3ng/ml are the same as results obtained in our laboratory (C Pitcher, personal communication) using exactly the same method as described in the present study to determine the concentration of SLT I which results in 50% inhibition of protein synthesis of Vero cells.

The IC_{50} value of 0.1ng/ml for the action of SLT I on Vero cells determined in the present study is the same as the 100% cytotoxic dose value (CD_{100}) reported by Kongmuang et al. (1988) for the action of SLT I in both its nicked (proteolytically cleaved SLT I A subunit) and unnicked forms on Vero cells. 0.1ng/ml was also the concentration of ST required to cause 50% cell death of HeLa S_3 cells reported by Olsnes et al. (1981). With regard to the cytotoxic nature of ST on Vero cells both Eiklid and Olsnes (1980) and Sandvig and Brown (1987) report ID_{50} values of 0.6ng/ml. The IC_{50} value of 0.1ng/ml reported in the present study for the action of SLT I on Vero cells is therefore, directly comparable to those values determined for the cytotoxicity of ST. In order to compare

directly the cytotoxic effect of ST and SLT I on Vero cells however, it would be necessary to carry out the cytotoxicity assays simultaneously, under the same conditions and using the same materials and methods.

SECTION 3.2.2 Comparison of the RNA N-glycosidase activities of Shiga-like toxin I A subunit (SLT IA) and ricin A subunit (RA) on ribosomes from different sources.

Introduction

The sensitivity of ribosomes from a number of different eukaryotic sources to modification by ricin A subunit (RA) has been widely examined as have the factors affecting such sensitivity. However, it has been found that information regarding the susceptibilities of ribosomes to modification by Shiga-like toxin A subunit (SLT IA) has mainly been derived from knowledge of the activity of Shiga toxin A subunit (STA) and Shiga-like toxin II A subunit (SLT IIA).

Endo et al. (1988) demonstrated the modification of rat liver ribosomes by ST and SLT II. The work of Saxena et al. (1989) revealed modification of the ribosomes of Xenopus oocytes by ST, RA and SLT II. From these results it was assumed that since ST and SLT I were almost identical in sequence and immmunologically, SLT I would also modify rat liver ribosomes and those of Xenopus oocytes.

The RNA N-glycosidase activity of RA has been more closely studied than that of ST and SLT II. Following identification of the site of depurination of rat liver 28S ribosomal RNA (rRNA)

by RA (Endo *et al.* 1987), other workers examined the sensitivity of rabbit reticulocyte, yeast (*Saccharomyces cerevisiae*) and wheatgerm ribosomes to modification by RA (Osborn, 1990) by employing the aniline cleavage of modified ribosomal RNA (rRNA) assay first described by Endo *et al.* (1987). Stirpe *et al.* (1988) also employed this assay in order to compare the RNA N-glycosidase activity of a variety of ribosome inactivating proteins (RIPs) such as tritin, momordin and saporin on yeast and rabbit reticulocyte ribosomes with that of ricin. Additional studies to examine the precise site of depurination by the above mentioned RIPs lead Stirpe *et al.* (1988) to suggest that it is likely that all RIPs, both type I and II inactivate ribosomes by 'the same mechanism and with exactly the same specificity'.

Results shown here directly examine the RNA N-glycosidase activity of SLT IA on ribosomes from different sources and show a direct comparison with the RNA N-glycosidase activity of RA.

Results and discussion

The aniline assay developed by Endo *et al.* (1987) was employed in this study to determine the sensitivity of ribosomes isolated from different sources to modification by SLT IA. The assay works on the basis that release of the specific adenine residue from the large subunit (LSU) rRNA as a result of RNA N-

glycosidase activity by a RIP, also results in an increased susceptibility of the phosphodiester backbone of the modified rRNA to attack by nucleophiles. Thus subjection of RIP-modified rRNA to aniline-catalysed hydrolysis results in the preferential release of a characteristic fragment from the 3' end of the LSU rRNA which may be visualised following resolution by denaturing gel electrophoresis (2.6.1).

Recombinant RA (rRA) was the source of ricin A subunit used in this experiment to compare the RNA N-glycosidase activities of RA and SLT IA on ribosomes from a variety of sources. It differs from the native RA in the possession of a methionine residue at its N terminus and, unlike the native RA, it is not glycosylated.

It was found in the course of this study, that a loss of activity of SLT I was observed on incubation with preparations of ribosomes which did not contain any form of reductant. Thus SLT I holotoxin used in these experiments was pretreated with 2-mercaptoethanol to a final concentration of 6mM in order to ensure an activity equivalent to that found in the reducing environment of the cytosol.

Although SLT I does not contain any interchain disulphide bonds linking the A and B subunits, it does possess a single intrachain disulphide bond which links the A_1 and A_2 peptides of SLT IA. It is presumed that on cellular translocation of SLT

FIG 3.2.2 **Comparison of the activities of Shiga-like toxin I A subunit and ricin A subunit on ribosomes from different sources.**

In each case 30ug of ribosomes was incubated (30C, 20min) (2.11.1(i)) with 1ng recombinant ricin A subunit (rRA) and 0.4ug Shiga-like toxin I (SLT I), the latter of which had been pretreated with 2-mercaptoethanol to a final concentration of 6mM in order to ensure optimal activity and maximal reduction of the Shiga like toxin A subunit (SLT IA). The ribosomal RNA (rRNA) was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). It was then fractionated by denaturing gel electrophoresis and the resultant gel stained with ethidium bromide to visualise the fractionated rRNA (2.6.1).

A: tobacco leaf ribosomes

B: Escherichia coli ribosomes

C: Saccharomyces cerevisiae (yeast) ribosomes

D: wheatgerm ribosomes

E: rabbit liver ribosomes

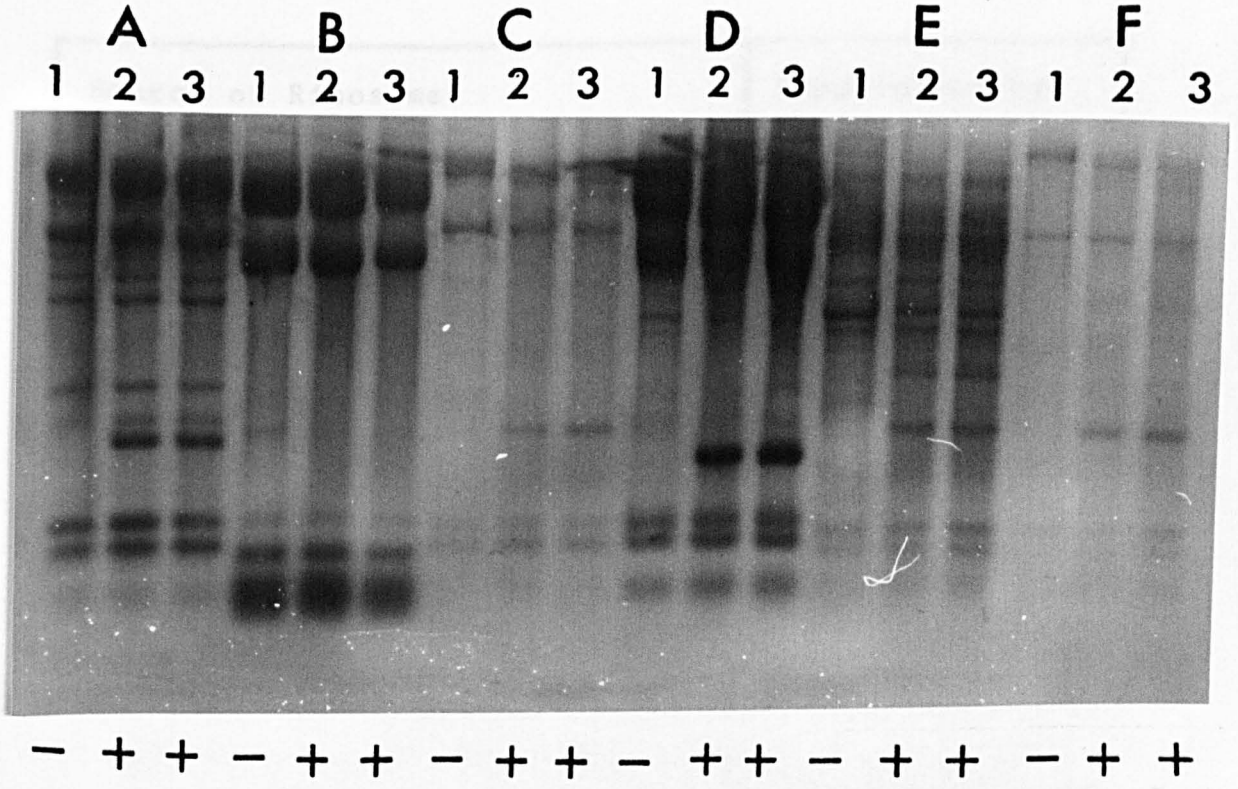
F: rabbit reticulocyte ribosomes

Lane 1: non aniline treated rRNA (-).

Lane 2: rRA modified rRNA.

Lane 3: SLT IA modified rRNA.

+ indicates treatment of rRNA with aniline reagent.


A B C D E F
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
− + + − + + − + + − + + − + + − + +

Source of Ribosome	Depurinated by: RICIN A	SLT IA
TOBACCO	+	+
E.coli	−	−
YEAST	+	+
WHEATGERM	+	+
RABBIT LIVER	+	+
RETICULOCYTE	+	+

FIG 3.2.3 Summary of the activity of Shiga-like toxin I A subunit (SLT IA) on ribosomes from different sources compared to ricin A subunit (RA) activity.

IA the intrachain disulphide bond is reduced. Reduction of this bond therefore, allows experimentation *in vitro* to be more closely representative of that which is proposed to take place *in vivo*.

FIG 3.2.2 shows the results of aniline cleavage of the rRNA isolated from a variety of different ribosomes following their incubation with SLT IA and rRA. These results are summarised in FIG 3.2.3.

Release of a characteristic fragment following aniline cleavage of the modified rRNA may be seen in lanes 2 and 3 of **A** (tobacco), **C** (yeast), **D** (wheatgerm), **E** (rabbit liver) and **F** (reticulocyte). Fragments released in each case are identical in size to those released from rRA-modified rRNA indicating that modification of the rRNA by SLT IA appears to be the same as that modified by RA. Such results suggest that SLT IA exhibits the same substrate specificities as does RA. These results show that in common with RA, SLT IA modifies rabbit reticulocyte ribosomes, tobacco ribosomes, *S.cerevisiae* (yeast) ribosomes and wheatgerm ribosomes. It is necessary to highlight here the apparent differences between for example, wheatgerm ribosomes and the yeast ribosomes with regard to the size of the fragment released following aniline cleavage of the rRNA. Such diffences are as a direct result of the variation in size of the LSU rRNA. Rat liver ribosomes (not shown here) for example, have a 28S LSU rRNA; following depurination by RA and

aniline cleavage a fragment of 390 nucleotides is released from the 3' end of the rRNA (Endo *et al*. 1987). In contrast, the yeast LSU rRNA is a 26S molecule and releases only a 368 nucleotide fragment following depurination by RA and aniline cleavage.

Also in common with RA (Hedblom *et al*. 1976, Stirpe and Hughes, 1989) is the apparent inability of SLT IA to modify *Escherichia coli* ribosomes. The insensitivity of *E.coli* ribosomes to modification by SLT IA, it could be argued, would be expected since SLT I is an *E.coli* produced protein and activity could in this case result in death of the producer strain. It is possible therefore, that the insensitivity of *E.coli* ribosomes to the RNA N-glycosidase activity of SLT IA is a protection mechanism. The apparent insensitivity of *E.coli* ribosomes to SLT IA is of particular relevance to this study since as is seen later it facilitates *in vivo* expression of recombinant SLT IA subunits (rSLT IAs).

E.coli ribosomes are not however, resistant to all RIPs. Recently Hartley *et al*. (1991) have demonstrated the sensitivity of *E.coli* ribosomes to type I RIPs such as dianthin 30 and 32 and pokeweed antiviral protein (PAP) and PAP-S; RIPs which by definition (ie type I RIPs), consist of an enzymatic A subunit alone.

The results presented here show eukaryotic ribosomes including mammalian ribosomes and plant ribosomes to be sensitive to modification not only by RA but also by SLT IA. E.coli ribosomes are however, insensitive to both. This suggests that SLT IA and RA are comparable with regard to their substrate specificities. Detailed kinetic analysis of SLT IA-catalysed depurination of wheatgerm ribosomes is presented in SECTION 3.2.4.

SECTION 3.2.3 Identification of the precise site of depurination of yeast ribosomes by Shiga-like toxin I (SLT I).

Introduction

By direct sequencing of modified RNA, Endo et al. (1987, 1988) had successfully identified the exact site of depurination of rat liver 28S ribosomal RNA (rRNA) by ricin, Shiga toxin (ST) and Shiga-like toxin II (SLT II) as being adenine residue 4324 (A_{4324}). Depurination of rat liver rRNA by SLT I was also assumed to occur at the same site since ST and SLT I are regarded as being essentially identical (Strockbine et al. 1988).

Osborn (1990), having initially demonstrated the modification of 26S rRNA isolated from Saccharomyces cerevisiae ribosomes by ricin A subunit (RA) went on to identify the exact site of depurination. Modification of the 26S rRNA was found to occur at adenine residue 3024 (A_{3024}), a site analogous to A_{4324} in rat liver 28S rRNA within a highly conserved loop structure 368 nucleotides from the 3' end of the S.cerevisiae 26S rRNA. By direct sequencing of modified rRNA in order to identify the missing base, Stirpe et al. (1988) also found A_{3024} of S.cerevisiae 26S rRNA to be the site of depurination of a number of other ribosome inactivating proteins (RIPs).

Previously it had only been assumed that SLT I modification of the rRNA of susceptible ribosomes was identical to that of ST and hence ricin (Endo et al. 1988). It was decided therefore, that the precise site of depurination of S.cerevisiae 26S rRNA by SLT I would be investigated and compared directly to that modified by ricin A subunit (RA) as identified by Osborn (1990).

In identification of the RA modified site in S.cerevisiae 26S rRNA, Osborn (1990) developed a method which combined the use of primer extension and dideoxysequencing of the modified rRNA. This method, which proved much less time consuming than the direct rRNA sequencing method employed by Endo et al. (1987, 1988) and Stirpe et al. (1988)), was the method chosen for use in the present study.

The method employs reverse transcriptase in the production of cDNA from the modified S.cerevisiae 26S rRNA and is based on the observation that reverse transcriptase is unable to read certain chemically modified bases in an RNA template (Hagenbuchle et al. 1978, Youvan and Hearst, 1979). The inability to read modified bases results in the reverse transcriptase pausing or terminating which in turn results in the appearance of a single-length DNA band. This band corresponds to the length of the cDNA from the 5' end of the primer to the nucleotide immediately preceding the modified base. Osborn (1990) worked upon the basis that primer extension

of a RA modified rRNA template would result in the appearance of a band distinct to those resulting from extension of unmodified rRNA.

Results and discussion

Already in this study (SECTION 3.2.2), the ribosomes of S.cerevisiae have been shown to be susceptible to modification by SLT I.

Primer extension of SLT I / rRA modified S.cerevisiae 26S rRNA.

The ^{32}P 5' end labelled 17 oligomer primer FIG 3.2.4 was kindly donated by R Osborn (Warwick) and is complementary to a region of the S.cerevisiae 26S rRNA approximately 70 nucleotides 3' to the putative site of depurination by SLT I and RA. The positions to which the primer anneals on the S.cerevisiae 26S rRNA are given as numbers below the 5' and 3' ends of the primer.

The sequencing gel FIG 3.2.5 shows the products of primer extension following reverse transcription of unmodified S.cerevisiae 26S rRNA in the presence of the dideoxynucleoside triphosphates as indicated by tracks G, A, C and T. Track 3 also shows the result of primer extension of unmodified S.cerevisiae 26S rRNA, whilst track 1 SLT IA subunit modified

FIG 3.2.4 Yeast primer used to determine the site of depurination by Shiga-like toxin I A subunit (SLT IA) in yeast ribosomal RNA. Kindly donated by R Osborn (Warwick).

FIG 3.2.5 **Sequencing gel showing the products of primer extension of Shiga-like toxin I A subunit (SLT IA) and ricin A subunit modified yeast ribosomal RNA.**

60ug yeast ribosomes were incubated with 0.4ug Shiga-like toxin I, with 1ng recombinant ricin A subunit (rRA) or with neither of these at 30°C for 60min. The yeast ribosomal RNA (rRNA) was then extracted (2.4.1) and used as the template for primer extension by reverse transcriptase as described (2.5.9). The products of primer extension are seen here following fractionation on a 8% polyacrylamide gel and visualisation by autoradiography (2.10.3(iii)).

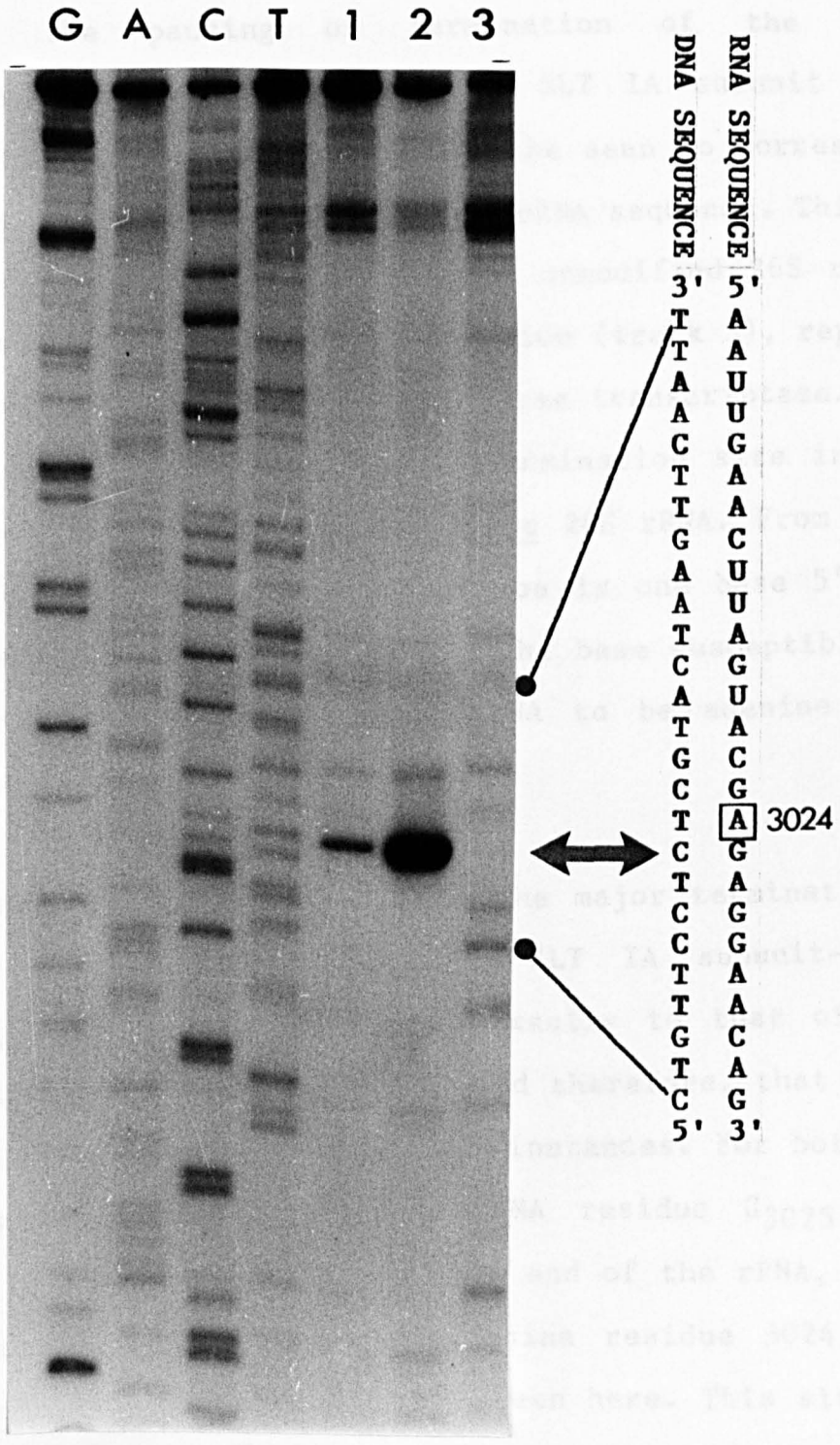
G,A,C,T denote the sequencing reactions carried out on unmodified yeast rRNA template in the presence of the dideoxynucleotide indicated at a concentration of 25uM.

1: SLT IA modified and aniline cleaved yeast rRNA template.

2: RA modified and aniline cleaved yeast rRNA template.

3: unmodified yeast rRNA template.

⟷ denotes primer extension termination site.


G A C T 1 2 3
RNA SEQUENCE 5' AAUUGAACUUAGUACGAGGAACAG 3'
DNA SEQUENCE 3' TTAACTTGAATCATGCTCCTTGTC 5'
3024

rRNA and track 2 RA modified rRNA. Bands seen to be present from primer extension from unmodified rRNA template are as a result of the pausing or termination of the reverse transcriptase. However, in both the SLT IA subunit and RA modified rRNA tracks a major band may be seen to correspond to residue G_{3025} of the S.cerevisiae 26S rRNA sequence. This band, which is not seen to be present when unmodified 26S rRNA was used as the template for primer extension (track 3), represents a major termination site for the reverse transcriptase. Osborn (1990) also observed such a major termination site in primer extension of rRA modified S.cerevisiae 26S rRNA. From this he inferred that the site of depurination is one base 5' to the observed major termination site, ie the base susceptible to RA modification in S.cerevisiae 26S rRNA to be adenine residue 3024.

In this study it can be seen that the major termination site for reverse transcriptase in the SLT IA subunit-modified S.cerevisiae 26S rRNA corresponds exactly to that of the RA modified 26S rRNA. It may be concluded therefore, that the site of depurination is the same in both instances. For both toxins termination appears to occur at RNA residue G_{3025}. Primer extension is complementary to the 3' end of the rRNA, the site of depurination is, therefore adenine residue 3024, ie one base 5' to the site of termination seen here. This site occurs within the highly conserved 5' GAGAGG 3' loop structure of the 26S rRNA.

Thus, as was originally inferred by Endo et al. (1988) from studies of the depurination of rat liver ribosomes by ST, SLT II and ricin, SLT I (with regard to yeast ribosomal rRNA at least) also modifies the rRNA at precisely the same site as does ricin ie in this case A_{3024} of S.cerevisiae 26S rRNA. It is now highly likely that the analogous adenine residue occurring within the highly conserved GAGAGG rRNA loop is the likely target site of modification by ricin and all other known RIPs with catalytic site homologies. However, this would exclude RIPs such as α sarcin. Unlike RA catalysed hydrolysis of a single N-glycosidic bond of adenine residue 4324, α sarcin hydrolyses a single phosphodiester bond in 28S rRNA between guanine residue 4325 and adenine residue 4326. In addition sarcin shares no homology with the ricin A subunit-like family of enzymes.

SECTION 3.2.4 Kinetic comparison of Shiga-like toxin I A subunit (SLT IA)-catalysed depurination with that of ricin A subunit (RA).

Introduction

As previously discussed, direct comparison between the activities of Shiga-like toxin I A subunit (SLT IA) and ricin A subunit (RA) has been limited. The following experiments attempt to compare kinetically, SLT IA catalysed depurination of salt washed wheatgerm ribosomes with the findings of Osborn (1990) for RA.

Studies by Osborn (1990) revealed a differential sensitivity between wheatgerm ribosomes and rabbit reticulocyte ribosomes to RA-catalysed depurination of approximately 1000 fold which was found not to be attributable to supernatant factors. In order to study the activity of RA on wheatgerm ribosomes more closely, Osborn (1990) carried out kinetic experiments using salt washed ribosomes in order to eliminate bound elongation factors, which may lead to aberrant results. Thus it was hoped that in this way any observed differences in sensitivity could be attributed to either differences in binding affinity of RA for wheatgerm ribosomal RNA (rRNA) and rabbit rRNA or in the rate of catalysis.

The kinetic experiments carried out allowed the binding affinity of the enzyme (RA) for its substrate (wheatgerm rRNA) to be determined. The Michaelis - Menton equation for first order kinetics predicts that if the rate of dissociation (k_2) of the enzyme substrate complex is greater than the rate of product formation (k_3) then the K_m value derived is a measure of the binding affinity of the enzyme for its substrate. By comparison of the dissociation constant (k_d) for RA and rat liver ribosomes of 2uM (Hedblom et al. 1976) with the K_m value of 2.6uM for the same reaction (Endo and Tsurugi, 1988), Osborn (1990) reasoned that K_m and k_{cat} values obtained from examination of RA-catalysed depurination of wheatgerm rRNA would be effective indicators of whether differences in sensitivity of ribosomes to RA were binding or catalytic in nature.

In the examination of SLT IA-catalysed depurination of wheatgerm rRNA, it was also assumed that the K_m value derived would be a direct measure of the binding affinity of the enzyme (SLT IA) for its substrate (rRNA) as was the case with RA.

Endo and Tsurugi (1988) had previously measured the rate of depurination of rat liver ribosomes by RA by calculating the amount of the characteristic 390 base fragment released following aniline cleavage of the depurinated ribosomal RNA (rRNA). In this study, radioactively labelled ribosomes had been used and the amount of aniline fragment released measured

from radioactive counts. The percentage depurination was calculated by expression of this figure as a ratio to the 5.8S rRNA.

Osborn (1990) also used the aniline assay as used by Endo and Tsurugi (1988) to measure the extent of depurination of wheatgerm ribosomes by RA. The amount of aniline released fragment and 5.8S rRNA however, was determined directly from ethidium bromide stained gels on the assumption that labelling of the rRNA by ethidium bromide is directly proportional to the length of the RNA fragments.

A Molecular Dynamics computing densitometer was employed to scan negatives of rRNA gels and its volume integration option used to generate meaningful data. This data could then used to calculate the relative amounts of each rRNA fragment. Osborn (1990) was able to derive an equation to calculate the percentage depurination from the data generated from scanning of the RNA gel. Derivation of the equation required that the lengths of wheat 5S and 5.8S rRNA (120 and 164 nucleotides respectively) and the approximate length of the aniline fragment released from the 3' end of wheat 25S rRNA following depurination (366 nucleotides) be known.

Equation 1:

$$\frac{\text{RELATIVE AMOUNT ANILINE FRAGMENT}}{\text{RELATIVE AMOUNT 5.8S rRNA}} \times \frac{164}{366} \times 100 = \%\ \text{depurination}$$

The above equation eliminates variations which may arise as a result of the differences in loading of RNA in the tracks, by expressing the aniline fragment relative to the 5.8S rRNA which is present in equimolar amounts to the 25S rRNA (Rubin, 1973).

It was decided that application of the kinetic experiments as described by Osborn (1990) and also used successfully by Chaddock (1991) in studies on RA mutants, would provide a valuable insight into the depurination of ribosomes by SLT IA. In addition, use of salt washed wheatgerm ribosomes would provide a direct comparison between RA-catalysed depurination of wheatgerm ribosomes (Osborn, 1990) and SLT-IA catalysed depurination.

Results and Discussion

The initial substrate (ribosome) and enzyme (SLT IA) concentrations used to investigate the kinetics of SLT IA catalysed depurination of salt washed wheatgerm ribosomes were based upon those described by Osborn (1990). Salt washed

wheatgerm ribosomes were prepared as described (2.3(ii)) and used in the kinetic experiments in concentrations ranging from 0.24uM to 1.43uM. For the following experiments it is assumed that wheatgerm ribosomes have a molecular weight of 4.2×10^6 the same as that published for yeast ribosomes (Wool, 1979).

As previously described, prior to incubation of SLT I holotoxin with ribosomes the holotoxin was pretreated with 2-mercaptoethanol to a final concentration of 6mM to accommodate for the lack of a reducing agent such as 2-mercaptoethanol in the preparation of the salt washed wheatgerm ribosomes. FIG 3.2.6 demonstrates the <u>in vitro</u> activity of SLT IA on salt washed wheatgerm ribosomes (lane 1) in comparison to that of RA (lane 2).

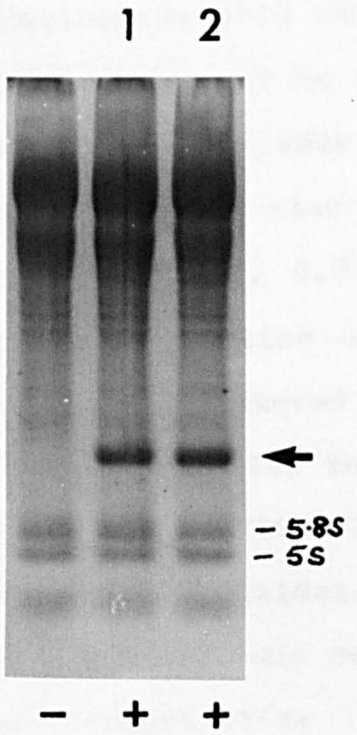
With regard to quantifying SLT IA concentrations, the holotoxin was regarded as having a molecular weight of approximately 69.5KDa. It was assumed that for any given concentration of holotoxin the 32KDa SLT I A subunit and the SLT I B subunit pentamer (5 x 7.5KDa = 37.5KDa) each comprised approximately 46% and 54% of that concentration. Thus the concentration of SLT IA subunit in each case was determined by calculating 46% of the given concentration of SLT I holotoxin. SLT IA concentrations in the range of 0.015uM - 0.1uM were used in the following kinetic experiments.

FIG 3.2.6 **Investigation of the activity of Shiga-like toxin I A subunit on wheatgerm ribosomes in comparison to that of ricin A subunit.**

0.4ug of pure Shiga-like toxin I (SLT I) was incubated with 30ug wheatgerm ribosomes for 20min at 30C (2.11.1(i)). The resultant ribosomal RNA (rRNA) was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). Fractionation and visualisation of the resultant rRNA was then carried out (2.6.1).

Lane **1** shows wheatgerm rRNA following incubation with pure SLT I.
Lane **2** shows wheatgerm rRNA following incubation with recombinant ricin A subunit (rRA).

+ indicates treatment of rRNA with aniline reagent.
- indicates that following incubation with SLT I and extraction the rRNA was not subjected to aniline cleavage.
➡ indicates the characteristic fragment released following aniline cleavage of toxin-modified rRNA.


1
2
5·8S
5S
–
+
+

Salt washed wheatgerm ribosomes at a concentration of 1.27uM (160ug in a final reaction volume of 30ul) were incubated with a range of concentrations of SLT IA (0.015uM - 0.1uM) over a time course. This allowed the concentration of SLT IA which would give a linear rate of depurination and the time span over which this would remain linear to be determined. FIG 3.2.7 shows the percentage depurination with time when [ribosomes] = 1.27uM and [SLT IA] = 0.033uM. It may be seen that the rate of depurination remains approximately linear for the first 10min. The initial rate of depurination was also examined at different concentrations of SLT IA - 0.015uM, 0.033uM, 0.05uM, 0.067uM with a constant ribosome concentration of 1.27uM (160ug in a final volume 30ul). Aliquots were removed at two time points in order to allow a value for the initial rate of depurination to be determined. The resultant plot (FIG 3.2.8) shows the initial rate of depurination to remain approximately linear with regard to the concentration of SLT IA. It was not possible to examine the initial rate of depurination at higher SLT IA concentrations than 0.067uM due to the low concentration of the SLT I holotoxin preparation. The SLT IA concentration chosen for use in the experiments to determine K_m and k_{cat} was 0.033uM.

A range of concentrations of salt washed wheatgerm ribosomes from 0.35uM (45ug in a final reaction volume 30ul) to 1.43uM (180ug in a final reaction volume 30ul) were incubated with 0.033uM SLT IA for 10min. Aliquots were removed from the

FIG 3.2.7 **Time course for depurination of wheatgerm ribosomes by Shiga-like toxin I A subunit.**

160ug of salt washed wheatgerm ribosomes in a final reaction volume of 30ul (final concentration 1.27uM), were incubated at 30°C for 20min with Shiga-like toxin I A subunit to a final concentration of 0.033uM (2.11.1(i)). From the total reaction volume of 30ul aliquots of 3ul were removed at various time points and the ribosomal RNA (rRNA) extracted (2.4.1). This was then subjected to aniline cleavage (2.5.1) and fractionated by denaturing gel electrophoresis (2.6.1). Scanning of the rRNA using a Molecular Dynamics computing densitometer and the application of **EQUATION 1** to the data generated, allowed the percentage depurination in each case to be determined.

This experiment was carried out in excess of six times. The resultant plot was achieved at least three times!

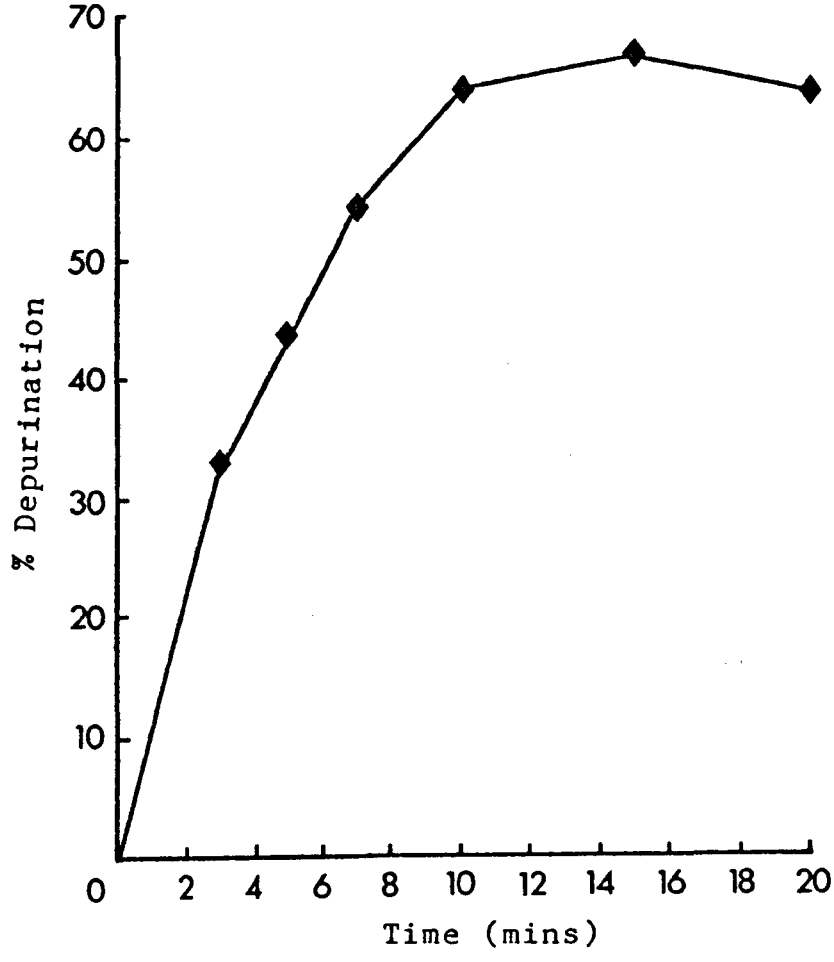
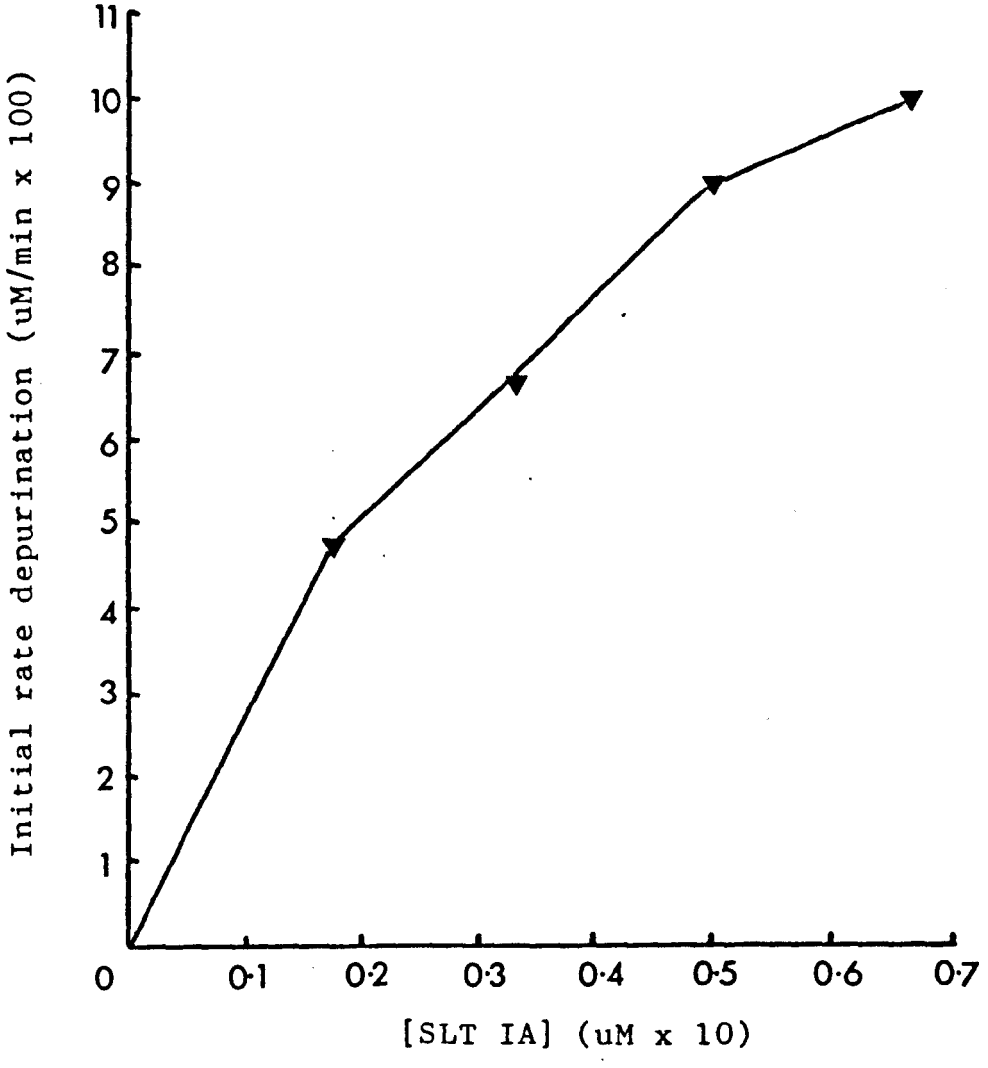

70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
% Depurination
Time (mins)

FIG 3.2.8 Examination of the effect of Shiga-like toxin I A subunit concentration on the initial rate of depurination.

160ug of salt washed wheatgerm ribosomes in a final reaction volume of 30ul (final concentration 1.27uM), were incubated with a range of concentrations of Shiga-like toxin I A subunit (SLT IA) from 0.015 - 0.067uM (2.11.1(i)). At intervals of 5 and 10min aliquots of 3ul were removed from the reaction (30ul total volume) and the ribosomal RNA (rRNA) extracted (2.4.1). This was treated with aniline reagent (2.5.1) and fractionated by denaturing gel electrophoresis (2.6.1). Based on the assumption that the reaction rate remained linear over the first 10min of the reaction, the initial rate of depurination for each concentration of SLT IA was calculated from the percentage depurination at the two time points.

This experiment was carried out a total of three times. The results are representative of data generated from the scanning of two gels.


Initial rate depurination (uM/min x 100)
11
10
9
8
7
6
5
4
3
2
1
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
[SLT IA] (uM x 10)

reaction at two time points, 5min and 10min, in order to allow a value for the initial rate of depurination to be calculated for each reaction. The rRNA was then extracted, subjected to treatment with aniline, fractionated by denaturing gel electrophoresis and the resultant gel carefully stained with ethidium bromide. Scanning of the negative photograph of the gel and application of **EQUATION 1** to the data obtained from the scanning allowed the percentage depurination at each time point to be determined. The initial rate of depurination for each [SLT IA] was then determined by application of these values to the following:

$$\frac{\text{sum \% depurination (2 time points)}}{2} = \text{average \% depurination}$$

$$\frac{\text{average \% depurination}}{\text{time (min)}} = \text{\% depurination / min}$$

$$\frac{\text{\% depurination} \times \text{ribosome concentration (uM)}}{100 \times \text{time (min)}}$$

INITIAL RATE DEPURINATION (uM/min)

In order to produce meaningful experimental data by scanning with the densitometer, great care had to be taken in both gel preparation and in its staining with ethidium bromide in order

to visualise the resolved RNA. With regard to the latter, staining and destaining had to be carefully monitored, since it was found that diffusion of RNA bands occurred relatively quickly. Such diffusion resulted in an inability to distinguish between the 5 and 5.8S rRNA bands. Thus, the generation of meaningful data by scanning the gel was impaired.

Use of a computer programme 'Enzkin 9' allowed determination of K_m and V_{max} from the experimental data via a number of different methods. Statistically, the Cornish - Bowden plot provides the best method of analysis since median values and not mean values of K_m and V_{max} are generated. Thus, unlike the use of the Lineweaver - Burke plot, the analysis is virtually unaffected by one or two aberrant values. FIG 3.2.9 shows the plots obtained. FIG 3.2.10 displays the values of K_m and V_{max} 0.978uM and 0.128umol/ min respectively obtained by Cornish - Bowden analysis in comparison to that derived by Osborn (1990) for RA-catalysed depurination of salt washed wheatgerm ribosomes.

The K_m and V_{max} values for the depurination of salt washed wheatgerm ribosomes by SLT IA are of a similar order to those values derived by Osborn (1990) for depurination of salt washed wheatgerm ribosomes by RA. The slightly lower K_m value for depurination by SLT IA (K_m = 0.978uM) indicates a higher affinity of SLT IA for its substrate than that of RA (K_m = 2.1uM). From the V_{max} value the turnover number (k_{cat}) may be

FIG 3.2.9 Determination of K_m and V_{max} for the depurination of wheatgerm ribosomes by Shiga-like toxin I A subunit using Cornish - Bowden and Lineweaver - Burke plots.

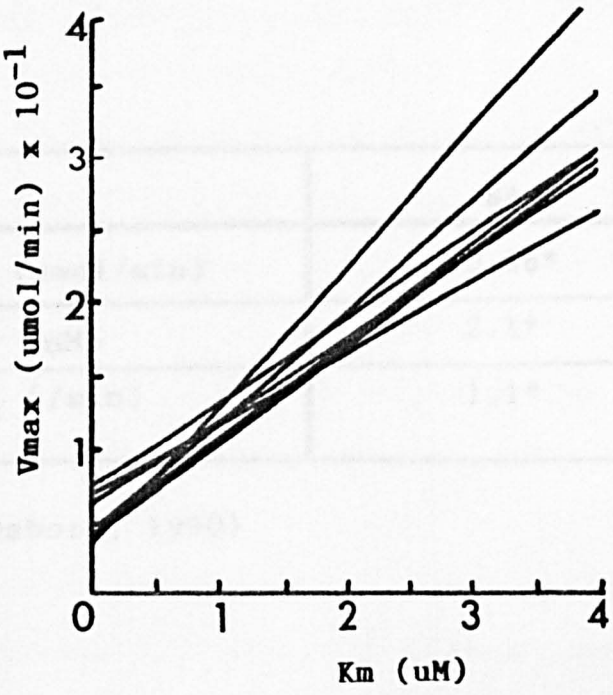
Salt washed wheatgerm ribosomes in concentrations ranging from 0.35uM - 1.43uM (45 - 180ug in a final reaction volume of 30ul) were incubated at 30°C (2.11.1(i)) with 0.033uM Shiga-like toxin I A subunit (SLT IA). Aliquots, each of 15ug were removed from the reaction at 5 and 10min and the ribosomal RNA (rRNA) extracted (2.4.1). This was then subjected to aniline cleavage (2.5.1) and fractionated by denaturing gel electrophoresis (2.6.1). Scanning of the resultant rRNA gel allowed the percentage depurination for each time point to be calculated using EQUATION 1. From this the initial rate of depurination for each ribosome concentration was determined as described in the text. The resultant data was applied to Lineweaver - Burke and Cornish - Bowden analysis in order to calculate values of K_m and V_{max}.

The resultant plots were generated from a single experiment.

1: Cornish - Bowden plot

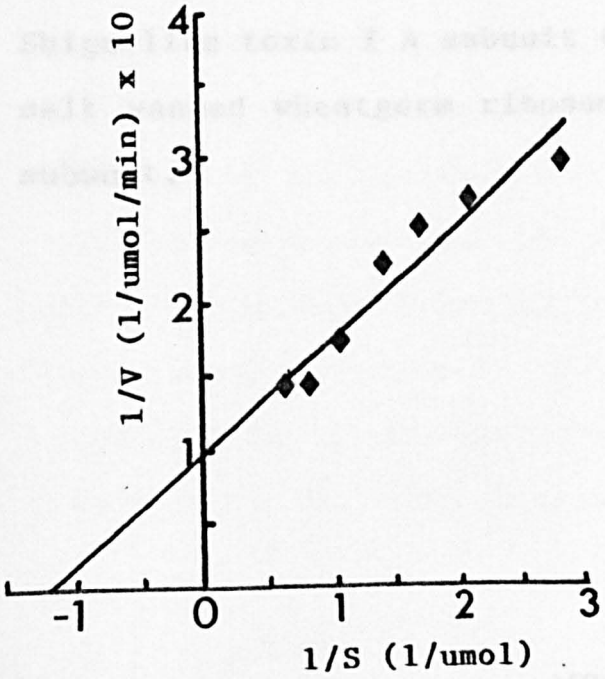
2: Lineweaver - Burke plot

1 CORNISH-BOWDEN PLOT



Vmax = 0.128 umol/min
Km = 0.978 uM

2 LINEWEAVER-BURKE PLOT



Vmax = 0.105 umol/min
Km = 0.826 uM

	RA	SLT IA
Vmax (umol/min)	0.06*	0.128
K_m (uM)	2.1*	0.98
K_{cat} (/min)	1.1*	3.9

* (Osborn, 1990)

FIG 3.2.10 Summary of the K_m, V_{max} and k_{cat} values derived for Shiga-like toxin I A subunit (SLT IA) catalysed depurination of salt washed wheatgerm ribosomes compared to those of ricin A subunit.

calculated giving a value of 3.9 min^{-1}, a value almost four times greater than the value of 1.1 min^{-1} for RA catalysed depurination of wheatgerm ribosomes as determined by Osborn (1990). Kinetic analysis of RA-catalysed depurination of yeast (Saccharomyces cerevisiae) ribosomes by Chaddock (1991) revealed a binding affinity of RA for yeast rRNA (K_m = 1.48uM) of a similar order to those of SLT IA (the present study) and RA (Osborn, 1990) for wheatgerm rRNA. However, a much greater turn over number (k_{cat} = 195min^{-1}) for depurination of yeast ribosomes by RA (Chaddock, 1991) was observed in this case which may serve to explain the differential sensitivity of yeast and wheatgerm ribosomes to depurination by RA.

The results of this investigation into the kinetics of the depurination of salt washed wheatgerm ribosomes by SLT IA suggest that the increased affinity (K_m) and a maximum velocity value (V_{max}) for SLT IA, which is almost double that of RA-catalysed depurination, are reflected by a turnover number almost four times as great as that for determined by Osborn (1990) for RA. Slight differences in SLT IA and RA-catalysed depurination of wheatgerm ribosomes are suggested by the findings of the present study. It is important to note however, that although the same protocols were followed to investigate SLT IA-catalysed depurination of wheatgerm ribosomes as had been used by Osborn (1990) to investigate RA-catalysed depurination of wheatgerm ribosomes, the complex nature of the experiments may also introduce a small degree of error. In

addition, it is also important to note that the salt washed wheatgerm ribosome substrate used may vary slightly in quality as a result of small differences in methods of preparation. Due to limited resources ie SLT I, the final experiment to generate data for the Cornish - Bowden analysis was carried out a total of three times. However, as a result of the complex nature of the kinetic assay, of the three resultant gels only one was suitable for scanning. K_m and V_{max} values presented here for SLT IA-catalysed depurination of salt washed wheatgerm ribosomes were therefore obtained from a single analysis. Due to the complex nature of the kinetic analysis, it is felt that differences (as revealed in this study) in the depurination of wheatgerm ribosomes by SLT IA (the present study) and RA (Osborn 1990) may in fact be attributable to experimental error and may therefore, be negligible.

Conclusion - Chapter 2

The results presented in this chapter provide a direct comparison between a number of biological activities of ricin and Shiga-like toxin I (SLT I), both of which are Type 2 ribosome inactivating proteins (RIPs) with a capacity to irreversibly inactivate eukaryotic ribosomes. The biological activities of ricin have been studied extensively and it is considered the proto-type member of the Type 2 RIPs. However, although SLT I has previously been compared to ricin, direct comparison between the two has been limited.

A direct comparison of the cytotoxicities, substrate specificities, precise site of depurination and the kinetics of depurination of salt washed wheatgerm ribosomes between SLT I and ricin is presented here. Sugar or receptor binding has not been analysed.

In comparison, the 50% cytotoxic dose value (IC_{50}) of 0.1ng/ml for the action of SLT I on Vero cells (SECTION 3.2.1) is seen to be approximately 10 fold less than that displayed by ricin (1 - 3ng/ml) (O'Hare *et al.* 1987, 1990, Yoshida *et al.* 1991). Results obtained by Olsnes *et al.* (1981) for the cytotoxic action of Shiga toxin (ST) on HeLa cells also report a reduction in the number of ST molecules required to cause 50% cell death compared with that of ricin. This is suggested to be

attributable to a greater binding affinity of ST for its cellular receptor than that of ricin for its. More recent studies however, present evidence to suggest that the differences in cytotoxicity of ricin and ST / SLT I may be attributable to any number of steps in the complex processes of binding, uptake and translocation of toxin. For example, ricin binds opportunistically to cell-borne glycolipids and glycoproteins terminating in galactose (Sandvig et al. 1991). Such opportunistic binding may not lead to endocytosis of the ricin molecule. In contrast, the binding of ST and SLT I to glycolipids terminating in Galα1-4Galβ is highly specific (Cohen et al. 1987, Lindberg et al. 1987, Lingwood et al. 1987). It is likely therefore, that in each case binding will lead to endocytosis of the ST / SLT I molecule. The existence of five potential receptor binding sites on the ST / SLT I B subunit pentamer observed from the crystal structure of the SLT I B subunit pentamer (Stein et al. 1992) may also contribute to the high specificity, tight binding of ST / SLT I to its cell-borne receptor.

There are of course other possibilities to explain the increased cytotoxicity of SLT I as compared to ricin. In both cases the processes following endocytosis remain unclear. Following absorptive endocytosis routing of ricin to the endoplasmic reticulum (ER) is thought to occur as a result of receptor exchange by the toxin in the trans-Golgi network (TGN) and interaction with proteins with KDEL-like sequences. Sandvig

et al. (1992) present evidence for the accumulation of ST in the ER following receptor mediated endocytosis (RME). The mechanism by which it reaches the ER is not known. However, it is suggested that as with ricin routing may occur via receptor exchange within the TGN or by interaction with proteins possessing KDEL-like sequences. A further suggestion is that transport of ST to the ER is mediated by glycolipids. As is evident, the process of intracellular routing may be complex.

Other factors which may contribute to the observed differences in cytotoxicity of ricin and SLT I, are those of processing, membrane translocation and differences in catalytic activity of the two toxins. The results of detailed kinetic analysis of the depurination of salt washed wheatgerm ribosomes by SLT IA (SECTION 3.2.4) may shed some light upon this matter.

Results in this section reveal a turnover number (k_{cat}) approximately four times that of ricin A subunit (RA) catalysed depurination of wheatgerm ribosomes as determined by Osborn (1990) ($3.9min^{-1}$ (SLT IA) and $1.1min^{-1}$ (RA)). This may be attributed to a maximum velocity value (V_{max} = 0.128umol / min) double that obtained for RA (0.06umol / min, Osborn, 1990) in addition to an increased binding affinity of SLT IA for its substrate (K_m = 0.978uM (SLT IA) compared to 2.1uM (RA - Osborn, 1990)). Thus it may be the case that the in vitro comparison of SLT IA and RA catalysed-depurination is reflected

in vivo, resulting in a reduction in the amount of SLT I required to give 50% cell death in Vero cells.

A comparison of the activities of SLT IA and RA on eukaryotic ribosomes isolated from different sources (SECTION 3.2.2) demonstrates that of the ribosomes examined, SLT IA has the same substate specificity as does RA. Whilst both RA and SLT IA may be seen to modify the ribosomal RNA (rRNA) of yeast, wheatgerm, rabbit reticulocyte, rabbit liver and tobacco leaf ribosomes, neither is able to modify the rRNA of *Escherichia coli* ribosomes. This is in contrast to recently characterised RIPs, dianthin 30, 32 and pokeweed antiviral proteins which can depurinate *E.coli* ribosomes (Hartley *et al*. (1991).

In addition to exhibiting the same substrate specificities as RA, examination of the precise site of depurination of yeast (*Saccharomyces cerevisiae*) ribosomes (SECTION 3.2.3) by SLT IA reveals that exactly the same adenine residue is removed (A_{3024}) as is removed by RA (Osborn, 1990).

To conclude, it would appear from investigations carried out here, that in essence the biological activity of SLT I is almost identical to that of ricin.

In addition, results presented in this study allow the activity of SLT I to be compared with published observations regarding the activity of ST.

With regard to RNA N-glycosidase activity of ST, Olsnes et al. (1981) and Reisbig et al. (1981) have reported the inhibition of protein synthesis in rabbit reticulocyte cell free systems by ST. Results from the present study (SECTION 3.2.2) also demonstrate the modification of rabbit reticulocyte ribosomes by SLT I. Comparison of the activities of ST and ricin by Endo et al. (1988) and Saxena et al. (1989) suggests that the two have the same substrate specificities. This was also the finding of the present study (SECTION 3.2.2) regarding the RNA substrate specificities of SLT IA and RA. It would appear, therefore, that ST / SLT I and ricin all exhibit the same substrate specificities and enzymatic activities.

This suggestion is further substantiated by Endo et al. (1988) who demonstrated that modification of rat liver ribosomes by ST occurred at the same site as that of ricin ie adenine residue 4324 of the 28S ribosomal RNA (rRNA). Results presented by this study (SECTION 3.2.3), reveal that the precise site of depurination by SLT I in yeast ribosomes is adenine residue 3024. This is the same site at which RA has been shown to modify yeast 26S rRNA (Osborn, 1990). Since studies reveal that ST depurinates rat liver ribosomes at exactly the same site as does ricin (Endo et al. (1988), and that SLT I depurinates yeast rRNA at exactly the same site as does ricin (the present study) it would appear that ST and SLT I modify the rRNA of susceptible eukaryotic ribosomes by depurination at the same adenine residue.

Examination of the cytotoxic effect of SLT I on Vero cells revealed a requirement of 0.1ng/ml SLT I to result in 50% inhibition of cell protein synthesis. This was also the 100% cytotoxic dose value for SLT I on Vero cells reported by Kongmuang et al. (1988) and by Olsnes and Eiklid (1980) for the cytotoxic effect of ST on HeLa S_3 cells. For the cytotoxic effect of ST on Vero cells both Eiklid and Olsnes (1980) and Sandvig and Brown (1987) report an ID_{50} value of 0.6ng/ml. It may be seen that results obtained in the present study regarding the cytotoxic activity of SLT I on Vero cells, is comparable to those obtained by other groups. In order to draw a direct comparison between the observed cytotoxicity of SLT I (this study) and ST (Sandvig and Brown, 1987) on Vero cells, it would be necessary to carry out the assays simultaneously using exactly the same materials and methods in each instance.

Recent kinetic studies of RA-catalysed depurination of eukaryotic ribosomes has revealed K_m values ranging from 0.1 - 2.6uM and k_{cat} values ranging from 201 - 1500min^{-1} (Endo and Tsurugi, 1988, Osborn, 1990, Chaddock, 1991 and Ready et al. 1991) depending on the source of ribosomes used and the assay conditions. In contrast, there has been little kinetic analysis of the depurination of eukaryotic ribosomes by ST / SLT I / SLT II. Reisbig et al. (1981) do however, report a turn over 40 ribosomes per minute for the action of the ST A_1 fragment on reticulocyte ribosomes. ST A_1 fragment used in this study was generated by pre-nicking of ST A subunit with trypsin followed

by reduction. Results presented in the present study for SLT IA-catalysed depurination of salt washed wheatgerm ribosomes give a k_{cat} value of $3.9min^{-1}$. In this study SLT I holotoxin was pretreated with 2-mercaptoethanol (final concentration 6mM) in order to examine activity of the holotoxin in a physiological environment, similar to the reducing conditions of the cytosol. In order to compare directly the kinetics of SLT I / ST-catalysed depurination of eukaryotic ribosomes the same assay conditions and same source of ribosomes must be used since there may be a marked difference in sensitivity of reticulocyte and wheatgerm ribosomes to depurination by SLT IA / ST A. With regard to this Osborn (1990) reported the differential sensitivity of reticulocyte and wheatgerm ribosomes to depurination by RA (reticulocyte ribosomes were 1000 times more sensitive than were wheatgerm ribosomes).

Results presented here do however, suggest that as proposed by Strockbine et al. (1988), ST and SLT I are the same.

CHAPTER 3

SECTION 3.3 **Production of recombinant Shiga-like toxin I A subunits.**

Introduction

It has been proposed and in some cases demonstrated, that bacterial toxins such as cholera toxin (CT) (Gill, 1976), Escherichia coli heat labile toxin (LT) (Spicer and Noble, 1982, Yamamoto et al. 1987), Pseudomonas exotoxin A (PE) (Jinno et al. 1989, Madhus and Collier, 1989, Ogata et al. 1990) and diphtheria toxin (DT), have a requirement for proteolytic processing in order to generate an enzymatic fragment with enhanced activity. This has also been suggested to be the case with Shiga toxin (ST) and Shiga-like toxin I (SLT I).

Olsnes et al. (1981) examined differences in the cytotoxic effects of nicked-reduced, nicked-unreduced and unnicked ST and reported there to be no difference in cytotoxicity for HeLa S_3 cells. In order to examine the effect of nicking and reduction on ST activity the toxin was treated with trypsin and reducing agents such as 5% 2-mercaptoethanol.

Kongmuang et al. (1988) report there to be no difference in the cytotoxicity of nicked and unnicked SLT I on Vero cells. Again, these effects were studied by incubation of toxin with trypsin in order to generate nicked SLT I.

Reisbig et al. (1981) used a variety of different chemical treatments including urea, sodium dodecyl sulphate (SDS), dithiothreitol (DTT), 2-mercaptoethanol and trypsin in order to compare the activity of isolated ST A_1 fragment (27.5KDa) to that of the untreated, intact whole ST A subunit (32KDa). Their findings were that the A_1 fragment alone was six times more efficient in the inactivation of rabbit reticulocyte ribosomes than was the unnicked A subunit and three times more active than trypsin / DTT / urea treated holotoxin. The increase in activity following such treatment they attribute to the splitting off of the A_2 peptide from the ST A subunit. This in turn results, they suggest, in the achievement of full activity of the A_1 fragment of ST.

It is evident from such studies that the A_1 and A_2 peptides have been generated via rather non specific chemical means. It was therefore decided that any role of the A_2 peptide in activity of the SLT I A subunit would be examined in this present study by the construction of recombinant SLT I A subunits (rSLT IAs). This would then allow any differences in enzymatic activity of the whole SLT I A subunit and the A_1 peptide to be examined both in vivo and in vitro without a prequisite for non specific chemical and enzymatic treatments of the proteins under examination.

Examination of the primary sequence of bacterial toxins such as PE (Jinno et al. 1989, Madhus and Collier, 1989, Ogata et al.

1990) revealed that, the region of the protein predicted to undergo proteolytic cleavage to generate a fragment with enhanced enzymatic activity was arginine-rich and bounded by cysteine residues capable of forming a disulphide bridge (FIG 1.7.2).

Calderwood et al. (1987) proposed that this may also be the case with the SLT I A subunit. It was suggested that cysteine (Cys) residues 242 and 261 bounded a region which, on proteolytic cleavage, would generate two peptides. The sizes of these fragments would correspond to those observed following careful digestion of SLT I A subunit with trypsin and resolution under reducing conditions by SDS polyacrylamide gel electrophoresis (PAGE). FIG 3.3.1 demonstrates the necessity not only for treatment of SLT IA with trypsin for production of the separate SLT IA_1 and SLT IA_2 peptides but also the requirement for reduction (lane 1). Neither alone results in separation of the two peptides (lanes 2 and 3).

Two arginine (Arg) residues exist within this proposed cleavage site (Arg^{248} and Arg^{251}). Spicer and Noble (1982) proposed that proteolytic cleavage of the A subunit of LT to generate A_1 and A_2 polypeptides occurred after Arg^{188}. In addition, Jinno et al. (1989) and Ogata et al. (1990), found that mutagenesis of Arg residues 276 and 279, (residues within domain II of PE) to glycine (Gly) greatly reduced the cytotoxicity of the toxin even though normal cell binding and enzymatic capabilities were

FIG 3.3.1 The effect of treating Shiga-like toxin I A subunit (SLT IA) with trypsin and reducing agent.

In each case 10ug purified Shiga-like toxin was subjected to different combinations of treatment with L-1-tosylamido-2-phenylethyl chloromethyl ketone treated-trypsin (Sigma) (50ug/ml final concentration (2.10.2) and SDS polyacrylamide gel electrophoresis (SDS PAGE) (2.10.3) under both reducing and non reducing conditions. Reducing conditions were achieved by the addition of 2-mercaptoethanol to the sample buffer to a final concentration of 0.75M. Treatment with trypsin was by incubation at 37°C for 15min (2.10.2). The resultant products were analysed by SDS PAGE (non reducing and reducing conditions) on a 15% gel followed by Coomassie staining (2.10.3(iii)).

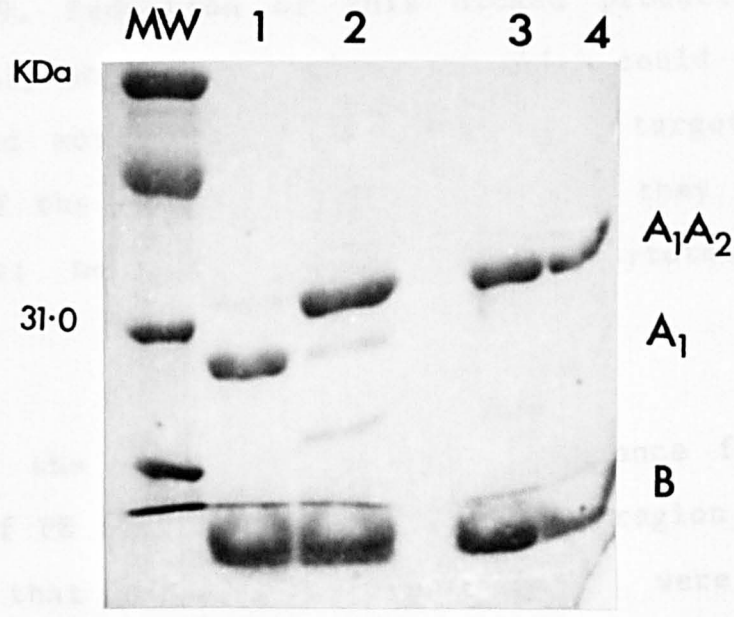
1: SLT I treated with trypsin and 2-mercaptoethanol (reducing).

2: SLT I treated with 2-mercaptoethanol alone (reducing).

3: SLT I treated with trypsin alone (non reducing).

4: SLT I untreated (non reducing).

MW: molecular weight markers.


MW
1
2
3
4
KDa
31·0
A_1A_2
A_1
B

evident. Again the Arg rich region within domain II of PE is bounded by Cys residues capable of forming a disulphide bond. Ogata et al. (1990) followed the processing of PE in mammalian cells and were able to conclude from their studies that native PE undergoes proteolytic cleavage within domain II close to Arg residue 279. Reduction of this nicked product generated an enzymatically active 37KDa fragment which could then enter the cytosol and act upon its intracellular target. Proteolytic cleavage of the domain II mutant, Gly^{276}, they found, did not take place; hence the reduction in cytotoxicity of the molecule.

It was on the basis of the above evidence for proteolytic cleavage of PE and LT within an Arg rich region bounded by Cys residues, that Arg residues 248 and 251 were identified as potential sites for cleavage in SLT I A subunit. It was decided that for the purpose of studying the role of the A_2 peptide in both the RNA N-glycosidase activity of SLT IA and the cellular uptake of the holotoxin these residues would be the targets for site directed mutagenesis in order to generate recombinant SLT I A fragments.

Recent work by Jobling and Holmes (1992) examined the interaction between the A_2 peptide of cholera toxin (CT) and its pentamer of B subunits. By constructing fusion proteins of bacterial alkaline phosphatase, maltose-binding protein and beta-lactamase, each possessing a C terminal CT A_2 peptide, it

was found that each of the fusion proteins were capable of associating with CT B subunits to form holotoxin-like chimeric molecules. In addition, the recent work of Streatfield et al. (1992) suggests a role for C terminal of E.coli heat labile toxin (LT) A subunit in promoting the oligomerisation of the B subunit pentamer of the toxin. On deletion of the last 14 C terminal residues of the LT A subunit (LTA) (-14), a significant reduction in assembly of the B subunit pentamer was observed. Deletion of only the last 4 amino acid residues of LTA (-4) however, resulted in assembly of an A_2-B_5 complex but with apparent reduced stability. These observations led to the suggestion that residues -14 to -4 of LTA are in some way involved in the promotion of B subunit oligomerisation, whilst the last 4 residues behave as an 'anchor' in stabilising the interaction between the A and B subunits during holotoxin assembly.

To date the role of the SLT IA_2 peptide in association of the SLT IB pentamer with the SLT IA subunit remains unclear. Thus, in addition to facilitating study of any contribution of the SLT IA_2 peptide in the RNA N-glycosidase activity and cell entry properties of the SLT I A subunit, it was decided that the recombinant SLT I A subunits constructed should also allow examination of the non covalent interaction between the SLT IA_2 peptide and the Shiga like toxin I B subunit (SLT IB) pentamer.

The following recombinant SLT I A subunits were generated:

(a) a truncated SLT IA comprising the A_1 fragment alone

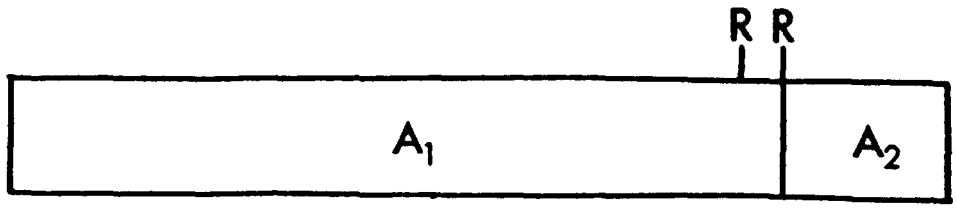
(b) a full length SLT IA rendered insusceptible to proteolytic processing.

A fragment of SLT IA A_2 alone was not successfully produced.

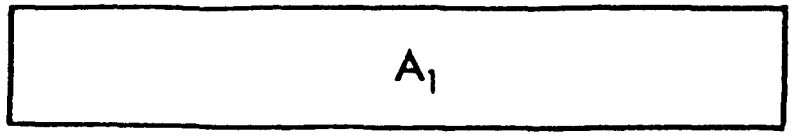
FIG 3.3.2 shows the recombinant SLT I A subunits generated.

Generation of the truncated SLT IA comprising the A_1 fragment alone was achieved by conversion of serine (Ser) residue 247 and Arg^{248} to STOP codons by site directed mutagenesis (2.5.8) using Oligonucleotide A_1 (FIG 3.3.3).

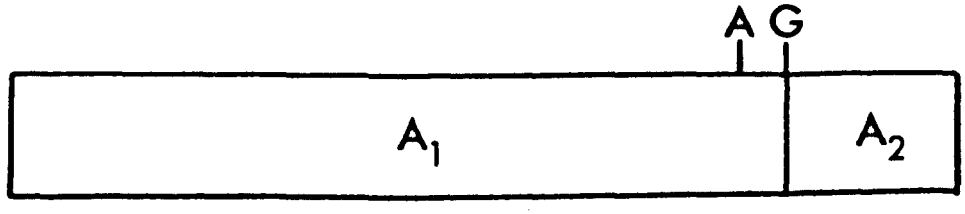
The full length, 'protease insensitive' SLT IA was generated by conversion of the Arg residues at the proposed site of proteolytic cleavage ie Arg^{248} and Arg^{251} to alanine (Ala) and glycine (Gly) respectively (FIGS 3.3.2 and 3.3.4). Both the size and the charge of the replacement residues were carefully considered in order to minimise any conformational changes in the subunit which may result in changes in its activity. Schlossman *et al*. (1989), in the mutagenesis of ricin A subunit (RA), reported a considerable difference in the activity of two mutants under examination. Replacement of



WILD TYPE SLT IA SUBUNIT



TRUNCATED SLT IA SUBUNIT A_1 ALONE



FULL LENGTH SLT IA SUBUNIT - 'PROTEASE INSENSITIVE'

R = Arginine

A = Alanine

G = Glycine

FIG 3.3.2 Recombinant Shiga-like toxin A subunits (rSLT IAs) designed to allow the role of the A_2 peptide in SLT IA RNA N-glycosidase activity to be determined.

OLIGONUCLEOTIDE A_1

5' CAT CAT CAT GCA TAG TGA GTT GCC AGA 3'

OLIGONUCLEOTIDE A_1A_2

5' CAT GCA TCG GCA GTT GCC GGA ATG GCA 3'

OLIGONUCLEOTIDE SEQ

1042 1060
5' ACA AGA CTC TGT TCG TGT A 3'

1042 - 1060 indicates the region within SLT IA to which sequencing primer OLIGONUCLEOTIDE SEQ is homologous.

◆ = mismatch

Fig 3.3.3 Oligonucleotides used in the production of recombinant Shiga-like toxin A subunits.

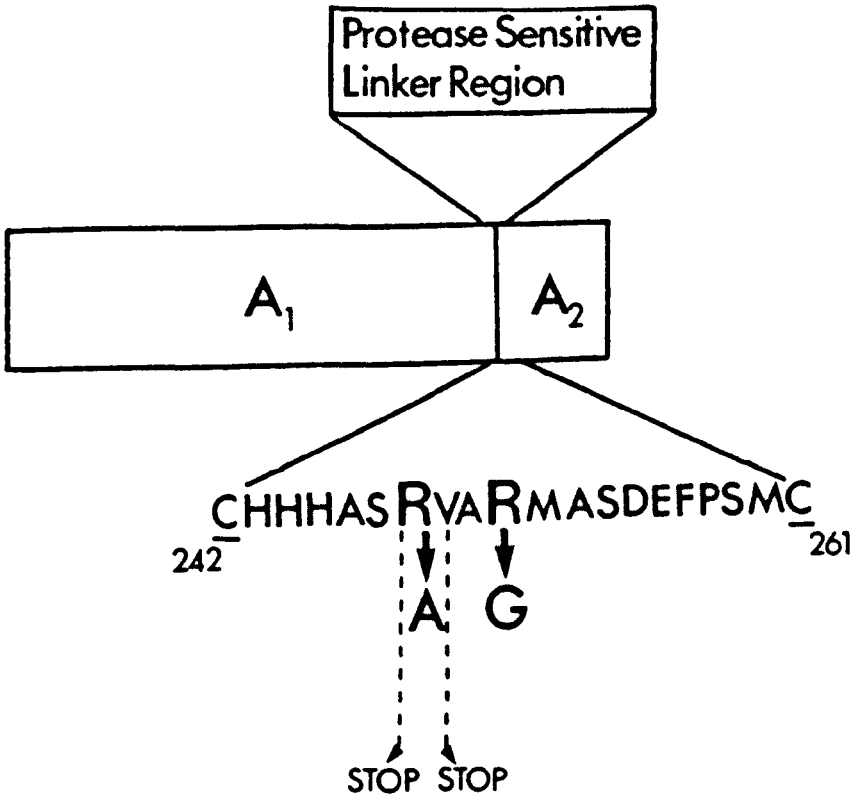


FIG 3.3.4 The proposed Shiga-like toxin I A subunit (SLT IA) protease sensitive region.

The amino acid sequence for this region is detailed with the cysteine residues thought to form a disulphide bridge at this region denoted C.

Arginine residues (R) and their conversions in this study to alanine (A) and glycine (G) by site directed mutagenesis are indicated by ⬇ ⬇

glutamic acid (Glu) residue 177 (at the proposed active site cleft of RA) with first Ala resulted in a 20 fold decrease in activity whilst replacement with aspartic acid (Asp) gave a reduction in activity of 80 fold. The latter substitution, they suggest, may result in a conformational change in the active site cleft as a result of the electrostatic interaction between the charged side chain of the Asp residue and an existing residue, Arg^{180}. This, they suggest, may account for the greater decrease in RA activity with substitution of a conservative Asp than is evident with the substitution of Glu^{177} with an uncharged Ala residue.

Ala and Gly were the residues chosen to replace Arg residues 248 and 251 in SLT IA in this study, since both are relatively small in size and neither possesses a charged side chain. Alanine residues are frequently used in protein engineering to introduce changes which are structurally non perturbing (Ashenazi et al. 1990, Kasturi et al. 1992).

The full length, 'protease insensitive' SLT IA was generated by site directed mutagenesis (2.5.8) using Oligonucleotide A_1A_2 (FIG 3.3.3).

Cloning steps involved in generation of the recombinant Shiga-like toxin I A subunits .

Plasmid pSC25 (FIG 3.3.5) expressing the whole SLT I A subunit under lac promotion was kindly provided by Dr S Calderwood (Boston USA).

(a) Cloning steps involved in the generation of the recombinant, truncated SLT IA (rSLT IA_1 (A_1 alone)).

A 976 base pair (bp) Pst I / Ssp I fragment was excised from pSC25 by restriction of the DNA (2.5.3) with the above mentioned endonucleases. This was then ligated (2.5.5) into the cloning site of bacteriophage M13mp18X (FIG 3.3.6) which had been restricted (2.5.3) and gel purified (2.4.4) as a Pst I / Sma I fragment. In this way a forced orientation cloning was achieved, eliminating the need for screening of successful recombinants. In addition, following successful mutation of the SLT IA DNA, it could be excised as Pst I / Eco RI fragment (Eco RI in this instance being a site in the M13 DNA) and substituted back into pSC25 for in vivo expression in E.coli.

Following ligation, the DNA was used to transform E.coli strain JM101 (2.7.3). Successful transformants were identified and single-stranded (ss) M13mp18X / SLT IA DNA prepared (2.4.2 (iv)) in readiness for site directed mutagenesis (SDM). SDM

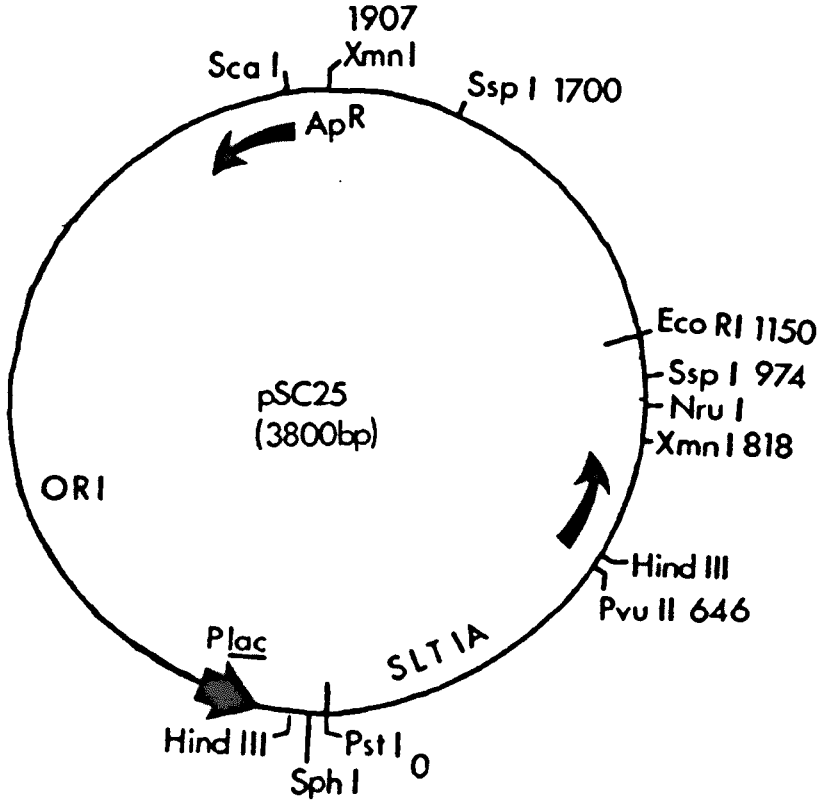


FIG 3.3.5 **Plasmid pSC25.**

Kindly donated by Dr S Calderwood (Boston, USA). The plasmid contains the DNA for the entire Shiga-like toxin I A subunit (SLT IA) under transcriptional control of the lacZ promoter (Hovde et al. (1988).

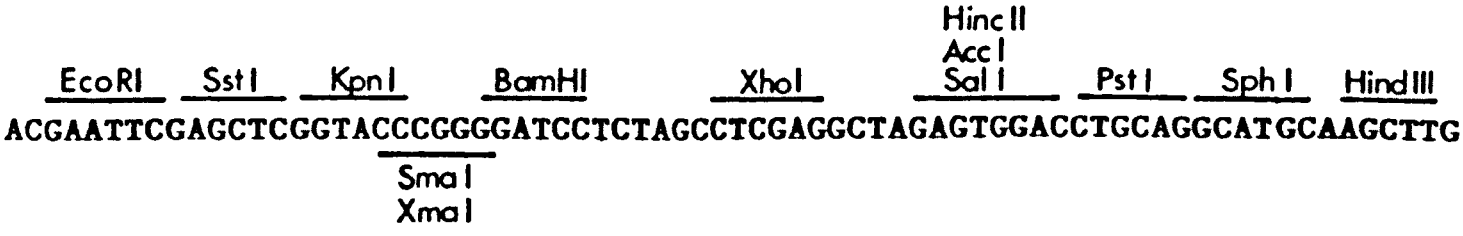


FIG 3.3.6 Bacteriophage M13mp18X cloning site.

(2.5.8) was carried out using Oligonucleotide A_1 (FIG 3.3.3) to instigate the required mutations. Putative mutants were then screened for possession of the desired mutations by dideoxy sequencing of the ss M13 / SLT IA DNA (2.5.8) using Oligonucleotide SEQ (FIG 3.3.3) complementary to bases 1042 to 1060 in the SLT IA DNA . This allows the sequence across the mutant region to be determined. FIG 3.3.7 shows the resultant dideoxy sequencing of a recombinant SLT IA with the correct nucleotide substitutions required to generate truncated SLT IA_1 (A_1 alone).

(b) Cloning steps involved in the generation of a recombinant, full length, 'protease insensitive' SLT IA_1A_2.

A 1150bp Pst I / Eco RI fragment was excised from pSC25 (FIG 3.3.5) by restriction of the DNA (2.5.3). Again this was ligated (2.5.5) into the cloning site of bacteriophage M13mp18X (FIG 3.3.6), which in this case had been restricted (2.5.3) as a Pst I / Eco RI fragment and gel purified (2.4.4). Once again forced orientation cloning of the SLT IA fragment ruled out the necessity for screening of successful recombinants. Following mutagenesis this fragment could also be easily excised and substituted back into pSC25 for in vivo expression studies.

Following ligation of the SLT IA fragment with M13mp18X steps taken were as described in (a) above. However, in this case SDM

FIG 3.3.7 **Dideoxysequencing of putative recombinant Shiga-like toxin A subunit (rSLT IAs) mutants.**

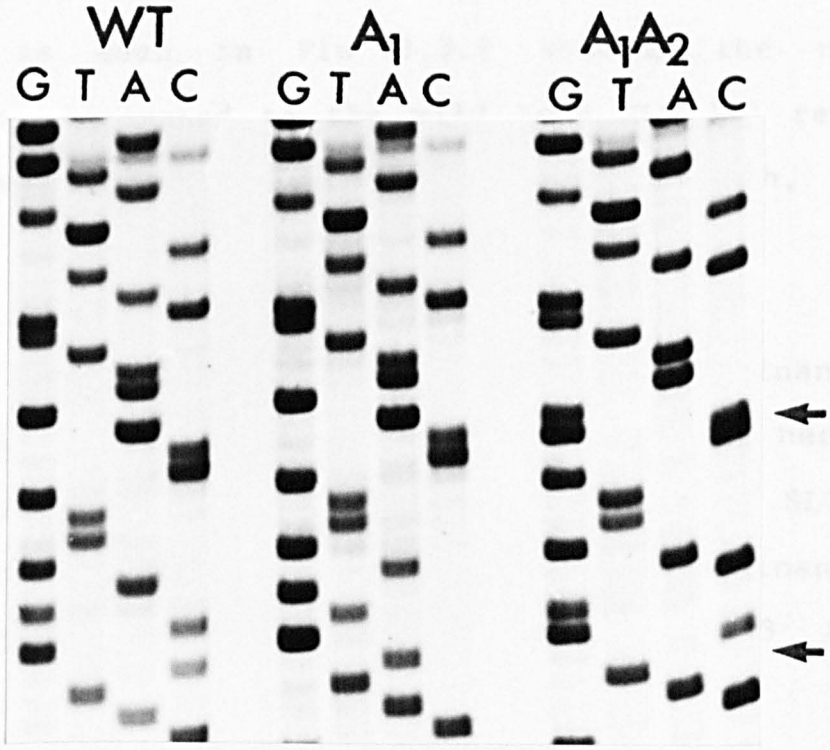
In each case 1pmol single stranded (ss) bacteriophage M13 / SLT IA DNA was used as template to which 0.5pmol primer (OLIGO SEQ FIG 3.3.3) was annealed as described (2.5.8(i)). Dideoxy sequencing of the DNA was then carried out using Sequenase^{TM} as detailed (2.5.8(i)). The products of dideoxy sequencing of the M13 / SLT IA putative mutants were fractionated on an 6% polyacrylamide gel (2.6.3) and visualised by autoradiography (2.10.3(iii)).

G,T,A,C denote the dideoxynucleotide relating to each termination reaction.

WT: rSLT IA (wild type).
A_1: rSLT IA_1 (truncated SLT I - A_1 alone).
A_1A_2: rSLT IA_1A_2 (full length SLT IA_1A_2 - 'protease insensitive').

⇉ indicates region of mutation.

----- indicates nucleotides separating sequences targetted for site directed mutagenesis.



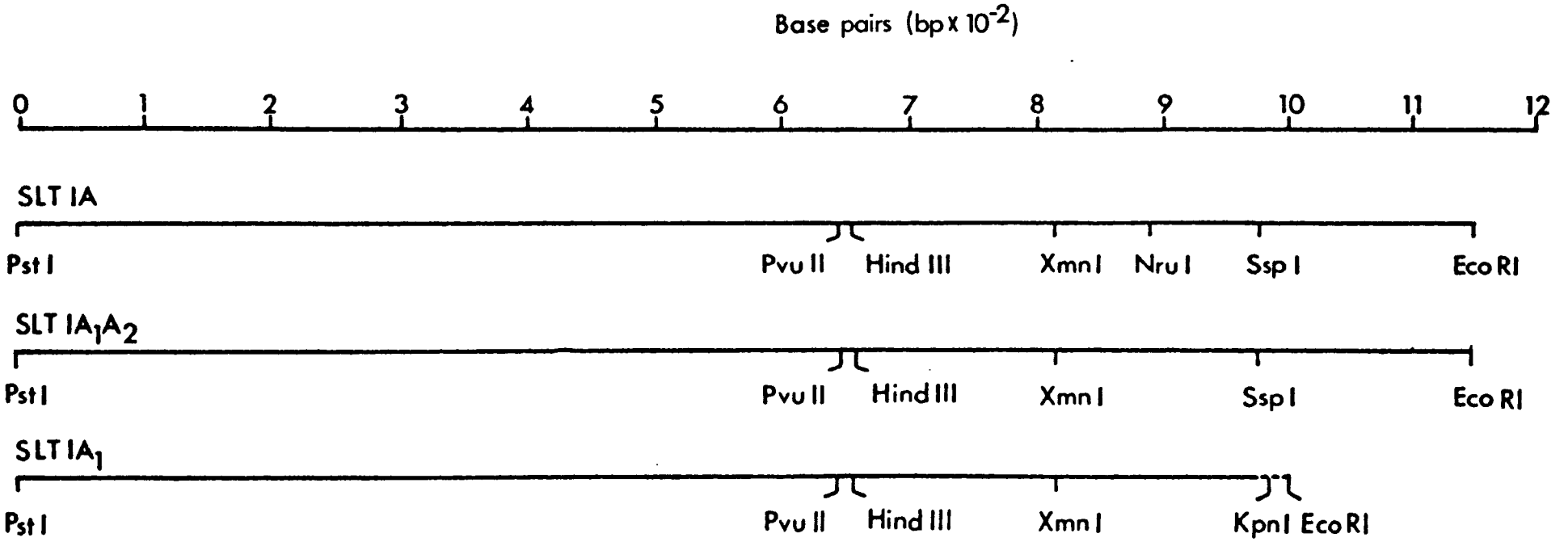
A T
C G C G A ------ A G A
G C G

A_1 ALONE
WILD TYPE
A_1/A_2
NON CLEAVABLE

(2.5.8) was achieved using Oligonucleotide A_1A_2 (FIG 3.3.3). Screening of putative mutants was again achieved by dideoxy sequencing of the ss M13 / SLT IA DNA (2.5.8) using Oligonucleotide SEQ (FIG 3.3.3). The resultant dideoxy sequencing is seen in FIG 3.3.7 showing the nucleotide substitutions (compared to the wild type SLT IA) required to successfully produce a recombinant, full length, 'protease insensitive' SLT IA_1A_2.

In order to proceed with expression of the recombinant SLT IAs in both in vitro and in vivo systems, it was first necessary to prepare replicative forms (RF) of the M13 / SLT IA DNA (2.4.2(iii)) in each case. The required recombinant SLT IA fragments could then be excised from the M13 / SLT IA constructs (FIG 3.3.8).

FIG 3.3.8 Recombinant Shiga-like toxin I A subunit (rSLT IA) DNA fragments excised from M13mp18X following site directed mutagenesis.



SECTION 3.3.1 *In vitro* expression of recombinant Shiga-like toxin I A subunits.

Introduction

Following the successful generation of recombinant Shiga-like toxin I A subunits (rSLT IAs) it was necessary to employ an expression system which would permit the sizing of the recombinant SLT IA protein products. In addition a system was required which would allow the relative sensitivity to proteolytic cleavage of the recombinant, full length, 'protease insensitive' SLT IA_1A_2 to be determined by comparison to that of the wild type SLT IA subunit. The RNA N-glycosidase activity of the recombinant SLT IAs was also an important aspect to be examined.

It was therefore, decided that *in vitro* expression of the recombinant SLT IAs would be employed in an initial attempt to address these questions.

Results and Discussion

Plasmid pGEM2 (FIG 3.3.9) was chosen as a suitable vector for *in vitro* expression of the rSLT IAs, since it provided suitable cloning sites for expression of the rSLT IAs under transcriptional control of the T7 promoter. In addition, this

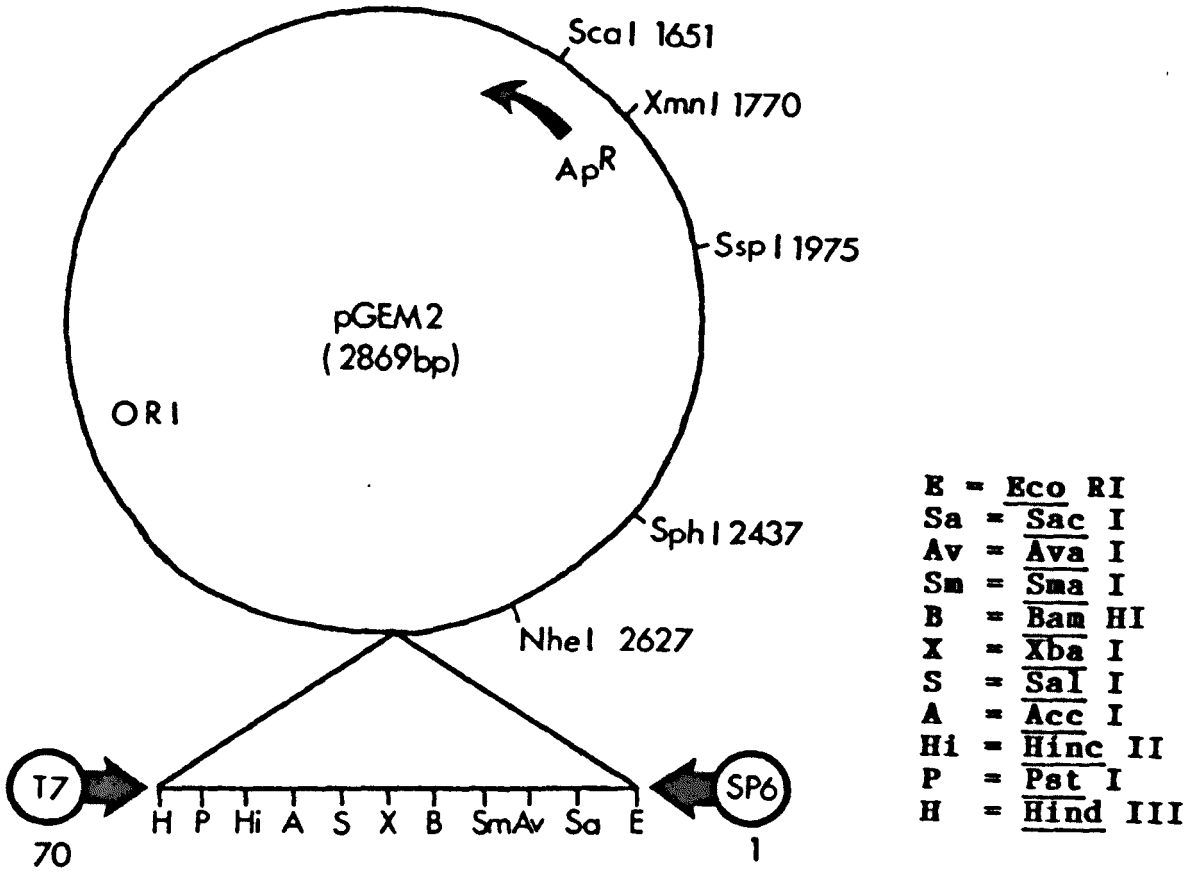


FIG 3.3.9 In vitro expression vector pGEM2.

particular vector had previously been used successfully by several members of our laboratory.

Steps involved in the generation of pGEM2 / SLT IA (wild type) (pGEM2WT), pGEM2 / truncated SLT IA_1 (A_1 alone (pGEM2A_1) and pGEM2 / full length, 'protease insensitive' SLT IA_1A_2 (pGEM2A_1A_2).

In order for the *in vitro* activities of the rSLT IAs to be compared directly, construction of vector pGEM2 carrying the wild type SLT IA subunit (pGEM2WT) was necessary in addition to the construction of pGEM2 vectors carrying the recombinant SLT IA subunits.

(a) Construction of pGEM2A_1.

A fragment of approximately 990bp was excised (2.5.3) from the M13mp18X / SLT IA_1 RF DNA (FIG 3.3.8) as a *Pst* I / *Eco* RI fragment thus releasing the mutant SLT IA_1 subunit. This was then ligated (2.5.5) into pGEM2 (FIG 3.3.9) which had also been restricted (2.5.3) using *Pst* I / *Eco* RI and gel purified (2.4.4). The resultant pGEM2 / SLT IA_1 DNA was used to transform *E.coli* strain JM101 (2.7.2). Forced orientation cloning of the SLT IA_1 fragment into pGEM2 eliminated the necessity for screening to determine fragment orientation. They were, however, screened for possession of the mutation required

for generation of the SLT IA subunit A_1 alone by dideoxy sequencing of the plasmid DNA (2.5.8(ii)). Large scale preparation (2.4.2(ii)) of pGEM2A_1 (FIG 3.3.10) was then carried out in order to yield purified plasmid DNA.

(b) Construction of pGEM2WT and pGEM2A_1A_2.

An 1150bp Pst I / Eco RI fragment was excised (2.5.3) from plasmid pSC25 (FIG 3.3.5) and ligated (2.5.5) into pGEM2 (FIG 3.3.9) exactly as described in construction of pGEM2A_1 in order to generate pGEM2WT (FIG 3.3.11).

Generation of pGEM2A_1A_2 (FIG 3.3.12) also involved the excision (2.5.3) of an 1150bp Pst I / EcoRI fragment which was then ligated (2.5.5)) into Pst I / Eco RI restricted (2.5.3) and gel purified (2.4.4) pGEM2 (FIG 3.3.9), however, in this case the source of the 1150bp fragment was M13mp18X / SLT IA_1A_2 RF DNA (FIG 3.3.8) which carried the correct mutation for generation of the full length, 'protease insensitive' SLT IA_1A_2 subunit.

Transformation of the ligated DNA in E.coli strain JM101 (2.7.3) and dideoxy sequencing of resultant plasmid DNA (2.5.8(ii)) in order to determine possession of the required rSLT IA DNA fragment followed by large scale preparation of the plasmid DNA was as described for construction of pGEM2A_1.

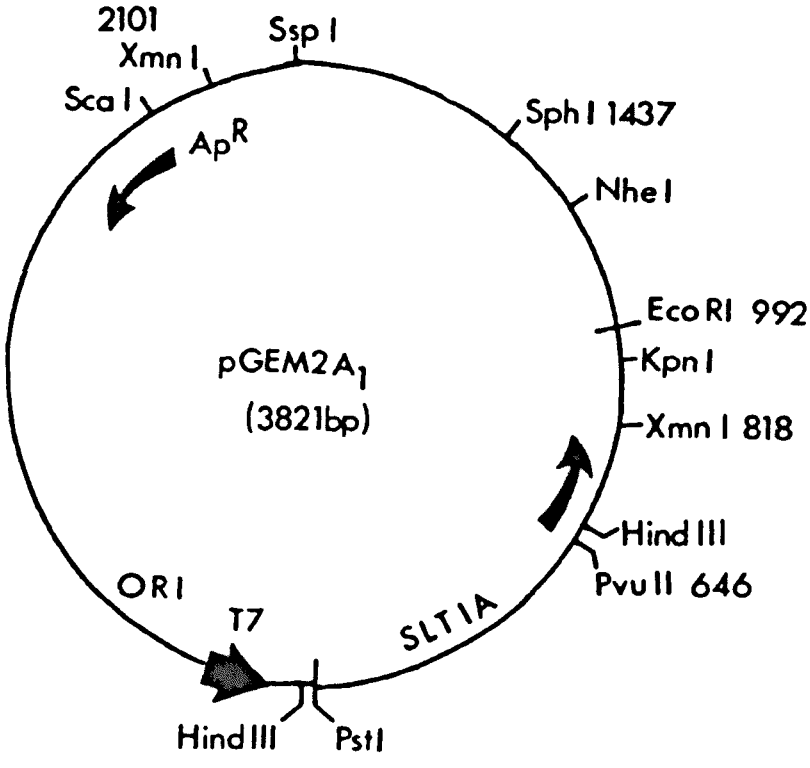


FIG 3.3.10 **Plasmid pGEM2A$_1$.**

Construction of pGEM2A$_1$ allowed *in vitro* expression of the truncated Shiga-like toxin I A subunit (SLT IA$_1$ - A$_1$ alone). A *Pst* I / *Eco* RI fragment of approximately 990 base pairs encoding the recombinant SLT IA$_1$ was ligated (2.5.5) with similarly restricted pGEM2 (FIG 3.3.9). The SLT IA$_1$ was transcribed under control of the pGEM2 T7 promoter.

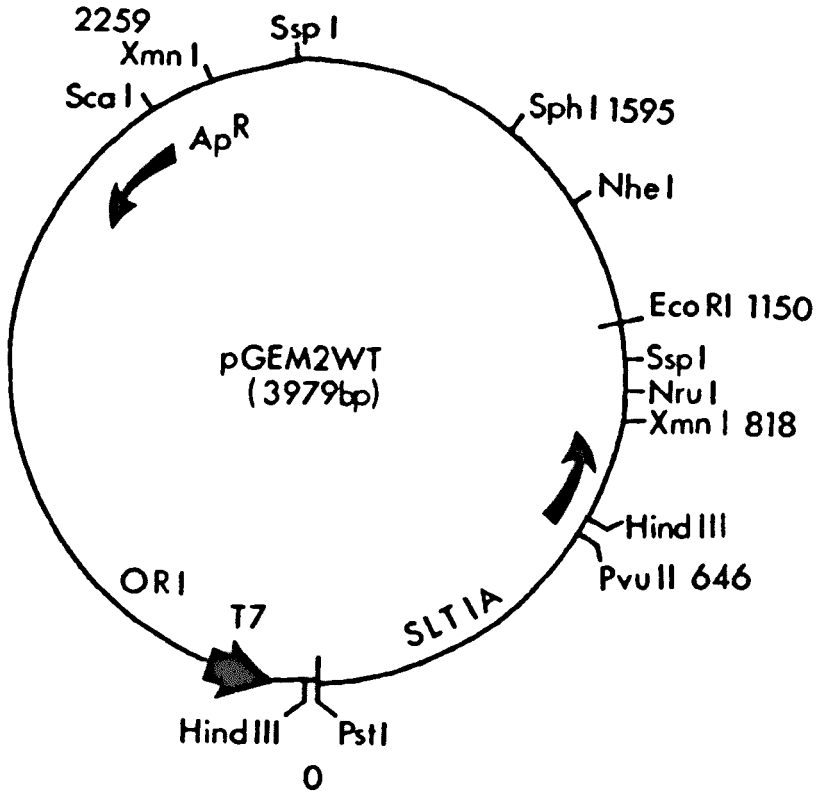


FIG 3.3.11 Plasmid pGEM2WT.

Construction of pGEM2WT allowed *in vitro* expression of the wild type Shiga-like toxin I A subunit (SLT IA) thus providing an experimental comparison between the wild type and recombinant SLT IA subunits. A *Pst* I / *Eco* RI fragment of approximately 1150 base pairs encoding the wild type SLT IA was ligated (2.5.5) with similarly restricted pGEM2 (FIG 3.3.9). The SLT IA wild type was transcribed under control of the pGEM2 T7 promoter.

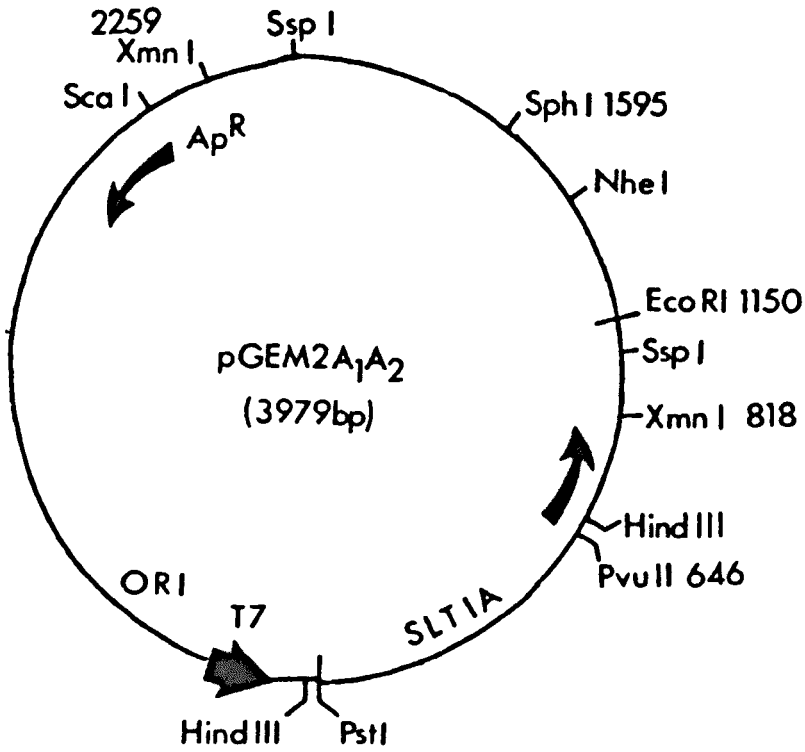


FIG 3.3.12 **Plasmid pGEM2A_1A_2.**

Construction of pGEM2A_1A_2 allowed <u>in vitro</u> expression of the full length protease insensitive Shiga-like toxin I A subunit (SLT IA_1A_2). A <u>Pst</u> I / <u>Eco</u> RI fragment of approximately 1150 base pairs encoding the recombinant SLT IA_1A_2 was ligated (2.5.5) with similarly restricted pGEM2 (FIG 3.3.9). The SLT IA_1A_2 was transcribed under control of the pGEM2 T7 promoter.

Prior to in vitro transcription (2.8.1) of the pGEM2 / rSLT IAs DNA, linearisation of the DNA was first necessary. This was achieved in all cases by restriction of the plasmid DNA (2.5.3) with Eco RI, a restriction endonuclease which cuts at a single site in all of the pGEM2 / rSLT IAs generated in this study. Following restriction, the DNA was extracted and precipitated (2.4.3) and its concentration determined spectrophotometrically (2.4.5). In each case 2ug of DNA was used in the transcription reaction (2.8.1).

In vitro translation of recombinant SLT IA transcripts.

(a) Wheatgerm translation.

Translation of the recombinant SLT IA transcripts in a wheatgerm translation system allowed the sizes and sensitivity to proteolytic cleavage of the rSLT IAs to be examined.

Following in vitro transcription (2.8.1) of the pGEM2 / rSLT IAs each resultant transcript was subjected to in vitro translation in a wheatgerm system (2.8.2(i)). Resolution of the resultant ^{35}S-methionine labelled translation products by SDS PAGE (2.10.3) on a 15% polyacrylamide gel (reducing conditions) followed by their visualisation by autoradiography (2.10.3(iii)) revealed (FIG 3.3.13) (by comparison with

FIG 3.3.13 **Sizing of recombinant Shiga-like toxin I A subunits (rSLT IAs) by translation in a wheatgerm cell free system.**

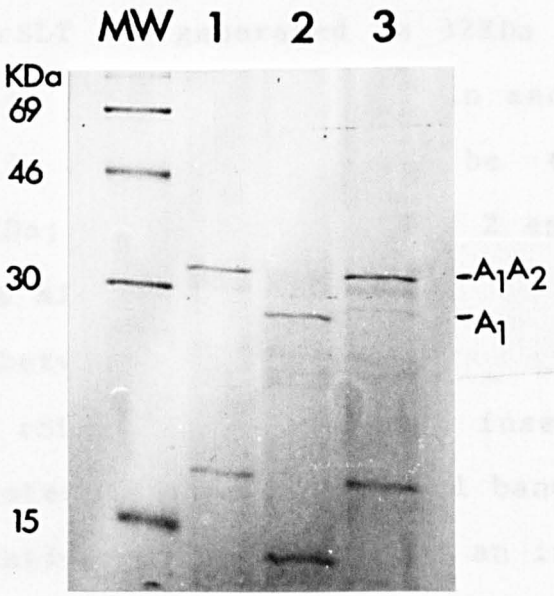
1ug of each rSLT IA mRNA transcript was translated in a wheatgerm cell free system as described (2.8.2(i)). Translation products were then resolved using a 15% polyacrylamide gel (2.10.3) (reducing conditions) and visualised by autoradiography (2.10.3(iii)).

1: rSLT IA (wild type) translation product.

2: rSLT IA_1 (truncated SLT I - A_1 alone) translation product.

3: rSLT IA_1A_2 (full length SLT IA_1A_2 - 'protease insensitive') translation product.

MW: molecular weight markers.


MW
1
2
3
KDa
69
46
30
15
A_1A_2
A_1

standard molecular weight (MW) markers) that they were indeed the sizes expected for rSLT IA (wild type) (32KDa) (lane 1), full length, 'protease insensitive' rSLT IA_1A_2 (32KDa) (lane 3) and truncated rSLT IA_1 (A_1 alone) (27-28KDa) (lane 2).

In addition to bands of sizes corresponding to those expected for each of the rSLT IAs generated ie 32KDa and 27 - 28KDa, a smaller translation product is evident in each case. The bands seen in lanes 1 and 3 appear to be the same size - approximately 20KDa; whilst that in lane 2 appears to be 4 - 5 KDa smaller. This size difference would appear to reflect the size difference between the rSLT IA_1 (A_1 alone) and rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') ie 4 - 5KDa. It is suggested that the additional bands may have arisen as a result initiation of translation at an internal initiation codon. Alternatively they may be C terminal proteolytic fragments generated as a result of non specific cleavage by serine proteases present in wheatgerm lysate.

In order to determine the sensitivity of the recombinant SLT IA translation products, each was incubated with different concentrations of trypsin (2.10.2) prior to resolution by SDS PAGE (2.10.3) on a 15% polyacrylamide gel under reducing conditions. Again the radioactively labelled wheatgerm translation products were visualised by autoradiography (2.10.3(iii)). FIGS 3.3.14 and 3.3.15 show the result of incubation of the recombinant SLT IA translation

FIG 3.3.14 Examination of the sensitivity to trypsin of recombinant Shiga-like toxin I A subunits (rSLT IAs) following wheatgerm lysate *in vitro* translation.

1ug mRNA transcript in each case was translated in wheatgerm cell free system as described (2.8.2(i)). The resultant translation products in each case were then incubated with L-1-tosylamido-2-phenylethyl chloromethyl ketone treated-trypsin (Sigma) (2.10.2) to a final concentration of 20ug/ml or 30ug/ml 37C for 15min. Products were resolved using a 15% polyacrylamide gel (2.10.3) (reducing conditions) and visualised by autoradiography (2.10.3(iii)).

In each case:

1: non trypsin treated translation product.

2: 20ug/ml (final concentration) trypsin added.

3: 30ug/ml (final concentration) trypsin added.

WT: rSLT IA (wild type).

A_1: rSLT IA_1 (truncated SLT I - A_1 alone).

A_1A_2: rSLT IA_1A_2 (full length SLT IA_1A_2 - 'protease insensitive').

MW: molecular weight markers.

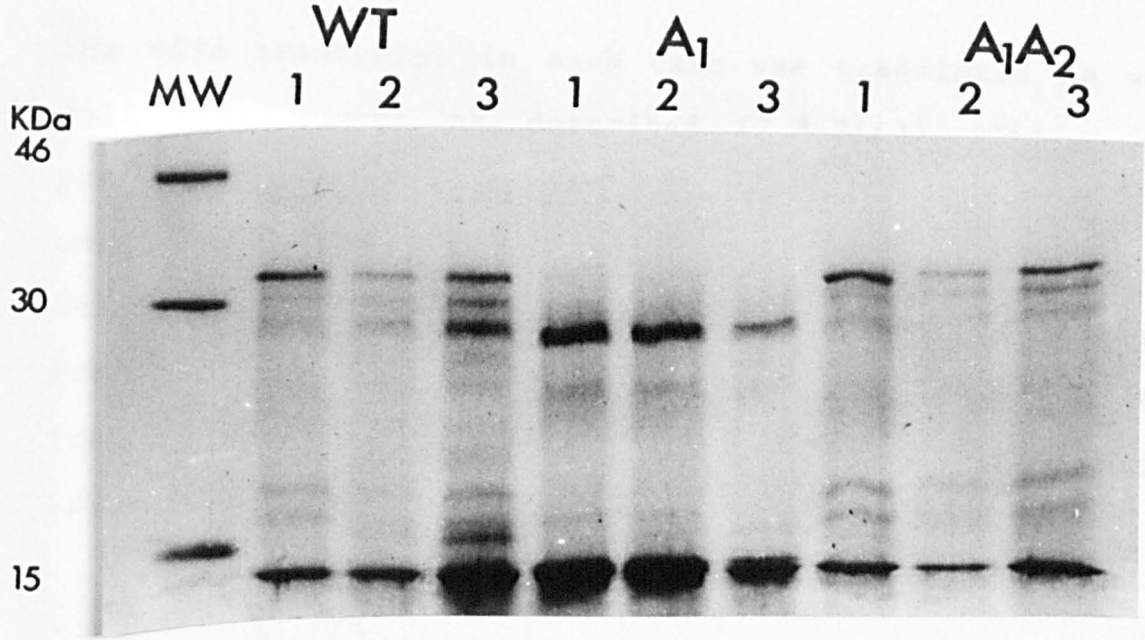

WT
A1
A1A2
MW
1
2
3
1
2
3
1
2
3
KDa
46
30
15

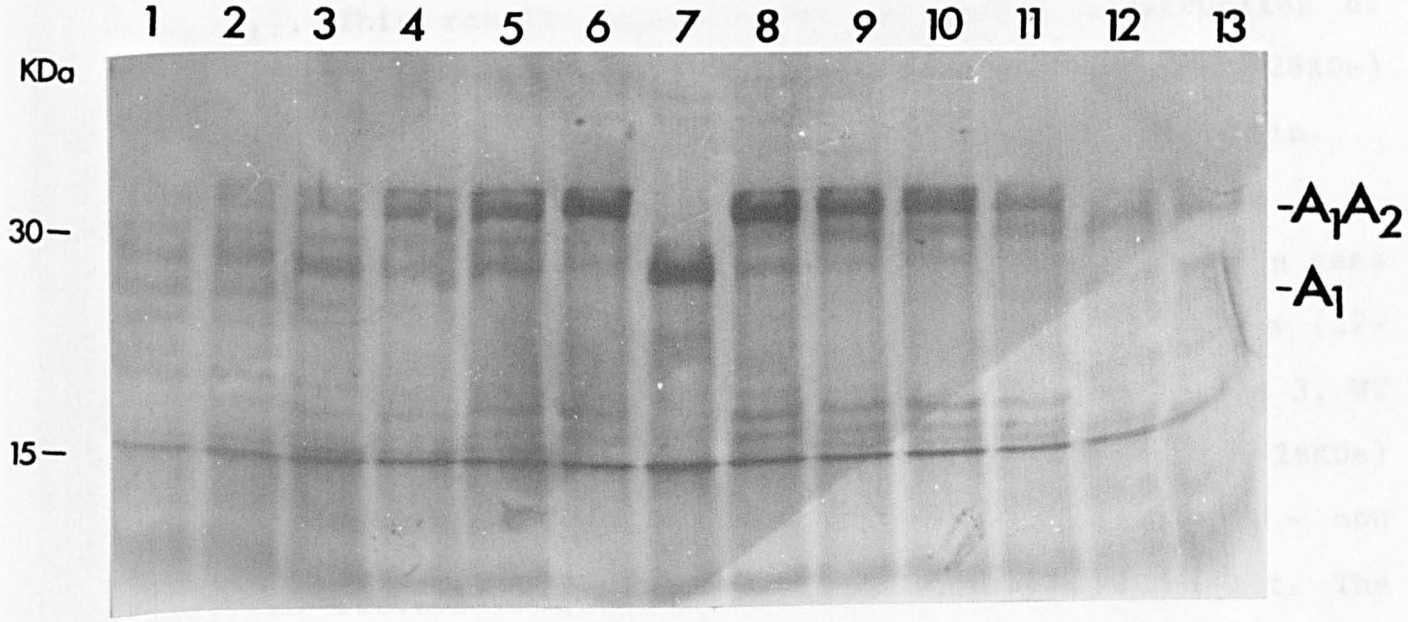
FIG 3.3.15 **Examination of the sensitivity of recombinant Shiga-like toxin I A subunits (rSLT IAs) to a range of concentrations of trypsin following wheatgerm lysate in vitro translation.**

1ug mRNA transcript in each case was translated in wheatgerm cell free system as described (2.8.2(i)). The translation products in each case were then incubated with a range of concentrations (0 - 50ug/ml) of 1-1-tosylamido-2-phenylethyl chloromethyl ketone treated-trypsin (Sigma) (2.10.2) for 15min. Products were resolved using a 15% polyacrylamide gel (2.10.3) (reducing conditions) and visualised by autoradiography (2.10.3(iii)).

Lanes 1 - 6 show the result of incubation of the **rSLT IA (wild type)** with **50, 40, 30, 20, 10, 0ug/ml** trypsin.
Lane 7 shows the **truncated rSLT IA_1 (A_1 alone).**

Lanes 8 - 12 show the result of incubation of the **full length rSLT IA_1A_2 ('protease insensitive')** with **0, 10, 20, 30, 40 and 50ug/ml** trypsin.

A_1A_2 = Full length SLT IA = 32KDa
A_1 = SLT IA_1 fragment alone = 27KDa


1 2 3 4 5 6 7 8 9 10 11 12 13
KDa
30—
15—
-A1A2
-A1

products with concentrations of trypsin in a range from 0 - 50ug/ml (FIG 3.3.15) and 0 - 30ug/ml (FIG 3.3.14).

It may be seen from FIG 3.3.14 that the size of the translation product identified as rSLT IA_1 (A_1 alone) (27-28KDa) is unchanged as a result of incubation with trypsin (lanes 2 and 3, A_1). This result suggests the successful construction of rSLT IA_1 (A_1 alone) since it is of the expected size (27-28KDa) and unaffected by treatment with a protease such as trypsin.

Incubation of the rSLT IA (wild type) (32KDa) with trypsin sees a reduction in size to that of the truncated A_1 alone (27-28KDa) at approximately 30ug/ml trypsin (FIG 3.3.14 lane 3, **WT** and FIG 3.3.15 lane 3). A faint band of this size (27 - 28KDa) is also evident however, in lane 1 **WT** of FIG 3.3.14 - non trypsin treated rSLT IA (wild type) translation product. The existence of a faint band corresponding to the size of rSLT IA_1 (A_1 alone) may again be as a result of cleavage of rSLT IA (wild type) by serine proteases present in wheatgerm lysate. At 30ug/ml trypsin (FIG 3.3.14 lane 3**WT** and higher FIG 3.3.15 lanes 3 - 1) the 27 - 28KDa (A_1 sized band) predominates. This indicates that rSLT IA (wild type) is undergoing proteolytic cleavage predominately at the proposed cleavage site.

With regard to the full length rSLT IA_1A_2 ('protease insensitive') (32KDa) a faint A_1 alone sized band (27 - 28KDa) is also evident in non trypsin treated rSLT IA_1A_2 ('protease

insensitive') (FIG 3.3.14 lane 1 A_1A_2 and FIG 3.3.15 lane 8). The intensity of this band is not however, seen to increase in intensity at following treatment of the translation product with trypsin. At higher trypsin concentrations (40 - 50ug/ml) (FIG 3.3.15 lanes 12 and 13) however, an intermediate sized fragment approximately 2 - 3KDa smaller than rSLT IA_1A_2 ('protease insensitive') (32KDa) is visible. This intermediate which is also to be seen following treatment of rSLT I (wild type) with 30ug/ml trypsin (FIG 3.3.14 lane 3 WT) may have arisen as a result of proteolytic cleavage of the rSLT IA_1A_2 ('protease insensitive') and rSLT IA (wild type) at a different arginine rich site less sensitive to cleavage by protease. It is possible that arginine residues 266 and 268 outside the disulphide bonded peptide of SLT IA may represent such a site. Cleavage close to these residues would result in a reduction in size of the rSLT IA_1A_2 similar to that seen in lanes 11 and 12 of FIG 3.3.15. Results seen here do, however, suggest that the full length, rSLT IA_1A_2 ('protease insensitive') construct is indeed less sensitive to proteolytic cleavage within the proposed cleavage site than is the wild type SLT IA subunit.

In the present study trypsin is used *in vitro* to mimic the proposed *in vivo* proteolytic processing of SLT IA by a specific intracellular (endosomal) protease. However, it may be difficult to mimic these intracellular events precisely using relatively crude *in vitro* methods. For example, native SLT IA produced *in vivo* possesses a disulphide loop between cysteine

residues 242 and 261, which is likely to be correctly surface-exposed to the specific intracellular protease responsible for proteolytic processing in vivo. It is unlikely during in vitro production of the rSLT IAs in a wheatgerm system, that this loop is formed. Results presented here do however, provide an indication of preferential cleavage at the proposed sites within the rSLT IAs generated. In the absence of microsequence data however this cannot be stated unambiguously.

(b) Reticulocyte lysate translation.

In vitro translation of the recombinant SLT IA transcripts in a reticulocyte lysate system allows the question of RNA N-glycosidase activity to be addressed. Activity may be examined in two ways using this system:

(i) Examination of the ribosomal RNA (rRNA) following translation (2.11.1(ii)). rRNA from reticulocyte ribosomes endogenous to the reticulocyte lysate translation reaction (2.8.2(ii)) may be extracted (2.4.1) following translation of a potentially toxic transcript and subjected to aniline cleavage (2.5.1). Resolution of the resultant rRNA (2.6.1) then reveals a characteristic fragment of approximately 390 bases if the

translation product did indeed possess RNA N-glycosidase activity.

(ii) Ability of the endogenous ribosomes to translate a second messagenger RNA (mRNA) encoding a non-toxic product following translation of a transcript encoding a toxic product (2.11.1(iii)). In other words, an attempt to synthesize a second translation product is made. This is not, however, possible if the endogenous reticulocyte ribosomes have been irreversibly inactivated by the RNA N-glycosidase activity of the first translation product. The results of the double translation may be examined by SDS PAGE (2.10.3) followed by autoradiography (2.10.3(iii)).

It is important to include a control transcript in both approaches in order to firmly establish that any depurination of the ribosomal RNA (rRNA) is as a direct result of translation of a product with RNA N-glycosidase activity. Thus in approach (ii), the inability to translate a second transcript may be determined as being a direct result of translation of the first transcript leading to depurination of the reticulocyte ribosomes. Lysozyme was chosen as the control transcript. It is a translation product which is not capable of inactivating the reticulocyte ribosomes. In addition it provides a second translation product (14KDa) which may be easily distinguished by SDS PAGE (2.10.3) and autoradiography

(2.10.3(iii)) from the initial translation products, the rSLT IAs, which range in size from 27-32KDa.

In vitro transcription and translation of the plasmid pTK_2Lys (Drummond et al. 1985) resulted in the non toxic control translation product lysozyme.

FIG 3.3.16 shows the extracted and aniline treated rRNA of the reticulocyte ribosomes following translation of the rSLT IA transcripts.

The appearance of the characteristic 390 base rRNA fragment released following aniline cleavage of depurinated rRNA (lanes 1, 2 and 3) indicates that all three of the rSLT IAs constructed possess RNA N-glycosidase activity.

FIG 3.3.17 demonstrates the inability of reticulocyte ribosomes to translate a second transcript ie pTK_2Lys after having first translated the rSLT IA transcripts. The pTK_2Lys translation product, lysozyme (14KDa) (L) is clearly visible in lanes 1 and 2, the latter of which is the result of translation of the pTK_2Lys transcipt alone. The translation products seen in lane 1 are as a result of translation of yeast preproalpha factor mRNA - a non toxic product (α) of 18.6KDa (May et al. 1989) followed by translation of the pTK_2Lys mRNA. The reduction in the amount of the second translation product, lysozyme would suggest a reduction in efficiency of the

FIG 3.3.16 **Extraction and aniline cleavage of rabbit reticulocyte ribosomal RNA following in vitro translation of recombinant Shiga-like toxin I A transcripts.**

1ug of each recombinant Shiga-like toxin I A subunit (rSLT IA) transcript was translated in vitro using rabbit reticulocyte lysate (non nuclease treated) (Promega) (2.11.1(ii)). The ribosomal RNA (rRNA) was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). The resultant rRNA was then fractionated by gel electrophoresis and visualised by staining the gel with ethidium bromide (2.6.1).

Lane 1: reticulocyte rRNA resulting from translation of rSLT IA (wild type).
Lane 2: reticulocyte rRNA resulting from translation of rSLT IA_1 (A_1 alone).
Lane 3: reticulocyte rRNA resulting from translation of rSLT IA_1A_2 ('protease insensitive').
Lane 4: non aniline treated extracted reticulocyte rRNA.
+ indicates treatment of rRNA with aniline reagent.
➡ indicates the characteristic fragment released following aniline cleavage of toxin-modified rRNA.

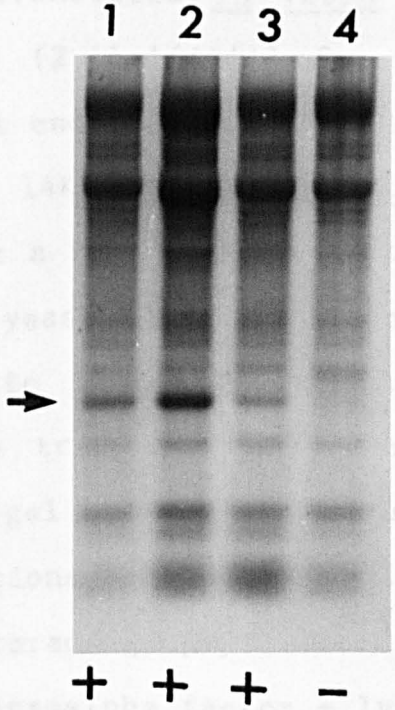

1 2 3 4
+ + + –

FIG 3.3.17 Examination of the ability of reticulocyte ribosomes to translate a second transcript following translation of recombinant Shiga-like toxin I A subunit transcripts.

1ug of each recombinant Shiga-like toxin I A subunit (rSLT IA) transcript was translated *in vitro* using rabbit reticulocyte lysate (Promega) (2.11.1(iii)) for 1 hour at 30°C. 1ug of a second transcript encoding a non toxic transcript, in this case lysozyme (approx 14KDa) (Drummond *et al*. 1985) was then added and incubated for a further 1 hour at 37°C. 1ug of messenger RNA (mRNA) encoding yeast preproalpha factor (May *et al*. 1989) a further non toxic product, was used as a control primary translation. The translation products were resolved by SDS polyacrylamide gel electrophoresis (SDS PAGE) (2.10.3) (reducing conditions) using a 15% polyacrylamide gel and visualised by autoradiography (2.10.3(iii).

Lane 1: Yeast preproalpha factor + lysozyme

Lane 2: Lysozyme alone

Lane 3: rSLT IA_1A_2 ('protease insensitive') + lysozyme

Lane 4: rSLT IA_1 (A_1 alone) + lysozyme

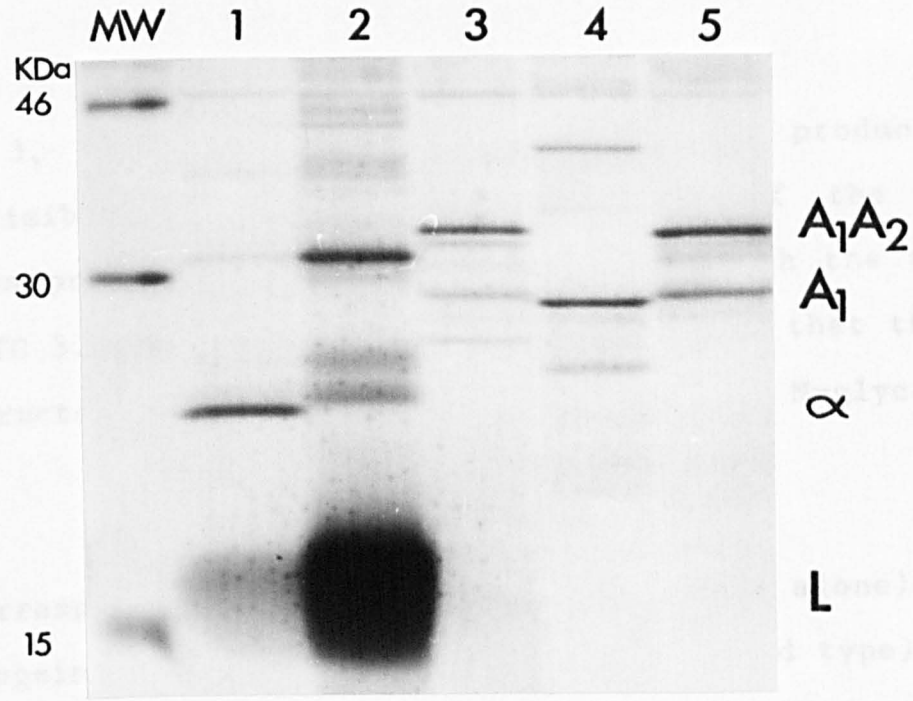
Lane 5: rSLT IA (wild type) + lysozyme

A_1A_2 = full length SLT IA = 32KDa.

A_1 = truncated SLT IA (A_1 alone) = 27KDa.

L: lysozyme

α: yeast preproalpha factor


MW
1
2
3
4
5
KDa
46
30
15
A1A2
A1
α
L

reticulocyte system in translation of a second mRNA. It is possible that this may be as a result of depletion of energy or amino acids following efficient translation of the first mRNA. In both lanes 1 and 2 a band indicating a translation product of approximately 31KDa may be seen, although this is much less evident in lane 1. The origin of this band is unknown.

In lanes 3, 4 and 5 the rSLT IA translation products are clearly visible. There is a disinct lack of the second translation product, lysozyme (L). Again, as with the results seen in FIG 3.3.16, this would appear to suggest that the rSLT IAs constructed in this study all possess RNA N-glycosidase activity.

A band corresponding to the size of rSLT IA_1 (A_1 alone) - 27 - 28KDa is again to be seen in lanes 5 rSLT IA (wild type) and 3 (rSLT IA_1A_2 ('protease insensitive')) of FIG 3.3.17 as was also the case in FIG 3.3.14 (lanes 3 **WT** and 3 $\mathbf{A_1A_2}$). Again this suggests cleavage of the rSLT IAs by serine proteases present in reticulocyte lysate. The A_1 size band in lane 3 (FIG 3.3.17) is much less evident than the corresponding band in lane 5. This adds to the suggestion that the rSLT IA_1A_2 ('protease insensitive') has a reduced susceptibility to proteolytic cleavage in comparison to that of rSLT IA (wild type). Alternatively the smaller rSLT IA bands seen may represent products of internal initiation of translation.

Interestingly, the various rSLT IA forms can be visualised in a reticulocyte lysate translation. This contrasts with ricin A subunit (RA) which can rarely be seen when RNA is translated in this system. It is possible that this indicates a greater senstivity of reticulocyte ribosomes to RA than to SLT IA.

It may be concluded from the above results therefore, that all of the recombinant SLT IA constructs exhibit RNA N-glycosidase activity. The assays described however, do not allow any accurate quantification of enzymatic activities.

SECTION 3.3.2 In vivo **expression of the recombinant Shiga-like toxin I A subunits.**

Introduction

In vivo expression of the recombinant Shiga-like toxin I A subunits (rSLT IAs) in Escherichia coli ultimately allowed the activities of the constructs to be examined in more detail. In vivo expression was also conducive to larger scale production of the rSLT IAs, a process which would eventually allow production of purified rSLT IA containing holotoxins. In this way the effect of the SLT IA A_2 peptide on the cytotoxic nature of the holotoxin could be addressed by comparing the cytotoxicities of recombinant, full length SLT IA_1A_2 ('protease insensitive')- and the recombinant, truncated SLT IA_1 (A_1 alone)-containing holotoxins with that of the recombinant, wild type SLT IA-containing holotoxin.

Results and Discussion

Using plasmid pSC25 (FIG 3.3.5) Hovde et al. (1988), excised the SLT IA DNA as a Pst I / Eco RI fragment, carried out the required mutagenesis for their study and substituted the mutated Pst I / Eco RI fragment back into pSC25 for in vivo expression in E.coli. Expression was achieved using the inducible lac promoter. Since Hovde et al. (1988) had

recombinant SLT IA in this way, it was decided that a similar strategy would also be employed for the in vivo expression of the rSLT IAs generated in the present study.

Generation of the pSC25 derivative plasmids.

(a) Construction of pSC25A_1 (FIG 3.3.18).

A fragment of 990bp was excised (2.5.3) from the M13mp18X / SLT IA_1 RF DNA (FIG 3.3.8) as a Pst I / Eco RI fragment thus releasing the mutant SLT IA_1 subunit. This was then ligated (2.5.5) into pSC25 (FIG 3.3.5) which had also been restricted (2.5.3) using Pst I / Eco RI and gel purified (2.4.4). The resultant pSC25 / rSLT IA_1 DNA was used to transform E.coli strain JM101 (2.7.2). The requirement for screening of transformants to determine fragment orientation had been eliminated by forced orientation cloning of the rSLT IA_1 fragment into pSC25, they were, however, screened for possession of the mutation required for generation of the SLT IA subunit A_1 alone by dideoxy sequencing of the plasmid DNA (2.5.8(ii)). Large scale preparation (2.4.2(ii)) of pSC25A_1 (FIG 3.3.18) was then carried out in order to yield purified plasmid DNA.

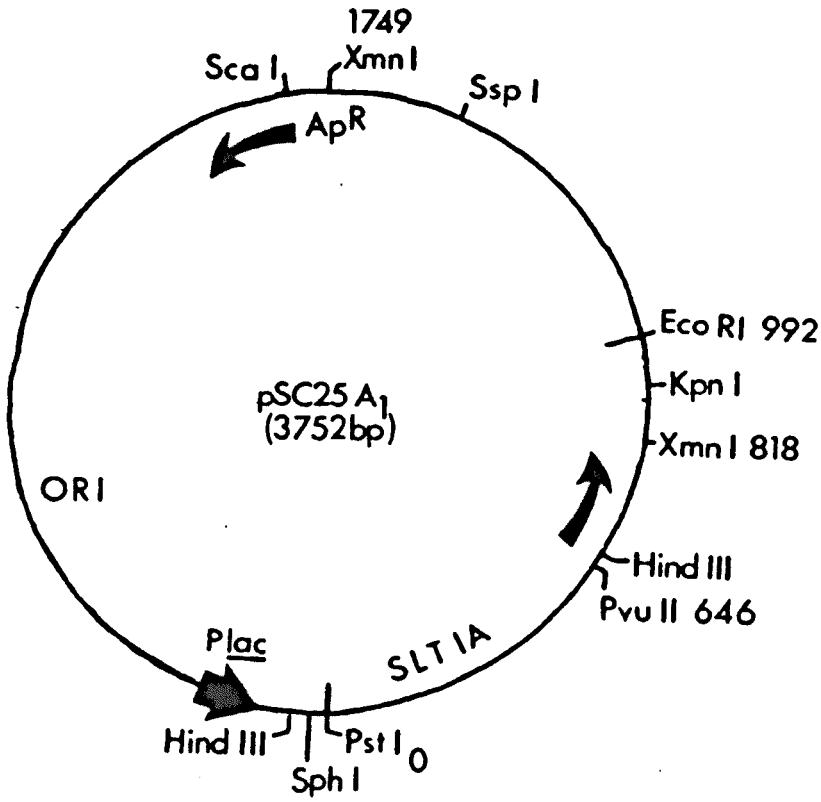


FIG 3.3.18 **Plasmid pSC25A_1.**

Derived from plasmid pSC25 (FIG 3.3.5) which was kindly donated by Dr S Calderwood (Boston, USA). The plasmid contains the DNA for the recombinant, truncated, Shiga-like toxin I A subunit (rSLT IA_1 (A_1 alone)) under transcriptional control of the lacZ promoter (Hovde et al. (1988). In order to generate pSC25A_1 a Pst I / Eco RI fragment of approximately 990 base pairs encoding the rSLT IA_1 (A_1 alone) DNA was ligated (2.5.5) with similarly restricted pSC25 (FIG 3.3.5).

(b) Construction of pSC25A_1A_2 (FIG 3.3.19).

Generation of pSC25A_1A_2 (FIG 3.3.19) involved the excision (2.5.3) of an 1150bp Pst I / Eco RI fragment from the M13mp18X / SLT IA_1A_2 RF DNA (FIG 3.3.18) which carried the correct mutation for generation of the full length, protease insensitive rSLT IA_1A_2. This was then ligated (2.5.5)) into Pst I / Eco RI restricted (2.5.3) and gel purified (2.4.4) pSC25 (FIG 3.3.5).

Transformation of the ligated DNA in E.coli strain JM101 (2.7.3) and dideoxy sequencing of resultant plasmid DNA (2.5.8(ii)) in order to determine possession of the required rSLT IA DNA fragment followed by large scale preparation of the plasmid DNA was as described for construction of pSC25A_1.

Each of the pSC25 derivatives (pSC25A_1 (FIG 3.3.18)and pSC25A_1A_2 (FIG 3.3.19) plus pSC25 (FIG 3.3.5) expressing the wild type SLT IA were used to transform E.coli strain JM101 (2.7.2) in readiness for expression in vivo (2.9). IPTG induced expression of pSC25 (FIG 3.3.5) , pSC25A_1 (FIG 3.3.18) and pSC25A_1A_2 (FIG 3.3.19) was carried out exactly as described by Hovde et al. (1988) in expression of SLT IA glutamic acid (Glu) 167 mutant (Asp^{167}).

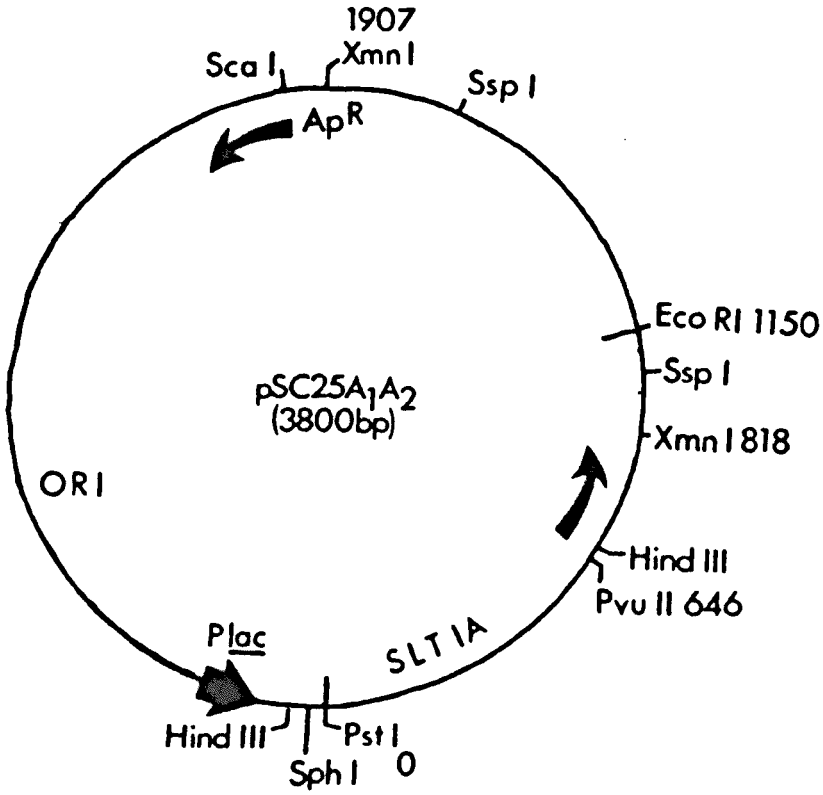


FIG 3.3.19 Plasmid pSC25A_1A_2.

Derived from plasmid pSC25 (FIG 3.3.5) which was kindly donated by Dr S Calderwood (Boston, USA). The plasmid contains the DNA for the recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit (rSLT IA_1A_2 (protease insensitive)) under transcriptional control of the lacZ promoter (Hovde et al. (1988). In order to generate pSC25A_1A_2 a Pst I / Eco RI fragment of approximately 1150 base pairs encoding the rSLT IA (protease insensitive) DNA was ligated (2.5.5) with similarly restricted pSC25 (FIG 3.3.5).

The SLT IA and SLT IB subunits each possess a signal peptide which causes them to be transported to the periplasm where assembly of the holotoxin takes place as is the case with E.coli heat labile toxin (LT). Hovde et al. (1988) prepared a periplasmic extract of recombinant SLT IA by incubation of the resultant cell pellet with polymyxin B (Griffin and Gemski, 1983). Osmotic release of the contents of periplasm (2.9.1) was however, the method chosen for preparation of periplasmic extracts in this particular study.

Successful expression of rSLT IA (wild type), rSLT IA_1 (A_1 alone) and rSLT IA_1A_2 ('protease insensitive') was determined by assay of the respective periplasmic extracts for RNA N-glycosidase activity. This was determined by incubation of a small amount of each periplasmic extract with yeast ribosomes (2.11.1) with the addition of vanadyl ribonuclease complex (VRC) to a final concentration of 2mM, in order to inhibit random degradation of the ribosomal RNA (rRNA) by ribonucleases indigenous to the periplasmic extracts.

FIG 3.3.20 shows the resultant yeast rRNA following its extaction (2.4.1), treatment with aniline reagent (2.5.1) and resolution by denaturing gel electrophoresis (2.6.1). The presence of the characteristic fragment released by aniline cleavage of depurinated rRNA (lanes 2, 3 and 4) indicates the successful in vivo expression of biologically active rSLT IAs

FIG 3.3.20 Examination of *in vivo* produced recombinant Shiga-like toxin I A subunits for RNA N-glycosidase activity.

10ul of each recombinant Shiga-like toxin I A subunit (rSLT IA)-containing periplasmic extract was incubated with 30ug yeast ribosomes for 20min at 30°C (2.11.1(i)). In order to reduce non specific degradation of ribosomal RNA (rRNA) vanadyl ribonucleoside complex (VRC) was also included at this stage (2mM final concentration). Prior to incubation with the ribosomes each of the extracts was preincubated with 2-mercaptoethanol to a final concentration of 6mM to ensure maximum activity of any SLT IA present. The resultant rRNA was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). The resultant rRNA was then fractionated and visualised (2.6.1).

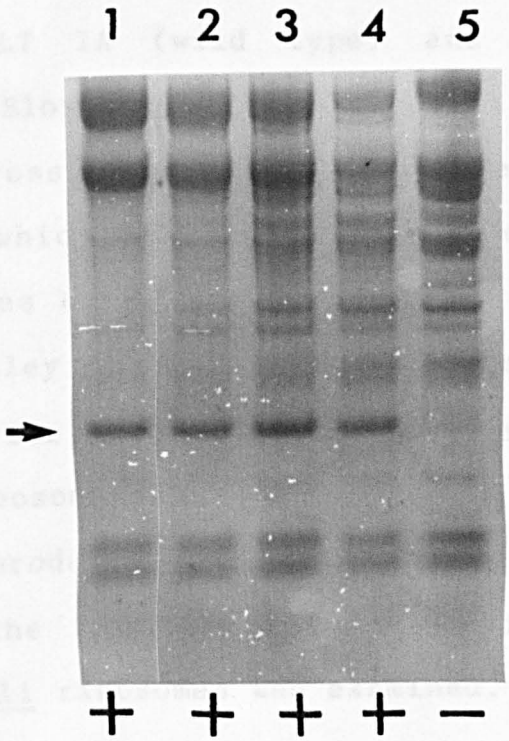
Lane 1 = positive control: rRNA resulting from incubation with pure SLT I.

Lanes 2, 3 and 4: rRNA following incubation with rSLT IA (wild type), rSLT IA_1 (A_1 alone) and rSLT IA_1A_2 ('protease insensitive') respectively.

+ indicates treatment of rRNA with aniline reagent.

- indicates non treatment of extracted rRNA with aniline reagent.

➔ indicates the characteristic fragment released following aniline cleavage of toxin-modified rRNA.


1 2 3 4 5
+ + + + −

since the same fragment is also seen to occur (lane 1) following modication of the rRNA by pure SLT I.

It was noticed during the culturing of E.coli JM101 expressing each of the rSLT IAs that those expressing the rSLT IA_1 (A_1 alone) appeared to grow more slowly in comparison to those expressing rSLT IA (wild type) and rSLT IA_1A_2 (protease insensitive). Slow growth of E.coli had also been observed by others in expression of Type I ribosome inactivating proteins such as those which are now known to be capable of inactivating E.coli ribosomes eg dianthins 30 and 32 and pokeweed antiviral proteins (Hartley et al. 1991). Thus we might propose that a possible role for the SLT IA A_2 peptide might be to protect homologous ribosomes from depurination during production of this E.coli-produced toxin. In order to examine this possibility, the RNA N-glycosidase activity of rSLT IA_1 (A_1 alone) on E.coli ribosomes was examined.

rSLT IA_1 (A_1 alone)-containing periplasmic extract was incubated with E.coli ribosomes (2.11.1) plus VRC (in order to eliminate unwanted ribonuclease degradation of the rRNA) the rRNA extracted (2.4.1) treated with aniline reagent (2.5.1) and examined by gel electrophoresis (2.6.1). FIG 3.3.21 shows the resultant RNA gel comparing non aniline treated E.coli rRNA, E.coli rRNA following incubation with pure SLT I and aniline treatment (lane 1) and E.coli rRNA following incubation with rSLT IA_1 (A_1 alone). No characteristic aniline-released

FIG 3.3.21 Investigation of the activity of recombinant, truncated Shiga-like toxin I A subunit (A_1 alone) on Escherichia coli ribosomes.

10ul of periplasmic extract (2.9.1) containing recombinant, truncated Shiga-like toxin I A subunit (rSLT IA_1 (A_1 alone) previously shown to exhibit RNA N-glycosidase activity (FIG 3.3.20), was incubated with 30ug Escherichia coli ribosomes for 20min at 30°C (2.11.1(i)). This followed preincubation of the extract with 2-mercaptoethanol to a final concentration of 6mM to ensure maximum activity of any SLT I present. The resultant ribosomal RNA (rRNA) was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). Fractionation and visualisation of the resultant rRNA was then carried out (2.6.1).

Lane 1 shows E.coli rRNA following incubation with pure SLT I.
Lane 2 shows E.coli rRNA following incubation with rSLT IA_1 (A_1 alone).

+ indicates treatment of rRNA with aniline reagent.
- indicates that following incubation with toxin and extraction the rRNA was not subjected to aniline cleavage.


1 2
– + +

fragment is visible indicating that, as is the case with pure SLT IA, (Chapter 2 SECTION 3.2.2), rSLT IA_1 (A_1 alone) is inactive on E.coli ribosomes.

Although successful expression of biologically active rSLT IAs was achieved, it was found that expression of rSLT IA_1 (A_1 alone) was either at very low levels only or that it was retrievable only at very low levels. The latter of which may be attributable to a reduction in stability of the recombinant protein often associated with truncated proteins. Attempts were made to increase the level of expression of rSLT IA_1 (A_1) by cloning the rSLT IA_1 (A_1) DNA into the high level expression vector pKK223.3 (Pharmacia). This vector has been used successfully by several members of our laboratory. In order to achieve such cloning it was necessary via polymerase chain reaction (PCR) to generate a rSLT IA_1 (A_1) DNA fragment possessing suitable restriction sites. A suitable fragment was generated, however successful ligation (2.5.5) with the pKK223.3 vector was never achieved. Unfortunately time did not permit further attempts to reclone or resolve the PCR problems encountered.

It was decided that studies into the role of the SLT IA_2 peptide in both the RNA N-glycosidase activity of SLT IA and its association with the SLT IB pentamer could be suitably examined using the two remaining rSLT IAs constructed. Thus attention, as will be seen in subsequent chapters, was focussed

upon the expression, purification and ultimately the cytotoxicies and *in vitro* activities of rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') in order to elucidate the consequences of an intact, non cleavable A_2 peptide on enzymatic activity and cytotoxicity.

Conclusion - Chapter 3

Recombinant Shiga-like toxin I A subunits (rSLT IAs) have been constructed for the purpose of studying any involvement of the A_2 peptide in the RNA N-glycosidase activity and cytotoxicity of of the SLT I. Results presented here serve to demonstrate the successful production of rSLT IAs, which are of expected size and which possess RNA N-glycosidase activity following expression in both *in vitro* and *in vivo* systems. In addition, the requirement for a recombinant SLT IA subunit with reduced sensitivity to proteolytic cleavage has also been met. Unfortunately the expression level of rSLT IA (A_1) alone) in *.coli* was too low to permit further analysis (data not shown).

As will be seen in subsequent chapters, the development of a successful purification scheme serves to allow a closer, more quantitative examination of the cytotoxicity of the 'non cleavable' rSLT IA_1A_2 ('protease insensitive') when reassociated with Shiga-like toxin I B subunit (SLT IB). This also allows the enzymatic activity of an unnickable SLT IA subunit (rSLT IA_1A_2 ('protease insensitive')) to be examined and compared with a wild type SLT IA subunit.

CHAPTER 4

SECTION 3.4.1 Purification of the Shiga-like toxin I B subunit (SLT IB).

Introduction

Several unsuccessful attempts to raise antisera to gel eluted SLT IA subunit in both rabbits and rats made it necessary to search for an alternative method by which recombinant Shiga-like toxin I A subunits (rSLT IAs) could be purified to homogeneity. The original strategy had proposed purification of the rSLT IAs via anti SLT IA affinity chromatography. Once purified their relative RNA N-glycosidase activities could be determined and, by reassociation with purified Shiga-like toxin I B subunit (SLT IB), their relative cytotoxicities could also be determined.

Weinstein *et al.* (1989), in production of hybrid Shiga toxin (ST) / SLT I / SLT II molecules, had successfully coexpressed A and B subunits each encoded by separate compatible plasmids with individual selective markers. Coexpression resulted in a cytotoxic product. The relevant plasmids - pDLW101 (Weinstein *et al.* 1989) encoding SLT IA and pJN26 (Newland *et al.* 1985) encoding SLT IB, were kindly provided by Dr C. Schmitt (Bethesda MD). The intention was to substitute rSLT IAs into pDLW101 and to coexpress them with SLT IB in order to produce a mutant holotoxin. This could then be purified to homogeneity

exploiting the highly specific binding of SLT IB to globotriose (Gb_3) via the method decribed by Ryd *et al.* (1989) in purification of the naturally occuring holotoxin. However, logistics prevented this approach, since coexpression would have required Category III containment; a facility which could not reliably be provided.

Several groups (Ito *et al.* 1988, Donohue-Rolfe *et al.* 1989), had reported the successful reassociation of ST / SLT I / SLT II A subunits with ST / SLT I / SLT II B subunits by mixing of the separate subunits together, resulting in the production of a cytotoxic product. In addition Takeda *et al.* (1981) had successfully demonstrated the association *in vitro* of the separated A and B subunits of cholera toxin (CT) and *Escherichia coli* heat labile toxin (LT) to generate biologically active, hybrid toxins. Again this was achieved by a simple mixing together of the relevant subunits.

Ito *et al.* (1988) had examined the formation of SLT I / SLT II hybrid molecule. This was achieved by mixing together the required combination of propionic acid treated, high performance liquid chromatography (HPLC) separated A and B subunits and dialysing for 16 hours in order to remove the denaturant. Product cytotoxicity was determined using Vero cells.

Donohue-Rolfe et al. (1989) successfully reassociated ST subunits which had undergone denaturation and chromatographic separation. If both subunits were still in a denatured state they were mixed together and dialysed for 36 hours prior to testing for cytotoxic product. If, however, both subunits had been renatured, they were simply mixed together and assayed for cytotoxicity immediately. Both procedures gave a cytotoxic product.

It was therefore decided that a similar approach would be taken in order to purify the rSLT IAs. In each case an excess of pure SLT IB would be mixed with periplasm containing each of the expressed rSLT IAs in the hope that reassociation would take place. The reassociated mutant holotoxins would then be purified by exploitation of the binding affinity of SLT IB for the Gb_3 molecule as described by Ryd et al. (1989).

In order to achieve this, production and purification of SLT IB was first necessary.

Results and Discussion

Dr S Calderwood (Boston USA) kindly provided pSBC32 (Calderwood et al. 1990) (FIG 3.4.1), a vector capable of high level expression of SLT IB.

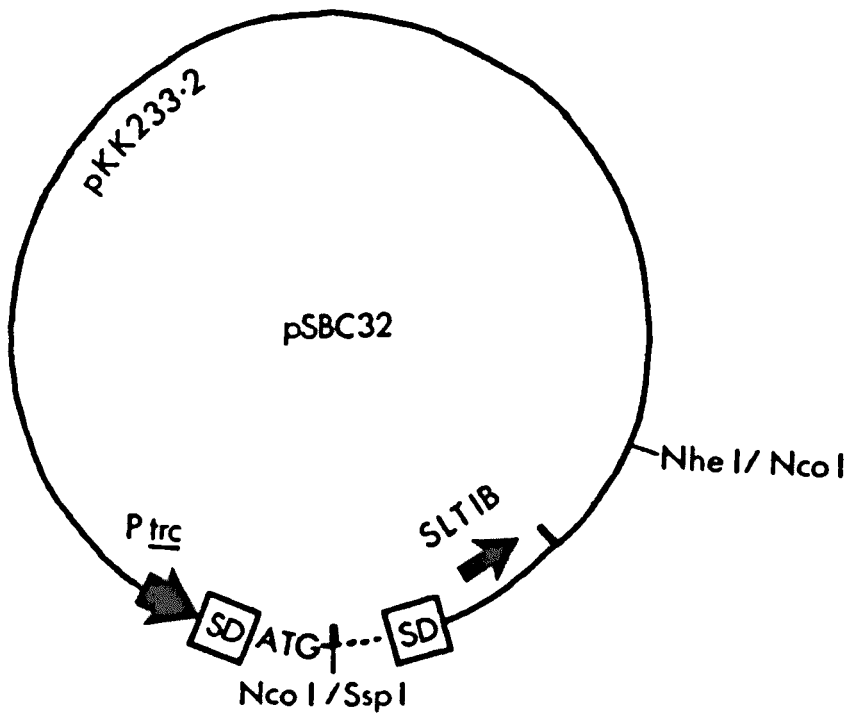


FIG 3.4.1 Plasmid pSBC32.

Kindly donated by Dr S Calderwood (Boston, USA), pSBC32 encodes the Shiga like toxin I B subunit (SLT IB) under control of the trc promoter of pKK233.2 (Calderwood et al. 1990).

A culture of E.coli JM105 carrying pSBC32 was grown up and expression induced with IPTG exactly as detailed by Calderwood et al. (1990). Preparation of a periplasmic extract was achieved by osmotic shock (2.9.1) as opposed to incubation of the culture with polymyxin B sulphate used by Calderwood. Osmotic shock preparation of periplasm reduced the production time of SLT IB by 24 hours. This resulted from there being no requirement for the removal of the polymyxin B from the periplasm preparation prior to the purification. Such removal would normally require an overnight dialysis step.

The periplasm preparation resulting from osmotic shock was applied to the Gb_3-Fractogel column (Biocarb) immediately and following washing of the column with phosphate buffered saline pH7.2 (PBS) the bound SLT IB was eluted using 6M guanidine HCl pH6.7 as described by Ryd et al. 1989 for elution of ST. Following overnight dialysis to remove guanidine HCl from the resultant fractions, they were analysed by SDS polyacrylamide gel electrophoresis (SDS PAGE) against pure SLT I and periplasmic extracts (FIG 3.4.2). Fractions 1 and 2 (lanes 3 and 4 respectively) may be seen to contain a band which migrates to the same point as the B subunit of the purified SLT I control (lane 1).

Protein determination by the Bio-Rad (2.10.1) procedure revealed a SLT IB concentration of 2mg / ml which could

FIG 3.4.2 Purification of Shiga-like toxin I B subunit (SLT IB).

A periplasmic extract of E.coli JM105 expressing plasmid pSBC32 (FIG 3.4.1) encoding SLT IB, was prepared (2.9.1). This was applied to a globotriosylceramide (Gb_3) column allowing a single step purification of SLT IB by exploitation of the high specificity binding of SLT IB to the Gb_3 molecule. Elution of the bound SLT IB was achieved with guanidine hydrochloride (2.12). The elution products were then analysed by SDS polyacrylamide gel electrophoresis (SDS PAGE) (2.10.3) on a 15% gel (reducing conditions) in comparison with purified SLT I holotoxin and the pre-column periplasmic extracts. The resolved proteins were visualised by Coomassie staining (2.10.3(i)) of the gel.

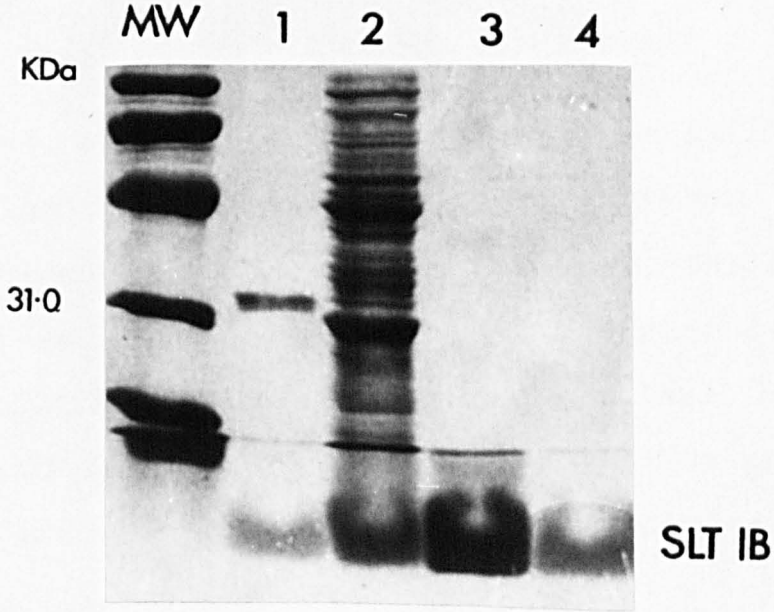
1: Pure Shiga-like toxin I (approx. 4ug).

2: E.coli JM105 + pSBC32 (FIG 3.4.1) periplasmic extract.

3: Gb_3 column fraction 1.

4: Gb_3 column fraction 2.

MW: molecular weight markers.


MW
1
2
3
4
KDa
31·0
SLT IB

then be used in order to attempt purification of the recombinant SLT IA subunits.

SECTION 3.4.2 **Reassociation of recombinant wild type Shiga-like toxin I A subunit and full length, 'protease insensitive' Shiga-like toxin I A subunit with Shiga-like toxin I B subunit.**

Introduction

Reassociation of the recombinant, wild type Shiga-like toxin I A subunit (rSLT IA (wild type)) and recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit (rSLT IA_1A_2 protease insensitive) with purified Shiga-like toxin I B subunit (SLT IB) was achieved using the simple but successful method described by Ito *et al.* (1988) and Donohue-Rolfe *et al.* (1989) of simply mixing the component subunits together and testing for product cytotoxicity.

Results and Discussion

In this study only the SLT IB subunit preparation was of a known concentration as determined by Bio-Rad Assay (2.10.1) SECTION 3.4.1. In both rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') periplasm preparations (2.9.1) (each a 1ml preparation resulting from a 1L culture), the concentration of rSLT IA (wild type) or rSLT IA_1A_2 (protease insensitive) respectively was unknown.

To 500ul of each periplasm extract was added an excess (approximately 200ug) of purified SLT IB subunit plus phenyl methyl sulphonyl fluoride (PMSF) to a final concentration of 1mM in order to inhibit the action of contaminating serine proteases. The mixture was mixed overnight at 4°C in order to promote reassociation of the SLT I subunits.

The resultant mixture was then applied directly to the 1ml Gb_3-Fractogel column (Biocarb) in order to allow purification of any reassociated product and free SLT IB. In each case the column was washed with phosphate buffered saline (PBS) pH7.2 in order to remove any unbound proteins and bound product was eluted using 6M guanidine HCL as described by Ryd et al. (1989) in purification of Shiga toxin (ST).

Following overnight dialysis against PBS to remove guanidine HCl from the resultant fractions analysis was by SDS polyacrylamide gel electrophoresis (SDS PAGE) (2.10.3). FIG 3.4.3 shows the resultant purified rSLT IA (wild type) containing holotoxin (lane 2) in comparison to purified SLT IB (lane 1), pure SLT I (lane 3) and periplasmic extracts containing rSLT IA (wild type) with added SLT IB (lane 4).

FIG 3.4.4 shows the resultant purified reassociated rSLT IA_1A_2 ('protease insensitive') containing holotoxin (lane 1) in comparison with pure SLT I (lane 2).

FIG 3.4.3 **15% SDS polyacrylamide gel showing the stages in purification of the reassociated wild type recombinant Shiga-like toxin I A subunit (rSLT IA (wild type) containing holotoxin.**

1ml of a periplasm preparation (2.9.1) containing an unknown concentration of wild type recombinant Shiga-like toxin I A subunit (rSLT IA (wild type)), was mixed with approximately 200ug of purified Shiga like toxin I B subunit (SLT IB) (2.13) with the addition of phenyl methyl sulphonyl fluoride (PMSF) to a concentration of 1mM. Following overnight mixing at 4C the mixture was applied to the globotriose (Gb_3)-Fractogel column (Biocarb) used in the purification of SLT I (Chapter 1). Bound products were eluted using 6M guanidine HCl pH6.7 as described by Ryd *et al.* (1989) in the purification of Shiga toxin (ST). Products were resolved using a 15% polyacrylamide gel and visualised by silver staining (2.10.3(ii)).

Lane **1** = purified SLT IB

Lane **2** = purified reassociated rSLT IA (wild type) containing holotoxin

Lane **3** = purified SLT I

Lane **4** = periplasm proteins containing rSLT IA (wild type)

Lane **5** = molecular weight markers

A and **B** represent **SLT IA** and **SLT IB** respectively.

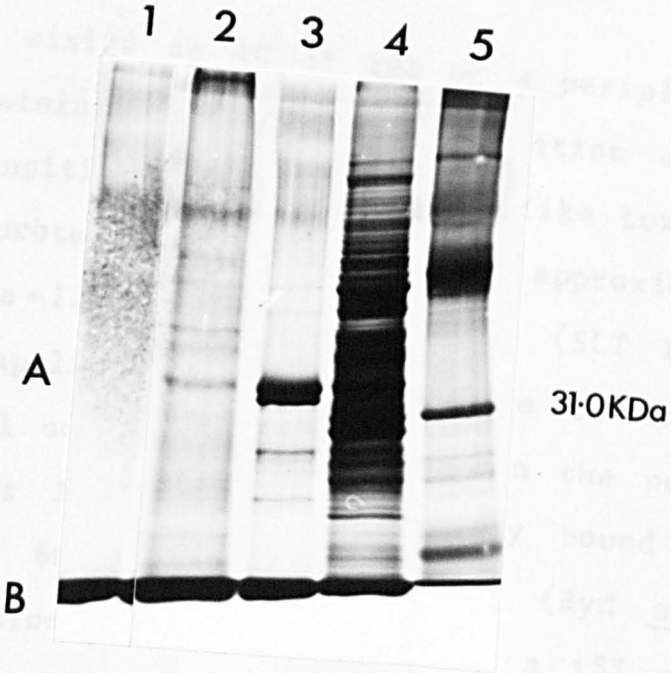
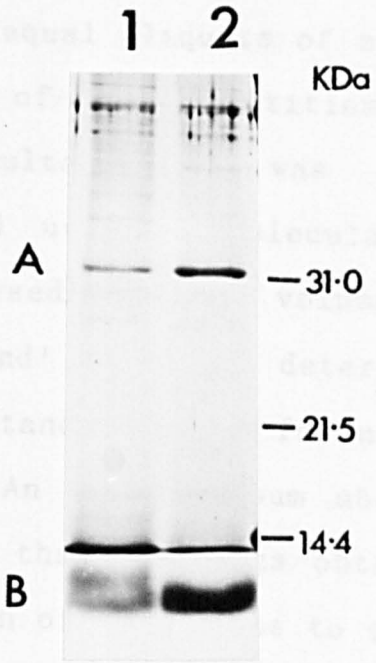

1
2
3
4
5
A
B
31·0KDa

FIG 3.4.4 **15% SDS polyacrylamide gel showing purified reassociated full length, 'protease insensitive' recombinant Shiga - like toxin I A subunit (rSLT IA_1A_2 ('protease insensitive')) containing holotoxin.**

An overnight mixing at 4C of 1ml of a periplasm preparation (2.9.1) containing an unknown concentration of full length, protease insensitive recombinant Shiga-like toxin I A subunit (rSLT IA_1A_2 (protease insensitive)) with approximately 200ug of purified Shiga-like toxin I B subunit (SLT IB) (2.13) was followed by application of the mixture to the globotriose (Gb_3)-Fractogel column (Biocarb) used in the purification of SLT I (Chapter 1). Eluted of affinity bound products was achieved using 6M guanidine HCl pH6.7 (Ryd et al. 1989). Resultant fractions were resolved using a 15% polyacrylamide gel and visualised by silver staining (2.10.3(ii)).

Lane 1 = purified reassociated rSLT IA_1A_2 ('protease insensitive') containing holotoxin
Lane 2 = purified SLT I
A and B represent SLT IA and SLT IB respectively.

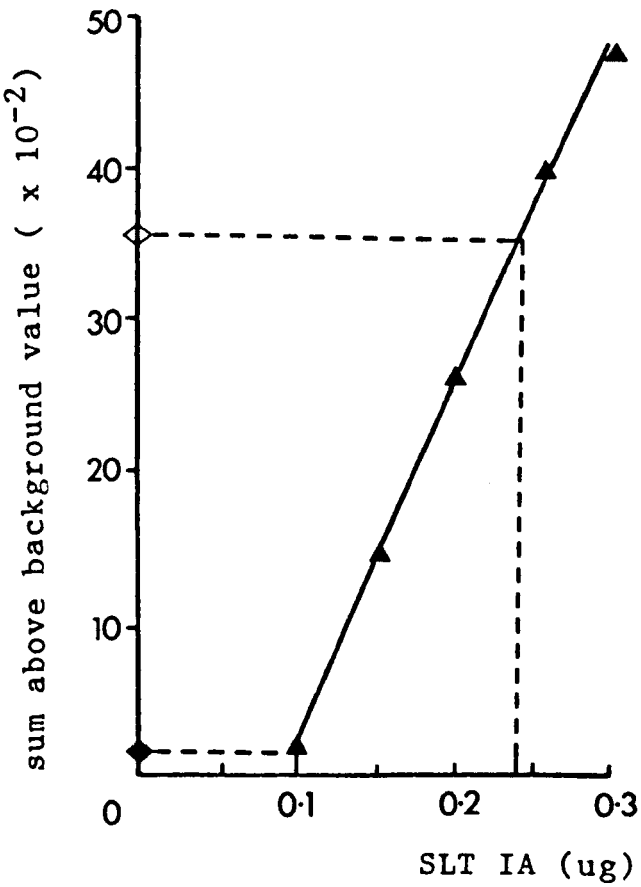

1
2
KDa
A
31·0
21·5
14·4
B

In each case it may be seen that an excess of SLT IB subunit is present in each preparation. For this reason quantitation of rSLT IA (wild type) or rSLT IA_1A_2 ('protease insensitive') respectively was determined by densitometry in comparison to aliquots of SLT IA subunit of known amount. This was achieved by SDS polyacrylamide gel electrophoresis (SDS PAGE) (reducing conditions) of three equal aliquots of each rSLT IA-containing holotoxin and a range of known quantities of native SLT I (0.05 - 0.3ug). The resultant gel was then silver stained (2.10.3(ii)), scanned using a Molecular Dynamics computing densitometer and analysed using the volume integration package. A 'sum above background' value was determined for each SLT IA and rSLT IA band. A standard curve for native SLT IA was then plotted (FIG 3.4.5). An average 'sum above background' value was obtained from the three readings obtained for each of the rSLT IAs. By comparison of this value to those of native SLT IA it was possible to determine the concentration of rSLT IA in each reassociated holotoxin. FIG 3.4.5 shows the standard curve derived for known amounts of SLT IA and the derivation of rSLT IA concentrations from this.

It should be noted that in each case prior to purification of the reassociated holotoxins using the Gb_3-Fractogel column (Biocarb), the column was thoroughly 'cleansed' of any residual bound SLT I. This was achieved using 6M guanidine HCl pH6.7 and PBS. Prior to each purification of reassociated holotoxin, 7 - 8 1ml guanidine HCl fractions were collected from the column

FIG 3.4.5 **Determination of the concentration of each rSLT IA .**

In each case three equal aliquots of 100ul of rSLT IA-containing holotoxin were resolved by SDS polyacrylamide gel electrophoresis (SDS PAGE) (2.10.3) (reducing conditions). In addition a range of known quantities (0.05 - 0.3ug) of native SLT IA were also subjected to SDS PAGE. The resultant gel was silver stained (2.10.3(ii)), scanned and analysed using a Molecular Dynamics computing densitometer and its volume integration package. A 'sum above background' value was found for each SLT IA and rSLT IA gel band. Those values obtained for native SLT IA were then plotted against amount of SLT IA (ug) to generate a standard curve. An average value was determined from the 'sum above background' values determined for each rSLT IA. By comparison to the standard curve it was possible to determine the amount of rSLT IA in each 100ul aliquot of rSLT IA-containing holotoxin and hence the concentration.



◇ average value rSLT IA_1A_2 ('protease insensitive')

◆ average value rSLT IA (wild type)

and analysed for the presence of SLT I by (i) ability to depurinate yeast ribosomes (2.11.1(i)) (FIGS 3.4.6A and 3.4.6B) and (ii) SDS PAGE (2.10.3) (FIG 3.4.7).

FIGS 3.4.6A and 3.4.6B demonstrate the lack of any biological activity in 'clean' column fractions eluted prior to purification of reassociated rSLT IA (wild type) and rSLT IA_1A_2 (protease insensitive) containing holotoxins respectively. In both cases there would appear to be no evidence of residual, biologically active SLT I in any of the eluted fractions as indicated by the lack of the characteristic aniline-released rRNA fragment (lanes 2 - 8 FIG 3.4.6A) (lanes 1 - 7 FIG 3.4.6B).

SDS PAGE analysis of 'clean' column fractions (FIG 3.4.7) prior to purification of reassociated rSLT IA_1A_2 ('protease insensitive') containing holotoxin also reveals a distinct lack of any residual SLT I (lanes 2 - 8) in comparison with purified rSLT IA (wild type) containing holotoxin (lane 1).

The results of this 'cleaning' procedure serve to demonstrate that this method of column regeneration leaves no detectable residual SLT I bound to the column. Thus any purified product detected in the above purification of reassociated rSLT IA-containing holotoxin is just that.

FIG 3.4.6B **Aniline assay to detect residual Shiga-like toxin I (SLT I) RNA N-glycosidase activity in Gb_3-Fractogel column fractions following column regeneration prior to use in the purification of reassociated recombinant full length, protease insensitive Shiga-like toxin I A subunit containing holotoxin.**

10ul of each 1ml fraction eluted from the globotriose (Gb_3)-Fractogel column (Biocarb) (using 6M guanidine HCl pH6.7) was incubated with 30ug yeast ribosomes at 30C for 20min (2.11(i)). The ribosomal RNA (rRNA) was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). The resultant rRNA was then fractionated by gel electrophoresis and visualised by staining with ethidium bromide (2.6.1).

Lanes **1 - 7** = rRNA following incubation of yeast ribosomes with Gb_3-Fractogel column fractions **1 - 7.**
Lane **8** = positive control. Resultant rRNA following incubation of yeast ribosomes with pure SLT I.
Lane **9** non aniline treated, SLT I modified yeast rRNA.
+ indicates treatment of yeast rRNA with aniline reagent.
➡indicates characteristic fragment released following aniline treatment of depurinated rRNA.

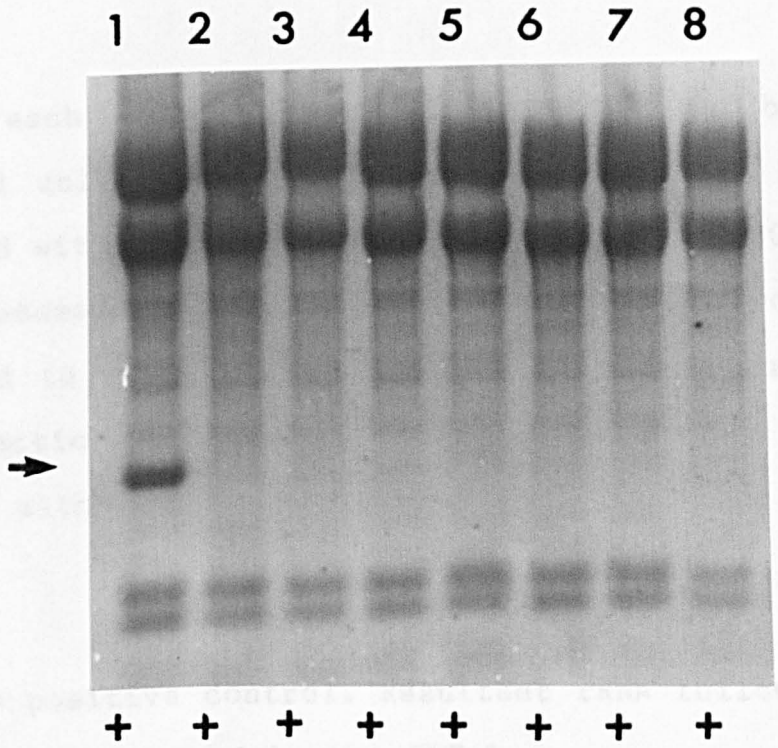

1 2 3 4 5 6 7 8
+ + + + + + + +

FIG 3.4.6A **Aniline assay to detect residual Shiga-like toxin I (SLT I) RNA N-glycosidase activity in Gb_3-Fractogel column fractions following column regeneration prior to use in the purification of reassociated recombinant wild type Shiga-like toxin I A subunit containing holotoxin.**

10ul of each 1ml fraction eluted from the globotriose (Gb_3)-Fractogel column (Biocarb) (using 6M guanidine HCl pH6.7) was incubated with 30ug yeast ribosomes at 30C for 20min (2.11(i)). The ribosomal RNA (rRNA) was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). The resultant rRNA was then fractionated by gel electrophoresis and visualised by staining with ethidium bromide (2.6.1).

Lane 1 = positive control. Resultant rRNA following incubation of yeast ribosomes with pure SLT I.
Lanes 2 - 8 = rRNA following incubation of yeast ribosomes with Gb_3-Fractogel column fractions 1 - 7.
+ indicates treatment of yeast rRNA with aniline reagent.
➡indicates characteristic fragment released following aniline treatment of depurinated rRNA.

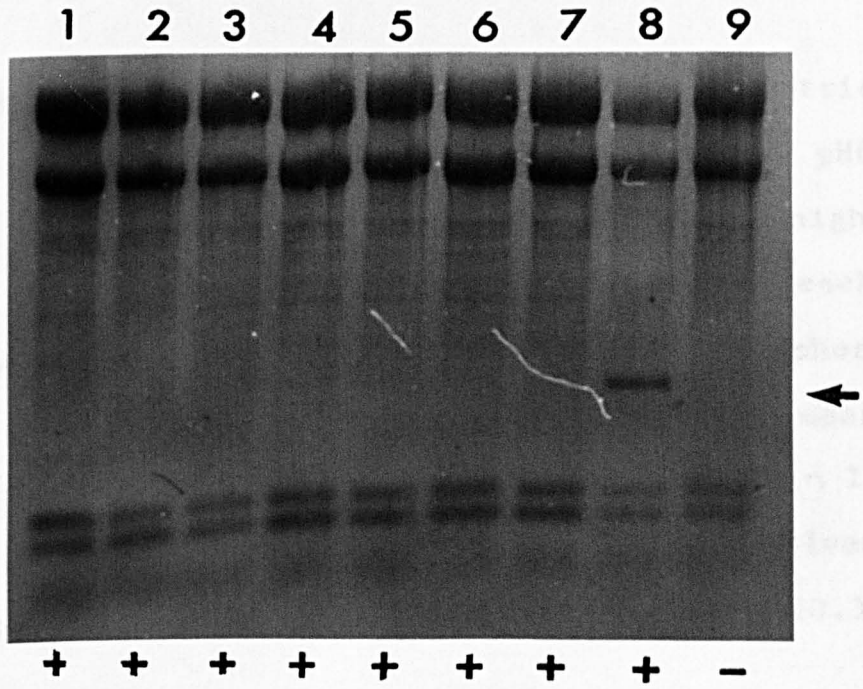
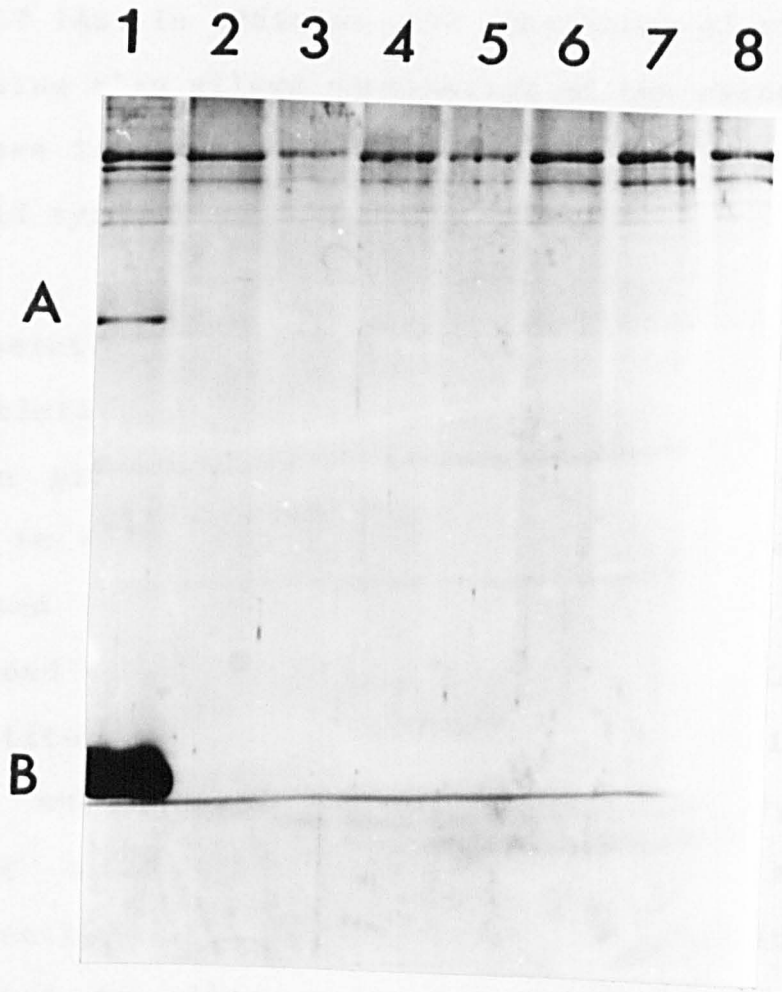

1 2 3 4 5 6 7 8 9
+ + + + + + + + −

FIG 3.4.7 SDS polyacrylamide gel electrophoresis (SDS PAGE) analysis of Gb_3-Fractogel column factions following 'cleaning'.

Residual bound proteins were eluted from the globotriose (Gb_3)-Fractogel column (Biocarb) using 6M guanidine HCl pH6.7. Seven 1ml fractions were collected and dialysed overnight against phosphate buffered saline (PBS) pH7.2. 500ul of each fraction was analysed by SDS polyacrylamide gel electrophoresis (SDS PAGE) (2.10.3) on a 15% polyacrylamide gel in comparison with reassociated recombinant wild type Shiga-like toxin I A subunit (rSLT IA (wild type)) containing holotoxin. Resolved proteins were visualised by silver staining of the gel (2.10.3(ii)).

Lane **1** shows reassociated rSLT IA (wild type) containing holotoxin - **A** and **B** indicating SLT IA and B subunits respectively.

Lanes **2 - 8** = column fractions **1 - 7.**


1 2 3 4 5 6 7 8
A
B

Conclusion - Chapter 4

The successful reassociation of the rSLT IAs with purified SLT IB demonstrated here facilitated the subsequent purification of the rSLT IAs. In addition, the generation of rSLT IA-containing holotoxins also allows examination of the cytotoxic nature of a 'protease insensitive' SLT IA subunit in comparison to that of the wild type SLT IA subunit.

The generation of rSLT IA containing holotoxins may be seen to be a relatively simple procedure. However, although it may be seen to give relatively pure rSLT IA-containing holotoxins, yields in each case were very poor (1ug / ml rSLT IA (wild type) and 2.4ug / ml rSLT IA_1A_2 ('protease insensitive') as determined by densitometry in comparison to SLT IA standards). In particular, this presented certain limitations to the kinetic analysis (Chapter 5 SECTION 3.5.2), since it was possible only to examine a relatively small range of concentrations of rSLT IA in each case. Yields were however, sufficient to allow a limited examination of the relative biological activities of the rSLT IAs.

CHAPTER 5

SECTION 3.5.1 **Comparing the cytotoxicities of reassociated holotoxin containing recombinant, wild type Shiga-like toxin I A subunit and recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit.**

Introduction

As previously discussed, the work of Olsnes *et al*. (1981) on Shiga toxin (ST) and of Kongmuang *et al*. (1988) on Shiga-like toxin I (SLT I) has shown there to be no difference in cytotoxicity of pre-nicked and unnicked ST and SLT I respectively. In order to carry out studies on the effects of nicking and reduction on the ST / SLT I A subunits however, the toxins were pretreated with trypsin. Studies by Olsnes *et al*. (1981), also involved treatment of ST with reducing agents such as 5% 2-mercaptoethanol.

One of the main aims of this study was to investigate the consequences of unnicked toxin in cellular uptake and enzymatic activity without a requirement for chemical pretreatment of the proteins under investigation.

In vitro expression of the recombinant SLT IA subunits (rSLT IAs) generated in this study has shown the recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit (rSLT IA_1A_2 ('protease insensitive')) to be of the same

expected size as the recombinant, wild type Shiga like toxin I A subunit (rSLT IA (wild type)) ie 32KDa (Chapter 3 SECTION 3.3.2). An apparent reduction in the sensitivity of the rSLT IA_1A_2 ('protease insensitive') to cleavage by the protease trypsin has also been demonstrated (Chapter 3 SECTION 3.3.2).

Successful reassociation of both the rSLT IA (wild type) and also the rSLT IA_1A_2 ('protease insensitive') with purified SLT IB subunit (Chapter 4 SECTION 3.4.2) has provided a way in which the cytotoxic nature of the rSLT IA_1A_2 ('protease insensitive') may be compared to that of the rSLT IA (wild type). It is hoped that the observed reduction in susceptibility to proteolytic cleavage of rSLT IA_1A_2 ('protease insensitive') in vitro may also be the case in vivo, ie it will remain as a full length, unprocessed SLT IA_1A_2 subunit comprising both the A_1 and the A_2 in an unnicked state.

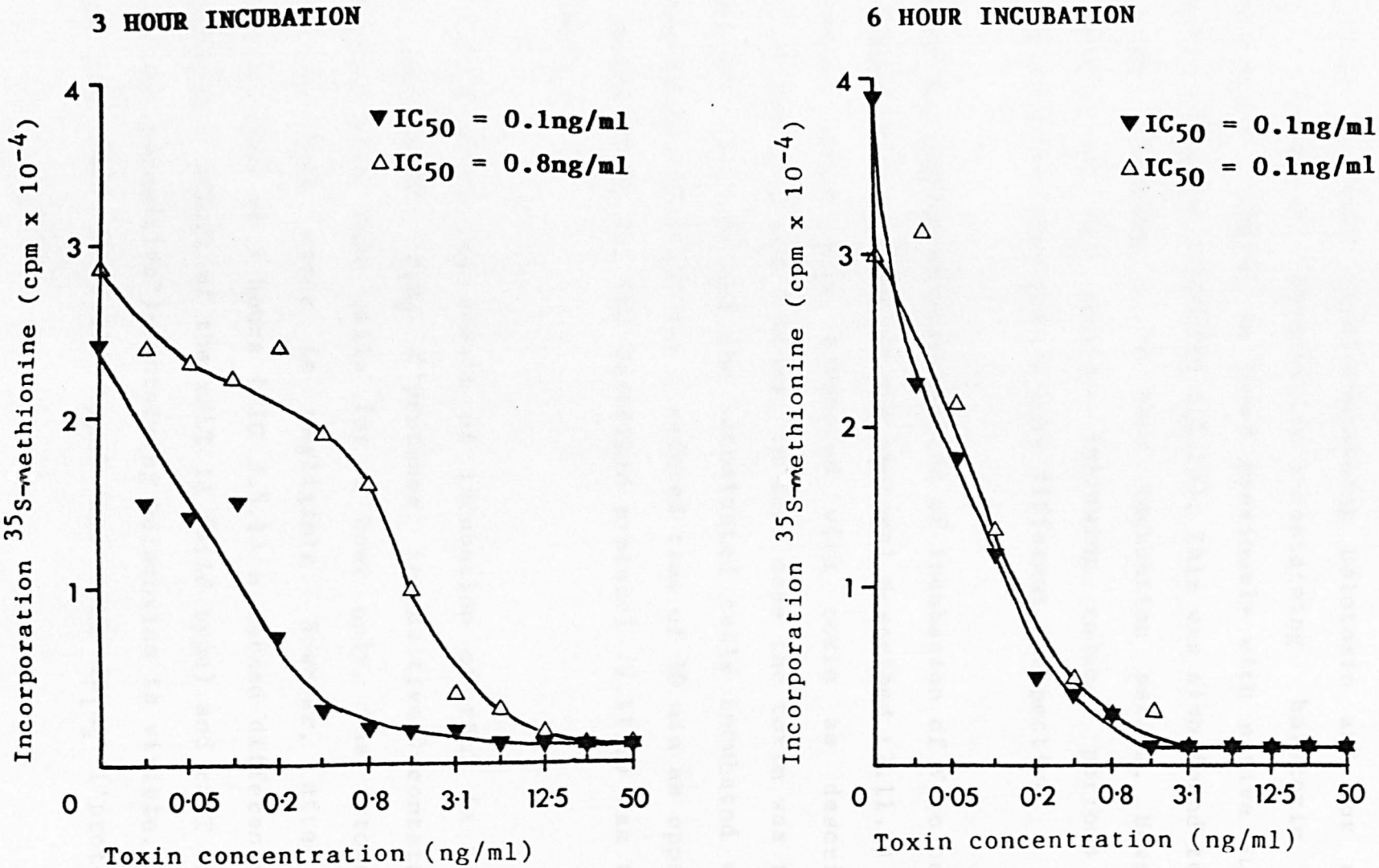
Results and Discussion

The cytotoxic nature of the reassociated, recombinant holotoxins was tested by incubation of Vero cells (2.11.2) with 0 - 50ng/ml for 1, 3, 6 and 24 hours. FIG 3.5.1 shows the results of 3 and 6 hour incubations on the protein synthesis of intoxicated Vero cells by measuring the incorporation of ^{35}S-methionine into cell protein.

FIG **3.5.1 Examination of the cytotoxic effect of reassociated, recombinant Shiga-like toxin I A subunit (rSLT IA)-containing holotoxins on Vero cells.**

0 - 50ng/ml of each recombinant Shiga-like toxin I A subunits (rSLT IAs) ie rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') was incubated with 1.2 - 1.5 x 10^4 cells in quadruplicate. Periods of cell intoxication of 3 and 6 hours were examined. The addition of 1uCi ^{35}S-methionine to each well allowed the cell viability to be determined via incorporation of isotope into cell protein. The 50% cytotoxic dose (IC_{50}) value was then determined for each of the rSLT IAs ie rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') on Vero cells at each incubation period.

This experiment was carried out twice.



▼= recombinant SLT IA (wild type)

△= recombinant SLT IA_1A_2 ('protease insensitive')

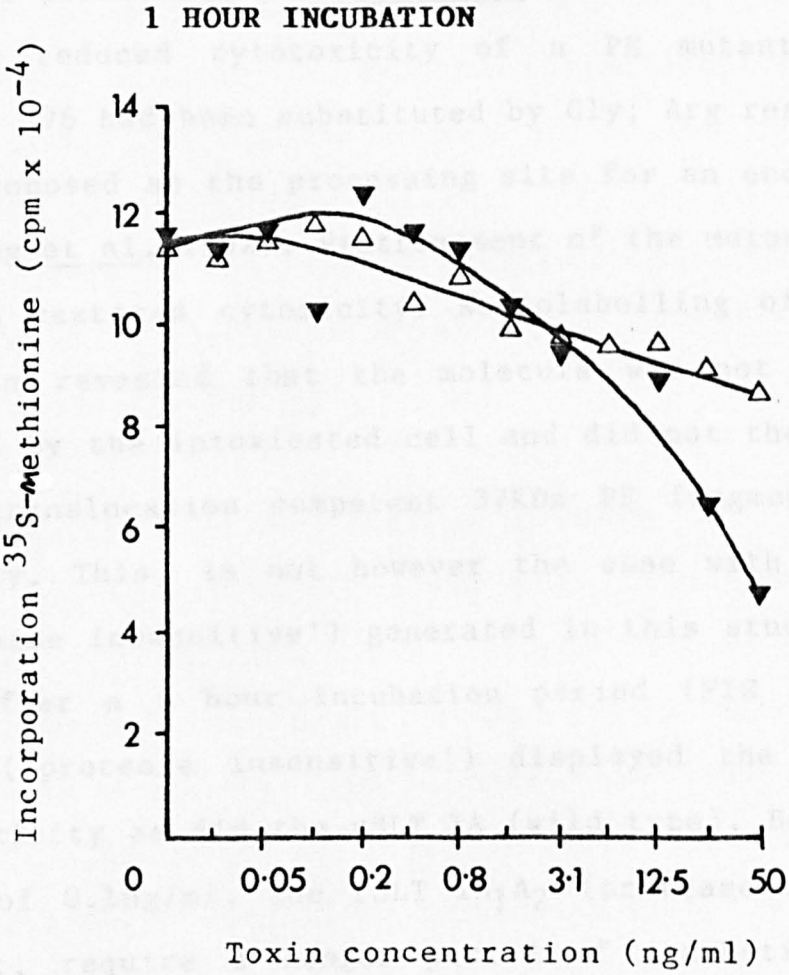
50% cytotoxic dose (IC_{50}) values obtained following an incubation period of 6 hours may be seen to be the same for both rSLT IA (wild type)-containing holotoxin and for rSLT IA_1A_2 ('protease insensitive')-containing holotoxin ie approximately 0.1ng/ml as found previously with native SLT I holotoxin (Chapter 2 SECTION 3.2.1)). This was also found to be the case following a 24 hour incubation period. However examination of the results following reduced periods of incubation with toxin give a very different perspective.

In order to examine shorter periods of incubation of Vero cells with toxin, modification of the protocol described (2.11.2) was necessary. Cells were incubated with toxin as described (2.11.2) for 1, 3 and 6 hours. In each case the toxin was then washed off (2.11.2) and the intoxicated cells incubated with ^{35}S-methionine (2.11.2) for a reduced time of 30 min as opposed to 2 hours (2.11.2). The described protocol (2.11.2) was then followed.

FIG 3.5.1A shows the result of incubation of rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive')-containing holotoxins with Vero cells for 1 hour only. The cytotoxic effect in both cases is negligible. However, after an incubation time of 3 hours (FIG 3.5.1) a marked difference in the cytotoxic effect of the rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive')-containing holotoxins is visible. IC_{50} values for rSLT IA (wild type) and rSLT IA_1A_2 ('protease

FIG **3.5.1A** **Examination of the cytotoxic effect of reassociated, recombinant Shiga-like toxin I A subunit (rSLT IA)-containing holotoxins on Vero cells.**

Concentrations of 0 - 50ng/ml of the recombinant Shiga-like toxin I A subunits (rSLT IAs) ie rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') were incubated in quadruplicate with 1.2 - 1.5 x 10^4 cells per well for a 1 hour period. The addition of 1uCi ^{35}S-methionine to each well following this incubation period allowed the extent of cell killing to be determined in each case. This was achieved by measuring the incorporation of isotope into cell protein.



▼= recombinant SLT IA (wild type)

△= recombinant SLT IA_1A_2 ('protease insensitive')

insensitive') are 0.1ng/ml and 0.8ng/ml respectively. A reduction in cytotoxicity of rSLT IA_1A_2 ('protease insensitive') may be attributed to a number of factors.

Jinno et al. (1989) and Ogata et al. (1990) in studies on the cellular processing of Pseudomonas exotoxin A (PE) reported the greatly reduced cytotoxicity of a PE mutant in which Arg residue 276 had been substituted by Gly; Arg residue 276 having been proposed as the processing site for an endosomal protease (Fryling et al. 1992). Pretreatment of the mutant molecule with trypsin restored cytoxicity. Radiolabelling of the mutant PE molecule revealed that the molecule was not proteolytically cleaved by the intoxicated cell and did not therefore generate the translocation competent 37KDa PE fragment required for toxicity. This, is not however the case with the rSLT IA_1A_2 ('protease insensitive') generated in this study. Results show that after a 6 hour incubation period (FIG 3.5.1) the rSLT IA_1A_2 ('protease insensitive') displayed the same degree of cytotoxicity as did the rSLT IA (wild type). Both gave an IC_{50} value of 0.1ng/ml. The rSLT IA_1A_2 (protease insensitive) did however, require a longer period of incubation in which to achieve this. Proteolytic cleavage would appear to be a rate limiting step in activity of the rSLT IA_1A_2 ('protease insensitive').

The observed lag may have arisen as a result of suboptimal processing of the mutant molecule at a location within the

molecule other than that at which proteolytic processing of SLT IA would normally take place. Such proteolytic cleavage may generate a fragment with reduced RNA N-glycosidase activity or one which is less efficiently translocated. It is speculated that some sort of nicking is required in this instance, in order to liberate A_1 from the A_2-B_5 moiety which might impose a considerable barrier to membrane translocation and ribosome inactivation. Nicking, as occurs in PE and diphtheria toxin (DT), can occur intracellularly (Fryling et al. 1992) and probably precedes the reductive cleavage step which might occur elsewhere in the cell to physically liberate A_1 for membrane translocation (Pelham et al. 1992).

It is possible however, that mutagenesis of Arg residues 248 and 251 may be responsible, via their interactions with other residues, for conformational changes at the proposed active site of the SLT IA subunit resulting in a reduction in RNA N-glycosidase activity. Thus it became important to examine the enzymatic activity of the isolated reduced rSLT IA_1A_2 ('protease insensitive') in comparison with that of the rSLT IA (wild type).

SECTION 3.5.2 **Kinetic examination of the depurinating activities of reduced holotoxins containing recombinant, wild type Shiga-like toxin I A subunit and recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit.**

Introduction

Reassociation of recombinant wild type Shiga-like toxin I A subunit (rSLT IA (wild type)) and recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit (rSLT IA_1A_2 ('protease insensitive')) with Shiga-like toxin I B subunit (SLT IB) and the subsequent purification of the resultant holotoxins (Chapter 4 SECTION 3.4.2) allowed their relative cytotoxicities on Vero cells to be determined (Chapter 5 SECTION 3.5.1). As discussed in the previous section (SECTION 3.5.1), examination of such cytotoxicities revealed a marked reduction in the cytotoxic effect of the rSLT IA_1A_2 ('protease insensitive')-containing holotoxin following incubation with toxin for a period of 3 hours prior to radiolabelling. After a 6 hour incubation period prior to radiolabeling however, the latter was seen to demonstrate the same cytotoxic effect as did the rSLT IA (wild type)-containing holotoxin.

As discussed in the previous section, there may be several explanations for this; one being the possibility that mutagenesis of the SLT I A subunit (required in order to

generate the rSLT IA_1A_2) ('protease insensitive') may, via conformational changes to the subunit, have reduced its RNA N-glycosidase activity. Thus it was decided that the enzymatic activities of rSLT IA (wild type) and the rSLT IA_1A_2 (protease insensitive) would be examined kinetically.

Once again, as described in Chapter 2 SECTION 3.2.4 in the kinetic comparison of SLT IA-catalysed depurination of salt washed wheatgerm ribosomes with that of ricin A subunit, the protocol was based on that of Osborn (1990).

Results and Discussion

The experimental conditions described in SECTION 3.2.4 of this study examining the kinetics of SLT IA-catalysed depurination of salt washed wheatgerm ribosomes, provided a basis from which suitable enzyme and substrate concentrations could be determined. Again salt washed wheatgerm ribosomes were chosen as the substrate for the kinetic investigations since they may be produced in large, relatively undegraded quantities.

Also in common with previous experiments in this study was the pretreatment of the reassociated recombinant holotoxins with 2-mercaptoethanol to a final concentration of 6mM to ensure complete reduction analogous to the situation in the cytosol of a cell.

In this case the concentration of each rSLT IA was determined by SDS polyacrylamide gel electrophoresis (SDS PAGE) (2.10.3) of 3 equal aliquots of each reassociated rSLT IA holotoxin and a range of known amounts of native SLT I holotoxin. The resultant gel was then silver stained (2.10.3(ii)) and scanned using a Molecular Dynamics computing densitometer and analysed using the volume integration package. The concentration of each rSLT IA ie rSLT IA (wild type) and rSLT IA_1A_2 (protease insensitive) was determined from a standard curve plotted from the data for the known amounts of native SLT IA (FIG 3.4.5, SECTION 3.4.2).

(i) Recombinant SLT IA (wild type)-catalysed depurination of wheatgerm ribosomes.

FIG 3.5.2 demonstrates the RNA N-glycosidase activity of rSLT IA (wild type) containing holotoxin on salt washed wheatgerm ribosomes (lane 2) compared with that of pure SLT I (lane 1).

1.27uM (160ug in a final reaction volume of 30ul) salt washed wheatgerm ribosomes was incubated with a range of concentrations of rSLT IA (wild type) 0.005uM - 0.01uM in order to determine a concentration which would give a linear rate of depurination with regard to time. Due to the relatively dilute nature of the reassociated rSLT IA (wild type) holotoxin and the constraints of the reaction volume, the range of

FIG 3.5.2 Investigation of the activity of reassociated, recombinant, wild type Shiga-like toxin I A subunit-containing holotoxin on wheatgerm ribosomes.

0.05ug of reassociated, recombinant, wild type Shiga-like toxin I A subunit (rSLT IA (wild type))-containing holotoxin previously shown to be cytotoxic to Vero cells (FIG 3.5.1) was incubated with 30ug wheatgerm ribosomes for 20min at 30°C (2.11.1(i)). This followed preincubation of the reassociated holotoxin with 2-mercaptoethanol to a final concentration of 6mM to ensure optimal activity and maximal reduction of any SLT I present. The resultant ribosomal RNA (rRNA) was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). Fractionation and visualisation of the resultant rRNA was then carried out (2.6.1).

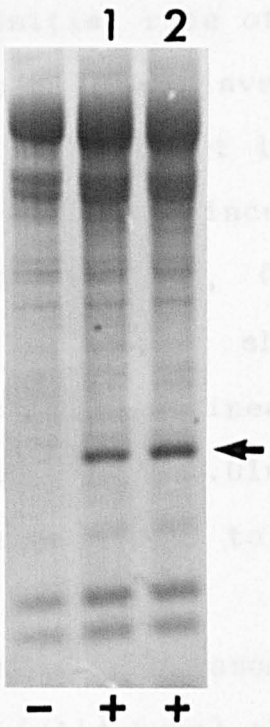
Lane 1 = positive control: wheatgerm rRNA following incubation with pure SLT I.

Lane 2 shows wheatgerm rRNA following incubation with reassociated rSLT IA (wild type) containing holotoxin.

+ indicates treatment of rRNA with aniline reagent.

- indicates that following incubation with toxin and extraction the rRNA was not subjected to aniline cleavage.

→ indicates the characteristic fragment released following aniline cleavage of toxin-modified rRNA.


1
2
–
+
+

concentrations of rSLT IA (wild type) which could be tested was limited. FIG 3.5.3 shows the percentage depurination against time when [rSLT IA (wild type)] = 0.01uM and the [ribosome] = 1.27uM. It would appear that at this enzyme concentration the rate of depurination remains approximately linear for 20min.

As stated earlier, the initial rate of depurination with regard to [rSLT IA (wild type)] was investigated. As above, the substrate concentration was kept at 1.27uM (160ug ribosomes in a final reaction volume 30ul) and incubated with concentrations rSLT IA (wild type) of 0.005uM, 0.0075uM and 0.01uM. The results represented in FIG 3.5.4 shows the initial rate of depurination to be approximately linear with regard to [rSLT IA (wild type)]. A concentration of 0.01uM rSLT IA (wild type) was used in the successive experiments to determine K_m and k_{cat}.

Concentrations of wheatgerm ribosomes incubated with this concentration of SLT IA (wild type) ranged from 0.32uM - 0.95uM (40 - 120ug ribosomes in a final reaction volume 30ul). In order to determine an average initial rate of depurination in each case, aliquots of the reaction mix were removed at 5min and 10min. The rRNA was then extracted, treated with aniline and the resultant rRNA resolved by denaturing gel electrophoresis. Following visualisation of the gel by ethidium bromide staining the gel was photographed and the negative photograph analysed using a Molecular Dynamics computing densitometer. As described in SECTION 3.2.4 **EQUATION 1** was

FIG 3.5.3 Time course for depurination of wheatgerm ribosomes by recombinant, wild type Shiga-like toxin I A subunit.

160ug of salt washed wheatgerm ribosomes (final concentration 1.27uM), were incubated at 30°C for 30min with recombinant, wild type Shiga-like toxin I A subunit (rSLT IA (wild type)) to a final concentration of 0.01uM (2.11.1(i)) in a total volume of 30ul. Aliquots of 3ul were removed at various time points and the ribosomal RNA (rRNA) extracted (2.4.1). This was then subjected to aniline cleavage (2.5.1) and fractionated by denaturing gel electrophoresis (2.6.1). Scanning of the rRNA using a Molecular Dynamics computing densitometer and the application of EQUATION 1 to the data generated, allowed the percentage depurination in each case to be determined.

This experiment was carried out four times. Results are representative of two successful analyses.

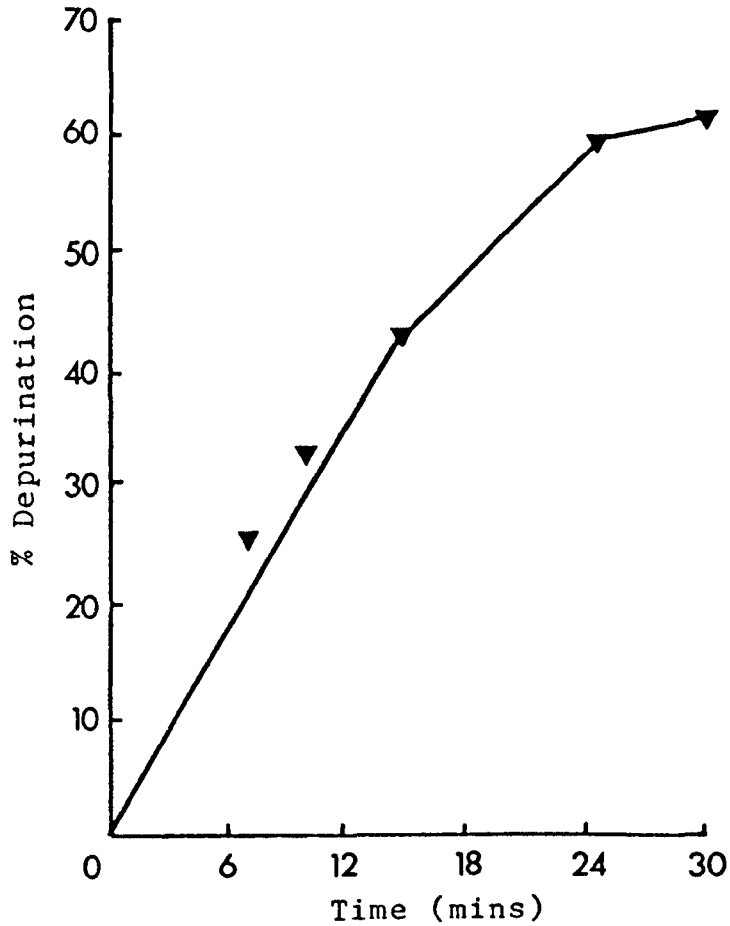
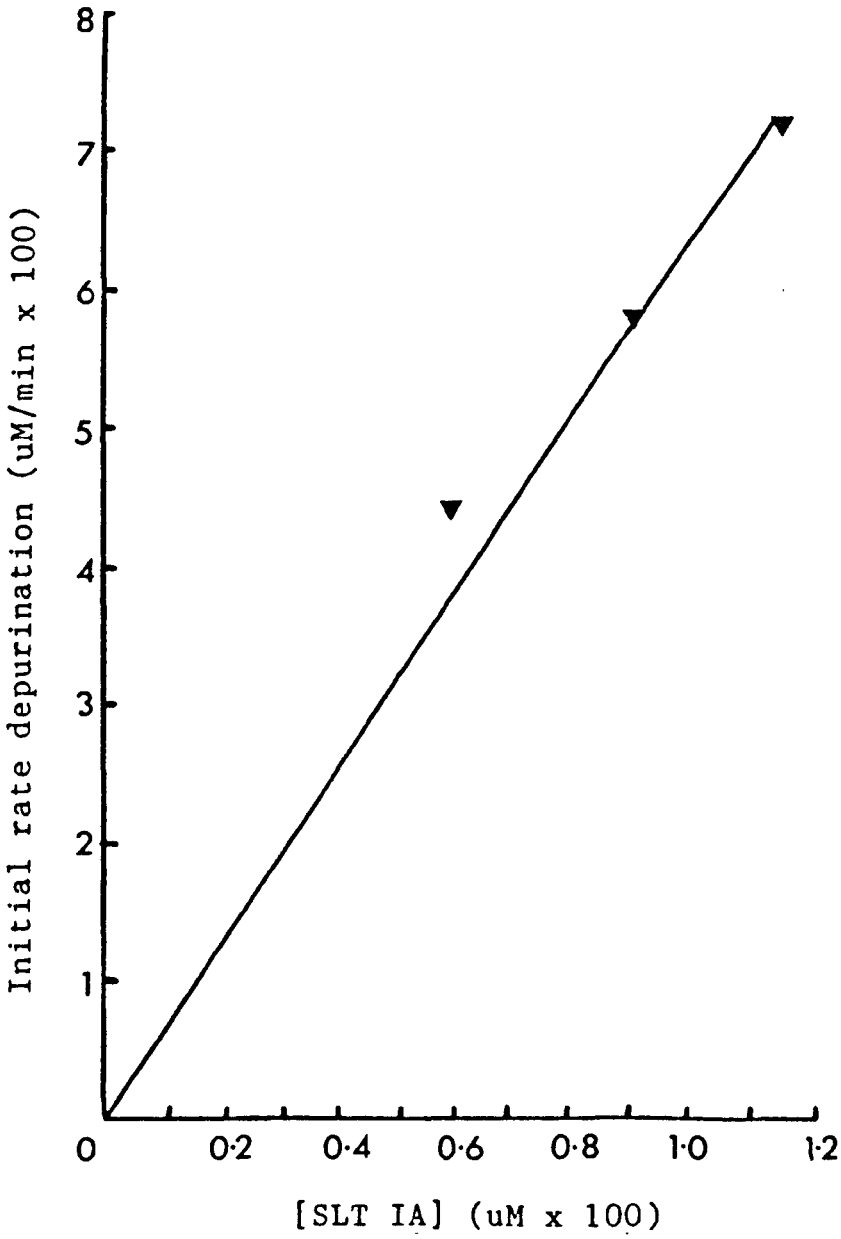

70
60
50
40
30
20
10
% Depurination
0
6
12
18
24
30
Time (mins)

FIG 3.5.4 Examination of the effect of recombinant, wild type Shiga-like toxin I A subunit concentration on the initial rate of depurination.

160ug of salt washed wheatgerm ribosomes (final concentration 1.27uM), were incubated with a range of concentrations of recombinant, wild type Shiga-like toxin I A subunit (rSLT IA (wild type)) from 0.005 - 0.01uM (2.11.1(i)). At 5 and 10min intervals 3ul aliquots were removed from the reaction (total volume 30ul) and the ribosomal RNA (rRNA) extracted (2.4.1). This was treated with aniline reagent (2.5.1) and fractionated by gel electrophoresis (2.6.1). Based on the assumption that the reaction rate remained linear over the first 10min of the reaction, the initial rate of depurination for each concentration of rSLT IA (wild type) was calculated from the percentage depurination at the two time points using EQUATION 1 and other equations as described in the text (SECTION 3.5.2(i)).

This experiment was carried out twice. Results seen here are representative of data generated in both cases.


Initial rate depurination (uM/min x 100)
8
7
6
5
4
3
2
1
0
0·2
0·4
0·6
0·8
1·0
1·2
[SLT IA] (uM x 100)

applied to the data obtained allowing determination of the initial rate of depurination for each ribosome concentration using the following:

$$\frac{\text{sum \% depurination (2 time points)}}{2} = \text{average \% depurination}$$

$$\downarrow$$

$$\frac{\text{average \% depurination}}{\text{time (mins)}} = \text{\% depurination / min}$$

$$\downarrow$$

$$\frac{\text{\% depurination x ribosome concentration (uM)}}{\text{100 x time (mins)}}$$

$$\downarrow$$

INITIAL RATE DEPURINATION

Again, as in Chapter 2 SECTION 3.2.4, the computer programme 'Enzkin 9' was employed in order to determine K_m and k_{cat} from the experimental data obtained. FIG 3.5.5 shows the resultant Lineweaver - Burke and Cornish - Bowden plots. The Cornish - Bowden method is considered statistically to be the best method of analysis. As previously discussed, the K_m and V_{max} generated by this method are median and not mean values with the result that an aberrant value will not distort the findings of the analysis.

FIG 3.5.5 Determination of K_m and V_{max} for the depurination of wheatgerm ribosomes by recombinant, wild type Shiga-like toxin I A subunit using Cornish - Bowden and Lineweaver - Burke plots.

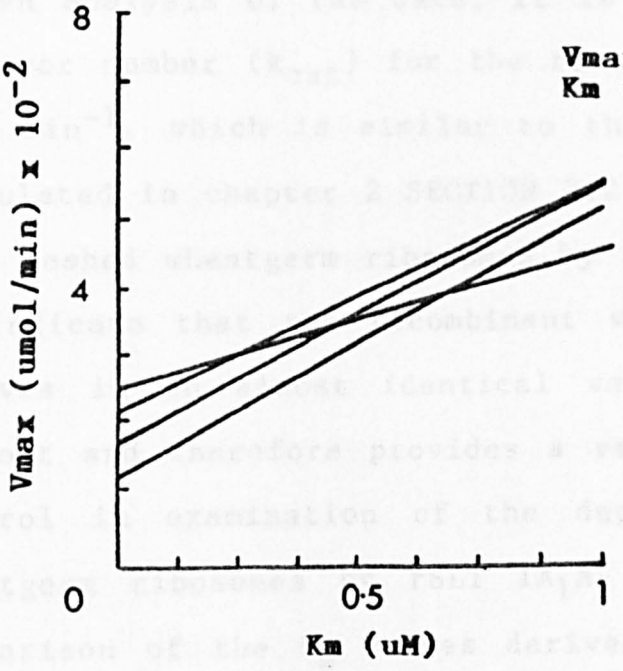
Salt washed wheatgerm ribosomes in concentrations ranging from 0.32 - 0.95uM (40 - 120ug ribosomes in a final reaction volume of 30ul) were incubated at 30°C, (2.11.1(i)) in a final volume of 30ul with 0.01uM reduced, recombinant, wild type Shiga-like toxin I A subunit (rSLT IA (wild type)). Aliquots, each of 14ug were removed from the reaction at 5 and 10min and the ribosomal RNA (rRNA) extracted (2.4.1). This was then subjected to aniline cleavage (2.5.1) and fractionated by denaturing gel electrophoresis (2.6.1). Scanning of the resultant rRNA gel allowed the percentage depurination for each time point to be calculated and thus the initial rate of depurination for each ribosome concentration to be determined using EQUATION 1 and other equations as detailed in SECTION 3.5.2 (i). The resultant data was applied to Lineweaver - Burke and Cornish - Bowden analysis in order to calculate values of K_m and V_{max}.

The resultant plots were generated from a single experiment.

1: Cornish - Bowden plot

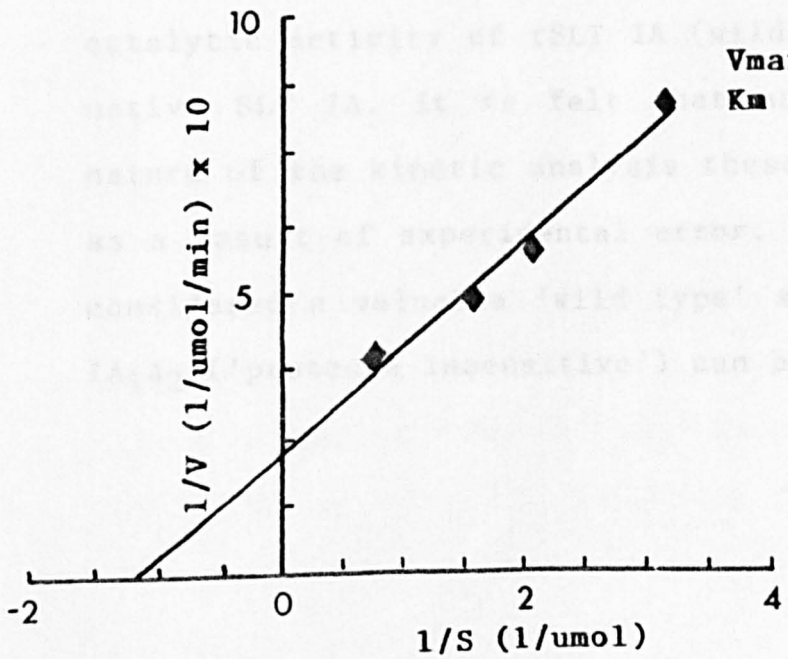
2: Lineweaver - Burke plot

1 CORNISH-BOWDEN PLOT



Vmax = 0.462 x 10^{-1} umol/min
Km = 0.844 uM

2 LINEWEAVER-BURKE PLOT



Vmax = 0.453 x 10^{-1} umol/min
Km = 0.829 uM

From the V_{max} value (0.462 x 10^{-1}umol/min) derived by Cornish-Bowden analysis of the data, it is possible to calculate the turnover number (k_{cat}) for the reaction. This is found to be 4.62 min^{-1}, which is similar to the k_{cat} value of 3.9 min^{-1} calculated in chapter 2 SECTION 3.2.4 for the depurination of salt washed wheatgerm ribosomes by native SLT IA. This serves to indicate that the recombinant wild type SLT I A subunit behaves in an almost identical way to the native SLT I A subunit and therefore provides a valuable 'wild type' SLT IA control in examination of the depurination of salt washed wheatgerm ribosomes by rSLT IA_1A_2 ('protease insensitive'). Comparison of the K_m values derived for rSLT IA (wild type) (0.844uM) and native SLT IA (0.978uM), indicating the binding affinity of the SLT IA for its substrate, also suggests that the rSLT IA is indeed acting as a 'wild type' SLT IA subunit. The maximum velocity (V_{max}) for rSLT IA catalysed depurination (0.462 x 10^{-1}umol/min) is however, lower than that of native SLT IA (0.128umol/min) indicating a slight reduction in catalytic activity of rSLT IA (wild type) compared to that of native SLT IA. It is felt that as a result of the complex nature of the kinetic analysis these slight differences may be as a result of experimental error. Thus, rSLT IA may still be considered a valuable 'wild type' standard against which rSLT IA_1A_2 ('protease insensitive') can be compared.

(ii) Recombinant SLT IA_1A_2 ('protease insensitive')-catalysed depurination of wheatgerm ribosomes.

FIG 3.5.6 demonstrates the RNA N-glycosidase activity of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin on salt washed wheatgerm ribosomes (lane 2) in comparison to that of pure SLT IA (lane 1).

Wheatgerm ribosomes at a concentration of 1.27uM (160ug in a final reaction volume of 30ul) were incubated with a range of concentrations of rSLT IA_1A_2 ('protease insensitive') from 0.005uM - 0.02uM in order to determine a concentration of enzyme which would give a linear rate of depurination with regard to time. FIG 3.5.7 represents the results obtained when [substrate] = 1.27uM and [enzyme] = 0.01uM. Depurination appears to proceed at an approximately linear rate for up to 20min.

The initial rate of depurination with regard to rSLT IA_1A_2 ('protease insensitive') concentration was again investigated. 1.27uM (160ug in a final reaction volume 30ul) wheatgerm ribosomes were incubated with 0.005uM, 0.01uM and 0.02uM rSLT IA_1A_2 ('protease insensitive'). FIG 3.5.8 shows the results obtained. Again as in (i) above it would appear that there exists a linear rate of depurination with regard to enzyme concentration. For the following experiments to determine K_m

FIG 3.5.6 Investigation of the activity of reassociated, recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit-containing holotoxin on wheatgerm ribosomes.

0.05ug of reassociated, recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit ('rSLT IA_1A_2 (protease insensitive')-containing holotoxin, previously shown to be cytotoxic to Vero cells (FIG 3.5.1) was incubated with 30ug wheatgerm ribosomes at 30°C for 20min (2.11.1(i)). In order to ensure its maximal reduction, prior to incubation with ribosomes, the reassociated holotoxin was pretreated with 2-mercaptoethanol to a final concentration of 6mM. Following incubation with the reassociated holotoxin, the wheatgerm ribosomal RNA (rRNA) was then extracted (2.4.1) and subjected to aniline cleavage (2.5.1). Fractionation and visualisation of the resultant rRNA was then carried out (2.6.1).

Lane 1 = positive control: wheatgerm rRNA following incubation with pure SLT I.
Lane 2 shows wheatgerm rRNA following incubation with reassociated rSLT IA_1A_2 ('protease insensitive')-containing holotoxin.
+ indicates treatment of rRNA with aniline reagent.
- indicates that following incubation with toxin and extraction the rRNA was not subjected to aniline cleavage.
➤ indicates the characteristic fragment released following aniline cleavage of toxin-modified rRNA.

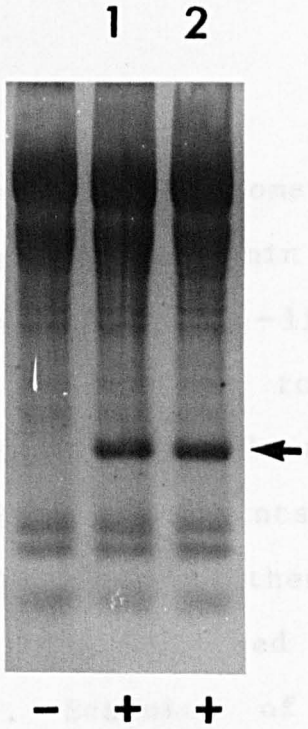

1
2
–
+
+

FIG 3.5.7 Time course for depurination of wheatgerm ribosomes by recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit.

160ug of salt washed wheatgerm ribosomes (final concentration 1.27uM), were incubated at 30°C for 30min with recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit (rSLT IA_1A_2 ('protease insensitive')) to a final concentration of 0.01uM (2.11.1(i)) in a total volume of 30ul. Aliquots of 3ul were removed at various time points and the ribosomal RNA (rRNA) extracted (2.4.1). This was then subjected to aniline cleavage (2.5.1) and fractionated by denaturing gel electrophoresis (2.6.1). Scanning of the rRNA using a Molecular Dynamics computing densitometer and the application of **EQUATION 1** to the data generated, allowed the percentage depurination in each case to be determined.

This experiment was carried out three times. Results are representative of two successful analyses.

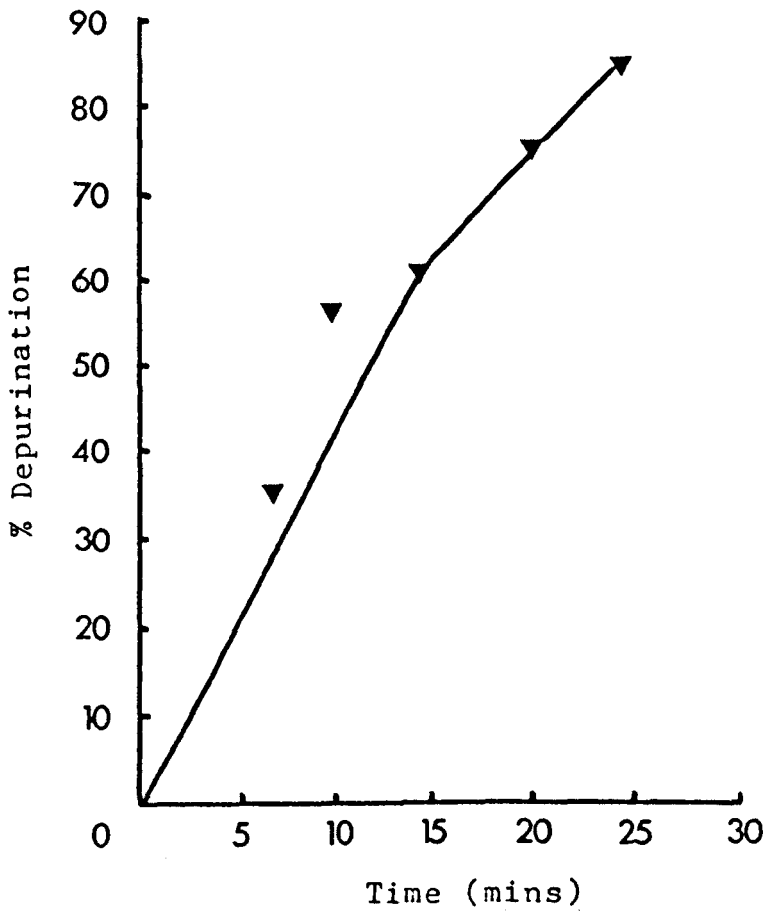
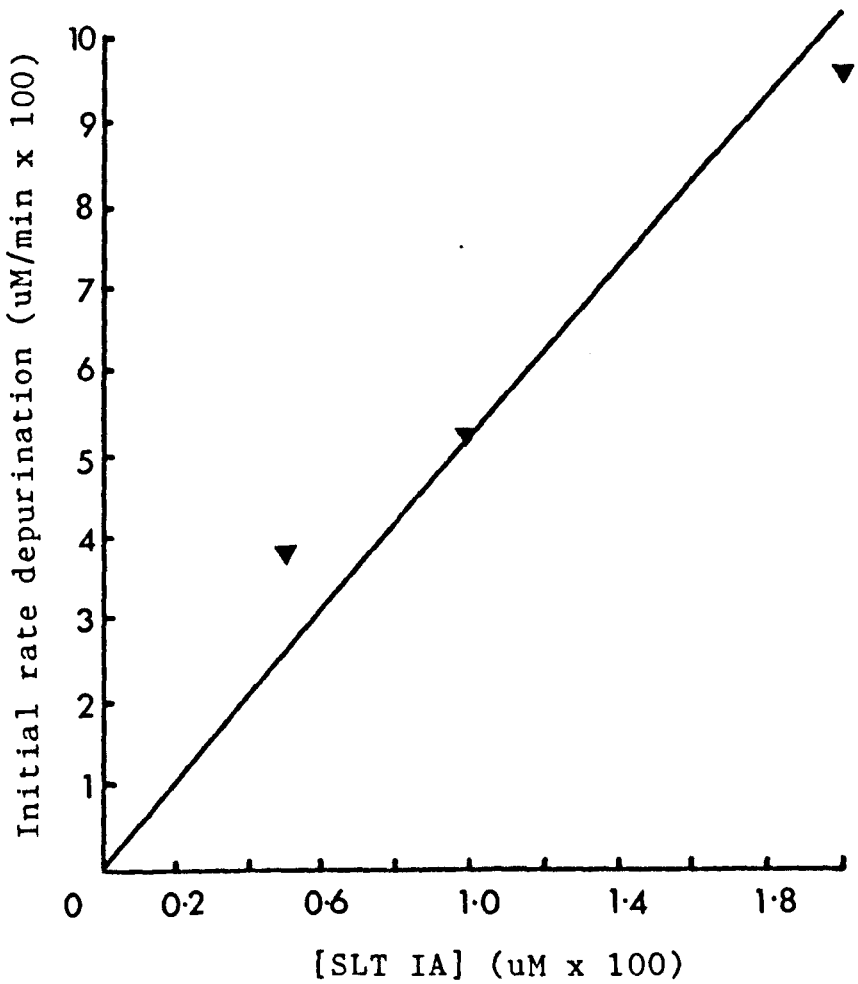

90
80
70
60
50
40
30
20
10
0
% Depurination
5
10
15
20
25
30
Time (mins)

FIG 3.5.8 Examination of the effect of recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit concentration on the initial rate of depurination.

160ug of salt washed wheatgerm ribosomes (final concentration 1.27uM) were incubated with a range of concentrations of recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit (rSLT IA_1A_2 ('protease insensitive')) from 0.005 - 0.02uM (2.11.1(i)). At 5 and 10min intervals 3ul aliquots were removed from the reaction (total volume 30ul) and the ribosomal RNA (rRNA) extracted (2.4.1). This was treated with aniline reagent (2.5.1) and fractionated by denaturing gel electrophoresis (2.6.1). Based on the assumption that the reaction rate remained linear over the first 10min of the reaction, the initial rate of depurination for each concentration of rSLT IA_1A_2 ('protease insensitive') was calculated from the percentage depurination at the two time points using EQUATION 1 and other equations descibed in the text (SECTION 3.5.2 (i).

This experiment was carried out twice. Results seen here are representative of data generated in both cases.


Initial rate depurination (uM/min x 100)
10
9
8
7
6
5
4
3
2
1
0
0·2
0·6
1·0
1·4
1·8
[SLT IA] (uM x 100)

and k_{cat} for the catalysed depurination of wheatgerm ribosomes a [rSLT IA_1A_2 ('protease insensitive')] of 0.01uM was used.

Concentrations of wheatgerm ribosomes incubated with 0.01uM rSLT IA_1A_2 ('protease insensitive') ranged from 0.16uM - 0.95uM (20 - 120ug in a final reaction volume 30ul). As previously described in this section (i), aliquots were removed from the reaction after 5min and 10min and the rRNA extracted, aniline treated and fractionated by denaturing gel, electrophoresis. The resultant gel was then analysed by scanning of a negative photograph of the ethidium bromide stained gel using a Molecular Dynamics computing densitometer and use of its volume integration programme. **EQUATION 1** and other equations (SECTION 3.5.1 (i)) were applied to the data obtained in order to generate an initial rate of depurination value for each ribosome concnetration. K_m and k_{cat} values for the reaction were derived as above by use of the 'Enzkin 9' computer programme.

FIG 3.5.9 shows both Lineweaver - Burke and Cornish - Bowden plots for the rSLT IA_1A_2 ('protease insensitive')-catalysed depurination of salt washed wheatgerm ribosomes.

Calculation of the turnover number (k_{cat}) for the reaction from the maximum velocity value (V_{max}) derived by Cornish-Bowden analysis of the data (0.233umol/min) gives a figure of $23.3min^{-1}$, which is approximately five times greater than the turnover

FIG 3.5.9 Determination of K_m and V_{max} for the depurination of wheatgerm ribosomes by recombinant, full length, 'protease insensitive' Shiga-like toxin I A subunit using Cornish - Bowden and Lineweaver - Burke plots.

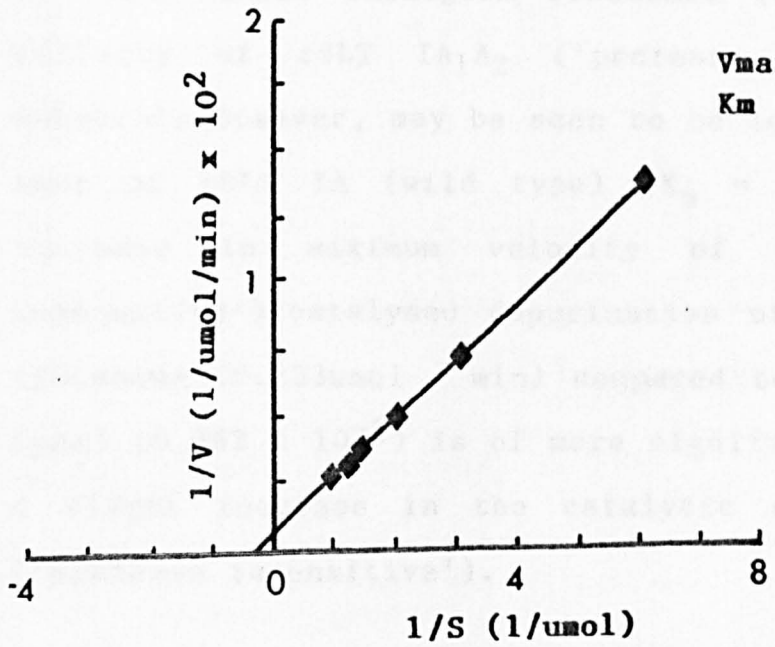
Salt washed wheatgerm ribosomes in concentrations ranging from 0.16 - 0.95uM (20 - 120ug ribosomes in afinal volume of 30ul) were incubated at 30°C, (2.11.1(i)) in a final volume of 30ul with 0.01uM recombinant, full length, 'protease insensitive' Shiga - like toxin I A subunit (rSLT IA_1A_2 ('protease insensitive')). Aliquots, each of 10ug were removed from the reaction at 5 and 10min and the ribosomal RNA (rRNA) extracted (2.4.1). This was then subjected to aniline cleavage (2.5.1) and fractionated by denaturing gel electrophoresis (2.6.1). Scanning of the resultant rRNA gel allowed the percentage depurination for each time point to be calculated and thus the initial rate of depurination for each ribosome concentration to be determined using EQUATION 1 and other equations as detailed in SECTION 3.5.2 (i). The resultant data was applied to Lineweaver - Burke and Cornish - Bowden analysis in order to calculate values of K_m and V_{max}.

The resultant plots were generated from a single experiment.

1: Lineweaver - Burke plot

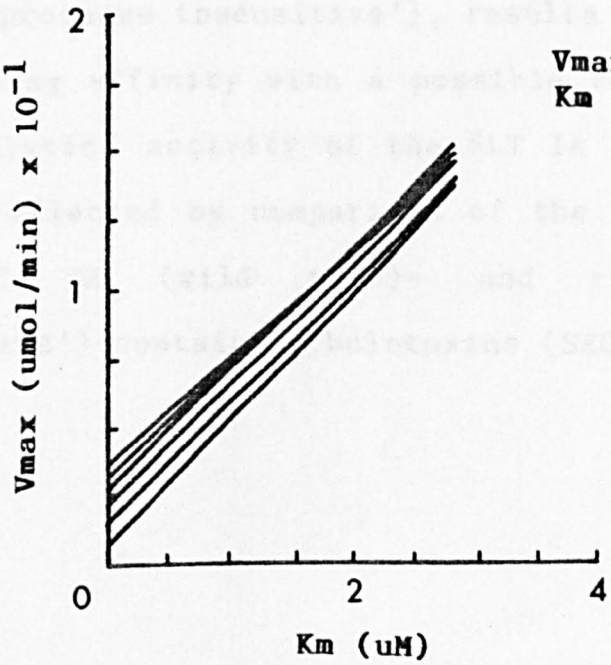
2: Cornish - Bowden plot

1 LINEWEAVER-BURKE PLOT



Vmax = 0.279 umol/min
Km = 5.89 uM

2 CORNISH-BOWDEN PLOT



Vmax = 0.223 umol/min
Km = 4.67 uM

number derived for rSLT IA (wild type)-catalysed depurination of salt washed wheatgerm ribosomes ($4.62min^{-1}$). The binding affinity of rSLT IA_1A_2 ('protease insensitive') for its substrate however, may be seen to be lower (K_m = 4.67uM), than that of rSLT IA (wild type) (K_m = 0.844uM). The observed increase in maximum velocity of rSLT IA_1A_2 ('protease insensitive')-catalysed depurination of salt washed wheatgerm ribosomes (0.233umol / min) compared to that of rSLT IA (wild type) (0.462×10^{-1}) is of more significance, since it implies a slight increase in the catalytic activity of rSLT IA_1A_2 ('protease insensitive').

FIG 3.5.10 summarises the K_m and V_{max} values for rSLT IA (wild type)- and rSLT IA_1A_2 ('protease insensitive')-catalysed depurination of salt washed wheatgerm ribosomes.

The experimental data obtained here would suggest that possession of the SLT IA_2 peptide, as demonstrated by rSLT IA_1A_2 ('protease insensitive'), results in a slight decrease in the binding affinity with a possible compensatory increase in the catalytic activity of the SLT IA subunit; a result which is not reflected by comparison of the relative cytotoxicities of rSLT IA (wild type)- and rSLT IA_1A_2 ('protease insensitive')-containing holotoxins (SECTION 3.5.1).

	Vmax (umol/min)	Km (uM)
rSLT IA (wild type)	0.453×10^{-1} 0.426×10^{-1}	0.829 (LB values) 0.844 (CB values)
rSLT IA_1A_2 ('protease insensitive')	0.279 0.233	5.890 (LB values) 4.670 (CB values)

LB = Lineweaver-Burke
CB = Cornish-Bowden

FIG 3.5.10 Summary of the V_{max} and K_m values derived for the depurination of salt washed wheatgerm ribosomes by recombinant Shiga-like toxin I A subunits.

Conclusion - Chapter 5

Results presented in SECTION 3.5.1 regarding the relative cytotoxicities of the rSLT IAs were found to be reproducible. As previously discussed, there are several explanations which may account for the observed reduction in cytotoxicity of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin compared to that of rSLT IA (wild type)-containing toxin. These include slower proteolytic processing of rSLT IA_1A_2 ('protease insensitive') at suboptimal cleavage sites, total lack of processing of the rSLT IA_1A_2 ('protease insensitive')-containing holotoxin and slower translocation of the molecule as a result of hindrance by the SLT IB subunit pentamer, which may remain associated with the unprocessed SLT IA subunit. Since the entire process of binding, uptake, processing and translocation of toxins is thought to be a highly complex procedure, it is not possible, by simply carrying out a cytotoxicity assay, to determine which of these explains the observed lag in activity of rSLT IA_1A_2 ('protease insensitive').

Detailed kinetic analysis of rSLT IA-catalysed depurination of salt washed wheatgerm ribosomes was carried out in order to eliminate reduced RNA N-glycosidase activity (caused by conformational changes induced during mutagenesis of SLT IA),

of rSLT IA_1A_2 ('protease insensitive') as an explanation for observed lag in cytotoxicity.

At first glance the kinetic analysis (SECTION 3.5.2), would appear to indicate a slight decrease in binding affinity of of rSLT IA_1A_2 ('protease insensitive') with a possible compensatory increase in catalytic activity compared to that of rSLT IA (wild type). This result is not however, reflected by the cytotoxicity assays carried out in SECTION 3.5.1. The latter results show a distinct lag in activity of the rSLT IA_1A_2 ('protease insensitive')-containing holotoxin in comparison with that of rSLT IA (wild type)-containing holotoxin. The cytotoxic effect of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin would appear from these results (SECTION 3.5.1) to be eight times less than that of rSLT IA (wild type)-containing holotoxin.

Due to limitations of time, low yields of reassociated toxin and the breakdown of the densitometer, final experiments used to generate data for Lineweaver - Burke and Cornish - Bowden analyses presented in SECTION 3.5.2 were carried out only twice in the case of rSLT IA_1A_2 ('protease insensitive') and three times in the case of rSLT IA (wild type). As a result of the complex nature of the kinetic analysis in each case only one gel suitable for analysis was generated. Thus the K_m and V_{max} values for each rSLT IA were derived from a single experiment in each case. In addition, due to the complicated

nature of the kinetic analysis, it is felt that the observed differences in catalytic activity of rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') may be within the realms of 'experimental error'. Thus, it is felt that in order to substantiate the initial findings of the kinetic analyses and to correlate these results with those regarding the relative cytotoxicities of the rSLT IA-containing holotoxins, kinetic analysis of the rSLT IAs needs to be repeated.

SECTION 4 FINAL DISCUSSION - Chapters 3, 4 and 5

Final discussion - chapters 3, 4 and 5.

The aim of work carried out as described in chapters 3, 4 and 5 has been to study the contribution of a non cleavable A_2 peptide in the biological activity (ie cytotoxicity and catalytic activity) of the Shiga-like toxin I A subunit (SLT I A). This has been facilitated by the construction of recombinant Shiga-like toxin I A subunits (rSLT IAs) (Chapter 3) and by successful reassociation of rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') with purified Shiga-like toxin I B subunits. This allowed purification of the rSLT IAs (Chapter 4 SECTION 3.4.2) and ultimately comparison of their relative biological activities.

Unfortunately, an inability to express the truncated rSLT IA_1 (A_1 alone) or A_2 alone (data not shown) at sufficiently high levels *in vivo*, meant that investigation into the role played by the A_2 peptide in associating with the Shiga-like toxin I B subunit pentamer was not possible in the time available.

The relative cytotoxicities on Vero cells of rSLT IA (wild type) and rSLT IA_1A_2-containing holotoxins were examined (Chapter 5 SECTION 3.5.1). Following incubation periods of greater than 6 hours the cytotoxic effect of the wild type and the 'protease insensitive'-containing holotoxins were seen to be identical; revealing a 50% cytotoxic dose (IC_{50}) value of 0.1ng/ml in each case. This value agreed with that determined

for the cytotoxic effect of native SLT I on Vero cells (Chapter 2 SECTION 3.2.1) and also the values derived by Kongmuang et al. (1988) for pre-nicked (proteolytically cleaved) and unnicked SLT I. In both, the study of the cytotoxic nature of native SLT I (the present study) and that carried out by Kongmuang et al. (1988), Vero cells had been incubated with toxin for a period of 24 hours. This led to the conclusion by Kongmuang et al. (1988) that there was no difference in the cytotoxicities of nicked and unnicked SLT I as had also been the conclusion of Olsnes et al. (1981) with regard to nicked and unnicked Shiga toxin (ST). Again cells, in this case HeLa S_3, had been incubated with toxin for an 'overnight' period; ie a period in excess of 6 hours.

A similar scenario was reported by Tsuji et al. (1984) in studies to compare the effect of nicked (proteolytically cleaved) and unnicked Escherichia coli heat labile toxin (LT) on chinese hamster ovary cells (CHO). Following an incubation period of 18 hours, examination of the CHO cells revealed that the activity of the unnnicked LT was almost identical to that of the nicked form. However, shorter incubation periods revealed a distinct lag in appearance of biological activity of the unnicked form of the toxin.

The effect of shorter incubation times on the biological activity of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin was therefore examined. The cytotoxic effect

following 1 and 3 hour incubation periods was examined (FIGS 3.5.1A and 3.5.1 respectively). After a period of only 1 hour the cytotoxic effect of both rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive')-containing holotoxins was seen to be negligible. However, after 3 hours, a distinct lag was evident in the case of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin. IC_{50} values for rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') were determined as being 0.1ng/ml and 0.8ng/ml respectively. Although an eight fold difference may not at first glance appear to be significant, this reflects a decrease in cytotoxicity of almost an order of magnitude below that of the extreme potency displayed by the wild type SLT IA. A similar difference in the potency of ricin A subunit, described as significant, was observed following the addition of -KDEL to its C terminus (Wales et al. 1992). In this case there was a seven fold increase in potency of the molecule.

Several suggestions were put forward at this stage to explain the observed lag in the biological activity of the protease insensitive ie unnicked rSLT IA_1A_2, with regard to previous studies of other bacterial toxins, such as Pseudomonas exotoxin A (PE) and LT, also with a prerequisite for proteolytic processing.

It was suggested that proteolytic processing of the rSLT IA_1A_2 ('protease insensitive') may be the rate limiting step, resulting in the observable lag in cytotoxic potency.

Ultimately in the case of rSLT IA_1A_2 ('protease insensitive'), proteolytic processing may occur not at the usual site for proteolytic cleavage, generating the expected A_1 and A_2 peptides of 27KDa and 5KDa respectively, but at a site less accessible or favourable for cleavage. Such suboptimal processing may in turn result in production of a fragment of reduced translocation-competence and / or with lower RNA N-glycosidase activity.

The possibility of proteolytic cleavage of rSLT IA_1A_2 ('protease insensitive') at a site less susceptible to cleavage may be substantiated by results generated earlier in this study (Chapter 3 SECTION 3.3.1).

In vitro examination of the sensitivity of the rSLT IAs constructed revealed the full length, 'protease insensitive' rSLT IA_1A_2 to be less sensitive to proteolytic cleavage than the wild type rSLT IA, as required. However, at higher concentrations of trypsin (40 and 50ug/ml) a reduction in size of the rSLT IA_1A_2 ('protease insensitive') of 2 - 3KDa was evident. Thus, it may be the case that this situation is reflected in vivo. However, great care must be taken in extrapolating data from in vitro studies with trypsin and the proposed intracellular processing events. For a start, trypsin is not the likely intracellular processing enzyme, although a serine protease of some description does appear to be involved. Work by Fryling et al. (1992) on the intracellular processing

of PE suggests that the enzyme responsible for such processing is endosomal (in at least one cell line) with a low pH requirement. However, until the compartment-specific protease responsible is known, it is difficult to know how similar the protease behaves compared with trypsin. The lack of an optimal, surface-exposed arginine residue may be compensated intracellularly by cleavage elsewhere. The intracellular protease may however, select quite a different site to that selected by trypsin in vitro. If proteolytic processing of rSLT IA_1A_2 ('protease insensitive') occurs at a site other than that at which cleavage would normally occur, a fragment with reduced enzymatic activity may be generated. Alternatively, such a fragment may translocate less efficiently. Potential sites for cleavage by a serine protease, which would result in the observed size reduction of 2 - 3KDa in the size of rSLT IA_1A_2 ('protease insensitive') (32KDa) seen in vitro at high trypsin concentrations (FIG 3.3.15), occur after arginine residues 266 and 268 of the Shiga like toxin I A subunit.

As mentioned above, a reduced translocation efficiency may well explain the observed lag. Such reduction in translocation efficiency could be attributable to either total insusceptibility of the rSLT IA_1A_2 ('protease insensitive') to proteolytic processing resulting in an attempt to translocate a larger sized A fragment than usual, or the production of a fragment not ideally suited for the membrane transfer step. We have no idea which of the options is likely. When considering

the translocation step it is worth remembering that the SLT IB subunit pentamer is likely to remain associated with A_2 intracellularly (evidence for an exclusive A_2-B_5 association comes from studies with comparable toxins of this design - cholera toxin (CT) Jobling and Holmes (1992) and LT (Sixma et al. 1991, Streatfield et al. 1992). Membrane translocation of a holotoxin would be extremely unlikely. It is known that alteration of the PE cell binding domain by mutagenesis of the translocation domain-arginine residues, produces a toxin of greatly reduced potency (Jinno et al. 1989). Likewise, ricin A subunit (RA) fused directly with staphylococcal protein A (PA) has no cytotoxic effect even though enzymic activity is evident (O'Hare et al. 1990). In the latter case however, when a cleavable linker (diphtheria toxin (DT) loop) is included between RA and PA, the fusion protein is cytotoxic (O'Hare et al. 1990). This has been interpreted as meaning a block in translocation in the 'protease insensitive' fusion rather than in cell binding, endocytosis or catalytic activity. With this in mind, it would seem that attachment of A_2-B_5 to A_1 would permanently render SLT I a poor cytotoxin. In contrast, the time lag in cytotoxicity suggests a rate limiting step, such as a slower rate of translocation - perhaps of an imperfectly processed A_1 subunit, which nevertheless lacks most of A_2 and all of the B_5 pentamer.

Earlier evidence (Olsnes et al. 1981, Kongmuang et al. 1988)of pre-nicked versus unnicked ST / SLT I being of similar potency

is now thrown into doubt. In both cases these earlier experiments involved long periods of incubation with toxin. Under these circumstances any rate limiting step may have been overlooked. Results in the present study demonstrated that after incubation periods of 6 hours plus, both rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') appeared to have similar cytotoxicities. In addition, we know from RA-PA fusions that pre-nicking with trypsin is not necessary (O'Hare et al. 1990). A so called unnicked toxin can apparently become nicked intracellularly. Thus mutagenesis of the processing site(s) is the only valid approach in making comparisons.

It remains possible of course that the observed difference in potency can be explained by a simple reduction in the catalytic activity of rSLT IA_1A_2 ('protease insensitive') as a direct consequence of conformational changes to the A subunit as a result of the amino acid substitutions required to generate rSLT IA_1A_2 ('protease insensitive'). For this reason, kinetic studies of rSLT IA (wild type)- and rSLT IA_1A_2 ('protease insensitive')-catalysed depurination of wheatgerm ribosomes were undertaken.

rSLT IA (wild type)-catalysed depurination of wheatgerm ribosomes may be seen to be almost identical to that of native SLT IA on salt washed wheatgerm ribosomes (Chapter 2 SECTION 3.2.4) ie K_m and k_{cat} values are almost identical (K_m rSLT IA (wild type) = 0.844uM, K_m native SLT IA = 0.978uM and k_{cat} rSLT

IA (wild type) = 4.53min^{-1}, k_{cat} native SLT IA = 3.9min^{-1}) thus providing a valuable 'wild type' against which rSLT IA_1A_2 ('protease insensitive') values may be compared.

Experimental data obtained from studies to examine rSLT IA_1A_2 ('protease insensitive')-catalysed depurination of salt washed wheatgerm ribosomes (Chapter 5 SECTION 3.5.2) demonstrates a decrease in binding affinity (K_m = 4.67uM) and an apparent increase in turn over number (k_{cat} = 22.3min^{-1}) compared to those values derived for rSLT IA (wild type). The observed activity of rSLT IA_1A_2 ('protease insensitive') does not therefore, correlate with the different cytotoxicitiesof rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive')-containing holotoxins.

Kinetic analysis of rSLT IA_1A_2 ('protease insensitive')-catalysed depurination of salt washed wheatgerm ribosomes would appear to indicate a slight decrease in binding affinity with a possible compensatory increase in the catalytic activity compared to that of rSLT IA (wild type). As previously discussed, this result may not be reflected by the cytotoxicity assays carried out in SECTION 3.5.1 which indicate the potency of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin to be approximately eight times less than that of rSLT IA (wild type)-containing holotoxin.

Data regarding the cytotoxicity of rSLT IA_1A_2 ('protease insensitive') was found to be reproducible. However, logistics prevented substantiation of kinetic data regarding the catalytic activities of rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive'). The low yield of reassociated toxins in conjunction with the complex nature of the kinetic analysis limited the number of times that each part of the analysis could be carried out, as did the limited time available in the final stage of this study. Additional to these limitations was the breakdown of the Molecular Dynamics computing densitometer. Ultimately, data for Lineweaver - Burke and Cornish - Bowden analyses presented in SECTION 3.5.2 were derived from a single analysis in each case. Due to the complicated nature of the kinetic analysis, it is felt that the observed differences in catalytic activity of rSLT IA (wild type) and rSLT IA_1A_2 ('protease insensitive') may be within the realms of 'experimental error'. For these reasons it may be that the observed differences in catalytic activity of rSLT IA_1A_2 ('protease insensitive') and rSLT IA (wild type) are negligible. If this was shown to be the case, then the observed reduction in potency of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin (SECTION 3.5.1) could be attributed to a step in the complex process of intracellular processing and translocation.

It is felt therefore, that in order to draw comparisons between the findings of the kinetic analysis results regarding the

relative cytotoxicities of the rSLT IA-containing holotoxins, kinetic analysis of the rSLT IAs should be repeated.

In conclusion, it would appear from the experimental data that the the observed lag in cytotoxicity of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin may be attributable to a reduction in its susceptibility to intracellular proteolytic processing which may in turn affect the membrane translocation step.

In order to further investigate the proposed suboptimal processing of rSLT IA_1A_2 ('protease insensitive') it would be necessary to adopt an approach similar to that of Ogata et al. (1989) in their investigation into the intracellular processing and fate of PE. Ogata et al. (1989) followed the processing of ^3H labelled PE over a 4 hour incubation period with Swiss 3T3 cells. By immunoprecipitation of cell associated PE and SDS polyacrylamide gel electrophoresis (SDS PAGE) the presence and intensity of the major processed PE fragments was assessed at each time point. Radiolabelling of the rSLT IAs would allow a the time course for processing of SLT IA to be examined. This would also provide valuable information regarding the apparent lag in potency of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin (Chapter 5 SECTION 3.5.1). This would indicate whether or not this molecule is actually processed by the cell and if this is the case, over what period of time processing occurs in comparison to that of rSLT IA (wild type).

The intracellular location of the processing of PE was also examined by Ogata et al. (1989). Percoll gradient fractionation of L929 cells incubated with ^{25}I labelled PE for 15min allowed the location of processed PE fragments. These were found in endosomal but not lysosomal compartments. This approach could also be applied to determine the intracellular location of each of the rSLT IAs following incubation periods of up to and including three hours. In this way any differences in location of the rSLT IAs could be identified. This may provide information to explain the observed lag in activity of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin compared with that of rSLT IA (wild type).

Finally, Ogata et al. (1989) examined which processed form of PE reached the cytosol ie which fragment was translocated to the cytosol. Following incubation with radiolabelled PE, cells were homogenised and the cytosolic and membrane fractions separated by centrifugation. Immunoprecipitation and resolution by SDS PAGE under reducing conditions revealed that the major processed fragment of PE to be found in the cytosol was a 37KDa fragment indicating that this is the translocated PE fragment. Again, this approach could be used to identify the size of fragment which finally reaches the cytosol with regard to rSLT IA_1A_2 ('protease insensitive'). This could then be compared with the processed rSLT IA (wild type) fragment found in the cytosol indicating whether or not the final processed fragments are the same size or whether suboptimal processing of rSLT

IA_1A_2 ('protease insensitive') has led to a larger / smaller fragment than that of rSLT IA (wild type). This procedure would also allow purification of the major processed rSLT IA fragments to be found in the cytosol.

It may be seen therefore, that further experimentation similar to that carried out by Ogata et al. (1989) on the processing of PE, would allow the intracellular processing of the rSLT IAs to be examined in much greater detail. It is hoped that this would then provide some information to enable us to explain the results (SECTION 3.5.1) demonstrating the reduced potency of rSLT IA_1A_2 ('protease insensitive')-containing holotoxin in comparison to that of rSLT IA (wild type).

As a result of an inability to express the truncated rSLT IA_1 (A_1 alone) in the given time for this study, the exact contribution of the A_2 peptide towards catalytic activity of SLT IA remains to be examined, as does its role (if any) in interaction with the SLT IB subunit pentamer.

SECTION 5 **APPENDICES**

Appendix 1

Details of *Escherichia coli* and *Saccharomyces cerevisiae* strains used in the present study.

E.coli strains used:

JM101: *E.coli* *supE*, *thi*, Δ(*lac-proAB*, [F', *traD*36, *proAB*, *lacI*^{q}, *lacZ*ΔM15]

JM105: *E.coli* *thi*, *rpsL*(str^{r}), *endA*, *sbcB*15, *supE*, *hdsR*4, Δ(*lac-proAB*), [F', *traD*36, *proAB*, *lacI*^{q}, *lacZ*ΔM15]

***E.coli* serotype 026:H11 strain E3787** (SLT I producer only) (National Type Culture Collection)

S.cerevisiae strain used:

ABYS 1: *S.cerevisiae* *a*, *pra*1, *prb*1, *prc*1, *cps*1, *ade*

Appendix 2

Kinetic equations

The Lineweaver - Burke plot (ii) can be derived from the Michaelis - Menton equation (i).

(i) Michaelis - Menton equation: $v = \frac{Vmax \times [S]}{Km + [S]}$

(ii) Lineweaver - Burke: $1/v = (K_m/V_{max}) \times 1/[S] + 1/V_{max}$

The values for K_m and V_{max} are calculated from a plot of 1/v (y-axis) versus 1/[S] (x-axis), where the intercept on the y-axis = $1/V_{max}$ and the intercept on the x-axis = $-1/K_m$.

Appendix 2 continued

In order to obtain a Cornish - Bowden plot (iii), the Michaelis - Menton equation (i) may be rearranged.

(iii) Cornish - Bowden: $\mathbf{V_{max}/v - K_m/s = 1}$

This has the form of y/b - x/a = 1 which is the equation of a straight line in xy space, where b is the intercept of the line on the y-axis and a the intercept of the line on the x-axis. Any single observation of v and [S] are represented by a line passing through v on the V_{max} axis (y-axis) and -[S] on the K_m axis (x-axis). If such a line is drawn, then the common intercept has the coordinates K_m and V_{max}. The observations are subject to error and a cluster of intersections are obtained. The K_m and V_{max} determined are ranked in order and a median value of K_m and V_{max} taken for the reaction.

Appendix 3

Ranked values of K_m and V_{max} as determined by Cornish - Bowden analysis for the depurination of salt washed wheatgerm ribosomes by:

(i) Native SLT IA (Chapter 2 SECTION 3.2.4)

K_m (uM)	V_{max} (umol/min)
15.900	1.030
9.540	0.635
6.580	0.460
3.590	0.283
2.750	0.226
1.980	0.183
1.910	0.167
1.600	0.148
1.360	0.141
1.190	0.128
➡ 0.978	➡ 0.128
0.974	0.118
0.761	0.107
0.755	9.950×10^{-2}
0.679	9.130×10^{-2}
0.507	7.560×10^{-2}
0.384	6.750×10^{-2}
0.290	6.330×10^{-2}
0.194	6.210×10^{-2}
0.120	5.290×10^{-2}
-4.880×10^{-2}	4.580×10^{-2}

➡ indicates median values obtained.

(ii) Recombinant SLT IA (wild type) (Chapter 5 SECTION 3.5.2(i))

K_m (uM)	V_{max} (umol/min)
3.280	0.137
1.710	7.73×10^{-2}
→ 0.989	→ 5.36×10^{-2}
0.698	3.87×10^{-2}
0.451	3.39×10^{-2}
0.308	3.11×10^{-2}

(iii) Recombinant SLT IA ('protease insensitive') (Chapter 5 SECTION 3.5.2(ii)

K_m (uM)	V_{max} (umol/min)
132.00	5.760
29.20	1.290
16.30	0.728
10.40	0.477
7.07	0.327
6.33	0.291
5.71	0.266
→ 4.67	→ 0.223
4.51	0.211
4.46	0.209
4.44	0.208
3.39	0.168
1.96	0.112
1.95	0.112
1.94	0.112

→ indicates median values obtained.

SECTION 6 REFERENCES

Achstetter T, Emter O, Ehmann C, Wolf DH, (1984) **Proteolysis in eukaryotic cells.** J Biol Chem 259, 13334 - 13343.

Ashkenazi S, Cleary KR, Pickering LK, Murray BE, Cleary TG, (1990) **the association of Shiga toxin and other cytotoxins with the neurological manifestations of shigellosis.** J Infect Dis 161, 961 - 965.

Ashkenazi A, Presta LG, Marsters SA, Camerato TR, Rosenthal KA, Fendly BM, Capon DJ, (1990) **Mapping of the CD4 binding site for human immunodeficiency virus by alanine-scanning mutagenesis.** Proc Natl Acad Sci USA 87, 7150 - 7154.

Booth BA, Boesman-Finkelstein M, Finkelstein RA, (1984) **Vibrio cholerae haemagglutinin/protease nicks cholera enterotoxin.** Infect and Immun 45, 558 - 560.

Brown JE, Rothman SW, Doctor BP, (1980) **Inhibition of protein synthesis in intact HeLa cells by Shigella dysenteriae 1 toxin.** Infect and Immun 29, 98 - 107.

Brown JE, Ussery MA, Leppla SH, Rothman SW, (1980) **Inhibition of protein synthesis by Shiga toxin.** FEBS Letts 117, 84 - 91.

Brown JE, Griffin DE, Rothman SW, Doctor BP, (1982) **Purification and biological characterisation of Shiga toxin from Shigella dysenteriae 1.** Infect and Immun 36, 996 -1005.

Brunton JL, (1990) **The Shiga Toxin Family. Molecular nature and possible role in disease.** The Bacteria, vol XI, Chpt 18, 377 - , Academic Press Inc.

Burns DL, (1988) **Subunit structure and enzymatic activity of pertussis toxin.** Micro Sci 5, 285 - 287.

Calderwood SB, Auclair F, Donohue-Rolfe A, Keusch GT, Mekalanos JJ, (1987) **Nucleotide sequence of Shiga-like toxin I genes of Escherichia coli.** Proc Natl Acad Sci USA 84, 4364 - 4368.

Calderwood SB and Mekalanos JJ, (1987) **Iron regulation of Shiga-like toxin I expression is mediated by fur locus.** J Bact 169, 4759 -4764.

Calderwood SB, Acheson DWK, Goldberg MB, Boyko SA, Donohue-Rolfe A, (1990) **A system for the production and rapid purification of large amounts of the Shiga toxin / Shiga-like toxin I B subunit.** Infect and Immun 58, 2977 - 2982.

Capriolini A, Luzzi I, Rosmini F, Pasquini P, Cirrincione R, Gianviti MC, Matteucci MC, Rizzoni G, (1992) **Hemolytic-Uremic Syndrome and verocytotoxin-producing Escherichia coli infection in Italy.** J Infect Dis 166, 154 - 158.

Carroll SF and Collier RJ, (1987) **Active site of Pseudomonas aeruginosa enterotoxin A.** J Biol Chem 262, 8707 - 8711.

Carroll SF, Barbieri JT, Collier RJ, (1988) **Diphtheria toxin: purification and properties.** Methods in Enzymol 165, 68 - 76.

Chaddock JA (1991) **Structure-function relationships of ricin A-chain.** PhD thesis (University of Warwick)

Choe S, Bennett MJ, Fujii G, Curmi PMG, Kantarjieff KA, Collier RJ, Eisenberg D, (1992) **The crystal structure of diphtheria toxin.** Nature 357, 216 -222.

Chung DW and Collier RJ, (1977) **Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of Pseudomonas aeruginosa.** Infect and Immun 16, 832 - 841.

Cohen A, Hannigan GE, Williams BRG, Lingwood CA, (1987) **Roles of the globotriosyl- and galabiosylceramide in verotoxin binding and high affinity interferon receptor.** J Biol Chem 262, 17088 - 17091.

Collier RJ, (1975) **Diphtheria toxin: Mode of action and structure.** Bact Rev 39, 54 - 85.

Cordovez A, Prado V, Maggi L, Cordero J, Martinez J, Misraji A, Rios R, Soza G, Ojeda A, Levine MM, (1992) **Enterohemorrhagic Escherichia coli associated with Hemolytic-Uremic Syndrome in Chiulean children.** J Clin Micro 30, 2153 - 2157.

Cortina G and Barbieri JT, (1989) **Role of tryptophan 26 in the NAD glycohydrolase reaction of the S-1 subunit of pertussis toxin.**J Biol Chem 264, 17322 - 17328.

DeGrandis S, Ginsberg J, Toone M, Climie S, Friesen J, Brunton J, (1987) **Nucleotide sequence and promoter mapping of the Escherichia coli Shiga-like toxin operon of bacteriophage H-19B.** J Bact 169, 4313 - 4319.

Deresiewics RL, Calderwood SB, Robertus JD, Collier RJ, (1992) **Mutations affecting the activity of the Shiga-like toxin I A-chain.** Biochem 31, 3272 - 3280.

Donohue-Rolfe A and Keusch GT, (1983) **Shigella dysenteriae 1 cytotoxin: Periplasmic protein releasable by polymyxin B and osmotic shock.** Infect and Immun 39, 270 - 274.

Donohue-Rolfe A, Keusch GT, Edson C, Thornley-Lawson D, Jacewics M, (1984) **Pathogenesis of shigella diarrhea.** J Exp Med 160, 1767 - 1781.

Donohue-Rolfe A, Kelley MA, Bennish M, Keusch GT, (1986) **Enzyme-linked immunoabsorbant assay for shigella toxin.** J Clin Micro 24, 65 - 68.

Donohue-Rolfe A, Jacewicz M, Keusch GT, (1989) **Isolation and characterisation of functional Shiga toxin subunits and renatured holotoxin.** Mol Microbiol 3, 1231 - 1236.

Donohue-Rolfe A, Acheson DWK, Keusch GT, (1991) **Shiga toxin: Purification, structure and function.** Rev Infect Dis 13, S293 - 297.

Douglas CM and Collier RJ, (1987) **Exotoxin A of Pseudomonas aeruginosa: substitution of glutamic acid 533 with aspartic acid drastically reduces cytotoxicity and enzymatic activity.** J Bact 169, 4967 - 4971.

Drummond DR, Armstrong J, Colman A, (1985) **The affect of capping and polyadenylation on the stability, movement and translation of synthetic mRNAs in Xenopus oocytes.** Nucl Acids Res 13, 7375 - 7394.

Eiklid K and Olsnes S, (1980) **Interaction of Shigella shigae cytotoxin with receptors on sensitive and insensitive cells.** J Recept Res 1, 199 - 213.

Eiklid K and Olsnes S, (1983) **Animal toxicity of Shigella dysenteriae cytotoxin: evidence that the neurotoxic, enterotoxic and cytotoxic activities are due to one toxin.** J Immunol 130, 380 - 384.

Eiklid K and Olsnes S, (1983) **Entry of Shigella dysenteriae toxin into HeLa cells.** Infect and Immun 42, 771 - 777.

Endo Y, Mitsui K, Motizuki M, Tsurugi K, (1987) **The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes.** J Biol Chem 262, 5908 - 5912.

Endo Y and Tsurugi K, (1988) **RNA N-glycosidase activity of ricin A-chain.** J Biol Chem 262, 8128 -8130.

Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogaswara T, Igarashi K, (1988) **Site of action of a verotoxin (VT2) from Escherichia coli 0157:H7 and of Shiga toxin on eukaryotic ribosomes.** Eur J Biochem 171, 45 - 50.

Fontaine A, Arondel J, Sansonetti PJ, (1988) **Role of Shiga toxin in the pathogenesis of bacillary dysentery studied using a tox$^-$ mutant of Shigella dysenteriae 1.** Infect and Immun 56, 3099 - 3109.

Frankel A, Schlossman D, Welsh P, Withers D, Johnston S, (1989) **Selection and characterisation of ricin toxin A-chain mutants in Saccharomyces cerevisiae.** Mol and Cell Biol 9, 415 - 420.

Fryling C, Ogata M, FitzGerald D, (1992) **Characterization of a cellular protease that cleaves Pseudomonas exotoxin.** Infect and Immun 60, 497 - 502.

Furutani M, Kashiwagi K, Endo Y, Igarashi K, (1992) **Comparison of the modes of action of a verotoxin (a Shiga-like toxin) from Escherichia coli, of ricin and of α-sarcin.** Arch Biochem and Biophys 293, 140 - 146.

Gill DM, (1976) **The arrangement of subunits in cholera toxin.** Biochem 15, 1242 - 1247.

Gill DM, Clements JD, Robertson DC, Finkelstein RA, (1981) **Subunit number and arrangement in Escherichia coli heat-labile enterotoxin.** Infect and Immun 33, 677 - 682.

Griffin DE and Gemski P, (1983) **Release of Shiga toxin from Shigella dysenteriae 1 by polymyxin B.** Infect and Immun 40, 425 - 428.

Hagenbuchle O, Santer M, Steitz JA, Mans RJ, (1978) **Conservation of the primary structure at the 3' end of 18S rRNA from eukaryotic cells.** Cell 13, 551 - 563.

Hale TL, (1991) **Genetic basis of virulence in Shigella species.** Micro Rev 55, 206 - 224.

Hart JP, Monzingo AF, Donohue-Rolfe A, Keusch GT, Calderwood SB, Robertus JD, (1991) **Crystallization of the B chain of Shiga-like toxin I from Escherichia coli.** J Mol Biol 218, 691 - 694.

Hartley MR, Legname G, Osborn R, Chen Z, Lord JM, (1991) **Single-chain ribosome inactivating proteins from plants depurinate Escherichia coli 23S ribosomal RNA.** FEBS Letts 290, 65 - 68.

Hedblom ML, Cawley DB, Houston LL, (1976) **The specific binding of ricin and its polypeptide chains to rat liver ribosomes and ribosomal subunits.** Arch Biochem and Biophys 177, 46 - 55.

Helting T and Zwisler O, (1974) **Enzymatic breakdown of tetanus toxin.** Biochem and Biophys Res Comm 57, 1263 - 1270.

Hirst TR, Randall LL, Hardy SJS, (1984a) **Cellular location of heat-labile enterotoxin in Escherichia coli.** J Bact 157, 637 - 642.

Hirst TR, Sanchez J, Kaper JB, Hardy SJS, Holmgren J, (1984b) **Mechanism of toxin secretion by vibrio cholerae investigated in strains harboring plasmids that encode heat-labile toxins of Escherichia coli.** Proc Natl Acad Sci USA 81, 7752 - 7756.

Hofstra H and Witholt B, (1984) **Kinetics of synthesis, processing and membrane transport of heat-labile enterotoxin a periplasmic protein in Escherichia coli.** J Biol Chem 259, 15182 - 15187.

Holmes DS and Quigley M, (1981) **A rapid boiling method for the preparation of bacterial plasmids.** Anal Biochem 114, 193 - 197.

Hovde CJ, Calderwood SB, Mekalanos JJ, Collier RJ, (1988) **Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I.** Proc Natl Acad Sci USA 85, 2568 - 2572.

Hunt CM, Harvey JA, Youngs ER, Irwin ST, Reid TM, (1989) **Clinical and pathological variability of infection by enterohaemorrhagic (verocytotoxin-producing) Escherichia coli.** J Clin Pathol 42, 847 - 852.

Hunt PD and Hardy SJS, (1991) **Heat-labile enterotoxin can be released from Escherichia coli by host intestinal factors.** Infect and Immun 59, 168 - 171.

Hwang J, FitzGerald DJ, Adhya S, Pastan I, (1987) **Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in Escherichia coli.** Cell 48, 129 - 136.

Igarashi K, Ogasawara T, Ito K, Yutsudo T, Takeda Y, (1986) **Inhibition of elongation factor 1-dependent aminoacyl-tRNA binding to ribosomes by Shiga-like toxin I (VT1) from Escherichia coli 0157:H7 and by Shiga toxin.** FEMS Micro Letts 44, 91 - 94.

Ito H, Yutsudo T, Hirayama T, Takeda Y, (1988) **Isolation and some properties of A and B subunits of VT2 and in vitro formation of hybrid toxins between subunits of VT1 and VT2 from Escherichia coli.** Microb Pathog 5, 189 - 195.

Jackson MP, Neill RJ, O'Brien AD, Holmes RK, Newland JW, (1987) **Nucleotide sequence analysis and comparison of the structural genes for Shiga-Like Toxin I and Shiga-Like Toxin II encoded by bacteriophages from Escherichia coli 933.** FEMS Micro Letts 44, 109 - 114.

Jackson MP, Deresiewics RL, Calderwood SB, (1990) **Mutational analysis of the Shiga toxin and Shiga-like toxin II enzymatic subunits.** J Bact 172, 3346 - 3350.

Janicot M, Fouque F, Desbuquois B, (1991) **Activation of rat liver adenylate cyclase by cholera toxin requires toxin internalization and processing in endosomes.** J Biol Chem 266, 12858 - 12865.

Jinno Y, Ogata M, Chaudhary VK, Willingham MC, Adhya S, FitzGerald D, Pastan I, (1989) **Domain II mutants of Pseudomonas exotoxin deficient in translocation.** J Biol Chem 264, 15953 - 15959.

Jobling MG and Holmes RK, (1992) **Fusion proteins containing the A2 domain of cholera toxin assemble with B polypeptides of cholera toxin to form immunoreactive and functional holotoxin-like chimeras.** Infect and Immun 60, 4195 - 4924.

Karch H, Heesemann J, Laufs R, O'Brien AD, Tacket CO, Levine MM, (1987) **A plasmid of enterohemorrhagic Escherichia coli 0157:H7 is required for expression of a new fimbrial antigen for adhesion to epithelial cells.** Infect and Immun 55, 455 - 461.

Karmali MA, Petric M, Lim C, Fleming PC, Arbus GS, Lior H, (1985) **The association between idiopathic Hemolytic Uremic Syndrome and infection by verotoxin-producing Escherichia coli.** J Infect Dis 151, 775 - 782.

Karmali MA, (1989) **Infection by verocytotoxin-producing Escherichia coli.** Clin Micro Rev 2, 15 - 38.

Kasturi S, Kihara A, FitzGerald D, Pastan I, (1992) **Alanine scanning mutagenesis identifies surface amino acids on domain II of Pseudomonas exotoxin required for cytotoxicity, proper folding and secretion into periplasm.** J Biol Chem 267, 23427 - 23433.

Katzin BJ, Collins EJ, Robertus JD, (1991) **Structure of ricin A-chain at 2.5Å.** Proteins: Struct, Funct, Genet 10, 251 - 259.

Keusch GT, (1981) Receptor-Mediated Endocytosis of Shigella cytotoxin p95 - 112 JL Middlebrook and LD Kohn (ed). Receptor-mediated binding and internalisation of toxins and hormones. Academic Press Inc. New York.

Kongmuang U, Honda T, Miwatani T, (1987) **A simple method for the purification of Shiga or Shiga-like toxin from Shigella dysenteriae and Escherichia coli 0157:H7 by immunoaffinity column chromatography.** FEMS Micro Letts 48, 379 - 383.

Kongmuang U, Honda T, Miwatani T, (1988) **Effect of nicking on Shiga-like toxin I of enterohaemorrhagic Escherichia coli.** FEMS Micro Letts 56, 105 - 108.

Konwalalchuk J, Speirs JI, Stavric S, (1977) **Vero response to a cytotoxin of Escherichia coli.** Infect and Immun 18, 775 - 779.

Koster FT, Boonpucknang V, Sujaho S, Gilman RH, Rahaman MM, (1984) **Renal histopathology in the Hemolytic-Uremic Syndrome following shigellosis.** Clin Nephrol 21, 126 - 133.

Kozlov YV, Kabishev AA, Lukyanov, Bayer AA, (1988) **The primary structure of the operons coding Shigella dysenteriae toxin and temperate phage H30 Shiga-I ike Toxin.** Gene 67, 213 - 221.

Kurazono H, Sasakawa C, Yoshikawa M, Takeda Y, (1987) **Cloning of a Verotoxin (VT1, Shiga-like toxin I) gene from a VT1-converting phage isolated from Escherichia coli 0157;H7.** FEMS Micro Letts 44, 23 - 26.

Levine MM, DuPont HL,Formal SB, Hornick RB, Takeuchi A, Gangarosa EJ, Snyder MJ, Libonati JP, (1973) **Pathogenesis of Shigella dysenteriae 1 (Shiga) dysentery.** J Infect Dis 127, 261 - 270.

Levine MM, (1987) **Escherichia coli that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent.** J Infect Dis 155, 377 - 389.

Levine MM, McEwen J, Losonsky G, Reymann M, Harari I, Brown JE, Taylor DN, Donohue-Rolfe A, Cohen A, Bennish M, Lim YL, Arnon R, (1992) **Antibodies to Shiga holotoxin and to two synthetic peptides of the B subunit in sera of patients with Shigella dysenteriae 1 dysentery.** J Clin Micro 30, 1636 - 1641.

Lindberg AA, Brown JE, Stromberg N, Westling-Ryd M, Schultz JE, Karlsson KA, (1987) **Identification of the carbohydrate receptor for Shiga toxin produced by Shigella dysenteriae type 1.** J Biol Chem 242, 1779 - 1785.

Lingwood CA, Law H, Richardson S, Petric M, Brunton JL, DeGrandis S, Karmali M, (1987) **Glycolipid binding of purified and recombinant Escherichia coli produced verotoxin in vitro.** J Biol Chem 262, 8834 - 8839.

Lopez EI, Diaz M, Grinstein S, Devoto S, Mendilaharzu F, Murray BE, Ashkenazi S, Rubeglio E, Woloj M, Vasques M, Turco M, Pickering LK, Cleary TG, (1989) Hemolytic Uremic Syndrome and diarrhea in Argentine children: the role of Shiga-like toxins. J Infect Dis 160, 469 - 475.

Louise CB and Obrig TG, (1992) **Shiga toxin-associated Hemolytic Uremic Syndrome: combined cytotoxic effects of Shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells in vitro.** Infect and Immun 60, 1536 - 1543.

Madshus IH and Collier RJ, (1989) **Effects of eliminating a disulphide bridge within domain II of Pseudomonas aeruginosa exotoxin A.** Infect and Immun 57, 1873 - 1878.

Marques LRM, Moore MA, Wells JG, Wachsmuth K, O'Brien AD, (1986) **Production of Shiga-like toxin by Escherichia coli.** J Infect Dis 154, 338 - 341.

Martin TE, Rolleston FS, Low RB, Wool IG, (1969) **Dissociation and reassociation of skeletal muscle ribosomes.** J Mol Biol 43, 135 - 149.

Maniatis T, Fritsch ES, Sambrook J, (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, NY.

May MJ, Hartley MR, Roberts LM, Krieg PA, Osborn RW, Lord JM, (1989) **Ribosome inactivation by ricin A chain: a sensitive method to assess the activity of wild-type and mutant polypeptides.** EMBO J 8, 301 - 308.

Merril CR, Goldman D, Sedman SA, Ebert MH, (1981) **Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins.** Science 211, 1437 - 1438.

Messing J, (1986) **New M13 vectors for cloning.** Methods in Enzymol 101, 20 - 78.

Middlebrook JL and Dorland RB, (1984) **Bacterial Toxins; cellular mechanisms of action.** Micro Rev 48, 199 - 221.

Moazed D, Stern S, Noller HF, (1986) **Rapid chemical probing of conformation in 16S ribosomal RNA and 30S ribosomal subunits using primer extension.** J Mol Biol 187, 399 - 416.

Moldave K and Sadnik I, (1979) **Preparation of derived and native ribosomal subunits from rat liver.** Methods in Enzymol LIX, 402 - 405.

Montfort W, Villafranca JE, Monzingo AF, Ernst SR, Katzin B, rutenber E, Xuong NH, Hamlin R, Robertus JD, (1987) **The three-dimensional structure of ricin at 2.8Å.** J Biol Chem 262, 5398 - 5403.

Moskaug JO, Sletten K, Sandvig K, Olsnes S, (1989) **Translocation of diphtheria toxin A-fragment to the cytosol.** J Biol Chem 264, 15709 - 15713.

Moskaug JO, Stenmark H, Olsnes S, (1991) **Insertion of diphtheria toxin B-fragment into the plasma membrane at low pH.** J Biol Chem 266, 2652 - 2659.

Neillands JB, (1982) **Microbial envelope proteins related to iron.** Ann Rev Micro 36, 285 - 309.

Newland JW, Strockbine NA, Miller SF, O'Brien AD, Holmes RK, (1985) **Cloning of Shiga-like toxin structural genes from a toxin-converting phage of Escherichia coli.** Science 230, 179 - 181.

Nilsson L and Nygard O, (1986) **The mechanism of the protein-synthesis elongation cycle in eukaryotes.** Eur J Biochem 161, 111 - 117.

O'Brien AD and Holmes RK, (1987) **Shiga and Shiga-like toxins.** Micro Rev 51, 206 - 220.

O'Brien AD, LaVeck GD, Griffin DE, Thompson MR, (1980) **Purification and characterization of a Shigella dysenteriae 1-like toxin produced by Escherichia coli.** Infect and Immun 40, 675 - 683.

O'Brien AD and LaVeck GD, (1982) **Immunochemical and cytotoxic activities of Shigella dysenteriae 1 (Shiga) and Shiga-like toxins.** Infect and Immun 35, 1151 - 1154.

O'Brien AD, LaVeck GD, Thompsom MR, Formal SB, (1982) **Production of Shigella dysenteriae type 1-like cytotoxin by Escherichia coli.** J Infect Dis 146, 763 - 769.

O'Brien AD and LaVeck GD, (1983) **purification and characterisation of a Shigella dysenteriae 1-like toxin produced by Escherichia coli.** Infect and Immun 40, 675 - 683.

Obrig TG, Moran TP, Brown JE, (1987) **The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis.** Biochem J 244, 287 - 294.

Obrig TG, Del Vecchio PJ, Brown JE, Moran TP, Rowland BM, Judge TK, Rothman SW, (1988) **Direct cytotoxic action of Shiga toxin on human vascular endothelial cells.** Infect and Immun 56, 2373 - 2378.

Ogata M, Chaudhary VK, Pastan I, FitzGerald DJ, (1990) **Processing of Pseudomonas exotoxin by a cellular protease results in the generation of a 37,000Da toxin fragment that is translocated to the cytosol.** J Biol Chem 265, 20678 - 20685.

O'Hare M, Roberts LM, Thorpe PE, Watson GJ, Prior B, Lord JM, (1987) **Expression of ricin A chain in Escherichia coli.** FEBS Letts 216, 73 -78.

O'Hare M, Brown AN, Hussain K, Gebhardt A, Watson G, Roberts LM, Vitetta ES, Thorpe PE, Lord JM, (1990) **Cytotoxicity of a recombinant ricin-A-chain fusion protein containing a proteolytically-cleavable spacer sequence.** FEBS Letts 273, 200 - 204.

Olsnes S and Eiklid K, (1980) **Isolation of Shigella shigae cytotoxin.** J Biol Chem 255, 284 - 289.

Olsnes S, Reisbig R, Eiklid K, (1981) **Subunit structure of shigella cytotoxin.** J Biol Chem 256, 8732 - 8738.

Osborn RW (1990) **The action of ricin A-chain on eukaryotic ribosomes.** PhD thesis (University of Warwick)

Osborn RW and Hartley MR, (1990) **Dual effects of ricin A chain on protein synthesis in rabbit reticulocyte lysate.** Eur J Biochem 193, 401 - 407.

Ostroff SM, Tarr PI, Neill MA, Lewis JH, Hargrett-Bean N, Kobayashi JM, (1988) **Toxin genotypes and plasmid profiles as determinants of systematic sequelae in Escherichia coli 0157:H7 infections.** J Infect Dis 160, 994 - 998.

Pai CH, Kelly JK, Meyers GL, (1986) **Experimental infection of infant rabbits with verotoxin-producing Escherichia coli.** Infect and Immun 51, 16 - 23.

Papini E, Schiavo G, Tomasi M, Colombatti M, Rappuoli R, Montecucco C, (1987) **Lipid interaction of diphtheria toxin and its mutants with altered B fragment.** Eur J Biochem 169, 637 - 644.

Pappenheimer AM, (1977) **Diphtheria toxin.** Ann Rev Biochem 46, 69 - 94.

Pelham HRB, Roberts LM, Lord JM, (1992) **Toxin entry ; how reversible is the secretory pathway?** Trends in Cell Biol 2, 183 - 185.

Petric M, Karmali MA, Richardson S, Cheung R, (1987) **Purification and biological properties of Escherichia coli verocytotoxin.** FEMS Micro Letts 41, 63 - 68.

Prior TI, FitzGerald DJ, Pastan I, (1992) **Translocation mediated by domain II of Pseudomonas exotoxin A: transport of barnase into the cytosol.** Biochem 31, 3555 - 3559.

Pitcher C personal communication.

Ready MP, Kim Y, Robertus JD, (1991) **Site-directed mutagenesis of ricin A-chain and implications for the mechanism of action.** Proteins: Struct, Funct, Genet 10, 270 - 278.

Reisbig R, Olsnes S, Eiklid K, (1981) **The cytotoxic activity of Shigella toxin.** J Biol Chem 256, 8739 - 8744.

Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, Blake PA, Cohen ML, (1983) **Hemorrhagic colitis associated with a rare Escherichia coli serotype.** New Eng J Med 308, 681 - 685.

Riley LW, (1987) **The epidemiological, clinical and microbiological features of hemorrhagic colitis.** Ann Rev Micro 41, 383 - 407.

Rubin GM, (1973) **The nucleotide sequence of Saccharomyces cerevisiae 5.8S ribosomal ribonucleic acid.** J Biol Chem 248, 3860 - 3867.

Ryd M, Alfredsson H, Blomberg L, Andersson A, Lindberg AA, (1989) **Purification of Shiga toxin by α-D-galactose-(1→4)-β-D-galactose-(1→ 4)- β-D-glucose-(1→) receptor ligand-based chromatography.** FEBS Letts 258, 320 - 322.

Sandvig K, Olsnes S, Pihl A, (1976) **Kinetics of binding of the toxic lectins abrin and ricin to surface receptors of human cells.** J Biol Chem 251, 3977 - 3984.

Sandvig K, Olsnes S, Pihl A, (1978) **Binding, uptake and degradation of the toxic proteins abrin and ricin by toxin-resistant cell variants.** Eur J Biochem 82, 13 - 23.

Sandvig K and Olsnes S, (1981) **Rapid entry of nicked diphtheria toxin into cells at low pH.** J Biol Chem 256, 9068 - 9076.

Sandvig K and Brown JE, (1987) **Ionic requirements for entry of Shiga toxin from Shigella dysenteriae 1 into cells.** Infect and Immun 55, 298 - 303.

Sandvig K, Olsnes S, Brown JE, Petersen OW, Van Deurs B, (1989) **Endocytosis from coated pits of Shiga toxin: a glycolipid-binding protein from Shigella dysenteriae 1.** J Cell Biol 108, 1331 - 1343.

Sandvig K, Prydz K, Van Deurs B, (1991) **Endocytosis and intracellular transport of glycolipid-binding ligand Shiga toxin in polarized MDCK cells.** J Cell Biol 113, 553 - 562.

Sandvig K, Garred O, Prydz K, Kozlov JV, Hansen SH, Van Deurs B, (1992) **Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum.** Nature 358, 510 - 513.

Saxena SK, O'Brien AD, Ackerman EJ, (1989) **Shiga toxin, Shiga-like toxin II variant and ricin are all single-site RNA N-glycosidases of 28S RNA when microinjected into Xenopus oocytes.** J Biol Chem 264, 596 - 601.

Schlossman D, Withers D, Welsh P, Alexander A, Robertus J, Frankel A, (1989) **Role of glutamic acid residue 177 of the ricin toxin A chain in enzymatic inactivation of ribosomes.** Mol and Cell Biol 9, 5012 - 5021.

Schreier PH and Cortese R, (1979) **A fast and simple method for sequencing DNA cloned in the single-stranded bacteriophage M13.** J Mol Biol 129, 169 - 172.

Scotland SM, Willshaw GA, Smith HR, Rowe B, (1990) **Properties of strains of Escherichia coli 026:H11 in relation to their enteropathogenic or enterohemorrhagic classification.** J Infect Dis 162, 1069 - 1074.

Sekizaki T, Harayama S, Brazil GM, Timmis KN, (1987) **Location of stx, a determinant essential for high-level production of Shiga toxin by Shigella dysenteriae serotype 1, near pyrF and generation of stx transposn mutants.** Infect and Immun 55, 2208 - 2214.

Sherman F, Fink GR, Hicks JB, (1986) Methods in yeast genetics. Cold Spring Harbor Laboratory manual.

Sixma TK, Pronk SE, Kalk KH, Wartna ES, Van Zanten BAM, Witholt B, Wol WGJ, (1991) **Cholera toxin related heat-labile enterotoxin from Escherichia coli.** Nature 351, 371 - 377.

Spangler BD, (1992) **Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin.** Micro Rev 56, 622 - 647.

Spicer EK and Noble JA, (1982) **Escherichia coli heat-labile enterotoxin.** J Biol Chem 257, 5716 - 5721.

Stein P, Boodhoo A, Tyrrell GJ, Brunton JL, Read RJ, (1992) **Crystal structure of the cell-binding B oligomer of verotoxin-1 from Escherichia coli.** Nature 355, 748 - 750.

Stirpe F, Bailey S, Miller SP, Bodley JM, (1988) **Modification of ribosomal RNA by ribosome inactivating proteins from plants.** Nucl Acids Res 16, 1349 - 1357.

Stirpe F and Hughes RC, (1989) **Specificity of ribosome-inactivating proteins with RNA N-glycosidase activity.** Biochem J 262, 1001 - 1002.

Streatfield SJ, Sandkvist M, Sixma TK, Bagdasarian M, Hol WGJ, Hirst TR, (1992) **Intermolecular interactions between A and B subunits of heat-labile enterotoxin from Escherichia coli promote holotoxin assembly and stability in vivo.** Proc Natl Acad Sci USA 89, 12140 - 12144.

Strockbine NA, Marques LRM, Newland JW, Williams-Smith H, Holmes RK, O'Brien AD, (1986) **Two toxin-converting phages from Escherichia coli 0157:H7 strain 933 encode antigenically distinct toxins with similar biological activities.** Infect and Immun 53, 135 - 140.

Strockbine NA, Jackson MP, Sung LM, Holmes RK, O'Brien AD, (1988) **Cloning and sequencing of the genes for Shiga toxin from Shigella dysenteriae type 1.** J Bact 170, 1116 - 1122.

Takao T, Tanabe T, Hong YM, Shimonishi Y, Kurazono H, Yutsudo T, Sasakawa C, Yoshikawa M, Takeda Y, (1988) **Identity of molecular structure of Shiga-like toxin I (VT1) from Escherichia coli 0157:H7 with that of Shiga toxin.** Micro Pathog 5, 357 - 369.

Takeda Y, Honda T, Taga S, Miwatani T, (1981) **In vitro formation of hybrid toxins between subunits of Escherichia coli heat-labile enterotoxin and those of cholera enterotoxin.** Infect and Immun 34, 341 - 346.

Tesh VL, Samuel JE, Perera LP, Sharefin JB, O'Brien AD, (1991) **Evaluation of the roles of Shiga and Shiga-like toxins in mediating direct damage to human vascular endothelial cells.** J Inf Dis 164, 344 - 352.

Tesh VL and O'Brien AD, (1991) **The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins.** Mol Micro 5, 1817 - 1822.

Tiporizi S, Karch H, Wachsmuth KI, Robins-Browne RM, O'Brien AD, Lior H, Cohen ML, Smithers J, Levine MM, (1987) **Role of a 60-megadalton plasmid and Shiga-like toxins in pathogenesis of infection caused by enterohemorrhagic Escherichia coli 0157:H7 in gnotobiotic piglets.** Infect and Immun 55, 3117 - 3125.

Tiporizi S, Gibson R, Montanaro J, (1989) **Nature and distribution of mucosal lesions associated with enteropathogenic and enterohemorrhagic Escherichia coli in piglets and the role of plasmid-mediated factors.** Infect and Immun 57, 1142 - 1150.

Tsuji T, Honda T, Miwatani T, (1984) **Comparison of effects of nicked and unnicked Escherichia coli heat-labile enterotoxin on chinese hamster ovary cells.** Infect and Immun 46, 94 - 97.

Tweten RK, Barbieri JT, Collier RJ, (1985) **Effect of substituting aspartic acid for glutamic acid 148 on ADP-ribosyltransferase activity.** J Biol Chem 260, 10392 - 10394.

Vandeyar MA, Weiner MP, Hutton CJ, Batt CA, (1988) **A simple and rapid method for the selection of oligodeoxynucleotide-directed mutants.** Gene 65, 129 -133.

Van Deurs B, Tonnessen TI, Petersen OW, Sandvig K, Olsnes S, (1986) **Routing of internalized ricin and ricin conjugates to the golgi complex.** J Cell Biol 102, 37 - 47.

Van Deurs B, Sandvig K, Petersen OW, Olsnes S, Simons K, Griffiths G, (1988) **Estimation of the amount of internalized ricin that reaches the trans-golgi network.** J Cell Biol 106, 253 - 267.

Wales R, Chaddock JA, Roberts LM, Lord JM, (1992) **Addition of an ER retension signal to the ricin A chain increases the cytotoxicity of the holotoxin.** Exp Cell Res 203, 1 - 4.

Weinstein DL, Holmes RK, O'Brien AD, (1988) **Effects of iron and temperature on Shiga like toxin production by Escherichia coli.** Infect and Immun 56, 106 - 111.

Weinstein DL, Jackson MP, Perera LP, Holmes RK, O'Brien AD, (1989) **In vivo formation of hybrid toxins comprising Shiga toxin and the Shiga-like toxins and role of the B subunit in localization and cytotoxic activity.** Infect and Immun 57, 3743 - 3750.

Williams-Smith H, Green P, Parsell Z, (1983) **Vero cell toxins in Escherichia coli and related bacteria: transfer by phage and and conjugates and toxic action in laboratory animals, chicken and pigs.** J Gen Micro 129, 3121 - 3137.

Wilson BA, Reich KA, Weinstein BR, Collier RJ, (1990) **Active-site mutants of diphtheria toxin: effects of replacing glutamic acid-148 with aspartic acid, glutamine or serine.** Biochem 29, 8643 - 8657.

Wool IG, (1979) **The structure and function of eukaryotic ribosomes.** Ann Rev Biochem 48, 719 - 754.

Yamamoto T, Gojobori T, Yokota T, (1987) **Evolutionary origin of pathogenic determinants in enterotoxigenic Escherichia coli and Vibrio cholerae 01.** J Bact 169, 1352 - 1357.

Yoshida T, Chen C, Zhang M, Wu HC, (1991) **Disruption of the golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin and Pseudomonas exotoxin.** Exp Cell Res 192, 389 - 395.

Youvan DC and Hearst JE, (1979) **Reverse transcriptase pauses at N^2-methylguanine during in vitro transcription of Escherichia coli 16S ribosomal RNA.** Proc Natl Acad Sci USA 76, 3571 - 3574.

Yu J and Kaper JB, (1992) **Cloning and characterisation of the eae gene of enterohemorrhagic Escherichia coli 0157:H7. Mol Micro 6, 411 - 417.**