Chemical and biological characterization of the cytolytic protein enterolobin from seeds of enterolobium contortisiliqumm

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Chemical and Biological Characterization of the Cytolytic Protein Enterolobin from Seeds of *Enterolobium contortisiliquum*

By

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This thesis is entirely the result of my work. It has not been submitted for a degree in this or any other university, and is not being submitted for any other degree.

Marcelo Valle de Sousa

This thesis and all my subsequent work are and will always be dedicated and offered to my parents, *Odimar de Araújo Sousa* and *Magaly Valle de Sousa*, and to *The Brazilian People*.

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Table of Contents

List of Tables and Schemes

List of Figures

Abbreviations

Amino acid residues (IUPAC-IUB Protein Code)*:

A	Ala	Alanine
B	Asx	Aspartic acid or Asparagine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Ans	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
X	XXX	Unknown
Y	Tyr	Tyrosine
Z	Glx	Glutamic acid or Glutamine
*	End	Terminator

Others:

A:	absorbance
AA:	amino acid
AN:	acetonitrile
AU:	arbitrary units
AUS:	arbitrary unit scale
BTCI:	black-eyed bean trypsin and chymotrypsin inhibitor
ConA:	concanavalin A
Da:	Dalton
DABITC:	4,*N*,*N*-dimethylaminoazobenzene-4'-isothiocyanate
DABTH:	dimethylaminoazobenzene thiohydantoin
DABTZ:	dimethylaminoazobenzene thioazolinone
DEAE:	diethylaminoethyl
DIDS:	diisothiocyanostilbene disulphonate
DTT:	dithiothreitol

*From IUPAC-IUB Commission on Biochemical Nomenclature (CBN). A One Letter Notation for Amino Acid Sequences. Tentative Rules. - A document approved by IUPAC-IUB in March 1968 and published in *Pure Appl. Chem.* **31**, 639-645 (1972).

EDTA:	ethylenediamine tetraacetic acid
EM:	electron microscopy
FITC:	Fluorescein isothiocyanate
FPLC:	fast liquid protein chromatography
HPLC:	high performance liquid chromatography
KDa:	kiloDalton
M.W.:	molecular weight
NEM:	*N*-ethylmorpholine
PAF:	platelet activating factor
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PC:	phosphatidylcholine
PCR:	polymerase chain reaction
PHA:	phytohaemagglutinin
PIR:	Protein Identification Resource
PITC:	phenylisothiocyanate
PVDF:	polyvinylidene difluoride
RBC:	red blood cell
RC:	reduced and S-carboxymethylated
RCA:	*Ricinus communis* agglutinin
rh:	relative humidity
RP:	reverse phase
SDS:	sodium dodecyl sulphate
% T:	percentage of transmittance
TEMED:	N,N,N',N'-tetramethylene diamine
TFA:	trifluoroacetic acid
TLC:	thin layer chromatography
TPCK:	1-chloro-3-tosylamido-4-phenylbutan-2-one
TNF-a:	tumor necrosis factor a
Tris:	tris-(hydroxymethyl) aminomethane
UPGMA:	unweighted pair-group method by arithmetic averaging
v:	volume
w:	weight
WGA:	wheat germ agglutinin
x g:	times the acceleration of the gravity

Abstract

Enterolobin, a 55 KDa haemolytic protein from *Enterolobium contortisiliquum* seeds, was purified and further characterized. Its partial amino acid sequence was determined by using both manual and automatic methods of sequence determination. A number of 271 residues could be placed by means of overlaps. A computational search of these overlapped peptides against the PIR database revealed that some residues of one peptide had short sequence matches to the bacterial pore-forming aerolysins from *Aeromonas hydrophyla* and *Aeromonas sobria* and to colicin Ib from *Escherichia coli*. A subsequent search for similar sequence patterns stored in the PROSITE database showed that the predicted cytolytic site of enterolobin and the aerolysins have similarities to the pattern of the *tonB*-dependent receptors of bacteria. This pattern occurs not only in these membrane receptor proteins, but also in colicins and other pore-formers and transport proteins. Hydropathy profile analyses made for aerolysins and the predicted cytolytic sites demonstrated their overall hydrophilic character with some short stretches of hydrophobic regions.

Studies on the kinetics of haemolysis of normal red cells by enterolobin in the presence of possible effectors such as Ca^{2+}, EDTA, galactose, choline, phosphatidylcholine, cholesterol, ricin and with trypsinised erythrocytes were performed. These analyses indicated that the membrane receptor for enterolobin was probably a protein, perhaps the band 3 anion-exchanger protein. Optical and electron microscopic observations of the effects of enterolobin and gold-enterolobin on the membranes of red cell and cancer cells were carried out, and revealed severely damaged membranes after contact with the cytolysin. Gold-enterolobin had a random distribution around holes on the red cell membrane. Bioassays showed that enterolobin was toxic to larvae of the insect *Callosobruchus maculatus* but not to those of *Spodoptera litorallis*. Assays of *in vitro* proteolysis using larval gut enzymes showed that only *S. litorallis* proteases could digest enterolobin. The mechanism of toxicity of enterolobin did not involve any damage to the microvillar membranes of the epithelial gut cells of *C. maculatus* as shown by electron microscopy.

1. *Introduction*

1.1 Concepts

The disruption of the organization of the cytoplasmic membrane with leakage of intracellular material leading to a loss of morphological and/or functional integrity of a cell is called cytolysis. Lysis is a more general term that can be applied to the disorganization of the cellular membrane, but also to types of membranes other than the cytoplasmic one. Haemolysis is the lysis of erythrocytes, or, as more commonly known, red blood cells.

A large number of substances with the capacity to cause cell lysis is known. The chemical nature of these compounds is very diversified, and includes saponins, lysophospholipids, ionic and non-ionic detergents, antibiotics, etc. (Assa *et al.*, 1973; Thelestan and Möllby, 1979; Zalavsky *et al.*, 1978). Besides these low molecular weight substances, there are also many cytolytic peptides and proteins produced by living organisms as it will be shown in this introduction.

Erythrocytes have long been used to test cytolytic effects. Indeed, these cells are very good models because they are easily available, contain an internal marker - haemoglobin - and have a well characterized membrane. Moreover, haemolysis occurs with almost all lytic agents with few exceptions (Alouf, 1976).

The aim of this introduction is to give an outline of the cytolytic proteins and peptides found in biological systems, focusing attention in particular on the novel group of plant cytolytic proteins (phytocytolysins), and especially enterolobin (Sousa, 1988; Sousa and Morhy, 1989), the first haemolytic/cytolytic protein to be isolated from plants.

1.2 Occurrence of Cytolytic Proteins and Peptides

Although cytolytic peptides and proteins seem to have an ubiquitous distribution among living organisms, they have been more often studied in bacteria, fungi, stinging arthropods, stinging marine invertebrates, poisonous reptiles, and mammals. Some examples of each group are described below.

1.2.1 Bacteria

A number of reviews have appeared on several aspects of the bacterial cytolytic proteins and peptides (reviews in Alouf, 1976; Bernheimer, 1970, 1974 and 1983; Bernheimer and Rudy, 1986; Sousa *et al.*, 1990). A complete issue of *Methods in Enzymology* was dedicated to the purification, assays and applications of microbial toxins including cytolysins (Harshman, 1988).

The most studied cytolytic proteins and peptides from bacteria are those from the genera *Streptococcus, Staphylococcus, Clostridium and Bacillus.* They also occur in *Vibrio, Pseudomonas, Listeria, Aeromonas, Escherichia* and many others. For example, Keen and Hoffman (1989) reported a *Legionella pneumophila* extracellular protease exhibiting haemolytic and other cytotoxic activities. Even the soil bacterium *Rhizobium leguminosarum* has a nodulation gene (*nodO*) that encodes a protein that is similar to *E coli* haemolysin (Economou *et al.*, 1990)

The bacterial cytolysins are generally classified in two groups, the thiol-activated cytolysins and other cytolysins. The cytolysins of the former group lose their cytolytic activity in the oxidized state and are activated by thiol compounds. Their site of interaction in the membrane is known to be cholesterol. They show close relationship to each other (Table 1). The second group is formed by cytolysins which are not activated by thiol compounds (Table 2). The cytolysins of this second group exhibit many differences to each other.

Table 1:

Examples of thiol-activated bacterial cytolysins*

Genus	Species	Cytolysin
Streptococcus	*pyogenes*	streptolysin O
	pneumoniae	pneumolysin
Bacillus	*cereus*	cereolysin
	thuringiensis	thuringiolysin O
	alvei	alveolysin
	laterosporus	laterosporolysin
Clostridium	*bifermentans*	bifermentolysin
	botulinum	botulinolysin
	histolyticum	histolyticolysin (ε-toxin)
	novyi type A *(oedematiens)*	oedematolysin O (γ-toxin)
	perfringens	perfringolysin O (θ-toxin)
	septicum	septicolysin
	tetani	tetanolysin
	chauvoei	chauveolysin
Listeria	*monocytogenes*	listerolysin

(*)From Bernheimer and Rudy (1986).

Table 2:

Examples of bacterial cytolysins not activated by thiols*

Genus	Species	Cytolysin
Staphylococcus	*aureus*	α–toxin
		β-toxin
		γ-toxin
		δ-toxin
		leucocidin
Streptococcus	sp.	streptolysin S
Clostridium	*perfringens*	α-toxin (phospholipase C)
	hemolyticum	lysin
	oedematiens	β-toxin
		γ-toxin
	septicum	α-toxin
	chauvoei	α-toxin
Bacillus	*subtilis*	surfactin
	thuringiensis	second haemolysin
	cereus	phospholipase D
Pseudomonas	*aeruginosa*	haemolysin
	fluorescens	haemolysin
Aeromonas	*hydrophyla*	aerolysin
	sobria	haemolysin[1]
	salmonicida	haemolysin[2]
Escherichia	*coli*	haemolysin

(*) Adapted from Bernheimer (1974). [1]Ljungh and Wadström (1982). [2]Titball and Munn (1983).

1.2.2 Protozoans

Cytolysins have also been found in protozoa. A 13-14 KDa cytolytic protein, probably involved in the pathogenesis of amoebic dysentery, is produced by the parasite *Entamoeba histolytica* (Lynch *et al.*, 1982; Young *et al.*, 1982). These results have been extended to another haemolysin-producing protozoan, *Naegleria fowleri* (Lowrey, D.M. and Young, J.D.-E., unpublished observations *apud* Young *et al.*, 1988). Also, *Trypanosoma cruzi*, the causative agent of Chagas' disease, secretes a haemolysin of 70-75 KDa that forms lesions of 10 nm on target membranes, and resembles perforin and component C9 of the human complement (reviewed by Andrews, 1990).

1.2.3 Lichens and Fungi

Cytolytic peptides and proteins have been frequently found in fungi. For example, alamethicin, suzukacillin and trichotoxin are antibiotic peptides isolated from different strains of the unicellular fungus *Trichoderma viride* (Irmsher and Jung, 1977). Volvatoxin is a cardiotoxin with M.W. = 24 000 from the mushroom *Volvariela volvacea*. Pleurotolysin is a 12 050 Da dimeric protein from the edible mushroom *Pleurotus ostreatus* (Bernheimer and Avigad, 1979). Phallolysin, a toxin from the poisonous mushroom *Amanita phalloides*, is one of the most studied fungal cytolysins. It has a M.W. = 33 000 and pIs of 8.06 and 7.49 (Seeger, 1975a,b). This protein is encountered in multiple forms and has some similarities with the staphylococcal α-toxin (Faulstich *et al.*, 1983). New additions to the group of cytolysin from fungi are always appearing in the literature. Recently, a haemolysin of

40 KDa has been purified from the poisonous mushroom *Rhodophyllus rhodophylius* (Suzuki *et al.*, 1990a).

A novel haemolysin from *Parmelia pulla*, purified by Hunaiti *et al.* (1988), was probably the first protein haemolysin isolated from lichens. It has a MW of 32 600 composed of two subunits alpha and beta of 18 000 and 14 000 Da respectively.

Also very recently, a new haemolysin has been discovered and purified from the culture filtrate of *Streptomyces* sp. strain no. A-6288, a bacterium possesing some characteristics of unicellular fungus, isolated from a soil sample (Suzuki *et al.*, 1990b). This cytolytic toxin had a molecular weight determined by gel filtration as 45 000.

1.2.4 Metazoans

Most of the metazoan phyla, such as porifera, coelenterata, anellida, mollusca, arthropoda and echinodermata, posseses cytolysins (reviewed by Canicatti, 1990). According to this author, they are generally calcium-dependent and heat-labile proteins, and are important factors in invertebrate defense, predation and humoral immunity. Canicatti (1990) discusses in his review the possibility of the evolution of a common ancestral gene which led to both invertebrate and vertebrate pore-forming proteins in their immune systems. His hypothesis was based on analogies of some invertebrate cytolysins with human complement and perforin (see Section 1.2.6). This may be true for the particular class of cytolytic proteins reviewed by him. However, at least arthropods produce predominantly small peptide cytolysins

rather than proteins, which excludes most of them from the above mentioned class.

As marine invertebrates, specially cnidarians (sea anemones), and arthropods are the most representative groups of cytolysin-containing invertebrates, their cytolytic peptides and proteins are described below in greater detail.

1.2.4.1 Cnidarians and other Marine Invertebrates

Among the marine invertebrates, the principal sources of cytolysins are the cnidarians (Coelenterates) (Bernheimer and Rudy, 1986). The toxin from nematocysts of the sea anemone *Stoichactis helianthus* is a basic protein with M.W. = 16 000 (Bernheimer and Avigad, 1975). The Atlantic Portuguese man-of-war, *Physalia physalis*, also contains in its nematocysts a lethal and cytolytic glycoprotein of high molecular weight (240 000) (Tankun and Hessinger, 1981). Table 3 lists known sea anemone cytolytic toxins, all of them having molecular weights between 10 to 30 KDa and basic pIs, except for metridiolysin with 80 KDa and pI 5.0 (Bernheimer and Avigad, 1978).

Besides the cnidarians, many other sea animals produce cytolysins (Canicatti, 1990). For example, the mucus secreted from the skin of the marine worm *Cerebratulus lacteus* contains cytotoxins with molecular weights around 10 500 Da (Ken and Blumenthal, 1978). Also, haemolysins occur in the coelomic fluid of three species of sea urchins, *Anthocidaris crassipina, Pseudocentrolus depressus* and *Hemicentrolus pulcherrimus* (Ryoyama, 1973). A cytolytic, but not

haemolytic, glycoprotein (dollabelanin P) has been recently purified from the purple fluid of the sea hare *Dolabella auricularia* (Yamazaki *et al.*, 1989) This invertebrate of the class Mollusca discharges a purple fluid from the purple gland when disturbed.

Table 3:

Cytolytic toxins from sea anemones*

Species	Toxin
Actinia cari	cariotoxin I
	cariotoxin II
Actinia equina	equinatoxin
	equinatoxin I
	equinatoxin II
	equinatoxin III
Actinia tenebrosa	tenebrosin A
	tenebrosin B
	tenebrosin C
Aiptasia pallida	fraction III
	fraction IV
Anthopleura japonica	*A. japonica* haemolysin 1
	" haemolysin 2
Anthopleura xanthogrammica	*A. xanthogrammica* lysin
Condylactis gigantea	*Condylactis* toxin
Epiactis prolifera	epiactin A
	epiactin B
	epiactin C
Metridium senile	metridiolysin
Parasicyonis actinostoloides	parasitoxin
Pseudactinia varia	variolysin
Radianthus macrodactylus	*R. macrodactylus* toxin
Stoichactis helianthus	*S. helianthus* toxin
=*Stichodactyla helianthus*	*Stichodactyla* cytolysin I
	" cytolysin II
	" cytolysin III
	" cytolysin IV
Stoichactis kenti	kentin
Tealia lofotensis	*T. lofotensis* lysin

(*) Compiled by Sencic and Macek (1990).

1.2.4.2 Arthropods

Arthropods have long been used as a source for isolation of cytolytic peptides and proteins (Iwanaga, 1976; Cavagnol, 1977; Schimdt, 1982; Bernheimer and Rudy, 1986). Bees, wasps, ants and scorpions, but not spiders, are known to have phospholipase A_2 in their venoms. Sphingomyelinase D, found in the venom of the spider *Loxosceles recluse*, has a M.W. = 19 000 and causes calcium-dependent haemolysis (Forrester *et al.*, 1978).

Besides these enzymes, arthropod venoms usually contain toxic cytolytic peptides. Mellitin from the bee (*Apis mellifera*), barbatolysin from the red harvester ant (*Pogonomyrmex barbatus*), mastoparan from the wasp (*Vespula lewisii*), crabolin from the European hornet (*Vespa crabre*) and bambolitin from the bumblebee (*Megabombus pennsylvanicus*) are examples of those peptides considered by Bernheimer and Rudy (1986). Recently, some synthetic amphiphilic peptides were constructed carrying mellitin-like activity (Katsu *et al.*, 1990).

Gooding (1977) described the presence of a haemolysin in the midgut of *Glossina morsitans,* a hematophagous fly and vector of the sleeping disease. Another hematophagous insect, *Rhodnius prolixus*, the vector that transmits Chagas' disease, possesses a haemolytic and trypanosomalytic peptide (Azambuja *et al.*,1989).

1.2.5 Reptiles, Amphibians and Fish

Not surprisingly, several cytolytic polypeptide fractions have been obtained from snake (Elapidae) venoms. Several terms have been used to describe them such as: direct lytic factors (DHF) (Aloof-Hirsh *et al.*, 1968; Condrea *et al.* 1964a,b; Yukel'son *et al.* 1975); cobramines (Larsen and Wolff, 1968); cytotoxin P (Braganca *et al.*, 1965); cardiotoxins (Louw and Visser, 1977); and cytotoxins (Dimari *et al.*, 1975).

These toxins have been classified as belonging to a congeneric group of cytolysins, because of their uniform structures and modes of action (Bernheimer and Rudy, 1986). Iwanaga (1976) described them as similar, strongly basic, peptides with molecular weights ranging from 5 700 to 6 700 (52 to 60 amino acid residues). Their chemical and pharmacological properties have been reviewed by Lee (1972). Phospholipase A_2 is also generally present in snake venoms in addition to cytolytic peptides. It appears to act synergistically with them (Harvey *et al.*, 1983; Yukel'son *et al.*, 1977).

Amphibians seem to contain cytolysins as well. A 87 000 Da acidic haemolytic protein was purified from the skin secretion of *Bombina variegata* (Mar and Michl, 1976). Sebben *et al.* (1989) demonstrated the presence of a protein fraction with haemolytic activity in the skin secretion of the frog *Odontophrynus cultripes*.

Cytolysins from fish are also known. For example, the arabian gulf catfish, *Arius thalassinus*, produces a toxic gelly secretion from its epidermis that contains an acidic haemolytic protein of 34 KDa with broad specificity (Al-Lahham *et al.*,, 1987). The secretion of this

toxic and haemolytic gel by the fish is induced after a fright or a shock as a mechanism of self-defense.

1.2.6 Humans

There are some proteins from the human immune system which have the ability to lyse cells. The most studied of these proteins belongs to the complement system, and have been investigated for a long period (reviews in Cooper, 1971; Fearon and Wong, 1983; Frank *et al.*, 1965; Mayer, 1965; Porter and Reid, 1979; Reid and Porter, 1981). Recently, Kipnis and Dias da Silva (1989) reviewed the action of complement on *Trypanosoma cruzi*. Complement causes the lysis of both animal cells and infectious agents. It is a system of about 20 distinct glycoproteins present in extracellular fluids. Some of these proteins are enzymes and cooperate with immunoglobulins in the immune system. Besides, Salama *et al.* (1988) demonstrated that some potent haemagglutinating antibodies can cause complement-independent haemolysis as well.

Another group of cytolytic glycoproteins are the lymphotoxins from human lymphoid cells. They are glycoproteins, and are divided in four groups of molecular weights - 200 KDa, 70-90 KDa, 30-50 KDa, and 15-20 KDa (Toth and Granger, 1979).

More recently, a novel cytolytic protein found in cytotoxic T lymphocytes and natural killer cells is gaining attention. This protein is called pore-forming protein or, simply, perforin (reviewed by Tschopp and Nabholz, 1990; Young and Cohn, 1986; Young *et al.* 1988; Young, 1989). Perforin exists in a 70 000 Da monomeric form. It can

lyse tumor cells and other target cells by a calcium-dependent polymerization of the monomers, forming pores through the membrane.

1.3 The novel group of plant cytolytic peptides and proteins

Surprisingly, only a few plant cytolytic peptides and proteins have been studied so far. Only one peptide, the *Pyrularia pubera* thionin (Vernon et. al, 1985) and two proteins, crotin (Banerjee and Sen, 1981) and enterolobin (Sousa, 1988; Sousa and Morhy, 1989) have been purified. More detailed description of each of them, especially enterolobin that is the object of this current work, are given below.

Certainly, many other distinct cytolytic peptides and proteins may be found in plants. The increased detection, purification, and chemical and biological characterization of these substances will permit further comprehension of their mechanisms of action, roles in the plants and practical applications.

1.3.1 *Pyrularia* Thionin

Pyrularia thionin is a 5 280 Da basic peptide with 47 amino acid residues purified from the nuts of the fern *Pyrularia pubera*

(Vernon *et al.*, 1985). *Pyrularia pubera* is a parasitic plant from the order Santalaceae, which contains the mistletoes. This thionin is a haemolytic, cytolytic and neurotoxic peptide, and has close resemblance to the viscotoxins from the mistletoes, to crambin, and to the thionins from wheat and barley (Vernon *et al.*, 1985). The peptide has most haemolytic activity against red cells from man, followed by rabbit, guinea pig and pig. Little or no activity is shown against erythrocytes from sheep, horse, cow or mouse. Recently, Bloch and Richardson (1991) purified and sequenced three small α-amylase inhibitors from sorghum with sequence similarities to the thionins, but no cytolytic activity has been found for this thionin-like inhibitor yet.

The *Pyrularia* thionin has no phospholipase activity itself , but acts synergistically with bee phospholipase A2 (Osorio e Castro *et al.*, 1989). It also activates membrane phospholipase A2, which promotes the release of free fatty acids, but this release is not totally responsible for the haemolysis.

Thionin and snake venom cardiotoxin seem to have the same binding site on the membrane, most likely a protein as concluded from kinetic analysis (Osorio e Castro and Vernon, 1989). Subsequent changes in the conformation of membrane proteins would result in phospholipase A_2 activation, enhancing thionin haemolytic activity.

1.3.2 Crotin

Crotin is a non-toxic lectin from the seeds of *Croton tiglium* (Euphorbiaceae). Banerjee and Sen (1981) purified and characterized this protein. Although it agglutinates erythrocytes of

sheep, cow, buffalo and goat, it fails to agglutinate or lyse human (A, B, O groups), horse and dog erythrocytes. However, it causes agglutination and haemolysis of rabbit erythrocytes. No evidence has been shown in the literature of lytic activity of crotin towards cell types other than rabbit erythrocytes.

Crotin has a M.W.= 55 000 by SDS-PAGE but can undergo association to form oligomers of 400 000 Da. It is a glycoprotein with 0.75% of carbohydrate content and consists of two isoforms of pI 5.1 and 5.4. It contains five cysteine residues with two of them forming a disulphide bond.

Early results (Banerjee and Sen, 1981) suggested that the binding site for crotin on rabbit membrane might be a glycopeptide and might involve galactose residues. Also, there were indications that there might be divergent pathways for hemagglutination and haemolysis subsequent to lectin binding. Later experiments (Banerjee and Sen 1983) confirmed the previous supposition and suggested a disaccharide structure consisting two Gal or GalNAc residues as the binding moiety.

1.3.3. Enterolobin

A screening of seeds harvested from plants found in the central region of Brazil was carried out in 1986 in order to look for the presence of haemolysins in them (Sousa and Morhy, unpublished results). In that test, the saline extract of *Enterolobium contortisiliquum* (Vell.) Morong showed haemolytic activity. *E. contortisiliquum* is a tree belonging to the family Leguminosae, subfamily Mimosoideae. This plant is commonly known as "orelha-de-macaco", "orelha-de-negro",

"tamboril" or "tambouva". It is found in the Brazilian rain forests, principally in the Atlantic forest, from the northern state of Ceará to Uruguay and Paraguay (Rizzini and Mors, 1976). It is well adapted to the "Cerrado" ecosystem in central Brazil, and is recommended for reafforestation (Heringer, 1978). None of the products of *E. contortisiliquum* is used as food for human consumption. The fruit pod is toxic to cattle, but the nature of the toxin is not known (Hoehne, 1939; Santos *et al.*, 1975).

The haemolytic protein, which was named enterolobin, was purified by the following method: extraction with 0.15 M NaCl, precipitation with ammonium sulfate (0-33% saturation), batch separation with DEAE-cellulose and gel filtration on Sephadex G-100 or G-150 (Sousa, 1988; Sousa and Morhy, 1989).

Enterolobin was shown not to possess any phospholipase D activity in a chemiluminescence assay (Sousa and Morhy, 1989), despite the fact that phospholipase D can be haemolytic (Bernheimer and Rudy, 1986; Thelestan and Möllby, 1979) and that seeds of legumes are rich sources of this enzyme (Galliard, 1980; Heller, 1978).

Mouse erythrocytes were less susceptible to haemolysis by enterolobin than human and rabbit erythrocytes in a microtitration plate assay. The haemolytic activity was rapidly lost at temperatures above 55 ^{o}C and at acidic and basic pHs.

Enterolobin has a molecular weight of 55 000 when determined by gradient PAGE-SDS, 59 800 by gel filtration, and 51 300 by gel filtration in HPLC. Its isoelectric point is 7.0. It contains high percentages of Asp(Asx), Phe, Ser and Thr and low percentages of

Ala, Cys and Pro in relation to average proportions found in proteins (Sousa, 1988; Sousa and Morhy, 1989).

The characterization of enterolobin by ultraviolet spectroscopy (Sousa, 1988; Sousa *et al.*, paper in preparation) showed the presence of Trp and Tyr in a molar ratio Tyr/Trp of 1:1. Fluorescence studies indicated that Trp is in an environment of medium polarity. The quantum yield of enterolobin was similar to that from a standard Trp solution, indicating that there was no quenching of the fluorescent emission from Trp residues in enterolobin. Data from infrared and circular dichroism spectra showed the presence of both ordered and unordered secondary structures in enterolobin. Additionally, a tight conformation was inferred for enterolobin.

New studies are underway showing new properties of enterolobin (Castro-Faria-Neto *et al*, in the press; Cordeiro *et al.*, in the press). These studies have demonstrated that enterolobin is a very potent inducer of paw oedema. Furthermore, enterolobin-induced oedema is partially dependent on lipoxygenase metabolites and histamine, while PAF-acether (a lipid mediator involved in several physiopathological conditions, including inflammation) and prostaglandins do not seem to be important in this reaction. Enterolobin also causes pleural exudation and cellular infiltration, with a remarkable ability to attract polymorphonuclear neutrophils and eosinophils (Castro-Faria-Neto, *et al.*, in the press). Cordeiro *et al* (in the press) have stated that enterolobin could be a very useful pharmacological tool to investigate some aspects of the inflammatory reaction, since it has the general ability to induce oedema, exudation and cellular influx to the inflamed area.

Sousa *et al.* (paper in preparation) have found that enterolobin is cytolytic to most of the blood cells, except lymphocytes. This finding suggests an immediate practical application for enterolobin in the purification of lymphocytes. It could also attract groups interested in investigating of the molecular mechanism of lymphocyte resistance to lysis by enterolobin.

1.3 Classification of Cytolysins

Cytolytic peptides and proteins can be classified in different ways. One of these systems follows the origin of the agent as it was done above. A wider classification would be simply microorganism, plant and animal cytolysins.

Cytolytic peptides and proteins from microorganisms are the most studied ones. They have been investigated since the beginning of this century. Bernheimer (1970) reviewed extensively the research done on this class of proteins up to that year. Animal cytolysins also have been very much considered as it has been shown above in this introduction. The only group that has not yet been significantly explored is the plant cytolysins group.

An early classification of the bacterial cytolytic proteins only was made by Bernheimer (1970). As already mentioned above (Section 1.2.1), he classified them in two groups: (a) cytolytic toxins

activated by SH-compounds (oxygen-labile lysins); and (b) cytolytic toxins not activated by SH-compounds.

SH-dependent cytolysins lose their activity in the oxidized state, but are active in the presence of thiol reagents. They form a uniform group in some characteristics. All of them come from gram-positive bacteria, and have the same receptor on the target cells - cholesterol (Bernheimer, 1974, 1983). They have molecular weights ranging from 47 000 to 68 000 Da, and can form dimeric and oligomeric forms (Alouf, 1976). The other cytolytic bacterial proteins show no close relationship among them and are not activated by thiols, thus are classified in the separate group of SH-independent cytolysins. Their physical-chemical and biological properties are very diverse.

Thelestam and Möllby (1979), by means of methodical experiments, classified cytolytic agents (micro- and macromolecules) by the type of leakage caused to target cells by them. Five groups were identified: (a) detergent-like agents which cause non-specific solubilization of membrane constituents, e.g. *Pseudomonas* haemolysin; (b) molecules interacting only with certain constituents of the membrane and causing solubilization or degradation, e.g. phospholipase C from *Bacillus cereus* and mellitin; (c) cytolysins interacting with specific receptors on the cell membrane and giving rise to lesions of limited size, e.g. aerolysin, δ-toxin, cereolysin, θ-toxin, listeriolysin, streptolysin O, vibriolysin and direct lytic factors of snakes; (d) agents which induce small channels of defined size, e.g. nystatin, amphotericin B and α-toxin; and (e) proteins producing only a very limited increase in the permeabeality of the plasma membrane, e.g. phospholipase D from *Corynebacterium ovis*, phospholipase C from *S. aureus*, γ-toxin and streptolysin S.

More recently, two other more extensive classifications of the cytolytic peptides and protein have been made. (Bernheimer and Rudy, 1986; Bhakdi and Tranum-Jensen, 1987). These new classification systems are amalgamations of the previous ones with the introduction of new data derived from electron microscopy, protein chemistry, kinetics of cytolysis and other biochemical and biophysical techniques such as use of cytolysins labelled with fluorescent probes.

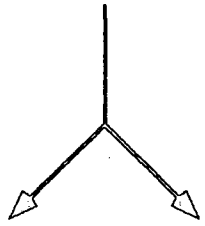
Bernheimer and Rudy (1986) introduced the general conceptual classification of cytolytic polypeptides producing actual or postulated discrete membrane lesions, and polypeptides not known to produce discrete lesions. They also subdivided each group into "congeneric" and "individualistic" agents, the former meaning those with similar physico-chemical characteristics and/or mechanisms of action, while the latter exhibits a high degree of individuality in their properties. This classification system is shown in Scheme 1.

Bhakdi and Tranum-Jensen (1987) suggested a way of classification also based on the type of pore: (a) cytolysins forming pores with recognizable ultrastructure, e.g. Staphylococcal α-toxin, streptolysin O, complement and perforin; and (b) cytolysins generating pores with no recognizable ultrastructure, e.g. cytolysin from gram-negative bacteria (such as *E.coli* haemolysin, *Pseudomonas aeruginosa* cytotoxin, *Aeromonas hydrophila* aerolysin and gramicidin A) and other pore-forming proteins (from sea anemone, fungi and amoeba). The authors define pores with recognizable structure as those that can be detected by electron microscopy.

Scheme 1

Classification of Cytolytic Peptides and Proteins

I. Polypeptides producing actual or postulated discrete membrane lesions



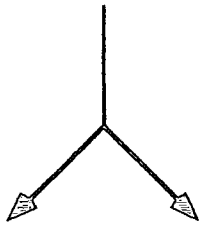
A. *Congeneric agents*

- SH-activated lysins
- Mellitin and other arthropods lysins
- Alamethicin
- Gramicidin
- Various sea anemone lysins
- δ-Toxin

B. *Individualistic agents*

- α-Toxin

II. Polypeptides not known to produce discrete membrane lesions



A. *Congeneric agents*

- Carditoxic peptides from snakes
- Phospholipase A2
- Phospholipase C
- Phospholiapse D
- Streptolysin S

B. *Individualistic agents*

- Aerolysin
- Vibrio cytolysins
- Physalia toxin
- Phallolysin
- Diamphotoxin

*Bernheimer and Rudy (1986)

1.4 Significance of the study of cytolysins

It has been shown in this introduction that biomolecules from different organisms, with distinct physical and chemical properties, and diverse mechanisms of action, can act as cytolysins.

Such variety stimulates studies aimed at characterizing and understanding these molecules to know about their biological and medical significance; relations between their structures, mechanisms of action and functions; and potentialities as biochemical, pharmaceutical and biotechnological tools.

The medical and pharmacological importance of cytolysins is clear since many of them are cytotoxins, neurotoxins and/or cardiotoxins. They can be harmful to man following microbial infection, ingestion of toxic mushrooms and plants, and inoculation of animal venoms. A deep knowledge of these cytolysins is therefore important for the development of appropriate medical treatment and control. On the other hand they could be exploited as medicines, if their activities are capable of being controlled and modified by protein engineering. Again, the same is obviously true for the human cytolysins, such as proteins of complement, lymphotoxins, perforin and tumor necrosis factors. Their study will lead to a better comprehension of the immunological system, and to their intelligent use in the prevention and treatment of pathological conditions.

Besides the importance of learning about the structure, mechanisms of action and function of cytolysins, a number of potential

applications of these substances has recently become evident. In a previous section of this introduction, it was observed, for example, that some fungal cytolytic peptides are antibiotics. Also, some cytolysins from snake venoms selectively lyse neoplastic cells when compared to other types of cells, thus being potential anti-tumor agents (Bragança *et al.*, 1965; Dimari *et al.*, 1975).

The diversity of cell lysis mechanisms mediated by cytolytic peptides and proteins makes them useful for the study of the ultrastructure of membranes and their role in cellular processes. As an example, Iwashita (1989) constructed a cholesterol-specific membrane probe deprived of cytolytic effect by chemical modification of a microbial cytolysin.

A concrete application of cytolysins was obtained through the construction of an immunocytolysin against neoplasic cells (Avila *et al.*, 1987). This immunocytolysin was built by coupling the cytolysin from the sea anemone (*Stoichactis helianthus*) to an anti-cancer cell immunoglobulin. The construction of cytolytic immunotoxins allows the destruction of specific cells such as tumor cells or pathogenic organisms.

The use of cytolytic peptides and proteins as biological tools is still in its infancy. New applications will certainly emerge with the development of research in this area.

1.5 Objectives

As it has been shown in this introduction, peptides and proteins with cytolytic activity are present in a variety of living forms as evolutionary diverse as microorganisms and man, including bacteria, fungi, sea anemones, stinging insects and snakes for example. Other groups of plant peptide and protein cytolysins have not been so thoroughly investigated. Because of their medical, pharmacological, biochemical and biotechnological importance, new efforts for the research on cytolytic peptides and proteins, principally on the new groups (e.g. from higher plants) should be stimulated.

The objectives of this present work were the determination, as far as possible, of the amino acid sequence of enterolobin; indication of the chemical nature of its binding site on the membrane; visualization of the pattern of the perforations on the membrane; and the demonstration of new biological properties towards other cells types and insect larvae.

2. Material and Methods

2.1 Materials

2.1.1 Seeds

The seeds of *Enterolobium contortisiliquum* (Vell.) Morong were collected from trees in Brasília, Brazil. Prior to use, the seeds were kept in a cold room at 4 °C. Voucher specimens of parts of *E. contortisiliquum* trees are conserved at the herbarium of the University of Brasília. Chick pea (*Cicer arietinum*) seeds were bought locally in Durham.

2.1.2 Erythrocytes

Human erythrocytes of different blood group types were obtained from the Hemocenter of Brasília (blood bank), Brasília, Brazil.

These erythrocytes were provided in 50 mM EDTA in 150 mM NaCl solution and kept at 4 °C for up to one week. In Durham, red cells were collected from M.V. Sousa at the Student Health Centre and maintained under the same conditions of conservation as above. Before being used, the sample was washed four times in 150 mM NaCl solution.

2.1.3 Cancer Cells

Cancer cells of strains L929 (murine fibroblast-like cell) and MRC5 (human lung fibroblast) were cell lines maintained at the Laboratory of Immunology of the Department of Biological Sciences, University of Durham. The culture medium for the cells was RPMI-1640, plus penicillin and streptomycin at 50 U/ml and 50 ug/ml respectively, 50 mM glutamine and 10 % foetal calf serum. All of the above products were tissue culture grade, and purchased from Gibco.

2.1.4 Insects

Cultures of *Callosobruchus maculatus*, originally from Campinas, Brazil, were maintained and reared in an insectary at 27 °C and 70% rh. Cultures of *Spodoptera littoralis* were supplied by Rhone Poulenc, being maintained and reared at 24 °C with a controlled day length of 16 h.

2.1.4 Enzymes and other Proteins

Only the highest quality enzymes and proteins were utilized in this work. Trypsin (TPCK-treated), chymotrypsin and elastase were from Sigma; *S. aureus* V-8 protease was from ICN ImmunoBiologicals; and pyroglutamate aminopeptidase, from Boehringer Mannhein.

Ovalbumin and bovine γ-casein were purchased from Sigma. TNF-α was obtained from NIBSC (National Institute of Biological Standard and Control). Ricin and BTCI (Black-eyed bean Trypsin and Chymotrypsin Inhibitor) were kindly provided in a pure state by Prof. L. Morhy from the Dept. of Cellular Biology of the University of Brasília, Brazil.

2.1.5 Reagents and Solvents

The reagents and solvents used for protein sequencing were all of sequencing grade. DABITC was from either Sigma or Fluka. PITC, TFA and pyridine were supplied by Rathburn or Applied Biosystems. The acetonitrile used for HPLC peptide separations was of HPLC grade from Rathburn. The solvents n-heptane, n-hexane, butyl-acetate and ethyl-actetae were products from BDH. Buffers and all other chemicals were of analytical grade from several suppliers.

2.2 Methods

2.2.1 Purification and Purity Analysis of Enterolobin

2.2.1.1 Purification of Enterolobin

The purification of enterolobin was performed according to Sousa and Morhy (1989). Decorticated *E. contortisiliquum* seeds were ground in an electric mill and 80 g of seed flour was extracted with 640 ml of 150 mM NaCl solution for 1 h, under agitation and at 4 °C. The extract was filtered through muslin and centrifuged at 16 300 x g, for 1 h, at 4 °C. The supernatant was then adjusted to 33 % saturation with ammonium sulphate . After 20 min, it was centrifuged at 7 000 x g, for 30 min, at 4 °C. The sediment was resuspended in 27 ml of saline solution, dialysed against distilled water (4 x 10 l) for 24 h and, then, centrifuged in a bench centrifuge for 10 min at room temperature. The supernatant was freeze-dried and this fraction was called F-1.

The following step was a DEAE-cellulose batch separation. In this adsorption process, 200 mg of fraction F-1 was added to 220 ml of activated DEAE-cellulose resin suspended in 220 ml of 50 mM Tris-HCl pH 8.0 buffer. After 1 h of equilibration at room temperature with sporadic gentle agitation, the resin was filtered through filter paper under vacuum. The non-adsorbed material in the filtrate was concentrated by freeze-drying down to 20 % of its original volume,

dialysed against distilled water (3 x 5 l) for 24 h, at 4 °C and, finally, fully freeze-dried (fraction F-2).

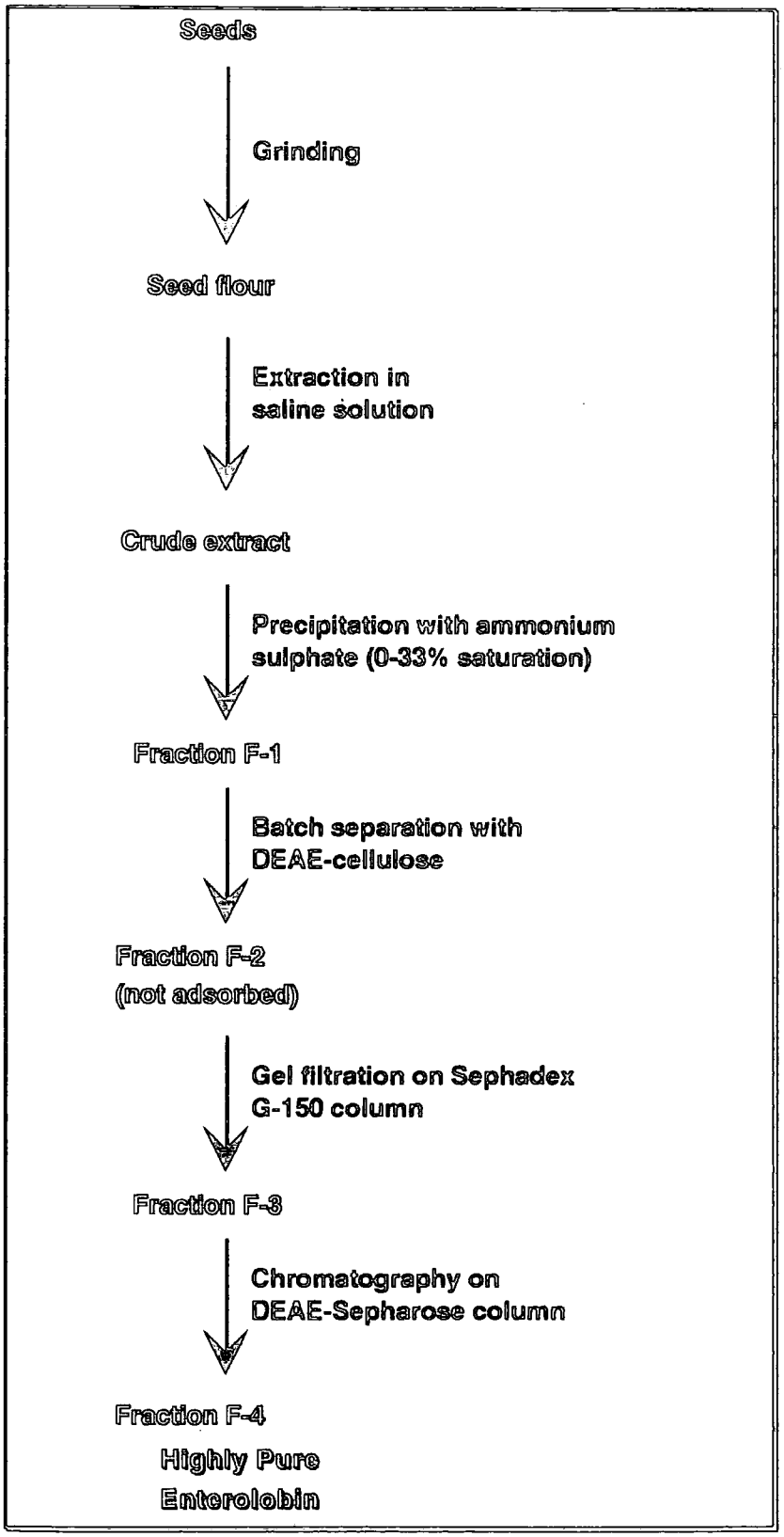
Subsequently, fraction F-2 was submitted to gel filtration on a column (1.9 x 40 cm) of Sephadex G-150 equilibrated in 50 mM tris-HCl pH 8.0. The sample was loaded as a solution of 30 mg in 2 ml of the equilibration buffer. The flow rate was kept at 15 ml/h. Fractions of 3 ml were collected and their absorbances, automatically read at 280 nm. The material from the peak containing enterolobin was dialysed against distilled water (3 x 2.5 l) for 24 h at 4 °C and freeze-dried as fraction F-3.

2.2.1.2 Further Purification of Enterolobin

In order to have a suitable purity for sequencing, enterolobin was further purified by anion-exchange chromatography on DEAE-Sepharose. The column of gel (2.5 x 15 cm) was equilibrated in 50 mM tris-HCl pH 8.5. The sample (20 mg of fraction F-3) was dissolved in 3 ml of equilibration buffer and applied to the column, which was then washed with 100 ml of the buffer. A linear gradient of 0 to 0.25 M NaCl in the equilibration buffer (400 ml total) was used for the elution step. The flow rate was 37.5 ml/h. Fractions of 2.5 ml were collected, and their absorbances read at 280 nm. The haemolytic activities present were determined according to the method described in the Section 2.2.1.3 below. The haemolytic fractions were pooled, dialysed and freeze-dried. This purest fraction (F-4) of enterolobin was used subsequently for the determination of the amino acid sequence. A fluxogram describing the method of purification of enterolobin is in Scheme 2.

Scheme 2

Purification of Enterolobin



Enterolobin (fraction F-4) was also submitted to reverse-phase HPLC on a Vydac 218TP5405 C-18 column (4.6 x 250 mm, 5 μm particle size, 300 Å pore size) from Technicol fitted to a Varian model 5000 HPLC chromatographer. One mg of enterolobin in 500 ul of 6 M guanidine-HCl in 0.1 % TFA was injected into the column, which was eluted with a linear gradient of 0 to 100% acetronitrile (in 0.1% TFA) in 60 min. The eluate was monitored at an absorbance of 214 nm.

A test tube test was previously made, in which filtered aliquots of 100 ul enterolobin (1 mg/ml 0.1% TFA) were added to a range of different concentrations of acetonitrile (0 to 100% in 10% intervals) in 0.1% TFA. The appearance of the resulting mixture was observed to detect any sign of protein precipitation.

2.2.1.3 Haemolytic Activity Assay

The determination of the haemolytic activity during the purification steps was carried out as previously described (Sousa and Morhy, 1989). Samples of 50 ul in 150 mM NaCl solution (saline) were two-fold serially diluted in saline on immunological microtitration U-plates at room temperature. To each well, 50 ul of a 3 % (v/v) suspension of human red cells (washed four times in saline) were added. After 1 hour, the highest dilution showing haemolysis was recorded. The inverse of this dilution was called the haemolysis titre.

2.2.1.4 Fast Liquid Protein Chromatography (FPLC)

A Pharmacia FPLC system fitted with an analytical Mono Q HR 5/5 column was used for evaluating the purity of enterolobin F-2 and F-3 fractions. The equilibration buffer was 20 mM tris-HCl pH 8.7. The column was eluted with a gradient of NaCl (0-0.1 M for 18 min, and 0.1-0.35 M for 1 min) in the same equilibration buffer. The flow rate was set to 1.0 ml/min. The absorbance was read on line at 280 nm.

2.2.1.5 Isoelectric Focusing

A method of isoelectric focusing (Wrigley, 1976) was employed to assess the state of purity of the further purified enterolobin fraction (F-4). The gel was prepared by mixing 4 ml of acrylamide/bisacrylamide (30:0.8), 1 ml Ampholine (LKB) (40 %), pH 6-8, 20 ml distilled water, 1 ml ammonium persulphate (1 %) and 15 ul TEMED. After the polymerization of the gel (15.0 x 15.0 x 0.2 cm), volumes of 5, 10 and 15 ul of enterolobin at 1 mg/ml in 10 % (w/v) sucrose were loaded into the wells. Hemoglobin was used as a marker of the focusing. The cathode solution was 0.4 % (v/v) triethylamine and the anodic solution, 0.2 % (v/v) sulphuric acid. The run was carried out at a voltage of 500 V for 3 h. The protein bands were then stained with 0.5 % (w/v) Coomassie Blue R-250 in methanol/acetic acid/water (45:5:40), and destained with this same mixture without Coomassie. The documentation of the results was done by both photography and laser densitometry. The laser densitometry scanning was performed in a LKB Ultroscan XL according to the manufacturer's recommendations.

photography and laser densitometry. The laser densitometry scanning was performed in a LKB Ultroscan XL according to the manufacturer's recommendations.

2.2.2 Determination of the Amino Acid Sequence of Enterolobin

2.2.2.1 Reduction and S-Carboxymethylation

The reduction and S-carboxymethylation of enterolobin (fraction F-4) was performed as described in Allen (1981). A sample of 20 to 30 mg of protein was dissolved in 200-300 ul of 6 M guanidine-HCl in 0.6 M tris-HCl pH 8.6, followed by the addition of 30 ul of 2-mercaptoethanol. The mixture was left under a stream of N_2 for 2-3 h, after which time, 300 ul of a freshly prepared solution of iodoacetic acid (0.286 g/ml in 1 M NaOH) was added. After 30 min of reaction in the dark at room temperature, the material was desalted in the dark by tangential dialysis against 10 l of distilled water. The sample was then freeze-dried.

2.2.2.2 Protein Cleavage Reactions

The enzymatic and chemical methods used for the cleavage of enterolobin into smaller peptide fragments were largely as described in Croft (1980), Allen (1981) and Aitken *et al.* (1988).

a) Trypsin (EC 3.4.21.4)

Trypsin is a proteolytic enzyme specific for the hydrolysis of lysyl and arginyl peptide bonds, except for lys-pro and arg-pro bonds. Occasionally, some cleavages occur at the carboxyl side of hydrophobic residues, due to chymotrypsin or ψ-trypsin contamination (Allen, 1981; Aitken *et al.* 1988).

The reduced and S-carboxymethylated protein (20 mg) was dissolved in 1 ml of 0.2 M N-ethylmorpholine-HCl pH 8.5 buffer.. Trypsin, dissolved in the NEM-buffer above, was added to yield a 2 % (w/w) enzyme/substrate ratio. After 3 h, at 37 °C, under agitation, the digest was submitted to gel filtration on Biogel P6 (Section 2.2.2.4 a). A small amount was used for peptide mapping by HPLC (Section 2.2.2.4 b).

b) Chymotrypsin (EC 3.4.21.1)

Chymotrypsin cleaves peptides bonds at the carboxyl side of tryptophan, tyrosine, phenylalanine, leucine and methionine residues. Occasionally, it cleaves at other sites. Cleavage does not occur where proline is following one of the above cited residues. The exact action of chymotrypsin is difficult to predict completely, and is partially dependent on the neighbouring residues (Allen, 1981).

The conditions of digestion by chymotrypsin were as described for trypsin above.

c) Elastase (EC 3.4.23.36)

Elastase, a serine protease, cleaves preferentially at small non-polar residues such as alanine and serine, but cleavages at glycine, valine and leucine have also been noticed (Aitken *et al.*, 1988)

The reduced and S-carboxymethylated enterolobin was dissolved in 750 ul of 0.1 M NH_4HCO_3 pH 8.1 to give a concentration of 30 mg/ml. Elastase was added at a 2% (w/w) enzyme/substrate ratio. The hydrolysis reaction took place at 37 °C, for 3 h, under agitation. After that, the mixture was applied to a BioGel P6 chromatography. A small amount was used for peptide mapping by HPLC.

d) Staphyloccocus aureus *V-8 protease (EC 3.4.21.19)*

The extracellular protease from *S. aureus* strain V-8 is specific for glutamyl peptide bonds, although it can cleave at aspartyl bonds under certain pH and buffer conditions (Aitken *et al.*, 1988).

For cleavage extended to aspartic acid residues, V-8 protease was added to RC-enterolobin (20 mg) in 1 ml of 0.1 M sodium phosphate buffer pH 7.8 containing 4 M urea to make 1% enzyme/substrate ratio. The reaction was allowed to occur for 48 h (with the addition of another same amount of enzyme after 24 h), at 37 °C, and under constant agitation. A small part of the mixture was applied to HPLC for peptide mapping, and the rest, submitted to gel filtration on BioGel P6.

e) Cyanogen bromide

Cyanogen bromide has been employed for many years for specific cleavage at the carboxyl side of methionine residues, albeit some other aspecific cleavages and side reactions can occur (Croft, 1980; Allen, 1981; Aitken *et al.*, 1988). Cleavages at tryptophanyl bonds have often been observed (Blumenthal *et al.*, 1975; Sanchez *et al.*, 1991).

Twenty mg (5 mg when for direct HPLC separation) of the reduced and S-carboxymethylated enterolobin were dissolved in 1 ml 70% (v/v) formic acid. A 500-fold molar excess of CNBr over methionine was added. The mixture was left standing for 36 h, at room temperature, in the dark, and under an atmosphere of nitrogen . Then, it was diluted in 15 ml of distilled water and freeze-dried twice and subjected to a gel-filtration on BioGel P 60 or reverse-phase HPLC chromatography.

2.2.2.3 Unblocking treatments

Two possible methods of unblocking obstructed N-terminal amino acids were attempted in order to remove the chemical blockage at the N-terminus of enterolobin. The first one was carried out with pyroglutamate aminopeptidase (EC 3.4.11.8) for the removal of pyrrolidone carboxylic acid (or simply pyroglutamic acid) residues from the N-terminal portion of proteins. The second one employed anhydrous TFA for unblocking proteins where the N-terminal residue has been acetylated.

a) Unblocking of pyroglutamyl N-terminal amino acid

This method was adapted from the one described in Zalut *et al.* (1980). Pyroglutamate aminopeptidase (400 ug) was dissolved in 1 ml of 0.1 M sodium phosphate pH 8.0 buffer, containing 10 mM EDTA and 0.7% (v/v) 2-mercaptoethanol. Half of the volume of the enzyme solution was added to 1 mg of enterolobin (fraction F-4) and the other half, to 3 mg of γ-casein (control). The reactions took place under nitrogen, at 4 °C for 24 h and at room temperature for 7 h. Then, the mixtures were dialysed and manually sequenced as in Section 2.2.2.5.

b) Unblocking of acetyl N-terminal amino acid

This reaction was performed according to a slightly modified protocol of Wellner *et. al* (1990). Two mg of enterolobin were dissolved in 50 ul of TFA under nitrogen, and left reacting for 3 min at 52 °C. The tube was then opened and dried for 5 min, at room temperature, under nitrogen, and for another 5 min at 52 °C. The tube was closed and left under nitrogen, for 40 h, at 52 °C. The protein was sequenced by the manual method (Section 2.2.2.5).

2.2.2.4 Separation of peptides

a) Gel filtration on BioGel

For the separation of groups of peptides produced by enzymatic cleavages a long column (1 x 200 cm) of BioGel P 6 was utilized. The equilibration and elution buffer was 50 mM NH_4HCO_3 pH 8.1. The flow rate was normally 10 ml/h, and the volume of each collected fraction was about 1.0 ml. The absorbances were read at 230

nm. The major peaks were lyophilised and stored for further separation by HPLC.

The larger peptides obtained from the CNBr reaction were applied to a column (1.6 cm x 90 cm) of BioGel P 60 in 70 % formic acid. The flow rate was 2.2 ml/h and the volume of the fractions was 1.1 ml. Alternatively, they were used directly for separation by HPLC.

b) High Performance Liquid Chromatography (HPLC)

HPLC was employed for peptide mapping and for the separation of peptides. A Varian model 5000 HPLC chromatography apparatus fitted with a reverse-phase C-18 Vydac 218TP5405 column (4.6 x 250 mm) from Technicol was used. Linear gradients of acetonitrile in 0.1 % TFA (v/v) were applied with slopes dependent on the complexity of the mixture of peptides. The samples were dissolved in 6 M guanidine-HCl in 0.1 % TFA for injection. The eluate was monitored at 214 nm.

2.2.2.5 Manual Sequencing

The DABITC/PITC double-coupling method (Chang *et al.* 1978; Chang, 1983) was routinely used for the sequence determination. The method was carried out as following:

a) Coupling:

The sample (at nmol level) was dissolved in 80 ul of 50 % aqueous pyridine solution (v/v) in a small pyrex tube (0.6 cm x 5 cm). After that, 40 ul (400 nmol) of DABITC (2.8 mg/ml in pyridine) was added. The mixture was purged with nitrogen, the tube was sealed, and

incubated at 52 °C for 50 min. After that, PITC (10 ul) was added and nitrogen flushed into the tube again. The tube was closed and mixed thoroughly (vortexed). The mixture was incubated for a further 20 min at 52 °C.

b) Washing:

The mixture was washed with heptane:ethyl acetate (2:1) (v/v) three times by adding the solvent, vortexing, separating the phases by brief centrifugation and aspirating off and discarding the upper (organic) phase. The lower (aqueous) phase was then dried exhaustively under vacuum.

c) Cleavage:

For peptides, 50 ul (100 ul for proteins) of anhydrous TFA was added to the tube which was flushed with N_2 and sealed. After 15 min at 52 °C, the TFA was removed by vacuum drying.

d) Extraction:

n-Butyl acetate (200 ul) and water (50 ul) were added, the tube was vortexed and centrifuged briefly. The upper (organic) phase was aspirated into a conversion tube. The organic extract and the remaining aqueous phase were then dried under vacuum. The dried "aqueous phase" was submitted to a further cycle.

e) Conversion:

The DABTZ-amino acid dried down from the organic extract was converted to the DABTH-amino acid by the addition of 50 ul of 50 % TFA to the conversion tube, which was sealed and incubated

at 80 °C for 10 min. After drying under vacuum, the DABTH-amino acid was ready for identification by TLC.

e) Identification

The identification of the DABTH-AA was performed by bidimensional TLC on 3 x 3 cm polyamide sheets. The sample and the marker (DABTIC-reacted diethylamine) were spotted at the bottom left corner of the sheet about 4 mm diagonally from the edge. The sheet was then chromatographed in the first solvent [acetic acid/water (2:1, v/v)] in a small covered beaker. After drying in a stream of warm air for 10 min, the sheet was then run in the second solvent [toluene/n-hexane/acetic acid (2:1:1, v/v/v)] perpendicularly to the first run. After drying the sheets, they were exposed to fumes of concentrated HCl making the DABTH-derivatives to become visible as coloured spots (red, purple or blue). The identification of the DABTH-AA spots was made by comparing them with standards (Fig. 1).

2.2.2.6 Automated Sequencing

An Applied Biosystems 477A automated protein sequencer coupled to a 120A analyser on-line was used for automatic sequencing of enterolobin and peptides. The sample was dissolved in 50% (v/v) TFA and directly loaded onto a glass fiber polybrene-treated membrane disc or blotted onto a PVDF membrane. The membrane disc was then set into the reaction chamber, and the machine run according to the manufacturer's instructions.

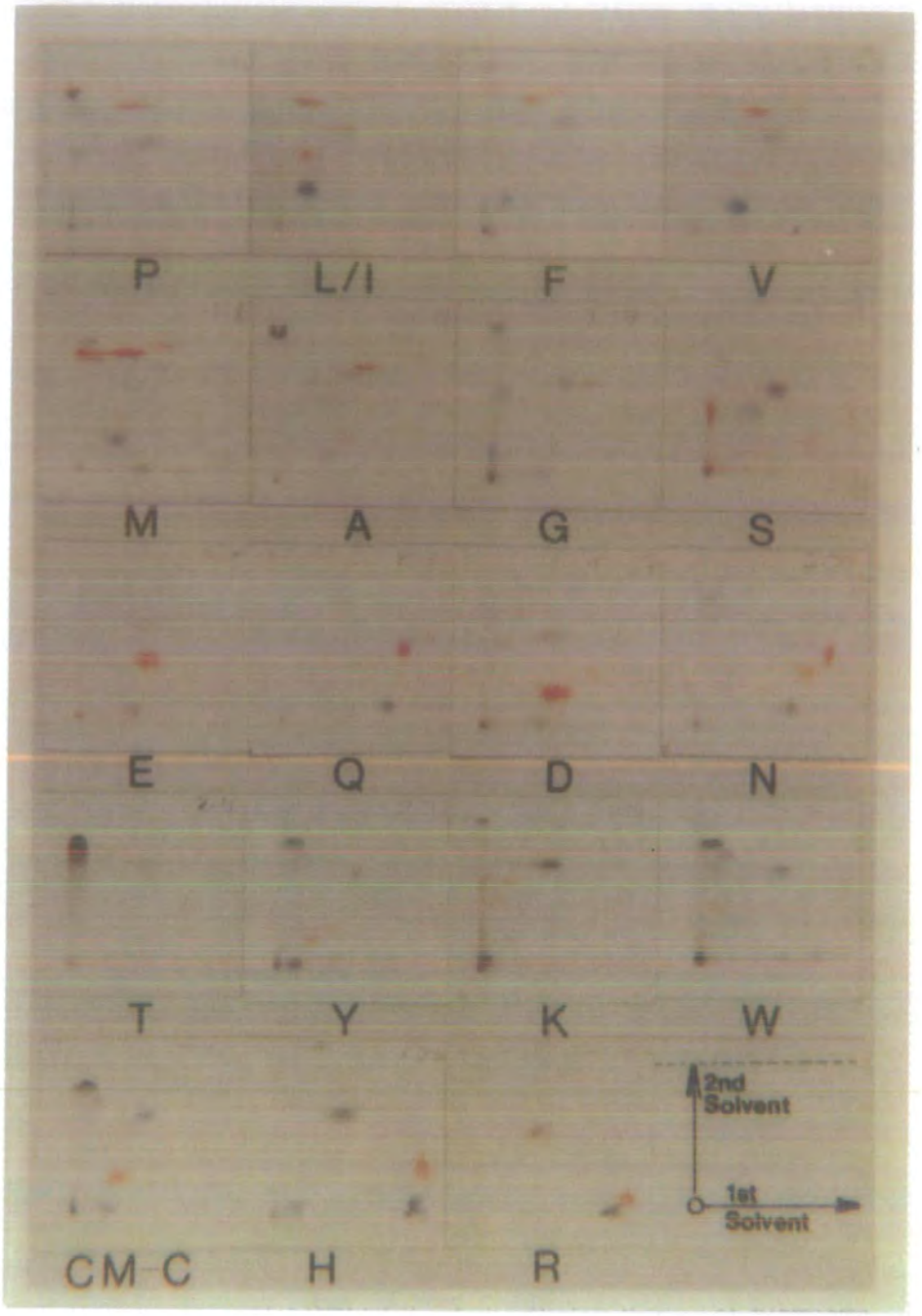


Figure 1: TLC of standards of DABTH-amino acid derivatives. The standards were produced by submitting the respective amino acids to the DABITC/PITC double-coupling sequencing method. The first solvent was glacial acetic acid:water (1:2 v/v). The second solvent was toluene:*n*-hexane:glacial acetic acid (2:1:1 v/v/v). The DABTH-derivatives are represented by the single letter code for amino acids. Photograph reprinted with the kind permission of Dr. A. Yarwood, Department of Biological Sciences, University of Durham.

2.2.2.7 Nomenclature of Peptides

The nomenclature of the peptides reflects both the method by which they were generated (first letter) and their order of elution during gel filtration (first number) and HPLC (second number). For peptides generated by CNBr digestions, only one number is used - numbers 1 to 4 for peaks obtained from gel filtration, and numbers 5 to 22 for peaks from HPLC separations.

Sequences obtained by overlapping of peptides are represented by the letters Ov followed by a number, as Ov-1, Ov-2, etc. The number refers exclusively to the chronological order in which the overlaps were found, having nothing to do with their position in the sequence of the protein.

Enzymes:	Trypsin	- T
	Chymotrypsin	- C
	Elastase	- E
	Staphylococcus aureus V-8 strain protease	- V
Chemical:	Cyanogen Bromide	- CN

For example, E-1.9 was a peptide obtained from the elastase digestion which appeared in peak 1 of the gel filtration and was eluted as peak 9 during HPLC.

For the CNBr digests, CN-1 to CN-11 are the peaks in the chromatogram originating from the gel filtration separation. The CNBr fragments separated by HPLC were called CN.H peptides. Thus the first peak was CN.H-1, the second, CN.H-2, and so on.

2.2.3 Computational Searches and Analyses of Peptide Sequences

2.2.3.1 Search of Matching Sequences

The sequenced regions of enterolobin obtained by the overlapped peptides (Ov) were searched for matches against the amino acid sequences of proteins deposited in the database PIR (Protein Identification Resource) databank release 26.0 (NBRF, National Biomedical Research Foundation, Washington, USA) by using the program FASTA version 1.4x (Pearson and Lipman, 1988). The program was run on an Apple Macintosh SE/30 microcomputer.

The program FASTA is a improved version of the program FASTP (Lipman and Pearson, 1985). FASTA is faster and more sensitive than its predecessor in making sequence searches through large databanks.

The search algorithm uses four steps for the determination of a score for pair-wise similarity between sequences. In the first step all identities or groups of identities between two DNA or protein sequences are located. Then, the ten best matched regions are used in the second step, which consists of rescoring these ten regions by means of a

previously determined scoring matrix as PAM250 (Dayhoff *et al.*, 1978) that allows for contributions such as conservative replacements. For each of these regions, a subregion with maximal score - initial region - is selected. The third step is the checking to see if several initial regions may be joined together, and the optimal alignment of initial regions as a combination of compatible regions with maximal score. The resulting score is used by the program to rank the library of sequences. In the last step, a final comparison considers all possible alignments of the query and library sequence that fall within a band centred around the highest scoring initial region. The result of this alignment is reported as the optimized score.

Other programs from the FASTA package were used. FINDP was used for finding sequences by key words in the PIR bank, and EXTRACTP was employed for downloading the sequences to the computer memory as readable files.

2.2.3.2 Pattern Search

Additionally, the overlapped peptides of enterolobin were searched against PROSITE version 6.10, February 1991, a database of known motifs or patterns identified in protein sequences (Bairoch, 1990). The program used for the search was MACPATTERN (Fuchs, 1991) written for running on Apple Macintosh microcomputers. The hardware used was an Apple Macintosh SE/30.

The algorithm utilized by the program for detecting patterns is based on the set-membership matrix concept (Cockwell and Giles, 1989), adopted for the pattern definition syntax of PROSITE.

Initially an index file must be created from the database to allow that the search be done faster. Then, after reading the index file, the program compares the query sequence to the patterns described in the database aiming to find any possible pattern in the sequence. The results are displayed in a separate window showing the patterns to which the sequence is related.

2.2.3.3 Alignment of Sequences

The alignment of the amino acid sequences of the aerolysins was performed by using the program CLUSTAL (Higgins and Sharp, 1989) running on a Apple Macintosh SE/30 microcomputer. Then, the peptides of enterolobin were manually aligned to the aerolysins sequences.

Firstly, the program calculates a matrix of similarity scores between all pairs of sequence to be aligned by the fast but approximate two-sequence alignment method of Wilbur and Lipman (1983). By using this matrix, it produces a hypothetical phylogenetic tree from the sequences by the UPGMA method (Sneath and Sokal, 1973). Then, the sequences are optimally aligned in groups corresponding to the branching order of the tree. The optimal alignments are carried out using the method of Myers and Miller (1988). The use of Myers and Miller's method made it possible to run a multiple alignment program, as CLUSTAL, on a microcomputer. With this method, the memory usage is a linear function of the length of the sequence, thus allowing it to be used in microcomputers, where there are limitations of memory space. The percentage of similarity between the aligned sequences was

calculated by the program ALIGN from the FASTA package. This program also uses the algorithm described in Myers and Miller (1988).

2.2.3.4 Determination of the Hydropathy Profile

The programs PLOT.A/HYD5, PLOT.A/H3 and PLOT.A/HYD from the compiled version of the MACPROT package (Markiewicz, 1990) were used for the analyses of the hydropathies of the amino acid sequences of aerolysins and the predicted cytolytic sites. This program calculates and plots the hydropathy profile of a sequence on the basis of given published hydropathy scales. An Apple Macintosh SE/30 was utilized for running the program.

The first scale used was a "consensus" scale (Eisenberg *et al.*, 1984) based on: (a) a theoretical scale to describe the energetic effects of the transfer of amino acids side chains to a hydrophobic from a hydrophilic phase; (b) the fraction of each type of residue that is found buried in globular proteins; (c) the distribution of residues between the interior and the exterior in the protein; (d) the free transfer energy from the aqueous phase to vapour. This consensus scale is normalized to the mean of 0.0 for 20 amino acids going from -2.53 (most hydrophilic) to 1.38 (most hydrophobic).

The other scale was that of Argos *et al.* (1982). This scale was the resultant from several sets of characteristics of amino acids as well as the previous scale: (a) the distribution of residues between the interior and exterior in the protein; (b) the estimated standard free energies of transfer for a residue in a helix in water to a helix in the non-aqueous phase; (c) the conformational propensity parameters for residues for the reverse turn configuration; (d) the bulkiness of residues; (e) the polarity of residues. The authors list the values

normalized to a mean of 0.0 for the above properties and use the added values in their analysis. For the current program, the scale goes from -4.92 (most hydrophilic) to 4.1 (most hydrophobic).

2.2.4 Kinetics of haemolysis

A continuous haemolytic assay was developed for the analysis of the kinetics of lysis of human red blood cell by enterolobin (fraction F-3).

A Perkin-Elmer model 554 double-beam spectrophotometer was employed in this method. The wavelength was set to 680 nm, the slit aperture was 2.0 and the recorder speed, 0.5 cm/min. All the experiments were performed at room temperature.

Four times washed RBC were daily prepared in 150 mM NaCl in 10 mM tris-HCl pH 7.4 (tris-buffered saline) to give a % T around 10.0. From this suspension, a haemolysed preparation (100% T) was made by lysing the red cells with water. Two 1 cm pathlength cuvettes were filled as follow. The control cuvette had 2.5 ml of the haemolysed RBC and 100 ul of the haemolytic sample (enterolobin fraction F-3 at 5 mg/ml). The control transmittance was then adjusted to 100%, corresponding to 100% haemolysis. The sample cuvette took 2.5 ml of RBC suspension and 100 ul of the enterolobin solution. This was quickly added and followed by rapid agitation with a Pasteur pipette to start the process. At this moment, the recorder was switched on. The transmittance was both automatically and manually (every 30 s) recorded.

Possible effectors such as Ca^{2+}, EDTA, galactose, choline, phosphatidylcholine, cholesterol and ricin were tested. Prior to the kinetic assays, suspensions of red cells (3% v/v) were prepared in 150 mM NaCl in 10 mM tris-HCl pH 7.4 containing the above effector substance (concentrations precisely specified in the legends of the figures in Section 3.7 of Results and Discussion). Enterolobin was also prepared in the same tris-buffered saline as the red cells, and kept in ice prior to use.

Trypsinised RBC were also used. They were produced by treating a 3% (v/v) suspension of red cells with 250 ug of trypsin from bovine pancreas (type III, Sigma) in 2.5 ml of tris-buffered saline for 30 min, at 37 °C, under gentle agitation. Then the suspension of red cells was washed five times with tris-buffered saline, and prepared as described in the above paragraphs. The cells were also, in an experiment, incubated in the presence of BTCI (1 mg), a trypsin and chymotrypsin inhibitor (Morhy and Ventura, 1987), to inhibit any remaining active trypsin after the trypsinisation.

2.2.5 Microscopic Observations of Enterolobin- and Gold-Enterolobin-treated Membranes

2.2.5.1 Optical Microscopic Observation of Haemolysis by Enterolobin

One drop of enterolobin (fraction F-3) at 1 mg/ml was mixed on a glass slide with one drop of 3% (v/v) suspension of erythrocytes, and immediately placed on the plate of a Nikon

microscope fitted with differential interference contrast filter (Nomarski optics). The haemolysis process was recorded on video-tape and photographed every 1 min until completion.

2.2.5.2 Labelling of Enterolobin by Colloidal Gold

Colloidal gold with average particle size of 12 nm (gold12) was prepared by reduction of $HAuCl_4$ with sodium ascorbate as described in Slot and Geuze (1984). Distilled water (5 ml), 1% $HAuCl_4$ (1 ml) and Na_2CO_3 pH 7.5 (1 ml) were put together at 0 °C. While mixing, 1 ml of 0.7% sodium ascorbate was added. A purple red colour formed in the solution. Then, the volume was adjusted to 100 ml with distilled water, heated to boiling, and left standing until cool.

The lowest protein concentration required to stabilize the colloidal gold (maintenance of its red colour) was pre-determined in a microtitration assay by following the guidelines of Hodges *et al.* (1984). In an immunological microtitration plate, 50 ul of enterolobin (fraction F-3) at a initial concentration of 1.0 mg/ml was two-fold serially diluted. To each well, 50 ul of gold colloid was added and the plate agitated by tapping. After 15 min, 10 ul of 10% NaCl was added to make a final concentration of 0.9% NaCl. The plate was agitated and, 5 min later, the stabilization point was read. This was the lowest enterolobin concentration in which the colour of the solution remained red. The controls were enterolobin plus gold plus water instead of NaCl solution (control 1) and water instead enterolobin plus gold plus NaCl solution (control 2).

The final protocol for gold labelling of enterolobin was then developed so that the final solution remained stable (red) at isotonic NaCl concentration, enterolobin kept its haemolytic activitity in a haemolytic concentration. In this way, 1 ml of colloidal gold was mixed with enterolobin F-3 (2.2 mg/ml in water), and 200 ul of 10% NaCl was added under agitation. The final mixture was gold-enterolobin (1.0 mg/ml) in 0.9% NaCl in 450 nM Na_2CO_3 pH 7.5. Gold-ovalbumin was also prepared in the same way to be used as a control.

2.2.5.3 Electron Microscopic Observation of Haemolysis by Enterolobin and Gold-enterolobin

A 1 ml sample of enterolobin (F-3) at 1 mg/ml was mixed with 1 ml of a 3% RBC suspension. At 2, 15 and 30 min of reaction, aliquots of 0.4 ml were removed. The control was RBC plus the same volume of saline. After the given times, the samples were washed 3 times in saline, and fixed by 0.5 ml 1% glutaraldehyde for 30 min. They were then washed 3 times with water, centrifuged, resuspended in a small volume, and mounted on Formvar-coated 200 mesh copper EM grids. After 5 min, any excess liquid was removed by blotting with filter paper. When dry, the samples were examined in a Phillips 400 electron microscope. Photomicrographs were taken using the automatic exposure setting.

The reaction of haemolysis by gold-enterolobin and the sample preparations were also carried out as above. Gold-ovalbumin was used as a control.

2.2.6 Cytotoxicity of Enterolobin to Cancer Cells

The cytotoxicity effect of enterolobin was tested on the standard and commonly used L929 and MRC5 fibroblast-like cell lines following a slightly modified method of Matthews and Neale (1987) as below.

Volumes of 100 ul of cell suspension (5 x 10^5 cells/ml of culture medium) were dispensed into the wells of a 96-well flat-bottom microtitration plate, leaving one row of eight wells without cells as a blank. A two-fold serial dilution of the protein sample was made in culture medium containing 2 ug/ml of actinomycin D, and 100 ul of each dilution was added in duplicate to the cells. Enterolobin fractions F-2 and F-3 were used at initial concentrations of 2 mg/ml and 500 ug/ml respectively. TNF-α, Tumor Necrosis Factor-α (from NIBSC), at an initial concentration of 100 U/ml (1 U = quantity of TNF that causes 50% death of L929 cells in standard assay), was the positive control. The negative control was tissue culture medium alone.

Then, the microtitre plate was incubated at 37 °C in a gassed incubator (5% CO_2 in air). After 3 days of incubation the assay was terminated by washing the plate with PBS, fixing for 15 min with formalin acetate fixative (90 ml 9% acetic acid, 8.2 g of 0.1 M sodium acetate, 100 ml 10% formalin and 1 l of water), staining for 30 min with Amido Black stain (same solution as the fixative less formalin plus 0.5 g of Amido Black) and drying it at 37 °C. After adding 100 ul of 0.38% NaOH to each well, the titres of cytotoxicity (inverse of the highest dilution of protein causing death to the cells) were recorded, and

the absorbances were read at 620 nm in a microplate reader. These assays were made in duplicate.

Videorecords and photographs of the effects of enterolobin and TNF-α on L929 cells were taken in a Nikon microscope using differential interference contrast (Nomarski optics).

L929 cells (5 x 10^5 cells/ml of culture medium) were incubated with TNF (100 U/ml) and enterolobin (500 ug/ml). At 0, 2 and 24 h of incubation, shots were taken in order to document the cytotoxic effects of these two proteins on the cells.

2.2.7 Toxicity of Enterolobin to Insect Larvae

2.2.7.1 Insect Feeding Trials

The effects of enterolobin on the survival and development of insect larvae were assessed by feeding them with artificial diets in which the protein had been incorporated. The two species used were the bruchid *Callosobruchus maculatus* (Coleoptera) and cotton worm *Spodoptera littoralis* (Lepidoptera).

The *C. maculatus* assays were performed using artificial seeds as in Gatehouse and Boulter (1983). The concentrations (% wt of sample/dry wt of diet) of enterolobin were 0.25%, 0.5%, 1.0%, 2.5%, 5.0%, 7.5% and 10% for fraction F-1, and 0.01%, 0.025%, 0.05%, 0.1%, 0.25% for fraction F-2. The control seeds contained no enterolobin . The required amount of enterolobin solution for a given

given concentration was added to 25 g of sieved chickpea meal followed by 24 ml of distilled water. The mixture suspension was homogenized and freeze-dried.

When dry, a small amount of water was added to the mixture, and five artificial seeds prepared for each concentration of protein. The artificial seeds were then dried over silica gel and equilibrated for seven days at 27 ºC, 70% rh. After that, they were individually covered with thin PVC plastic film and left in a *C. maculatus* colony for oviposition for 24 h and placed in individual glass vials closed by a filter paper stopper. The numbers of emerged adult insects were recorded daily. Forty two days after initial oviposition, the assays were terminated and the numbers of surviving larvae and pupae inside each pellet were also recorded.

For the *S. littoralis* trials, 300 ul of enterolobin solution were applied to a communion wafer to make a final concentration of 5% (w/w) for fraction F-1 and 0.1% (w/w) for fraction F-2. The wetted wafer was then offered to a third stage larva as the only food source. The wafer was replaced when totally consumed. Ten replicas (one larva/replica) were used for F-1 and six for F-2. Controls consisted of wafers wetted by water with no protein. The number of surviving larvae was recorded daily.

2.2.7.2 Assays of Enterolobin Digestion In Vitro

In vitro digestion assays of enterolobin by larval gut extracts were performed according to Gatehouse et al. (1990). The digestive tracts of late instar *C. maculatus* and third instar *S. littoralis* larvae were dissected at 0 °C in 1 mM DTT for *C. maculatus* (33

guts/100 µl), or water for *S. littoralis* (4 guts/ml). The guts were homogenized, centrifuged, and held on ice until required. For the *C. maculatus* assays, 40 ul of the larval gut extract were added to 80 ul of enterolobin (2 mg/ml in McIlvane's 0.2 M citrate-phosphate buffer pH 5.4) and 100 ul of the same buffer. For *S. littoralis*, the assay was carried out in the same way, but in 0.2 M glycine buffer pH 9.5. The mixtures were incubated at 33 ºC for time periods of 0, 1, 2, 4, 8, 24 and 48 h.

At the given time intervals, the reactions were stopped by the addition of an equal volume of SDS sample buffer [0.4 M tris-HCl pH 6.8, 4% (w/v) SDS, 20% (w/v) sucrose] and boiled for 2 min. The controls comprised enterolobin without gut extracts incubated at both pH values in the same conditions as above for 48 h. The samples were analysed by SDS-PAGE on mini-slab gels, pH 8.8, in the presence of 1% (v/v) 2-mercaptoethanol, followed by staining with Coomassie Blue R-250 (Laemmli, 1970). The proteolytic activities of the gut extracts were ascertained by using myoglobin as substrate under the same assay conditions of reaction as above.

2.2.7.3 Electron Microscopy Observation of Larval Guts

The ultrastructure of *C. maculatus* larvae gut cells was examined by transmission electron microscopy to evaluate any damage that could have been caused by enterolobin.

In a small volume beaker, 2 mg of enterolobin F-2 was mixed with 2 g of sieved chickpea meal to give 0.1% (w/w) protein.

The mixture was homogenized in 5 ml of water, freeze-dried, and left for 7 days for equilibration in an insectary at 27 °C, 70% r.h.. The control meal was devoid of enterolobin. The larvae were placed in contact with the meals for 6, 12, 24 and 48 h in the insectary. After each of these times, 6 larvae were removed and the insect digestive tracts were dissected into Karnovsky fixative (Karnovsky, 1965). The samples were fixed according to Karnovsky (Karnovsky, 1965) as follows.

The fixative was composed of two solutions. Solution A is made of 2 g of paraformaldehyde in 40 ml of warm water plus 2-6 drops of 1 N NaOH. Solution B was prepared with 10 ml of 25% gluteraldehyde and 50 ml of 0.2 M sodium cacodylate buffer pH 7.3. The solutions were kept separate and at 4 °C until just before use, when they are mixed well. The specimen was fixed in Karnovsky fixative for 1-1.5 h at 4 °C, and post-fixed in 1% buffered osmium tetroxide (2% osmium tetroxide mixed with an equal volume of 0.2 M sodium cacodylate pH 7.3). The sample was then dehydrated by three changes of 5 min in 70% and 95% ethanol and three of 10 min in 100% ethanol; infiltrated with three changes of 10 min in an intermediate solution of 100% ethanol/propylene oxide (1:1) and in propylene oxide only; infiltrated at 45 °C with propylene oxide/araldite (1:1) for 30 min and pure araldite for another 30 min. The sample was orientated and embedded in a suitable mould, covered with araldite and left polymerizing for 12 h at 45 °C and 24 h at 60 °C. Afterwards, the specimen was sectioned using an ultramicrotome, mounted on copper grids covered by Formvar film, and stained with lead citrate and uranyl acetate. A Philips 400 electron microscope was used for the examination of the samples.

3. Results and Discussion

3.1 Purification of Enterolobin

Enterolobin was purified by performing a slightly modified method based on Sousa and Morhy (1989). The method involved extraction in 0.15 M NaCl, fractionation with ammonium sulphate between 0 to 33% saturation, batch separation by adsorption on DEAE-celullose and gel filtration chromatography on a column of Sephadex G-150 (Fig. 2). The haemolytic activity was found in the larger peak of the chromatogram.

The pooled and freeze-dried material from this peak normally yielded a product after Sephadex gel filtration (fraction F-3) which was homogeneous (i.e., single band) when examined by gel electrophoresis under both non-denaturing and denaturing conditions, and stained by either Coomassie Blue or silver nitrate (Sousa, 1988; Sousa and Morhy, 1989).

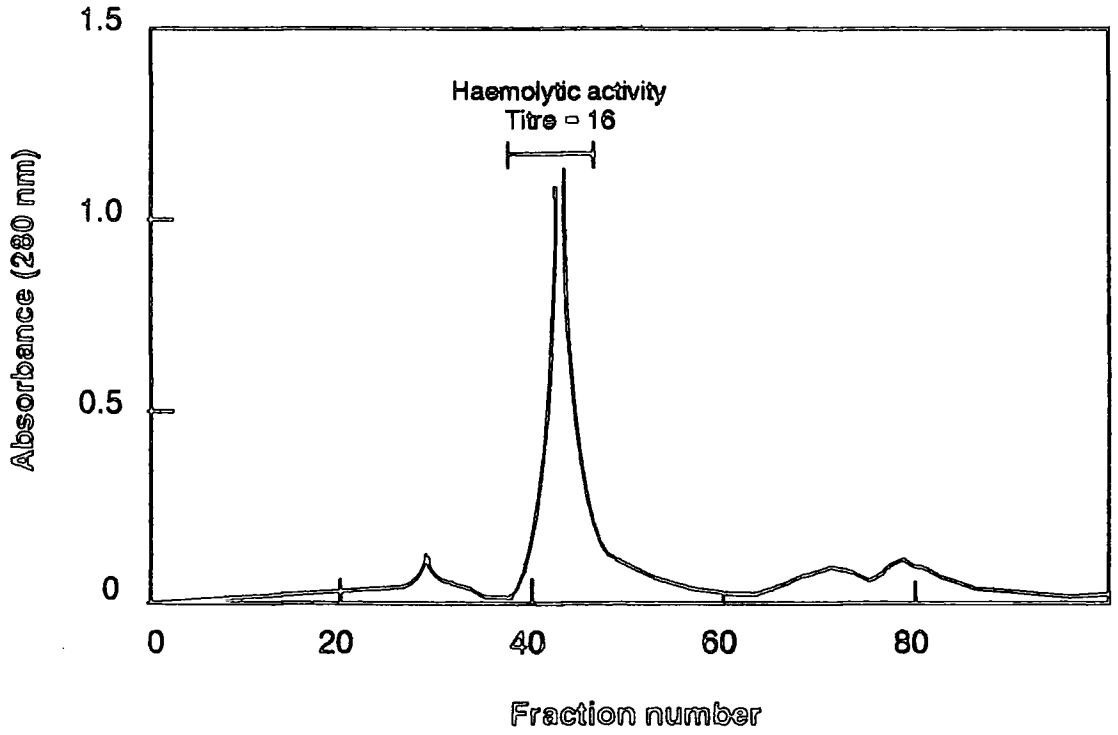


Figure 2: Gel filtration of enterolobin F-2 on Sephadex G-150. The column of Sephadex (1.9 x 40 cm) was equilibrated and eluted with 50 mM Tris-HCl pH 8.0. The sample was 30 mg of enterolobin (fraction F-2) dissolved in 1.5 ml of the same Tris-HCl buffer. The flow rate was 15 ml/h, and the volume of each fraction was 3 ml. (———) refers to A_{280}, and the peak with haemolytic activity is indicated in the figure with its titre of haemolysis. The peak with activity was collected and freeze-dried as enterolobin fraction F-3.

However, on some occasions, when the fraction F-3 was analysed by FPLC on a MonoQ column, it was found to contain some minor contaminants (Fig. 3). One should observe that the level of contamination was already extremely low in comparison with the peak carrying the haemolytic activity. The automatic integration of the peak carried out by the FPLC chromatographer computer demonstrated that the haemolytic peak was responsible for 91% of the total area.

Due to the very low level of the impurities, their detection by the sequencing methods used would be negligible and would not interfere with the correct interpretation of the results. A blind test performed concomitantly with several protein sequencing laboratories came to demonstrate that, on average, a 20% molar contamination in a sample represents no problem for making accurate assignments of the residues (Speicher *et al.*, 1990).

Nevertheless, since the aim was to obtain enterolobin as pure as possible for the work of determination of the sequence of enterolobin, and in order to avoid any doubt during the interpretation of the results, the protein was submitted to an additional step of purification. It consisted of a further anion-exchange chromatography on a DEAE-Sepharose column (Fig. 4). The peak containing the haemolytic activity was the larger one, and was eluted between 0.06 M and 0.09 M NaCl.

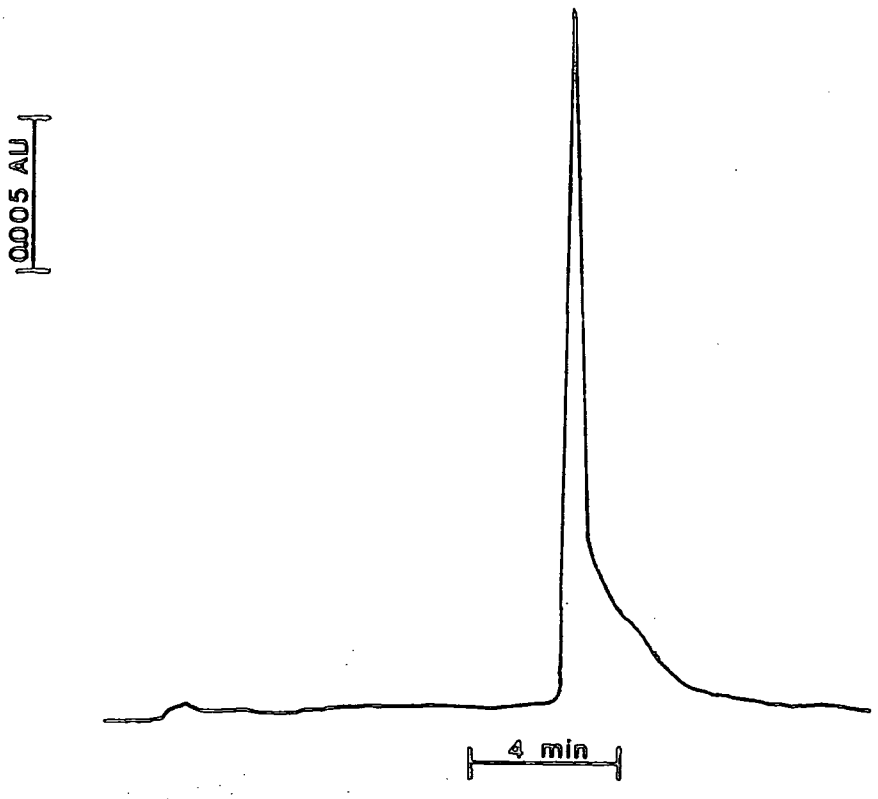


Figure 3: FPLC chromatography of enterolobin F-3. An aliquot (25 µl) from the pooled sample from the gel filtration chromatography was applied to a Mono Q HR 5/5 column equilibrated with 20 mM tris-HCl pH 8.7, and eluted with a gradient of NaCl (0-0.1 M in 18 min and 0.1-0.35 in 1 min). The flow rate was 1.0 ml/min, and the absorbance was recorded continuously at 280 nm at AUS 0.05.

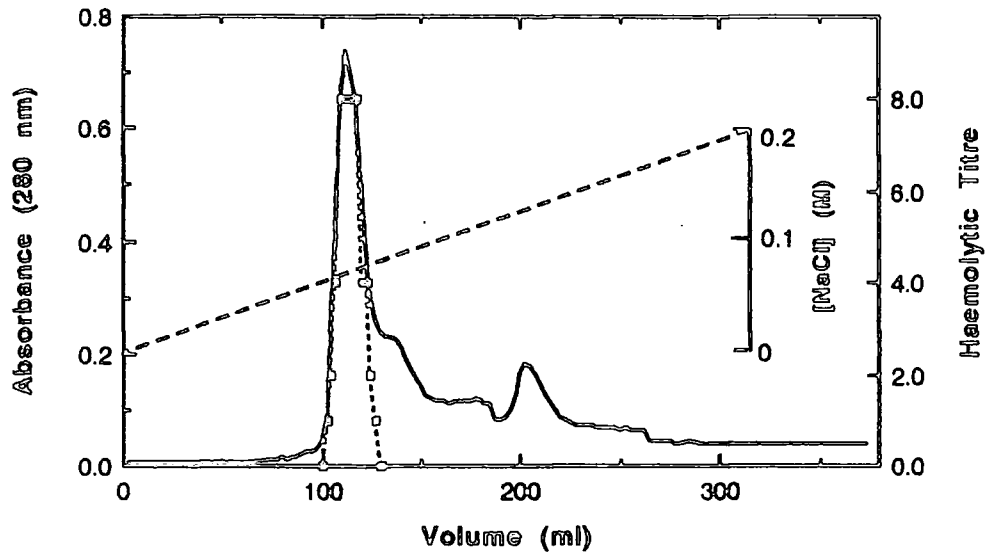


Figure 4: DEAE-Sepharose chromatography of enterolobin F-3. The pooled sample (20 mg dissolved in 3 ml of equilibration buffer) from the gel filtration chromatography was applied to a column (2.5 x 15 cm) equilibrated with 50 mM tris-HCl pH 8.5, and eluted with a linear gradient (- - -) of NaCl (0-0.25 M) at a flow rate of 37.5 ml/h. The absorbance (——) of the fractions was read at 280 nm. The haemolytic activity (-□--□-) was determined in a microtitration plate assay. The active peak was pooled and freeze-dried as enterolobin fraction F-4.

A sample from the pooled and freeze-dried haemolytic peak (no.1) from the DEAE-Sepharose chromatography was subjected to isoelectric focusing in a narrow range of pHs (6 to 8) (Fig. 5). Enterolobin has a pI=7.0 (Sousa and Morhy, 1989), and this characteristic was once more confirmed here. Only a single strong band was detected on the gel after staining with Coomassie Blue. The resulting stained gel was then scanned by a LKB laser densitometer (Fig. 6) in order to provide further evidence for the homogeneous state of enterolobin. This pure condition was thoroughly confirmed by the laser scanning, as long as just a single peak was detected by this rigorous analytical method. Enterolobin was then proved to be in a highly pure state suitable for initiating the sequencing work.

An attempt was made to use reverse-phase HPLC (Vydac C-18 column eluted with a gradient of 0 to 100% acetronitrile in 0.1% TFA) for the purification of enterolobin. However, no peaks were eluted from the column even at the higher acetonitrile concentrations. In fact, it was later shown in a quick test tube experiment, in which aliquots of enterolobin (in aqueous solution) were added to a range of increasing concentrations of acetonitrile (0 to 100% in 10% intervals) in 0.1% TFA, that enterolobin precipitates at acetonitrile concentrations as low as 20%.

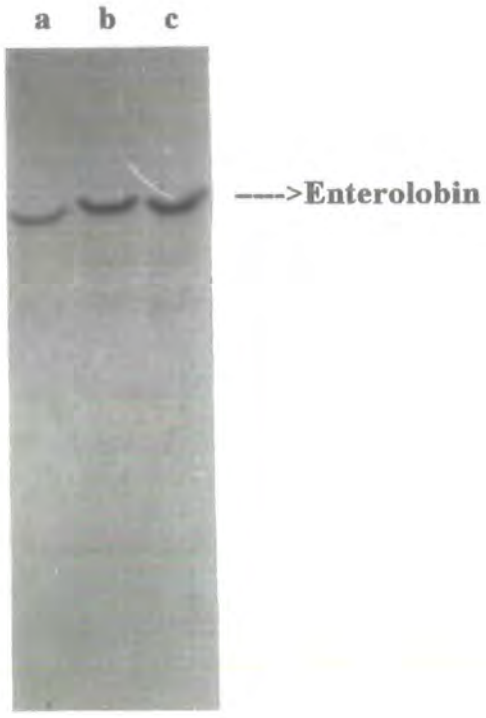


Figure 5: Isoelectric focusing of enterolobin F-4. The gel was a 7.5% acrylamide gel with a pH gradient from 6.0 to 8.0. Volumes of 5 (lane **a**), 10 (lane **b**) and 15 µl (lane **c**) of enterolobin F-4 (from the DEAE-Sepharose chromatography) at 1 mg/ml in 10 % sucrose were applied. Hemoglobin was used as marker of the focusing. The cathode solution was 0.4 % triethylamine and the anodic solution, 0.2 % sulphuric acid. The run was carried out at a voltage of 500 V for 3 h. The gel was stained with Coomassie Blue R-250, and was scanned by a LKB laser scanner..

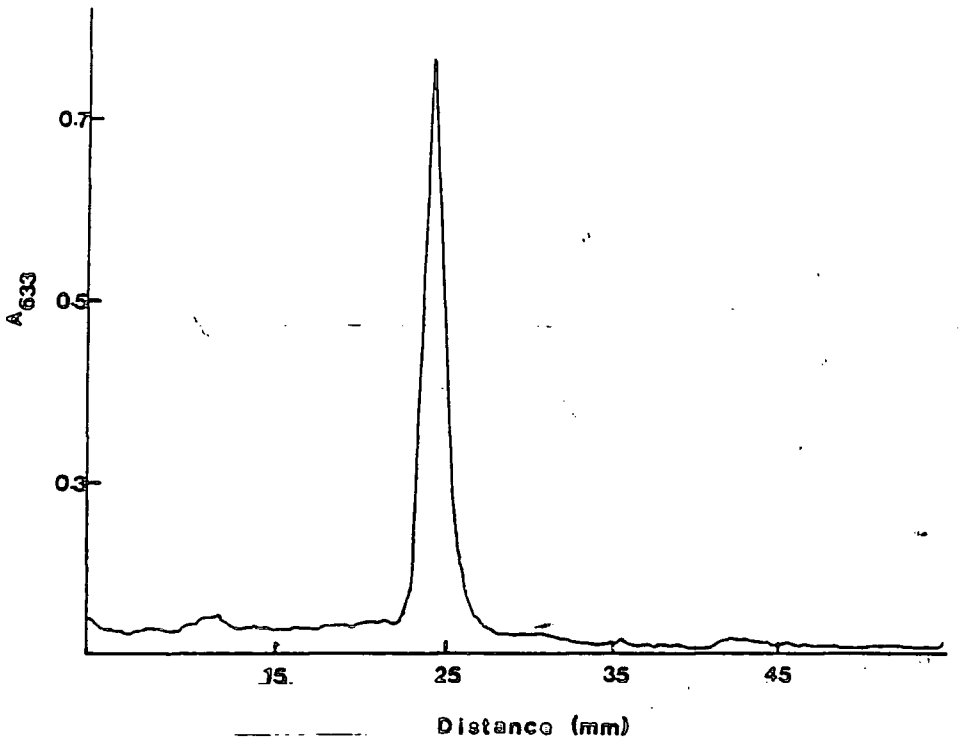


Figure 6: Densitometric scanning profile of isoelectric focusing gel of enterolobin F-4. The gel was scanned by a LKB Ultroscan XL after being stained with Coomassie Blue R-250. The figure represent the densitometric profile of lane c (15 ug of enterolobin F-4) from the gel in Figure 5. The absorbance (—) was read at 633 nm.

It was not unexpected that enterolobin would interact strongly with the Vydac C-18 hydrophobic adsorbent. Enterolobin is a high molecular weight protein (55 KDa by SDS-PAGE) (Sousa and Morhy, 1989), and such firm interactions of proteins with hydrophobic coatings can commonly occur, especially when C-18 packings are used in the columns (Roe, 1989), and has actually been observed for large proteins on several occasions in the Laboratories of Protein Chemistry of the Universities of Durham and Brasília. Also, enterolobin could have suffered denaturation and precipitation inside the column when the concentration of acetonitrile reached 20% during the application of the eluting gradient.

The amino acid composition (Table 4) of enterolobin (Sousa and Morhy, 1989) shows that there is a higher percentage of hydrophilic amino acid residues than hydrophobic ones. Besides, enterolobin has always been soluble in aqueous solutions. It suggests that enterolobin is a hydrophilic protein overall. Thus the precipitation caused by 20% acetonitrile is not surprising.

3.2 Peptide Maps

A series of preliminary experiments were carried out to determine which were the most suitable enzymatic and chemical methods for cleaving enterolobin to generate peptides applicable for amino acid sequencing. Small samples (1 mg) in the appropriate buffers were digested separately for the required times with 2% (w/w) of trypsin, chymotrypsin, V-8 protease (amount of protease doubled at 24 h

Table 4:

Amino Acid Composition of Entelorolobin*

Amino Acid	Percentage (%)
Ala	6.0
Arg	4.3
Asx	14.4
Cys	1.2
Glx	9.2
Gly	6.3
His	1.7
Ile	3.2
Leu	6.9
Lys	6.9
Met	0.9
Phe	4.9
Pro	3.4
Ser	9.5
Thr	8.9
Trp	3.4
Tyr	3.7
Val	5.2

* Sousa and Morhy (1989)

of digestion) and elastase. Enterolobin samples were also subjected to cleavages with a 500-fold excess of CNBr. The products of these cleavages were analysed by reverse-phase HPLC on an analytical C-18 Vydac column with gradients from 0 to 70 % acetronitrile in 0.1 % TFA. The peptide maps obtained are shown in Figs. 7 to 11.

The largest number of peaks (58 peaks) was produced by digestion with chymotrypsin (Fig. 7). This was expected since the amino acid composition analysis of enterolobin (Table 4) indicated that the molecule contains a elevated percentage of residues where chymotryptic cleavage can occur such as leucyl, tryptophanyl, tyrosyl and phenylalanyl. Chymotrypsin is well known as a proteolytic enzyme that is specific for cleaving on the C-terminal side of hydrophobic residues, especially phenylalanine, tryptophan, tyrosine, and leucine in order of reducing susceptibility (Aitken *et al.*, 1989).

Likewise, plausible figures for the number of peaks from the cleavages with elastase (49 peaks) (Fig. 8) and trypsin (34 peaks) (Fig. 9) were recorded. It is also in agreement with the amino acid composition of the cytolytic protein (Table 4) and the possible sites for cleavage by these enzymes. The amino acid analysis shows that there are a high percentage of residues that would be suitable sites for digestion with trypsin (mainly at Arg and Lys residues) and elastase (mainly at Ala and Ser residues) (Aitken *et al.*, 1989).

Not such expected results were obtained with the V-8 protease (Fig. 10). The enzyme, which is specific for glutamyl and aspartyl bonds (Aitken *et al*, 1989), yielded only 9 peaks during the peptide mapping. This is not in accord with the amino acid composition (Table 4) which shows aspartic and glutamic acid residues at a high

percentage in the protein. The availability of these residues was further confirmed by the amino acid sequence of the several peptides from enterolobin (Section 3.4). Consequently, a large number of peptides was to be expected from the incubation of enterolobin with the V-8 protease. However, there are reports in the literature stating that V-8 protease does not always attack all acidic residues even after prolonged incubation (Vandekerckhove and van Montagu, 1977), suggesting that the local amino acid environment may exert some influence on the efficiency of the hydrolytic reaction by V-8 protease. Additionally, the experience gathered at the Laboratories of Protein Chemistry of the University of Durham and the University of Brasília reinforce these observations on the V-8 protease.

From the chemical digestion with cyanogen bromide (CNBr), one should expect four peptides if the reaction had been completely specific for only the three Met residues found in the amino acid composition of enterolobin (Sousa and Morhy, 1989). However, the peptide map of the mixture of peptides obtained from the CNBr digestion of enterolobin showed at least 18 peaks in its profile (Fig. 11). It is known that CNBr can also cleave polypeptides at sites other than Met especially when large excesses of this reagent are used as was the case for the cleavage of enterolobin. These cleavages have been observed principally at Trp sites of some proteins (Blumenthal *et al.*, 1975; Sanchez *et al.*, 1991), and could have happened for enterolobin as well. Also the strongly acid conditions of the reaction can cause cleavages at Tyr and Trp residues occasionally (Aitken *et al.*, 1989).

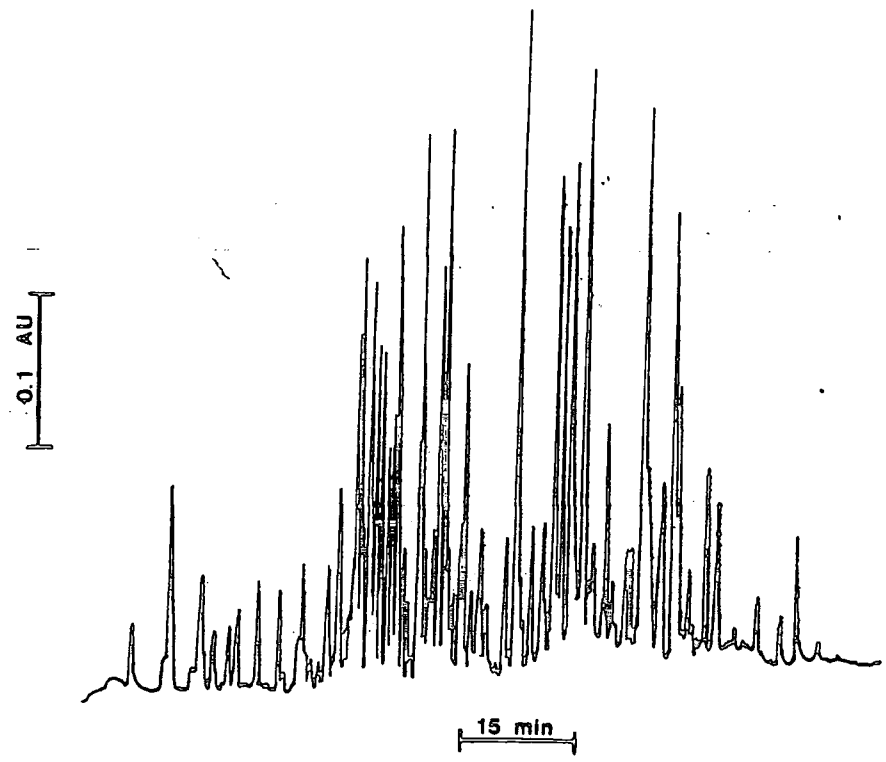


Figure 7: HPLC peptide map of enterolobin digested by chymotrypsin. RC-enterolobin F-4 was digested by chymotrypsin as described in Material and Methods. An aliquot of 500 µl (dissolved in 6 M guanidine-HCl in 0.1 % TFA) was injected to a reverse-phase C-18 Vydac column (4.6 x 250 mm) equilibrated with 0.1 % TFA. A linear gradient from 0 to 40 % acetronitrile in 0.1 % TFA (v/v) for 100 min was used to elute the peptides.The flow rate was 1.0 ml/min. The eluate was monitored at 214 nm at AUS = 0.5.

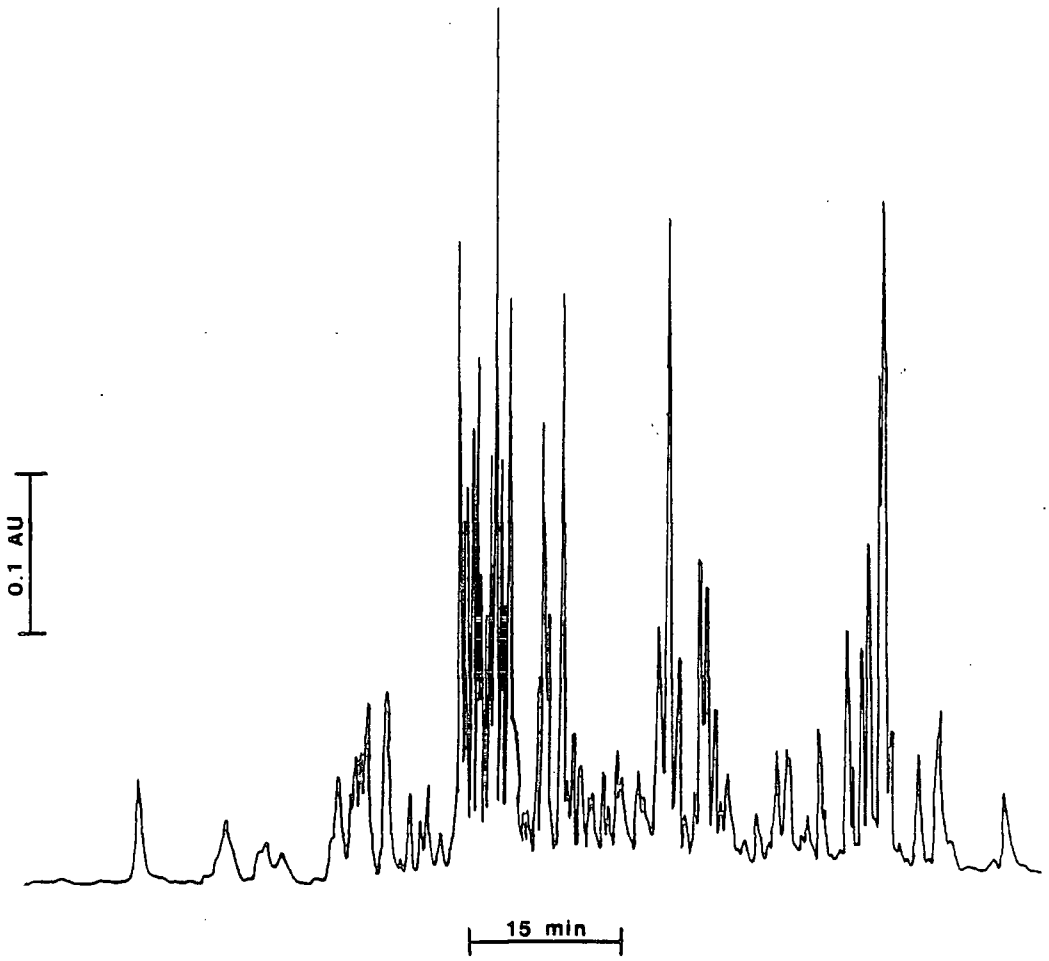


Figure 8: HPLC peptide map of enterolobin digested by elastase. RC-enterolobin F-4 was digested by elastase as described in Material and Methods. An aliquot of 500 µl (dissolved in 6 M guanidine-HCl in 0.1 % TFA) was injected to a reverse-phase C-18 Vydac column (4.6 x 250 mm) equilibrated with 0.1 % TFA. A linear gradient from 0 to 40 % acetronitrile in 0.1 % TFA (v/v) for 90 min was used to elute the peptides. The flow rate was 1.0 ml/min. The eluate was monitored at 214 nm at AUS = 0.5.

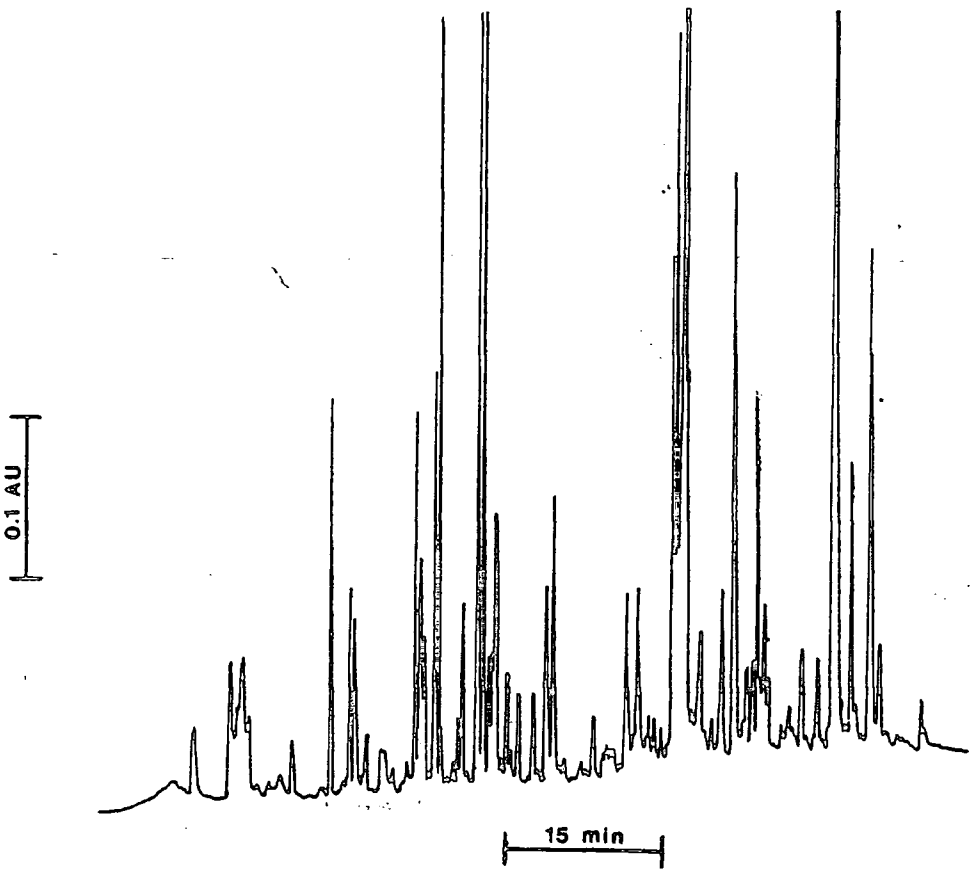


Figure 9: HPLC peptide map of enterolobin digested by trypsin. RC-enterolobin F-4 was digested by trypsin as described in Material and Methods. An aliquot of 500 µl (dissolved in 6 M guanidine-HCl in 0.1 % TFA) was injected to a reverse-phase C-18 Vydac column (4.6 x 250 mm) equilibrated with 0.1 % TFA. A linear gradient from 0 to 40 % acetronitrile in 0.1 % TFA (v/v) for 90 min was used to elute the peptides. The flow rate was 1.0 ml/min. The eluate was monitored at 214 nm at AUS = 0.5.

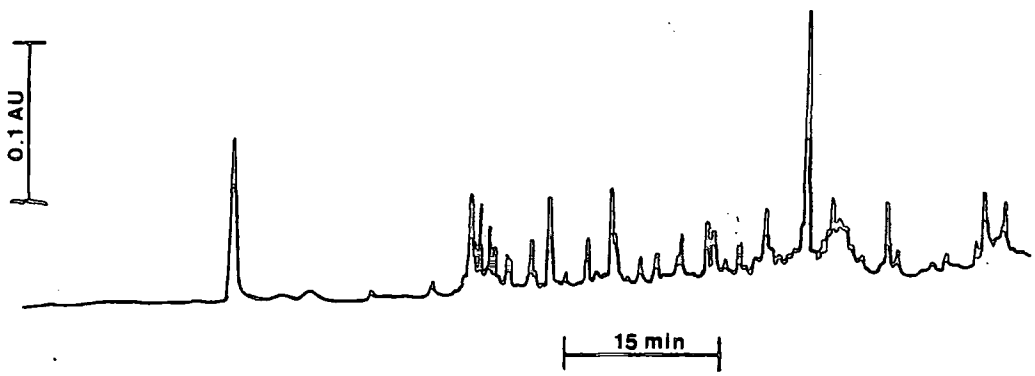


Figure 10: HPLC peptide map of enterolobin digested by *S. aureus* V-8 protease. RC-enterolobin F-4 was digested by V-8 *S. aureus* protease as described in Material and Methods. An aliquot of 500 µl (dissolved in 6 M guanidine-HCl in 0.1 % TFA) was injected to a reverse-phase C-18 Vydac column (4.6 x 250 mm) equilibrated with 0.1 % TFA. A linear gradient from 0 to 40 % acetronitrile in 0.1 % TFA (v/v) for 90 min was used to elute the peptides. The flow rate was 1.0 ml/min. The eluate was monitored at 214 nm at AUS = 0.5.

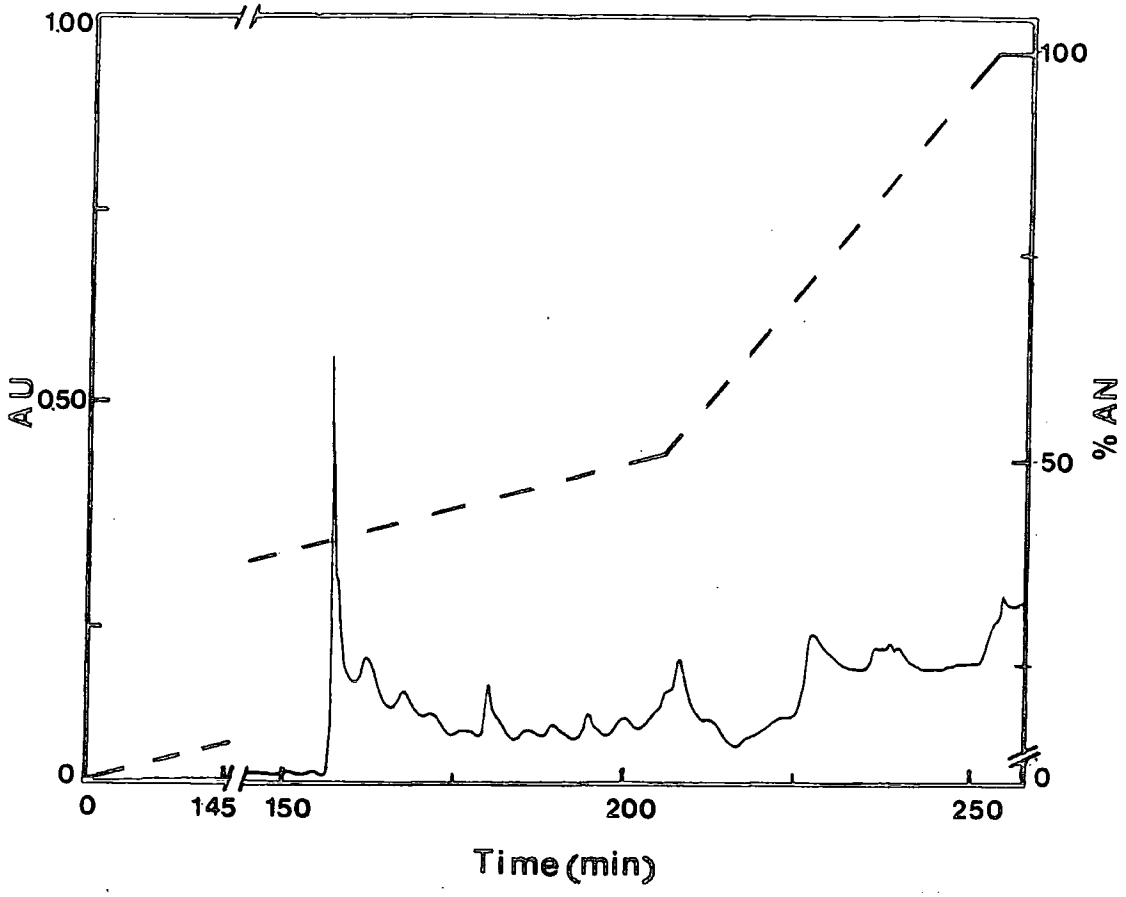


Figure 11: HPLC peptide map of enterolobin digested by CNBr. RC-enterolobin F-4 was digested by V-8 CNBr as described in Material and Methods. The sample (dissolved in 6 M guanidine-HCl in 0.1 % TFA) was injected to a reverse-phase C-18 Vydac column (4.6 x 250 mm) equilibrated with 0.1 % TFA. A gradient from 0 to 50 % acetronitrile in 0.1 % TFA (v/v) for 210 min and 50 to 100 % in 40 min was used to elute the peptides. The flow rate was 1.0 ml/min. The eluate was monitored at 214 nm at AUS = 1.0.

3.3 Separation of Peptides

The peptides obtained by the digestions of larger amounts of enterolobin (20-30 mg) with chymotrypsin, elastase, trypsin and *S. aureus* V-8 protease were initially fractionated by gel filtration on a column (1 x 200 cm) of Biogel P 6 (Figs. 12 to 15). Each of the fractions from the gel filtration was collected as indicated in the figures, freeze-dried, and then subjected to reverse-phase HPLC on a Vydac C18 column (some typical chromatograms are shown in Figs 16 to 19). The main peaks from the HPLC chromatographies were manually collected, freeze-dried, and submitted to sequence determination by the double-coupling DABITC-PITC manual method. The results of the manual sequencing are in Section 3.4.1. Some of these peptides were chosen for automatic sequence determination (Section 3.4.2).

Peptides produced by the CNBr digestion method were applied to a column (1.6 x 90 cm) of BioGel P 60 (Fig. 20). The peaks were collected, freeze-dried and submitted to sequence determination by the DABITC/PITC manual method. Four initial larger peaks were detected. Most probably, they correspond to the four peptides that were expected to be generated by cleavages at the three methionine residues of enterolobin. Lower molecular weight peptides were also produced by the reaction with CNBr (peaks 5 to 11) as previewed by the peptide mapping. Seemingly, they were the result of non-especific peptide cleavages that can occur with CNBr.(Blumenthal *et al.*, 1975; Aitken *et al.*, 1989; Sanchez *et al.*, 1991)

Alternatively, the CNBr peptides were directly applied to the reverse phase HPLC column. The HPLC chromatographer was

programmed to provide a low slope acetonitrile gradient (0 to 50 % acetronitrile in 0.1 % TFA (v/v) for 200 min and 50 to 100 % in 85 min) in order to allow an optimum separation of the fragments. The results of this chromatography are represented in Fig. 21. The peaks were carefully collected by hand, to avoid contamination from neighbouring peaks, and freeze-dried. Only well separated and defined peaks were selected to be sequenced automatically.

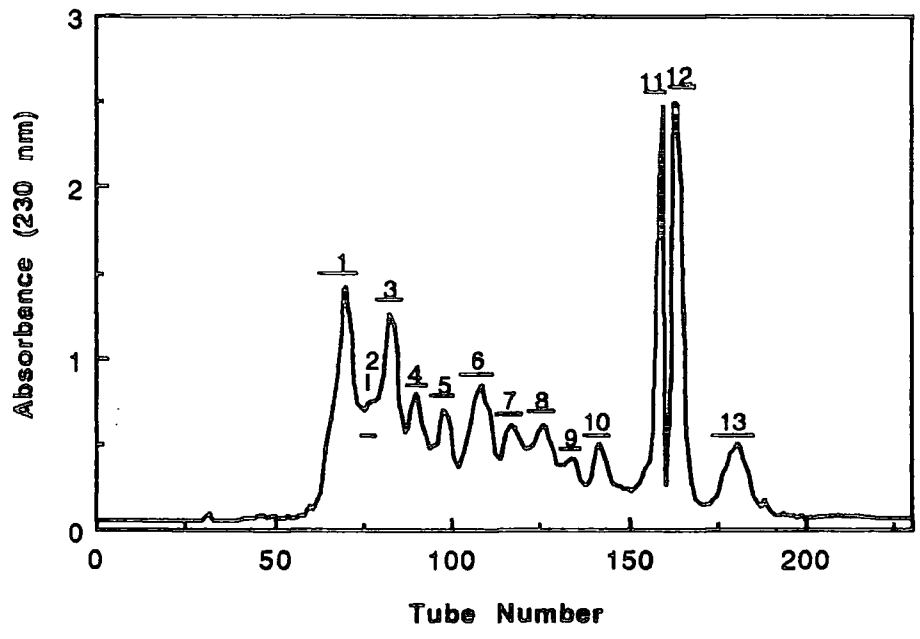


Figure 12: Gel filtration of the chymotryptic digest of enterolobin. RC-enterolobin F-4 was digested by chymotrypsin as described in Material and Methods. The digest was dissolved in 50 mM NH_4HCO_3 pH 8.1 and applied to a column (1 x 200 cm) of BioGel P 6 equilibrated with the same buffer. The absorbance (—) was read at 230 nm. The flow rate was 10 ml/h, and the volume of each fraction was 1.0 ml. The peaks were pooled as indicated in the figure by the bars and freeze-dried as peaks C-1 to C-13..

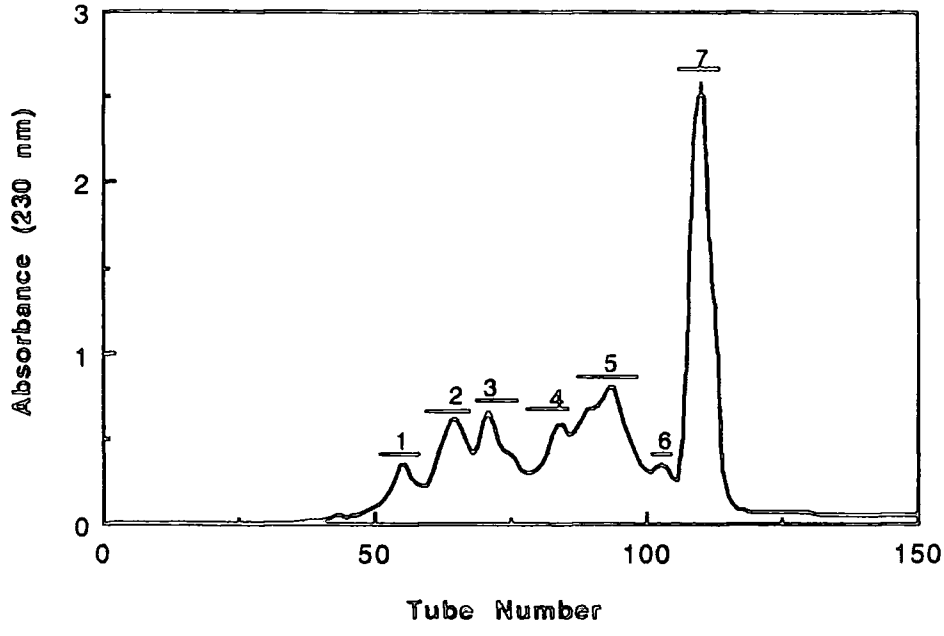


Figure 13: Gel filtration of the elastase digest of enterolobin. RC-enterolobin F-4 was digested by elastase as described in Material and Methods. The digest was dissolved in 50 mM NH_4HCO_3 pH 8.1 and applied to a column (1 x 200 cm) of BioGel P 6 equilibrated with the same buffer. The absorbance (—) was read at 230 nm. The flow rate was 10 ml/h, and the volume of each fraction was 1.0 ml. The peaks were pooled as indicated in the figure by the bars and freeze-dried as E-1 to E-7.

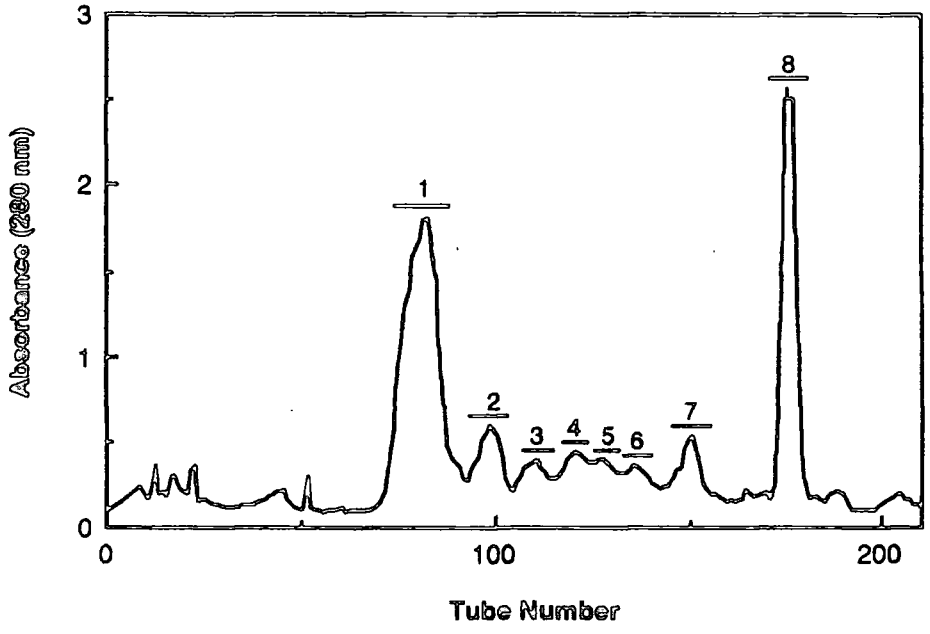


Figure 14: Gel filtration of the trypsin digest of enterolobin. RC-enterolobin F-4 was digested by trypsin as described in Material and Methods. The digest was dissolved in 50 mM NH_4HCO_3 pH 8.1 and applied to a column (1 x 200 cm) of BioGel P 6 equilibrated with the same buffer. The absorbance (—) was read at 230 nm. The flow rate was 10 ml/h, and the volume of each fraction was 1.0 ml. The peaks were pooled as indicated in the figure by the bars and freeze-dried as T-1 to T-8.

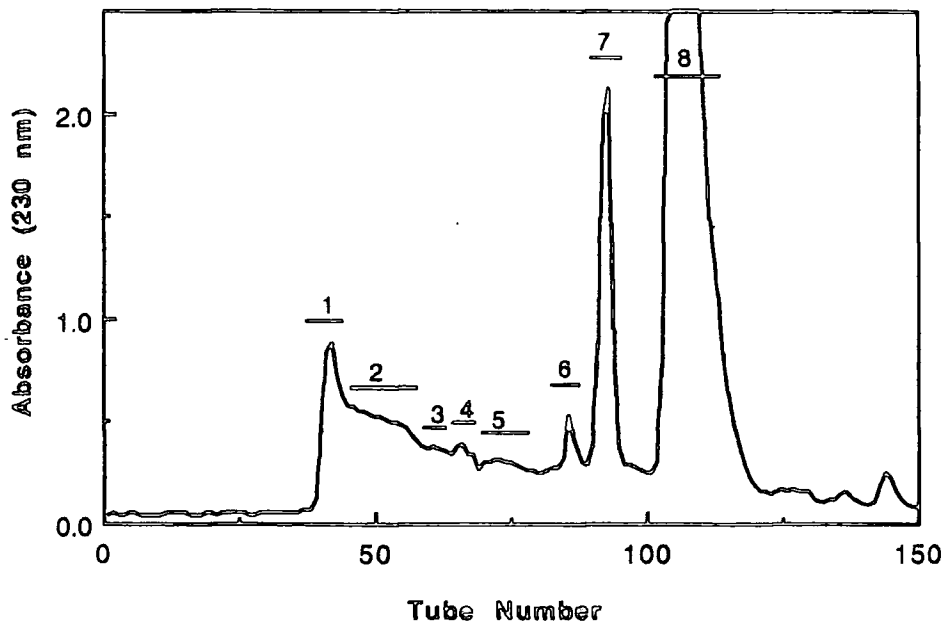


Figure 15: Gel filtration of the *S. aureus* V-8 protease digest of enterolobin. RC-enterolobin F-4 was digested by *S. aureus* V-8 protease as described in Material and Methods. The digest was dissolved in 50 mM NH_4HCO_3 pH 8.1 and applied to a column (1 x 200 cm) equilibrated with the same buffer. The absorbance (—) was read at 230 nm. The flow rate was 10 ml/h, and the volume of each fraction was 1.0 ml. The peaks were pooled as indicated in the figure by the bars and freeze-dried as V-1 to V-8.

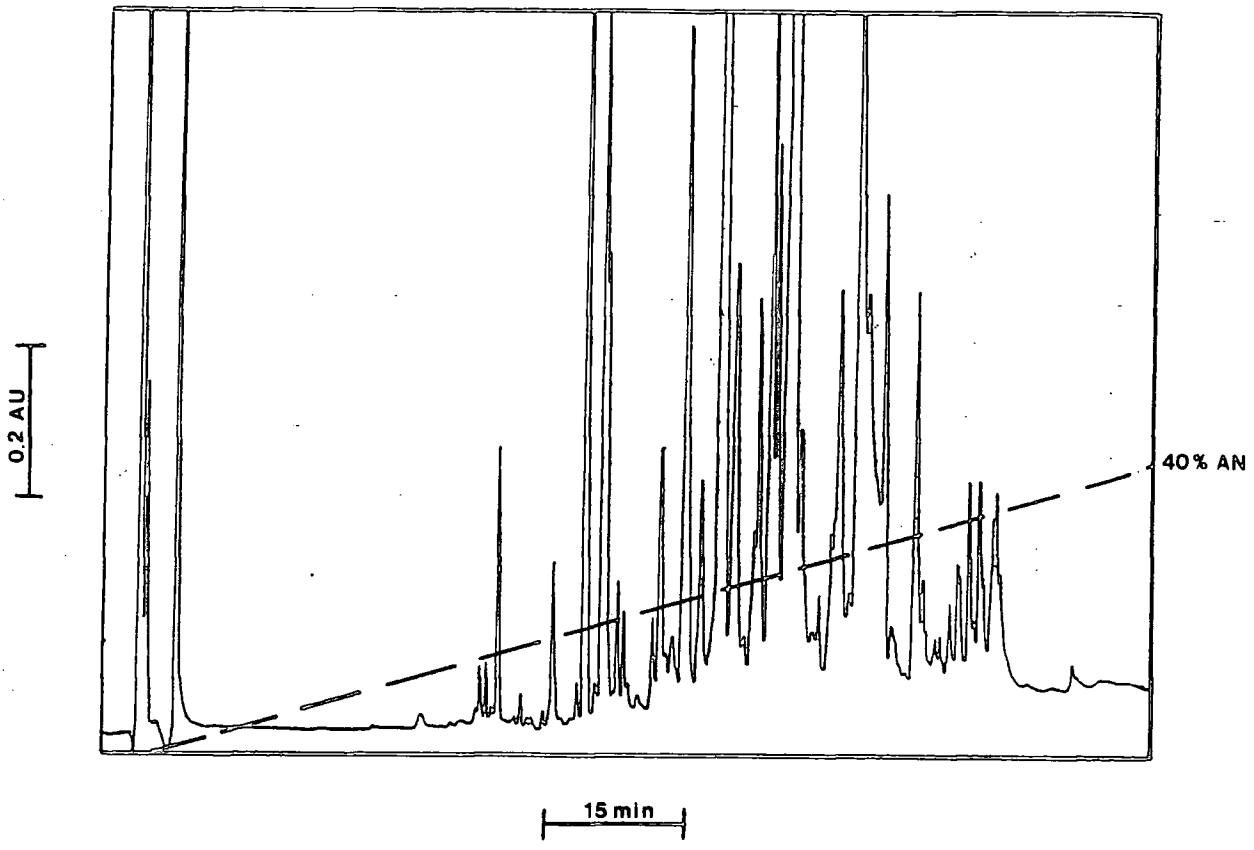


Figure 16: HPLC separation of fraction 1 from the gel filtration of the chymotryptic digest. Fraction 1 from the gel filtration of the chymotryptic digest of RC-enterolobin was dissolved in 6 M guanidine-HCl in 0.1 % TFA, and injected to a reverse-phase C-18 Vydac column (4.6 x 250 mm) equilibrated with 0.1 % TFA. A linear gradient (dashed line) from 0 to 50 % acetronitrile in 0.1 % TFA (v/v) for 150 min was used to elute the peptides. The flow rate was 10 ml/min. The eluate was monitored at 214 nm at AUS = 1.0. The peaks were manually collected and freeze dried as C-1.X, where X is the order of elution of the relevant peak.

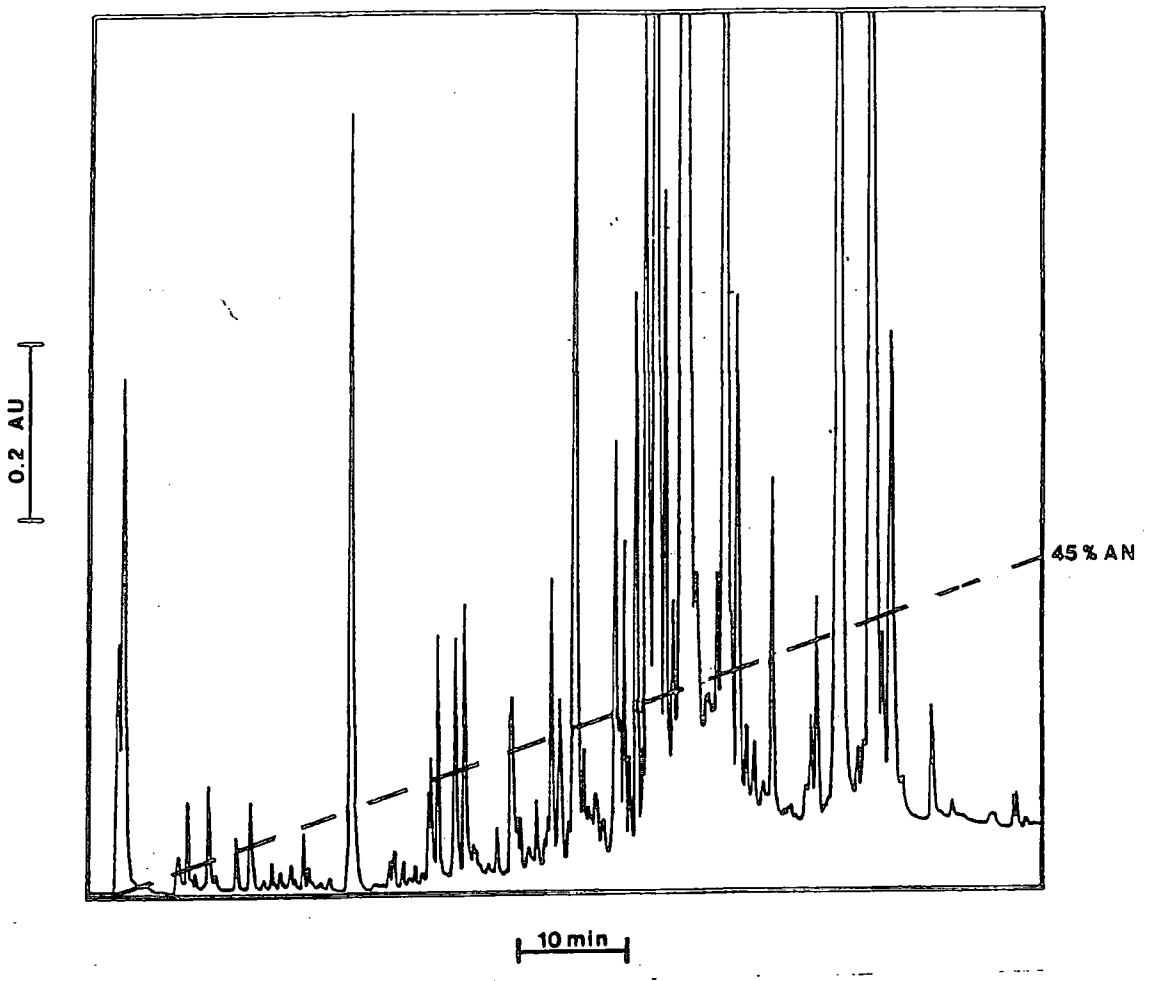


Figure 17: HPLC separation of fraction 1 from the gel filtration of the tryptic digest. Fraction 1 from the gel filtration of the tryptic digest of RC-enterolobin was dissolved in 6 M guanidine-HCl in 0.1 % TFA, and injected to a reverse-phase C-18 Vydac column (4.6 x 250 mm)) equilibrated with 0.1 % TFA. A linear gradient (dashed line) from 0 to 50 % acetronitrile in 0.1 % TFA (v/v) for 100 min was used to elute the peptides.The flow rate was 10 ml/min. The eluate was monitored at 214 nm at AUS = 1.0. The peaks were manually collected and freeze dried as T-1.X, where X is the order of elution of the relevant peak.

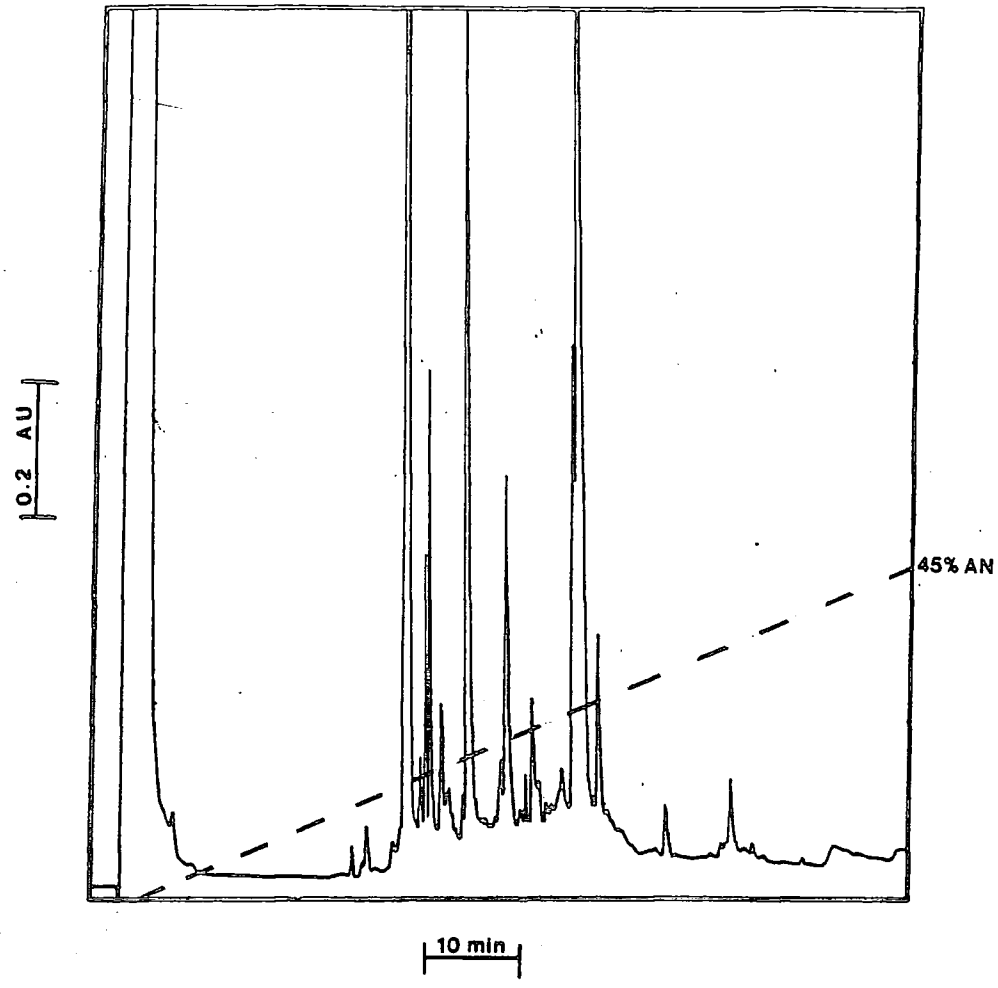


Figure 18: HPLC separation of fraction 1 from the gel filtration of the elastase digest. Fraction 1 from the gel filtration of the elastase digest of RC-enterolobin was dissolved in 6 M guanidine-HCl in 0.1 % TFA, and injected to a reverse-phase C-18 Vydac column (4.6 x 250 mm) equilibrated with 0.1 % TFA. A linear gradient (dashed line) from 0 to 50 % acetronitrile in 0.1 % TFA (v/v) for 100 min was used to elute the peptides. The flow rate was 10 ml/min. The eluate was monitored at 214 nm at AUS = 1.0. The peaks were manually collected and freeze dried as E-1.X, where X is the order of elution of the relevant peak.

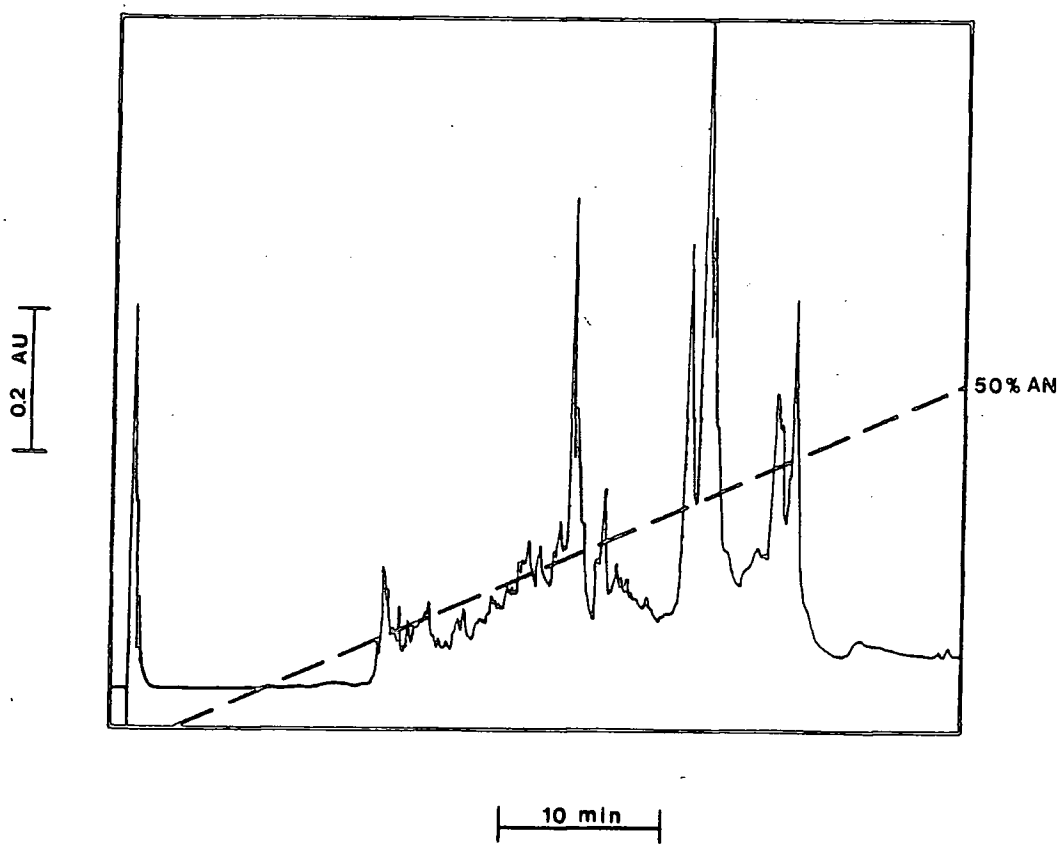


Figure 19: HPLC separation of fraction 1 from the gel filtration of the V-8 *S. aureus* protease digest. Fraction 1 from the gel filtration of the V-8 *S. aureus* protease digest of RC-enterolobin was dissolved in 6 M guanidine-HCl in 0.1 % TFA, and injected to a reverse-phase C-18 Vydac column (4.6 x 250 mm) equilibrated with 0.1 % TFA. A linear gradient (dashed line) from 0 to 50 % acetronitrile in 0.1 % TFA (v/v) for 100 min was used to elute the peptides.The flow rate was 10 ml/min. The eluate was monitored at 214 nm at AUS = 1.0. The peaks were manually collected and freeze dried as V-1.X, where X is the order of elution of the relevant peak.

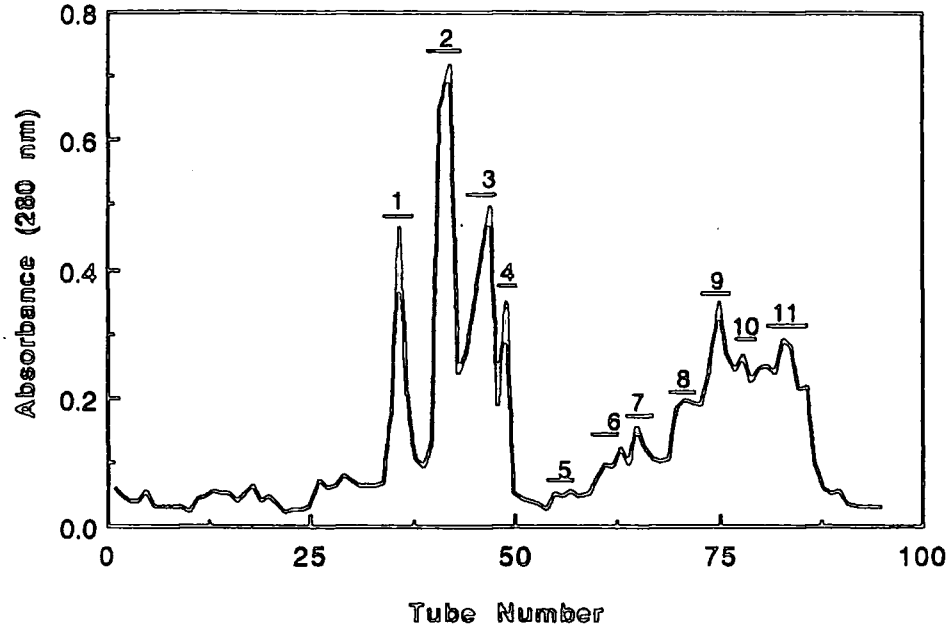


Figure 20: Gel filtration of the CNBr digest of enterolobin. RC-enterolobin F-4 was digested by CNBr as described in Material and Methods. The digest was dissolved in 70 % formic acid and applied to a column (1.6 cm x 90 cm) of BioGel P 60 equilibrated with the same solution. The absorbance (—) was read at 230 nm. The flow rate was 2.2 ml/h, and the volume of each fraction was 1.1 ml. The peaks were pooled as indicated in the figure by the bars and freeze-dried as CN-1 to CN-11

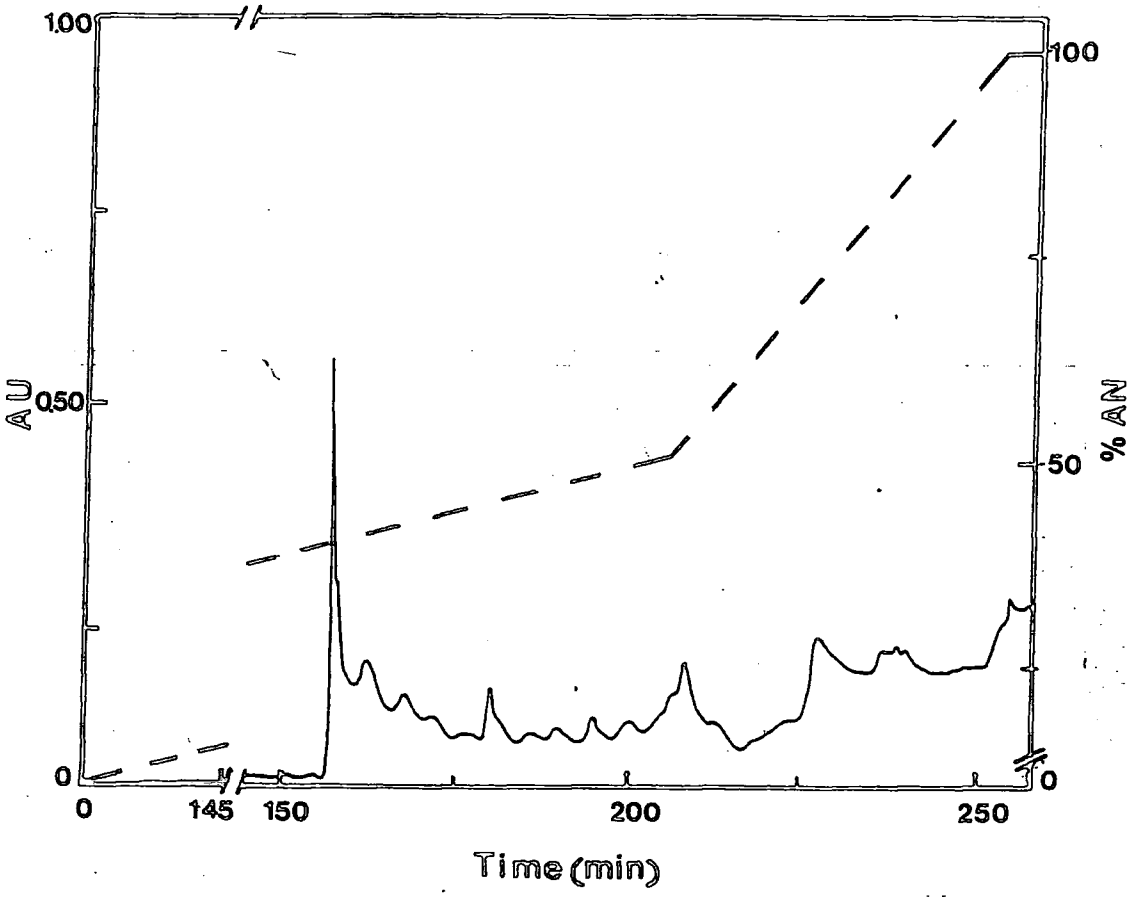


Figure 21: HPLC separation of the CNBr digest. The freeze-dried CNBr digest of RC-enterolobin F-4 was dissolved in 6 M guanidine-HCl in 0.1 % TFA, and injected to a reverse-phase C-18 Vydac column (4.6 x 250 mm) equilibrated with 0.1 % TFA. A gradient from 0 to 50 % acetronitrile in 0.1 % TFA (v/v) for 210 min and 50 to 100 % in 40 min was used to elute the peptides.The flow rate was 1.0 ml/min. The eluate was monitored at 214 nm at AUS = 1.0. The peaks were manually collected and freeze dried as CN.H-1.X, where X is the order of elution of the relevant peak.

3.4 Amino Acid Sequences of Peptides

3.4.1 Manually Sequenced Peptides

The amino acid sequences of peptides obtained by the enzymatic cleavage methods were determined by the manual DABITC/PITC double-coupling method. The results are shown in Tables 5 to 8.

The amino acid residues are represented by the standard single letter code (see Abbreviations). Only the most prominent residue found in each sequencing cycle is shown. The minor contaminants which were occasionally detected are ignored, since the significant sequence can be inferred from the dominant residues.

The peptides produced by the CNBr cleavage method and purified by gel filtration provided no useful sequence information and are not shown. Attempts to sequence them manually generated very weak spots on TLC or none at all.

It is not possible to differentiate leucine (L) from isoleucine (I) by the manual DABITC/PITC sequencing method used. These residues are then represented as L/I (leucine or isoleucine). A X was used when no residue was identified, or when it was uncertain. The concentrations (v/v) of acetonitrile, in which the peptides were eluted during HPLC, are given in percentage (%).

Table 5:

Amino Acid Sequence of Chymotryptic Peptides (C)

Peptide	Acetonitrile (%)	Sequence
C-1.3	15	TDGRS
C-1.4	20	L/IVAGSD
C-1.5	20	TQRDTL/I
C-1.6	20	TQRDTL/IT
C-1.10	22	EARDAGH
C-1.11	23	ASNDAGPT
C-1.12	24	AKEL/IGPG
C-1.13	25	WL/IVATG
C-1.14	25	QFAWN
C-1.16	26	RSFNHGEG
C-1.17	27	SPNKL/IT
C-1.18	27	L/IQFAWNSFGDPTVR
C-1.19	28	TQNHSWGFA
C-1.20	28	TQNHL/I
C-1.22	29	L/ISTN
C-1.23	31	NPTDL/I
C-1.25	31	GSPG
C-1.26	32	VPTG
C-1.30	36	ADH
C-1.31	36	TSD
C-2.2	20	ASDKNPDKDS
C-2.4	23	TNVQQVTYT
C-2.8	25	ATDGFTDT
C-2.9	28	L/IADTGTGV
C-2.12	28	RAPP
C-2.13	31	RSP
C-2.14	31	L/IDNH
C-2.16	31	RRSP
C-2.17	32	RSP
C-2.18	38	SPN
C-3.2	16	ASDKNPD
C-3.3	17	ASD
C-3.4	18	AGG
C-3.5	19	YL/IQ
C-3.7	19	TQRDTL/IT
C-3.8	20	TQRDTL/IT
C-3.11	21	TSTRV
C-3.14	23	ATTG
C-3.16	24	DSC
C-3.17	24	DSC
C-3.18	25	L/IKGE
C-3.20	27	L/IGXV
C-3.21	27	L/IPV
C-3.22	28	RRSPNP
C-3.23	30	TVL/I

C-3.24	31	L/IVNG
C-3.25	31	TL/IVDNEL/I
C-3.28	33	TL/IVDNEL/I
C-3.29	37	TL/IVDNEL/I
C-4.3	18	KGDRGG
C-4.4	18	SGGEVVSPG
C-4.5	19	RYL/IQEDVQV
C-4.6	19	TGRDTVTG
C-4.10	22	L/IRVQL/IGHG
C-4.11	22	ATTGT
C-4.12	24	L/IRVQL/IGHG
C-4.14	25	ATTGTL/I
C-4.20	30	WL/IL/IDFDN
C-4.21	31	RL/IL/IDA
C-4.21	32	RL/IAAPFDSC
C-4.23	33	FYXPL/I
C-5.1	11	KGDY
C-5.2	14	ETKEP
C-5.3	15	SDHRV
C-5.6	16	TDRN
C-5.9	17	AKFEVXE
C-5.10	18	KPL/IYCAD
C-5.13	19	TQRDTL/IT
C-5.14	19	TQRDTL/IT
C-5.17	22	RTXQADN
C-5.18	23	FKXPTL/IVNA
C-5.20	24	DVYTTNP
C-5.21	25	SVTTEP
C-5.22	26	KL/IYVPPRTTL/IV
C-5.24	28	TGP
C-5.27	33	FVTL/IL/IVDN
C-6.2	10	KVTNG
C-6.3	13	TVTDGSL/IRT
C-6.4	14	L/IWPRQ
C-6.5	15	SYDDDRL/I
C-6.6	15	TTHD
C-6.7	16	VEKQQV
C-6.8	16	TERDH
C-6.9	17	QRSDHF
C-6.11	17	NATV
C-6.12	18	KSGDNGQ
C-6.15	19	TYAEVN
C-6.18	22	NTGL/INSTHV
C-6.19	22	QTG
C-6.20	23	L/IVYP
C-6.24	27	ACL/IHQY
C-6.26	27	TFFTH
C-6.27	27	VKSD
C-6.28	27	TGL/IQ
C-6.29	30	WPL/IKVDNNNVV
C-6.30	32	TL/IVG
C-6.32	33	FWPL/IKVDNNNVV
C-7.1	9	SGAL/I
C-7.2	15	QVG
C-7.3	15	QL/IAPG
C-7.4	16	DYRTTQ
C-7.8	19	NL/IGNNN
C-7.9	19	KPVYVDAL/I

C-7.11	20	NFKYET
C-7.12	21	DHFG
C-7.15	24	KL/IVQ
C-7.16	25	KPVYVDADAATVR
C-7.18	28	KL/IL/I
C-7.19	30	GFVQ
C-8.2	9	SGP
C-8.7	15	L/IL/IRP
C-8.8	15	L/IL/IRPGSVRVVL/IT
C-8.9	16	TQNHG
C-8.10	17	DSR
C-8.11	17	DAR
C-8.12	19	WVEKHQV
C-8.15	26	AVVG
C-8.16	27	SDHFG
C-9.5	15	NAGVSTVSRT
C-9.6	16	SST
C-9.7	20	VGQGL/IVH
C-9.10	27	SDHFG
C-10.1	9	TVSG
C-10.2	17	NFKYET
C-10.3	17	GVTRY
C-10.5	18	RNL/IGN
C-10.6	18	RNGL/INNN
C-10.7	23	RL/IAAP
C-10.9	25	TVRD
C-10.10	26	TQNHF
C-10.11	27	VKSDHRF
C-10.12	31	SPN
C-11.2	7	KSNY
C-11.3	9	AL/INR
C-11.5	11	NHKY
C-11.6	14	AKT
C-11.7	16	KSS
C-11.9	18	RNL/IGNNN
C-11.11	19	TVRR
C-11.14	24	GAF
C-13.3	17	SAR

Table 6:

Amino Acid Sequence of Tryptic Peptides (T)

Peptide	Acetonitrile (%)	Sequence
T-1.5	12	TQEPNT
T-1.8	16	L/ITA
T-1.9	17	SPN
T-1.10	17	YL/IQ
T-1.11	20	SFN
T-1.12	21	HQYL/IQF
T-1.13	22	ATT
T-1.14	22	DTL/ITNGXQT
T-1.15	24	DTL/I
T-1.16	25	VGDPT
T-1.17	25	DTL/I
T-1.18	26	L/IPN
T-1.19	26	L/IPN
T-1.20	26	SPNPL/IGTD
T-1.21	26	SPNPL/IGT
T-1.23	27	HQYL/IQ
T-1.24	27	DTL/ITNGAQ
T-1.27	29	NNSDTL/I
T-1.28	30	SDTL/IF
T-1.35	34	SPNPL/IXT
T-1.38	36	NPDKDSYDVYT
T-1.40	37	NPD
T-2.3	15	TQKPN
T-2.16	27	L/IAAPFD
T-2.18	30	WWWWS
T-3.2	13	CCY
T-3.4	17	TSTL/INAG
T-3.6	19	TST
T-3.7	20	TSTL/INAG
T-3.8	20	TXCQTG
T-4.1	11	L/ITFTT
T-4.3	16	L/ISY
T-4.4	17	VDNGCYL/I
T-4.5	18	NL/IGNNNF
T-4.6	19	GDNNVVA
T-5.1	13	SDHFG
T-5.3	16	GDNGQ
T-5.4	16	L/IL/IDAH
T-5.6	17	GDNGQYL/ISAR
T-5.8	18	NL/IGNNNFC
T-6.2	6	YETN
T-6.3	7	YETN
T-6.4	11	SDHFG
T-6.5	11	SDHFG
T-6.9	15	GL/INGQYL/ISAR

T-6.10	19	L/IDTMAGVLNR
T-7.5	15	WL/IEG
T-8.8	10	L/IYQH
T-8.12	10	SGVQV
T-8.13	14	HVAFS
T-8.16	17	SSTCN
T-8.17	20	FVV
T-8.18	21	SSTCN

Table 7

Amino Acid Sequence of Elastasic Peptides (E)

Peptide	Acetonitrile (%)	Sequence
E-1.2	17	DSDDTTNNNSDT
E-1.4	18	SSPNPDD
E-1.5	18	SSPNPDT
E-1.9	20	WADSDDTTNNNSD
E-1.11	23	SSNPDTASS
E-1.14	36	SSNPD
E-1.15	27	SSNPDKDGXDVY
E-1.16	27	SSNPD
E-2.1	13	DADAEPSDT
E-2.2	14	SNDVGDXP
E-2.4	14	SGGETVN
E-2.5	16	TL/IPPTTPTNV
E-2.6	16	TL/IPPTTVT
E-2.7	17	STTVVETR
E-2.8	17	VGNPTT
E-2.9	17	TQRDTL/IT
E-2.10	17	TQRDTL/IT
E-2.11	18	YVDADAATVR
E-2.12	18	TYNVDGVL/IYT
E-2.13	19	TYNVDGV

E-2.14	19	TYNVDGVL/IYT
E-2.15	19	RYL/IQEDVQV
E-2.17	20	QFSGGET
E-2.18	21	EGKL/IEFG
E-2.19	21	RYL/IQEDVQ
E-2.20	22	TYNVDDTL/IYT
E-2.21	22	VGNEL/IFVTSG
E-2.22	22	QFSGGETVSP
E-2.24	23	STTDTVDV
E-2.25	24	RL/IDVPFSY
E-2.26	24	NCFG
E-2.27	28	GVN
E-2.28	28	APFDSCL/IF
E-2.29	29	AAPFDSCL/I
E-2.30	30	APFDSCL/I
E-2.31	31	AAAPFDSC
E-2.32	31	AAAPFDSC
E-2.33	33	TVV
E-3.1	11	YTEAT
E-3.2	12	YTE
E-3.3	14	NYTQEPNT
E-3.4	15	YVD
E-3.8	16	QFSGG
E-3.11	17	DL/INVP
E-3.13	18	GVAT
E-3.16	19	HDGPL/I
E-3.17	19	HDGPL/I
E-3.21	22	FKGDNG
E-3.22	22	FKGDNG
E-3.23	22	GTTGDGYL/I
E-3.25	24	DWFNFKYETKQE
E-3.26	25	GSDVP
E-3.27	26	GVP
E-3.28	29	L/IDT
E-4.6	11	VL/IKR
E-4.8	13	SPY
E-4.9	14	QFYT
E-4.11	16	SFQTR
E-4.12	16	VSPYF
E-4.13	17	YNET
E-4.15	17	KL/IRRVGN
E-4.16	18	VL/IGH
E-4.17	18	KWSC
E-4.18	18	RWVEKH
E-4.19	19	RNL/IGNNNFC
E-4.21	20	NYL/I
E-4.22	20	NFCHRVT
E-4.23	21	YQWGET
E-4.26	23	RGXS
E-4.29	24	L/IFGPY
E-4.33	26	CL/IG
E-4.36	28	GFL/IQ
E-4.37	29	CL/IWR
E-5.1	10	HYR
E-5.2	11	HYR
E-5.3	11	RNL/IGNNN
E-5.7	16	YTVS
E-5.8	16	HFYT

E-5.9	17	VSP
E-5.11	17	YNL/IGR
E-5.12	18	YWQTRNV
E-5.13	18	RWVEKH
E-5.14	19	L/IRNGNV
E-5.16	20	QYRGN
E-5.17	20	NYNHKL/IYN
E-5.18	21	YQXG
E-5.20	21	YSQH
E-5.21	22	RNL/I
E-5.23	23	RNL/IGNNNF
E-5.26	26	RWVEKH
E-5.27	25	RCCY
E-5.28	25	RCCY
E-5.28	25	NFR
E-5.29	27	NFRR
E-5.31	31	CL/IWR
E-5.33	31	FYP
E-6.1	15	KWN
E-6.7	24	RSPN
E-6.8	25	DHFG
E-6.11	30	QNH
E-7.4	17	RWT
E-7.11	25	WRD
E-7.12	25	WRD

Table 8:

Amino Acid Sequence of V-8 Protease Peptides (V)

Peptide	Acetonitrile (%)	Sequence
V-1.2	28	NVRNFR
V-1.3	28	NVRNFR
V-1.5	35	VL/IL/IPVTDR
V-1.6	36	VL/IL/IPVT
V-1.7	37	VL/IL/IPV
V-1.8	40	VL/I
V-1.9	41	VVG

V-1.10	42	TVT
V-2.2	16	TVTTAL/ID
V-2.6	16	YGTGER
V-2.7	17	DL/ID
V-2.8	18	VL/IT
V-2.9	19	ADTGTR
V-2.10	19	DVT
V-2.11	20	TDT
V-2.13	21	TDT
V-2.14	22	TDTG
V-2.15	22	DVGY
V-2.16	23	DVGY
V-2.17	24	DYDPD
V-2.18	24	DTL/I
V-2.19	24	DTL/I
V-2.21	25	DTL/I
V-2.24	28	TQVPG
V-3.2	15	DYTV
V-3.3	15	HL/IL/IR
V-3.5	16	GL/IPA

3.4.2 Automatically Sequenced Peptides

Some peptides of enterolobin obtained from the digestion with enzymes were chosen for automatic sequencing on an Applied Biosystems 477A sequencer on the basis of the following factors:

a) Purity of the peptide, as determined by its peak shape and manually determined sequence. The shape of its peak obtained during the HPLC chromatography should preferentially be gaussian with no shoulders. Also the manually determined sequence of the

peptide should contain no contamination, i.e., single amino acid residues should be found especially during the initial cycles of reaction.

b) Concentration of acetonitrile where the peptide was eluted. Assuming that larger peptides would have been eluted at higher concentrations of the eluent, peptides eluted at less than 20% would not be preferentially selected for automatic sequencing. However, peptides eluted at high acetonitrile concentrations were avoided. Less hydrophobic peptides were given preference for automatic sequencing, because they tend to be less susceptible to be washed out from the support at earlier cycles.

c) Number of hydrophobic residues found in the manual sequence. For the same reason as above, very hydrophobic peptides were rejected for automatic sequence.

d) Total number of residues manually sequenced. The larger the number of manually sequenced residues, the easier the comparative assessments of the automatically sequenced residue for the operator of the automatic sequencer.

e) The presence of prolines. The knowledge of the position of proline residues manually sequenced facilitates the programming of suitable cycles to deal with these residues. Prolines give poorer rates of cleavage during the cleaving step.

From the cleavage of enterolobin with CNBr, only well separated and defined peaks (by HPLC) were selected to be sequenced automatically. This way, peaks CN.H-1, 4, 10, 19 and 22 were submitted to automated sequencing, but only CN.H-10 produced some sequence. The other peaks failed to provide any sequence of amino

acids, probably due to chemical blockage at their N-terminus similar to the intact protein or derived from some secondary chemical modification on amino acid residues.

Table 9 shows the sequence of the peptides that were successfully sequenced by the automatic method.

Table 9

Automatically Sequenced Peptides

Peptide	Acetonitrile (%)	Sequence
C-1.19	28	TQNHSWIVAGSDEP
E-1.9	20	WADSDDTTNNNSDTLF
T-1.14	22	DTLTNGQGQYNDD
CN.H-1	44	VALAGHPLAGASNRNDLRV

3.4.3 Determination of the N-terminus Amino Acid

The automatic sequence determination by an Applied Biosystems pulsed-liquid sequencer failed to reveal the N-terminal residue of enterolobin. Several cycles were run on the machine, but no detectable peak corresponding to an amino acid residue was obtained.

This result is in agreement with those from the manual sequence determination that did not reveal any N-terminal residue as well, even when amounts as large as 2 mg of pure enterolobin were used.

Also after the two different unblocking treatments to remove the supposed blockage, the N-terminus could not be found.

The first treatment employed pyroglutamate amino-peptidase for the removal of N-terminal pyroglutamic acid residue (Zalut *et al.*, 1980), but no free residue from enterolobin could be found in the manual sequencing after this treatment. The treatment worked well, however, for the control protein γ-casein, which yielded a glutamic acid as its N-terminal residue as should be expected

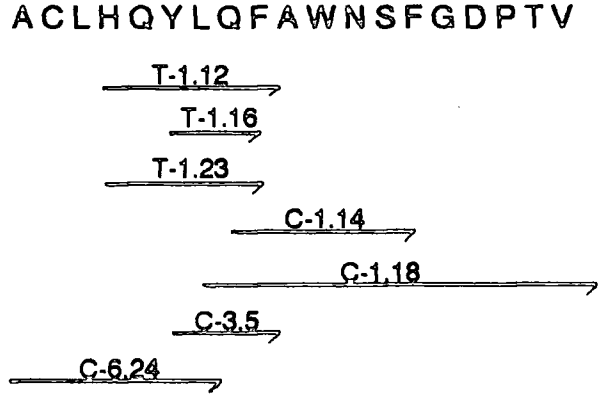
The second method used anhydrous TFA for unblocking proteins with acetylated N-terminal residues, especially acetylated serines and threonines (Wellner *et. al*, 1990). No strong residue could be identified by manual sequencing. Instead, some weak spots could be seen on the TLC sheets, probably arising from peptides produced by internal cleavages of enterolobin by the acid treatment.

Sousa and Morhy (1989) had previously obtained an indication that the N-terminus could be a glutamine. That early interpretation was probably an error due to the presence of a minor contamination, since they used enterolobin fraction F-3 analysed for purity by electrophoresis only. It is known now that enterolobin F-3 can sometimes contain some contamination, although at low concentrations (Section 3.1 above).

3.5 Overlaps among Peptides

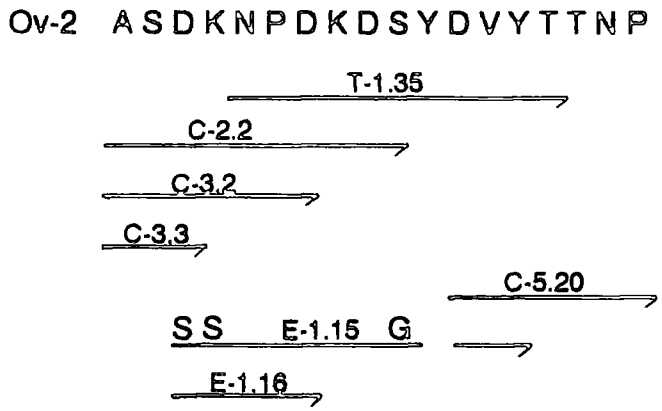
Twenty four overlaps (Ov-1 to Ov-24) from the enterolobin peptides could be assembled. Peptide Ov-24 resulted from the overlap of peptides Ov-4 and Ov-7. A total number of 271 residues were placed in overlaps. The sequenced peptides are indicated below the overlaps to which they contributed. The notation employed (see Section 2.2.2.7) constitutes the letters Ov and a number from 1 to 24.

The assembly of peptide Ov-1 was made only with the participation of tryptic and chymotryptic peptides. An unusual cleavage after a glutamine residue occurred for the tryptic peptide T-1.16. Leucine could be attributed as the residue at the fourth position of the peptide, since a cleavage by chymotrypsin (C-1.14) occurred at the carboxyl side of it. So far, chymotrypsin never has been found to cut at isoleucyl sites.

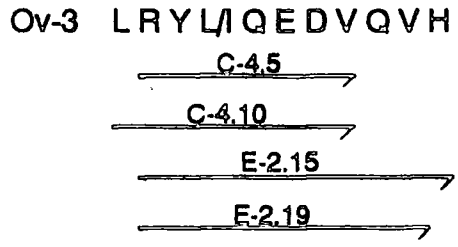


Peptide Ov-2 did not show any cleavage in unexpected places. However, there seems to be some microheterogeneity as shown

by the substitutions which occurred in E-1.15, from D K to S S and S to G. No tryptic peptide was found in Table 6 to fit after the second lysine residue in the sequence.

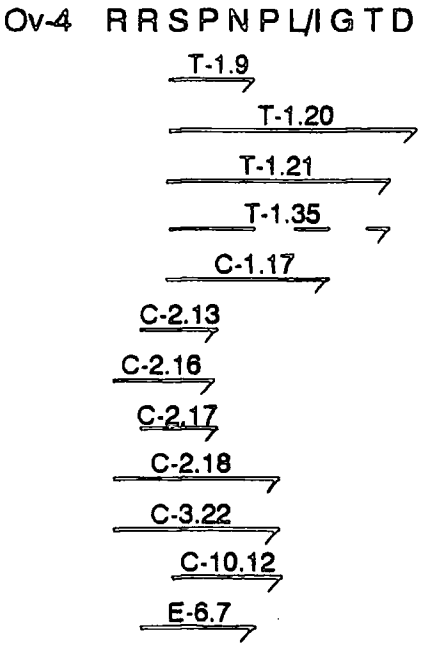


In peptide Ov-3, no uncommon cleavages happened. Similarly as in Ov-1, the first residue could be assigned as a leucyl based on the occurrence of a chymotryptic cleavage after it. As in Ov-2, there was no peptide among the tryptic ones in Table 6 that could be placed after the arginine residue in the second position of the peptide.



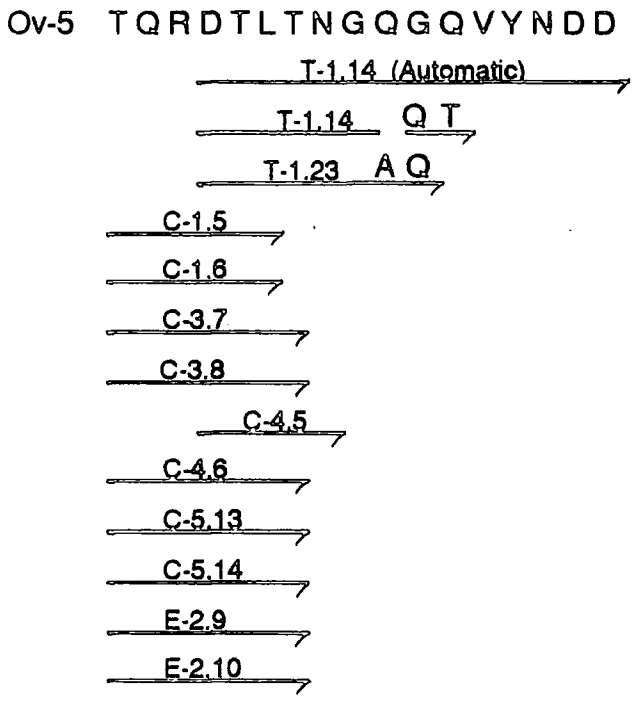
Some unusual chymotryptic cleavages were found to be located after arginine residues in peptide Ov-4. This type of behaviour

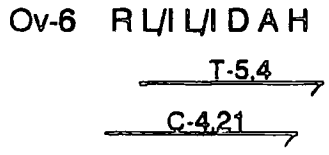
Some unusual chymotryptic cleavages were found to be located after arginine residues in peptide Ov-4. This type of behaviour has not frequently been observed for chymotrypsin in the literature. Likewise, elastase cleaved after arginine in E-6.7, what is not common. Nevertheless, chymotrypsin and elastase are proteolytic enzymes with a broad specificity.

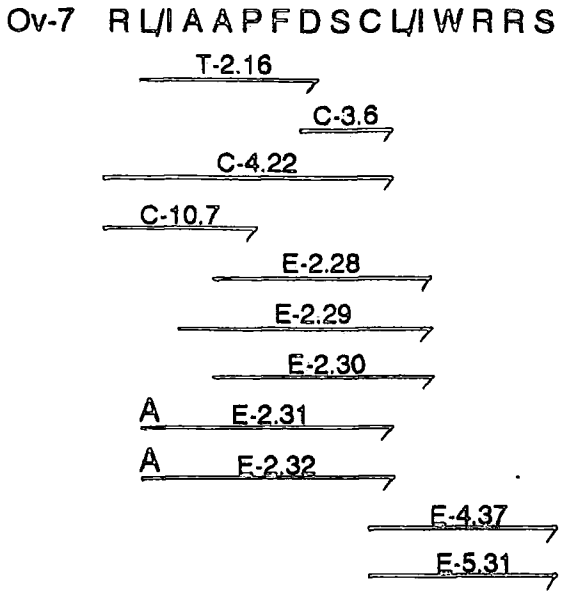


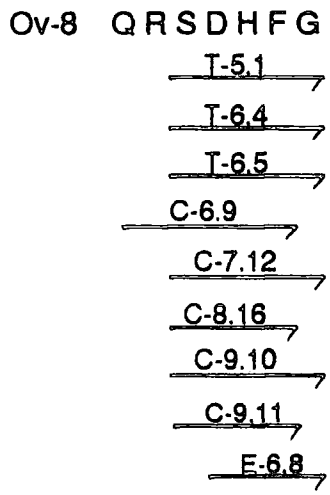
In Ov-5, peptide T-1.14 was sequenced both manually and automatically. There were two differences - G Q was found to be Q T in the manual sequencing. In T-1.23, two substitutions were also found. As the automated sequencing proved to give the same results as the manual one, the final assignment was based on the former method. Often, the last cycles in a manual sequencing are not fully reliable, so that the automatically assigned residues are given preference.

No special comments needs to be made for Ov-6 and Ov-7 and Ov-8, apart from the microheterogeneity in Ov-7 (L/I changed to A) which occurred in peptides E-2.31 and E-2.32.









Peptide Ov-9 showed unusual hydrolysis by trypsin at a serine residue (T-5.3, T-5.4 and T-6.9). There was a replacement from D to L/I in T-6.9. A leucine residue could be identified because of a chymotryptic cleavage after it in C-13.3.

Ov-9 F K S G D N G Q Y L S A R

T-5.3
T-5.6
L/I T-6.9
C-6.12
C-13.3
E-3.21
E-3.22

Again, nothing special can be mentioned about peptides Ov-10, Ov-11, Ov-12 and Ov-13, which had cleavages in expected places and no microheterogenic substitutions.

Ov-10 D Y R T T Q A D N

C-5.17
C-7.4

Ov-11 F W P L/I K V D N N N V

C-6.29
C-6.32

Ov-12 D W F N F K Y E T K Q E

T-6.2
T-6.3 N
C-7.11
C-10.2
E-3.25

Ov-13 R N L/I G N N N F C

T-4.5

T-5.8

C-7.8

C-10.5

C-10.6

C-11.9

E-4.19

E-5.3

E-5.23

A cleavage after arginine by chymotrypsin occurred for C-8.12 in peptide Ov-14 as seen in the examples above. Yet, a replacement of H by Q occurred in C-6.7.

A chymotryptic cleavage (C-9.5) allowed the determination of a leucyl in peptide Ov-15.

Ov-14 R W V E K H Q V

C-6.7 Q

C-8.12

E-4.18

E-5.13

E-5.26

Ov-15 T S T L N A G V S T V S R T

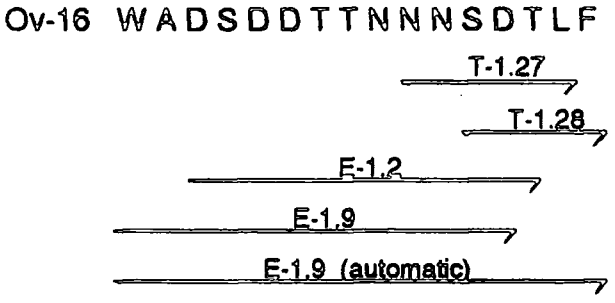
T-3.4

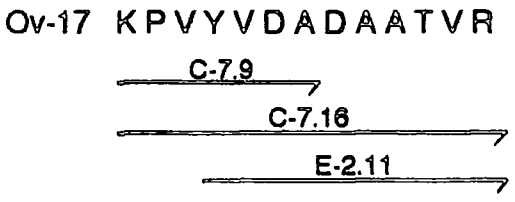
T-3.7

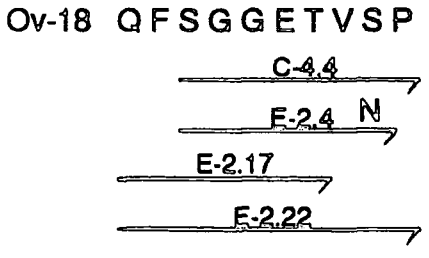
C-9.5

In Ov-16, particular observations must be made for the two cleavages by trypsin (T-1.27 and T-1.28) in different sites of asparagine residues. In this peptide, automatic sequencing confirmed the correctness of the manually determined residues.

No specific commentary can be made for Ov-17, Ov-19, Ov-20 and Ov-21, but in Ov-18, there was a microheterogeneity (S to N) in peptide E-2.4.







Ov-19 N Y T Q E P N T

T-1.5

T-2.3

E-3.3

Ov-20 L/I V A G S D V V

C-1.4

E-3.26

Ov-21 R C C Y

T-3.2

E-5.27

E-5.28

Also in Ov-22, the automated sequence corroborated the manual sequence. Some minor substitutions occurred in some peptides. Only expected cleavages happened in Ov-22 and Ov-23.

Ov-22 T Q N H S W I V A G S D E P

C-1.14

C-1.19 G F

C-1.19 (Automatic)

C-8.9 F

C-10.10 G

E-3.26

Ov-23 K S N Y N H K L/I Y N

C-11.2

E-4.21

E-5.17

E-11.5

Ov-24 was an overlap of two previously overlapped peptides, Ov-4 and Ov-7. This assembly was based on the overlap of the region R R S commonly occurred in both peptides, and also on sequence similarities to other two cytolysins as shown in Fig. 27 below.

Ov-24 R L/I A A P F D S C L/I W R R S P N P L/I G T D

Ov-4

Ov-7

3.6 Computational Analyses of the Amino Acid Sequences

3.6.1 Searches and Alignments

When new sequence information derived from peptides and proteins becomes available, it is a general procedure to search a database in order to detect any relatedness with sequences previously determined. After this initial search is done, other analyses of the sequences, such as optimal alignment, pattern search and hydropathy profiling, can follow.

Using this strategy, the Ov peptides from enterolobin were used for searching for matching sequences in the PIR database by using the program FASTA running on an Apple Macintosh SE/30 desktop computer.

Although peptide Ov-1 was found to have higher similarities scores to some proteins (S-locus-specific glycoprotein S13 precursor and glucose-transporter protein from yeast), it proved to have some correspondence to two functionally related proteins - the haemolytic/cytolytic proteins aerolysins from *Aeromonas hydrophila* and *Aeromonas sobria*. Both provided 45.5% similarity to Ov-1 in 11 amino acid overlaps:

```
          10
   ACLHQYLQFAWNSFGDPTV          Ov-1
      ..:.:..:.:.
ILWVGANDYLAYGWNTEQDAKRVRD       A. hydrophila aerolysin
 130       140       150

          10
   ACLHQYLQFAWNSFGDPTV          Ov-1
   ..: .:: .::
KPTSYLAHYLGYAWVGGNHSQYVGE       A. sobria aerolysin
 140       150       160
```

The amino acid sequences of the aerolysins were found in the PIR database by the program FINDP, and downloaded as readable files by the program EXTRACTP (Figs. 22 and 24). Their amino acid composition and molecular weights were calculated from the sequences by the program AA.DATA (Figs. 23 and 25). The aerolysin from *A. hydrophila* is a large protein with a molecular weight of 52.5 KDa and 463 amino acid residues. *A. sobria* possesses a slightly larger aerolysin of 53.2 KDa and 469 residues.

The alignment of the amino acid sequences of the two aerolysins was performed by the program CLUSTAL, and is shown if Fig. 26. They are highly homologous, which proteins what is not surprising, since they are from the same genus. The percentage of identity between them is 78% as calculated by the program ALIGN. Most of the non-identical residues are the result of conservative replacements.

Besides these two haemolysin-producing *Aeromonas*, other species in the genus were found to have cytolytic activities produced by them. *Aeromonas salmonicida*, a nonmotile major pathogen of fish, contains a T-lysin with haemolytic acitvity (Titball and Munn, 1983) that is secreted as an inactive precursor which is activated by an autogenous caseinase (Titball *et al.* 1985). Also, another extracellular haemolytic toxin, salmolysin, has been purified from *Aeromonas salmonicida* (Nomura, *et. al.*, 1988). *Aeromonas veronii* was found to be haemolytic as well. Neves *et al.* (1990) detected haemolytic activity in thirteen out of fourteen strains of *A. veronii* isolated from fresh and salt water in Rio de Janeiro, Brazil.

The aerolysin of *A. hydrophila* has been far more studied than those of *A. sobria* and other *Aeromonas* species. *A. hydrophila* is a gram-negative aquatic and motile bacteria that has been recognized as a pathogen to humans and other animals, principally fish (Khardori and Fainstein, 1988). In humans, it is able to cause gastrointestinal infections, extraintestinal infections, meningitis, osteomyelitis, septic arthritis, endocarditis, peritonitis and eye, urinary and other infections (Khardori and Fainstein, 1988). *A. hydrophila* toxins have been reviewed elsewhere (Ljungh and Wadström, 1982, 1982-3).

Bernheimer and Avigad (1974) carried out the partial purification and characterization of a haemolytic protein from the culture filtrates of *A. hydrophila.* which they named as aerolysin. The haemolysin was identified as having a molecular weight of 50-53 KDa by SDS-PAGE and gel filtration. Subsequently, Buckley *et al.* (1981) isolated a similar protein, which was also called aerolysin. This protein is secreted across the inner bacterial membrane as a preprotoxin that has its signal peptide removed cotranslationally (Howard and Buckley, 1985a). Then it is secreted to be later activated by proteolytic removal of a peptide from the C-terminal region (Howard and Buckley, 1985b).

The similarities between enterolobin and aerolysins are remarkable. They are all cytolytic proteins with close molecular weight. By gel filtration, enterolobin has a MW = 51 300 Da. By SDS-PAGE, it is a 55 000 Da. single chain polypeptide (Sousa and Morhy, 1989). The amino acid composition of enterolobin is also very close to those of aerolysins, revealing characteristic low level of Cys and high levels of Ser and Thr in relation to the average in other proteins.

```
         10        20        30        40        50
AEPVYPDQLRLFSLGQGVCGDKYRPVNREEAQSVKSNIVGMMGQWQISGL
ANGWVIMGPGYNGEIKPGTASNTWCYPTNPVTGEIPTLSALDIPDGDEVD
VQWRLVHDSANFIKPTSYLAHYLGYAWVGGNHSQYVGEDMDVTRDGDGWV
IRGNNDGGCDGYRCGDKTAIKVSNFAYNLDPDSFKHGDVTQSDRQLVKTV
VGWAVNDSDTPQSGYDVTLRYDTATNWSKTNTYGLSEKVTTKNKFKWPLV
GETQLSIEIRANQSWASQNGGSTTTSLSQSVRPTVPARSKIPVKIELYKA
DISYPYEFKADVSYDLTLSGFLRWGGNAWYTHPDNRPNWNHTFVIGPYKD
KASSIRYQWDKRYIPGEVKWWDWNWTIQQNGLSTMQNNLARVLRPVRAGI
TGDFSAESQFAGNIEIGAPVPLAADSKVRRARSVDGAGQGLRLEIPLDRE
ELSGLGFNKSASA
```

Figure 22: Amino acid sequence of aerolysin from *A. hydrophila*. The amino acid sequence (463 residues) was derived from the nucleotide sequence of the gene (Howard *et al*, 1987). The precursor amino acid sequence was found in the PIR database by the program FINDP from the FASTA package, and extracted by the program EXTRACTP from the same package. Subsequently, it was edited with the program AA.DATA from the MACPROT package to have its signal peptide removed.

RESIDUE	NUMBER	%
A	30	6.48
C	4	0.86
D	33	7.13
E	18	3.89
F	11	2.38
G	46	9.94
H	6	1.30
I	22	4.75
K	23	4.97
L	29	6.26
M	5	1.08
N	28	6.05
P	25	5.40
Q	20	4.32
R	24	5.18
S	37	7.99
T	29	6.26
V	34	7.34
W	18	3.89
Y	21	4.54
X	0	0.00
MOL. WEIGHT: 52532.59		

Figure 23: Amino acid composition of aerolysin from *A. hydrophila*. The composition was calculated from the amino acid sequence of aerolysin in figure 22 by the program AA.DATA from MACPROT. The resulting molecular weight of the protein was also determined by the program.

```
        10        20        30        40        50
AEPIYPDQLRLFSLGEDVCGTDYRPINREEAQSVRNNIVAMMGQWQISGL
ANNWVILGPGYNGEIKPGKASTTWCYPTRPATAEIPVLPAFNIPDGDAVD
VQWRMVHDSANFIKPVSYLAHYLGYAWVGGDHSQFVGDDMDVIQEGDDWV
LRGNDGGKCDGYRCNEKSSIRVSNFAYTLDPGSFSHGDVTQSERTLVHTV
VGWATNISDTPQSGYDVTLNYTTMSNWSKTNTYGLSEKVSTKNKFKWPLV
GETEVSIEIAANQSWASQNGGAVTTALSQSVRPVVPARSRVPVKIELYKA
NISYPYEFKADMSYDLTFNGFLRWGGNAWHTHPEDRPTLSHTFAIGPFKD
KASSIRYQWDKRYLPGEMKWWDWNWAIQQNGLATMQDSLARVLRPVRASI
TGDFRAESQFAGNIEIGTPVPLGSDSKVRRTRSVDGANTGLKLDIPLDAQ
ELAELGFENVTLSVTPARN
```

Figure 24: Amino acid sequence of aerolysin from *A. sobria*. The amino acid sequence (469 residues) was derived from the nucleotide sequence of the gene (Husslein *et al.*, 1988). The precursor amino acid sequence was found in the PIR database by the program FINDP from the FASTA package, and extracted by the program EXTRACTP from the same package. Subsequently, it was edited with the program AA.DATA from the MACPROT package to have its signal peptide removed.

RESIDUE	NUMBER	%
A	35	7.46
C	4	0.85
D	31	6.61
E	22	4.69
F	15	3.20
G	39	8.32
H	8	1.71
I	23	4.90
K	19	4.05
L	30	6.40
M	8	1.71
N	27	5.76
P	27	5.76
Q	18	3.84
R	25	5.33
S	37	7.89
T	31	6.61
V	35	7.46
W	17	3.62
Y	18	3.84
X	0	0.00
MOL. WEIGHT: 53241.49		

Figure 25: Amino acid composition of aerolysin from *A. sobria*. The composition was calculated from the amino acid sequence of aerolysin in figure 22 by the program AA.DATA from MACPROT. The resulting molecular weight of the protein was also determined by the program.

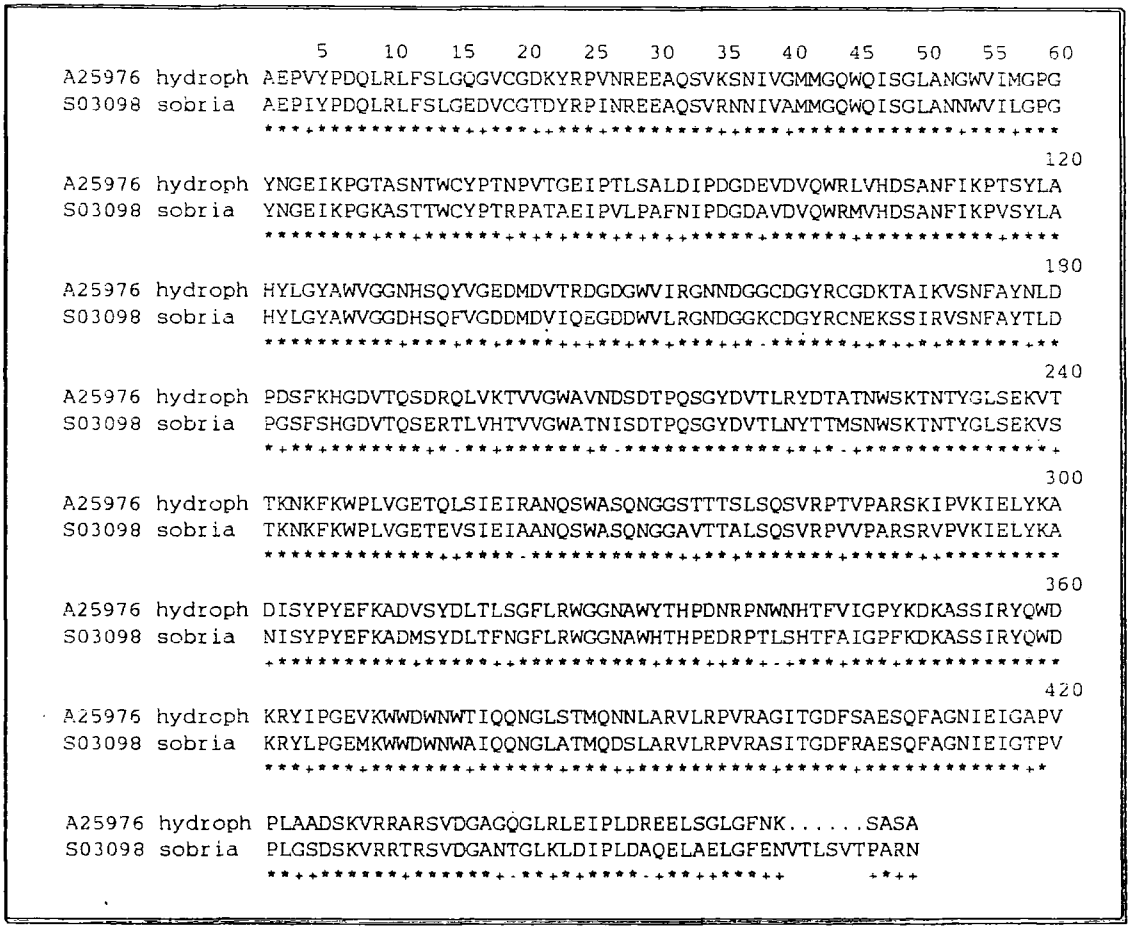


Figure 26: Alignment of amino acid sequences of aerolysins from *A. hydrophila* and *A sobria*. The figure is an output of the program CLUSTAL which was utilized for performing the alignment. The default settings of a fixed gap of 10 and a variable gap of 10 were used. A25976 and S03098 are the reference number for the aerolysins sequences of A. hydrophila and A sobria, respectively, in the PIR database. A * means identical matches across the sequences, + represents conservative substitutions, and -, non-conservative substitutions.

A tentative manual alignment of some enterolobin peptides with the automatically aligned sequences of the aerolysins was made (Fig. 27). It showed that in some regions there are very promising similarities. One of such similar regions is the predicted cytolytic site. The prediction of this putative cytolytic site was performed by analysing the common characteristics in the hydropathatic profiles, charge density distribution and helix amphiphilicities of several cytolytic proteins and peptides of reptilian, amphibian, insect and microorganisms (Kini and Evans, 1989).

From these results and considerations, it can be perhaps suggested that enterolobin and aerolysins could be homologous proteins, or, in other words, they could have evolved from a common ancestral gene. It is clear that they share a functional relationship and similarites in their amino acid sequences. However, local protein sequence similarity does not necessarily imply a structural relationship among proteins (Sternberg and Islam, 1990). For this reason, it is not possible to state that there is a tertiary structural relationship among these proteins, since no complete X-ray crystallography and/or NMR data are available for them yet.

It is known that only some still preliminary spectroscopic and X-ray analyses have been done for *A. hydrophila* aerolysin (Green and Buckley, 1990). These data reveal an almost entire hydrophilic β structure with no obvious transmembrane helices. The structure seems to be analogous to those of the porins from which aerolysin may have evolved (Green and Buckley, 1990). Porins are bacterial and mitochondrial proteins (30-36 KDa) that form stable aggregates and

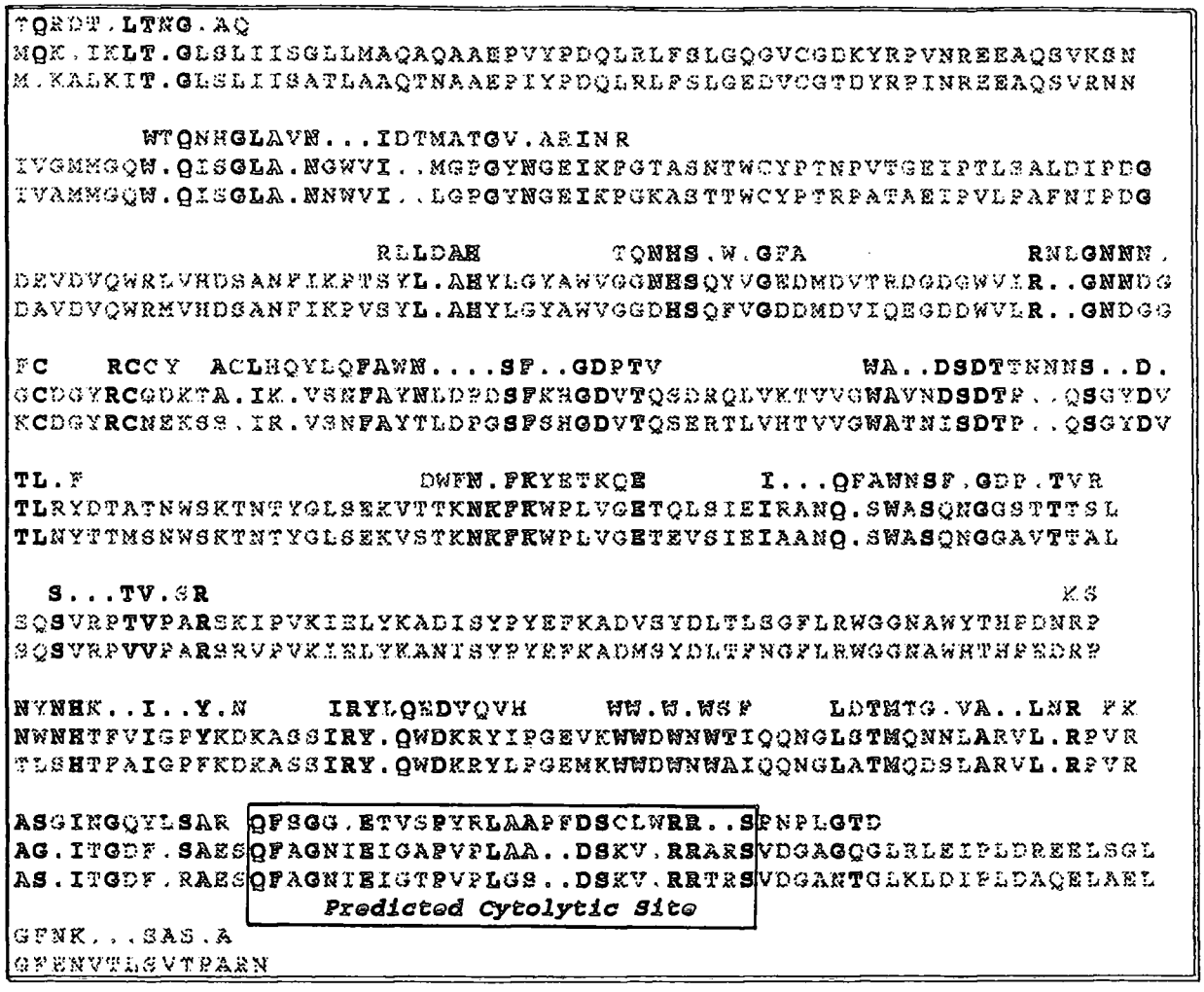


Figure 27: Possible manual alignment of the sequences of some enterolobin peptides with the sequences of aerolysins. Sequenced and overlapped enterolobin peptides (ent) are in the first row of sequences with aerolysin from *A. hydrophila* (hydr) in the second row and aerolysin from *A. sobria* (sobr) in the third one. Leucine (L) and isoleucine (I) in enterolobin are assumed to be equal to their correspondent matching I or L in the aerolysins, unless they are actually known. Black bold letters indicate identical residues, and grey letters are non-identical ones. The regions within the box are the cytolytic sites as tentatively predicted by Kini and Evans (1989).

transmembrane pores through the outer membranes of the bacteria and mitochondria to allow the transport of some solutes (Nikaido, 1982).

Program FASTA also detected similarities between peptide Ov-1 and colicin 1b, a channel-forming protein from *E. coli*, at 60% identity in 10 amino acid overlaps:

```
          10
  ACLHQYLQFAWNSFGD          Ov-1
   :::.:: :
IHICLEKYLFFKTIEEQL          Colicin Ib
  160       170
```

Colicins are proteins of a large family of bactericidal pore-forming proteins consisting of a single polypeptide of 50 000 to 80 000 Da, which are encoded by genes present in *Col* plasmids. Each plasmid also carries information to provide the colicin-producing bacteria with resistance to its own colicin (Luria and Suit, 1982). These proteins have specific receptors in the outer membranes of bacteria and are believed to be regulators of the transport of molecules into the cell. For a review see Neville and Hudson (1986).

In view of the encouraging results, another analytical approach, pattern search, was utilized. Patterns or motifs in proteins are distinguished from a set of aligned sequences with the use of pattern-recognition matrices (discriminators), and are representations of a consensus stretch (Fuchs, 1990; Attwood *et al*, 1991). The use of this type of approach to detect unsuspected but meaningful relationship among sequences of proteins is becoming rapidly a obligatory step in the analysis of sequences. The sequences of the aerolysins, the Ov enterolobin peptides and the predicted cytolytic sites of the three cytoysins in study were searched by the program MACPATTERN (Fig. 28)

```
Sequence file: ENTEROLOBIN PREDICTED CYTOLYTIC SITE
Pattern set:   All entries

Sequence 1 (24 residues):

Matching pattern CK2_PHOSPHO_SITE:
(    2) SGGE
Total matches: 1

Matching pattern tonB_DEPENDENT_RECEP:
(    1) FSGGETVSPYRLAAPFDSCLWRRS
Total matches: 1


429 patterns searched in 1 sequences, 25 residues.
2 hits identified.
```

a

```
Sequence file: Aeromonas hydrophila AEROLYSIN PREDICTED
CYTOLYTIC SITE

Pattern set:   All entries

Sequence 1 (24 residues):

Matching pattern tonB_DEPENDENT_RECEP:
(    1) FAGNIEIGAPVPLAADSKVRRARS
Total matches: 1


429 patterns searched in 1 sequences, 24 residues.
1 hits identified.
```

b

```
Sequence file: Aeromonas sobria AEROLYSIN PREDICTED CYTOLYTIC
SITE

Pattern set:   All entries

Sequence 1 (24 residues):

Matching pattern tonB_DEPENDENT_RECEP:
(    1) FAGNIEIGTPVPLGSDSKVRRTRS
Total matches: 1


429 patterns searched in 1 sequence, 24 residues.
1 hits identified.
```

c

Figure 28 a,b,c: Pattern analyses of the predicted cytolytic sites from enterolobin and aerolysins. Enterolobin (**a**), aerolysin of *A. hydrophyla* (**b**) and aerolysin of *A. sobria* (**c**). The description of the pattern *ton*b-dependent receptor is in (**d**) next page. The figures are outputs of the program MACPATTERN, which was used for carrying out the search of the sequences against the database PROSITE.

PS00430; ***tonB*_DEPENDENT_RECEP**}
{BEGIN}
****
**tonb*-dependent receptor proteins signature *
****

In Escherichia coli the *tonB* protein interacts with outer membrane receptor proteins that carry out high-affinity binding and energy dependent uptake into the periplasmic space of specific substrates that are poorly permeable through the porin channels or are encountered at very low concentrations. In the absence of *tonB* these receptors bind their substrates but do not carry out active transport. The *tonB* protein also interacts with some colicins. The proteins that are currently known to interact with *tonB* are:

Gene	Function or name.
btuB	Receptor for cobalamin.
fecA	Receptor for iron dicitrate.
fepA	Receptor for ferric enterochelin.
fhuA	Receptor for ferrichrome-iron.
fhuE	Receptor for ferric coprogen, ferrioxamine B, and rhodotrulic acid.
iutA	Receptor for ferric aerobactin.
cir	Receptor for colicin I (exact substrate not known).
cba	Colicin B, a channel forming colicin.
cda	Colicin D.
cma	Colicin M, inhibitor of murein biosynthesis.

All these proteins contains, at their N-terminus, a short conserved region, called the *tonB*-box, which is involved in the interaction of the protein with the *tonB* protein.

-Consensus pattern: <x(10,60)-[DEF]-T-[LIVMFY]-[LIVST]-V-x-[AGP]-[SANP]
-Sequences known to belong to this class detected by the pattern: ALL, except colicin D which has His in position 1 of the pattern.
-Other sequence(s) detected in SWISS-PROT: human alpha-2-macroglobulin, rat alpha-1-inhibitor III, and bovine copper superoxide dismutase.
-Last update: November 1990 / First entry.

d

Figura 28d: Continuation from previous page

Interestingly, all the three sequences were shown to have a common similarity to the *tonB*-dependent receptor pattern. The cytolytic site of enterolobin also has in addition a phophorylation site pattern. However, phosphorylation as well as glycosylation and acylation patterns are very commonly found in several proteins (Bairoch, 1990). The other Ov peptides proved to have only these common patterns (not shown). The two aerolysins also contain short sequences with these common patterns, besides naturally their own signature. This aerolysin pattern is also shared by the *Pseudomonas aeruginosa* cytotoxin (Bairoch, 1990).

When the information in Fig 28d is analysed, one can find again the presence of colicins, thus strengthening the hypothesis of colicin as another pore-forming protein sharing some function and sequence relationship to aerolysins and enterolobin.

Several receptors for nutrients were also detected in the pattern, mainly ferric receptors from bacteria. These receptors need a product from the *tonB* gene to carry out their functions (Bell *et al.*, 1990). These *tonB*-dependent transport proteins from *E. coli* possess highly conserved short regions alternated by long variable sequences (Nau and Konisky, 1989). The *E. coli* gene of the *tonB* protein has been sequenced and encodes a 26.1-26.6 Kda hydrophilic protein that is likely to be embedded in the inner or outer membrane of *E. coli* (Postle and Good, 1983)

Also present in the output in Fig. 28d is human α_2-macroglobulin, which was identified in the SWISS-PROT database as belonging to the same pattern. α-Macroglobulins have been conserved during evolution, and have been purified from invertebrates, reptiles,

birds, and many mammalian organisms The family is composed of high molecular weight (subunits of 185 KDa) proteinases inhibitors, such as α_2-macroglobulin and some components of the complement system, such as factors C3, C4 and C5 (Enghild *et al.*, 1990). Recently, Enghild *et al* (1990) demonstrated that a α-macroglobulin of the ancient invertebrate *Limulus polyphemus* (horsehoe crab) is not only a protease inhibitor, but also is a complement-like protein participating in the lytic system of the crab. Then *Limulus polyphemus* α-macroglobulin complement-like (limac) could be a link between the two sides of the α-macroglobulin family - the protease inhibitors and the complement.

There seem to be a common link among all these pore-forming protein considered above - enterolobin, aerolysins, porins, colicins, transport proteins in *E. coli*, α-macroglobulins and complement factors. The question whether they are evolutionary related has not been answered yet. Further analysis must be done using more powerful and sensitive software and faster hardware. Package of programs and databases such as OWL, SOMAP, ADSP from the University of Leeds and UWGCG from the University of Winsconsin, USA, running on DEC VAX operating VMS and SUN (or similar workstations) operating UNIX could be of great help to the analyses.

3.6.2 Hydropathy Profiles

The theoretical approach of analysing the hydropathy profiles of sequences has been used for some time for the prediction of surface-seeking, antigenic and membrane-interacting regions in proteins

(for review Eisenberg, 1984; Heijne, 1988). Being cytolytic proteins, enterolobin and aerolysin should somehow interact with the target membranes. In order to gain some indication about the regions of these proteins that could interact with membranes, analyses of their hydropathic characteristics were carried out.

To perform a hydropathy analysis, one should firstly choose an appropriate hydrophobicity scale, i.e., a scale that assigns hydrophobicity values to each of the twenty amino acids. Basically, there are three types of scales: (a) experimental ones constructed from physical-chemical properties of the amino acids; (b) statistical, from the knowledge derived from previously determined proteins structures; and (c) theoretical consensus that employs several scales to optimize a given parameter.

Although other scales such as Kyte and Doolittle's are more frequently used, the hydropathy analyses of aerolysins and enterolobin were done by applying the consensus scales of Eisenberg *et al.* (1984) and Argos *et al.* (1982). These two scales are highly optimized for detecting membrane-interacting sequence regions.

An important parameter that must be set during the analysis is the moving window size. This is the number of residues along the sequence that will be taken in account each time for the calculation of the average hydrophobic value for the first residue in the window. This window advances one residue at a time, and has the function of smoothing the hydrophobic plot in order to allow a better interpretation of the results. For example, windows with short sizes (6-7 residues) are set when the objective is to find turns in the sequence, while long windows (18-20 residues) are indicated for investigating membrane-

spanning stretches, since the width of biological membranes is equal to the length of 18-20 residues in average.

Using both Argos' and Eisenberg's scales with several window sizes, it was demonstrated that the two aerolysins are rather more hydrophilic than hydrophobic proteins with no region long enough to be considered as membrane-spanning (Figs 29 and 30). This result is in agreement with the data provided by preliminary crystallographic studies, which demonstrates that the aerolysin of *A. hydrophila* is a hydrophilic β-structured protein with no recognizable amphiphilic helices (Green and Buckley, 1990).

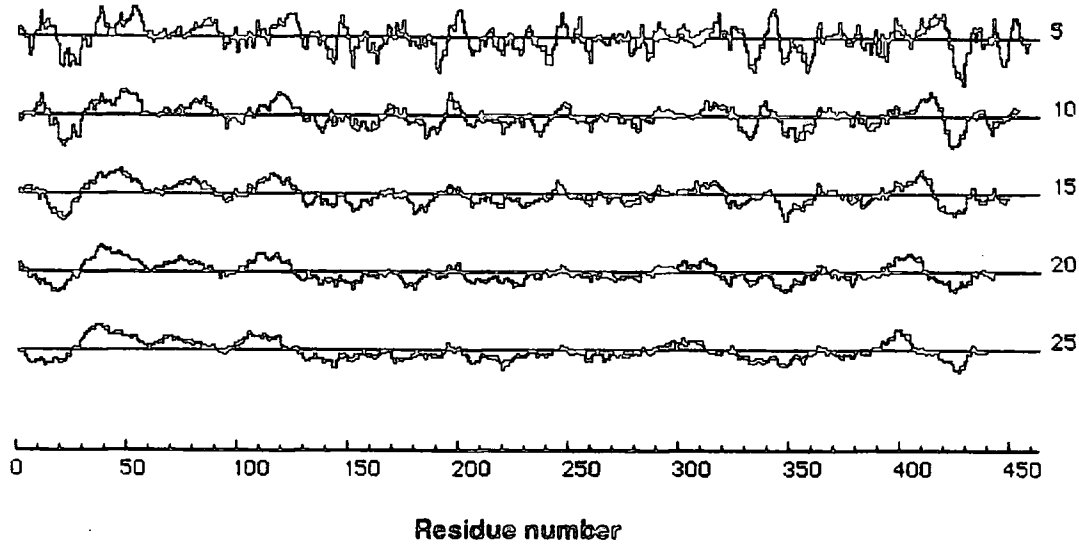
There seem to be, however, some regions which could be possible turns. More resolved plots were produced with a window size of 7 residues to permit a better interpretation of this fact (Figs 31 and 32). For both scales and both methods, the possible existence of turns related to proline residues (at positions 25, 59, 77, 86, 90, 115, 181, 248, 305, 347, 419, 421) was predicted satisfactorily. Proline is known as a turn former, and is not generally found in helices and β-sheets (Richardson and Richardson, 1989). The method did consider the two close proline residues (419 and 421) that are located in the predicted cytolytic site.

For the sake of comparison, the hydropathy profiles of the two aerolysins were ploted in the same graph (Fig. 33) Similarly to the sequence alignment, the hydropathic plots revealed two extremely homologous, mostly hydrophilic, proteins, which was absolutely expected. It would not be surprising if enterolobin had a hydrophilic profile resembling those of the aerolysins. The complete analysis of the hydropathic characteristics of enterolobin will be performed when its

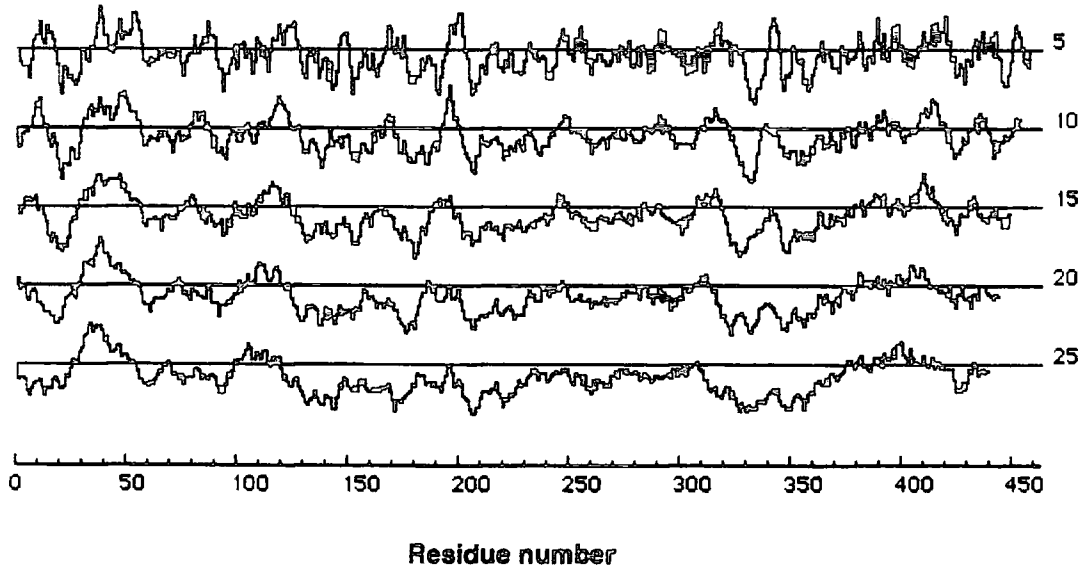
complete amino acid sequence is determined. Meanwhile, its predicted cytolytic site was compared to the sites of aerolysins (Fig. 34)

The predicted cytolytic sites of the two aerolysins shared a great uniformity in their hydropathy pattern as can be seen in Fig 34. The outline of the profile for enterolobin was similar to the others, although there were some differences, especially when the scale of Argos was used. In the aerolysins, there is a hydrophobic region followed by a hydrophilic one. As mentioned above, there are two proline residues (Pro_{419} and Pro_{421}) placed in the predicted cytolytic sites and related to possible turns. Enterolobin also has a proline residue aligned to Pro_{419}. It is not unreasonable to suppose that these prolines could actually form a turn or a distortion in the strucuture. This motif would be an 'amphipatic proline finger', which could behave like amphipathic helices (e.g. mellitin) in the disruption of membranes. However a hypothesis like this must be carefully built, since this concept of a common cytolytic region could be wrong for some cases.

The existence of a common cytolytic region in myotoxins, haemolysins, cardiotoxins and antibacterial peptides from several sources and diverse physical-chemical and biological characteristics was a proposal by Kini and Evans (1989). The authors employed several theoretical tools, including analysis of hydropathy. For *A. hydrophila* aerolysin, they described a region between residues Phe_{410} and Arg_{422} shown in Fig. 27. This generalization for the aerolysin has to be taken in consideration with care, since recent experiments of site-directed mutagenesis revealed the involvement of histidine residues in membrane binding and cytolysis by *A. hydrophila* aerolysin (Green and Buckley, 1990). These residues are His_{332} (binding) and His_{132} (cytolysis), and are distant from the discussed site, at least in the primary structure.

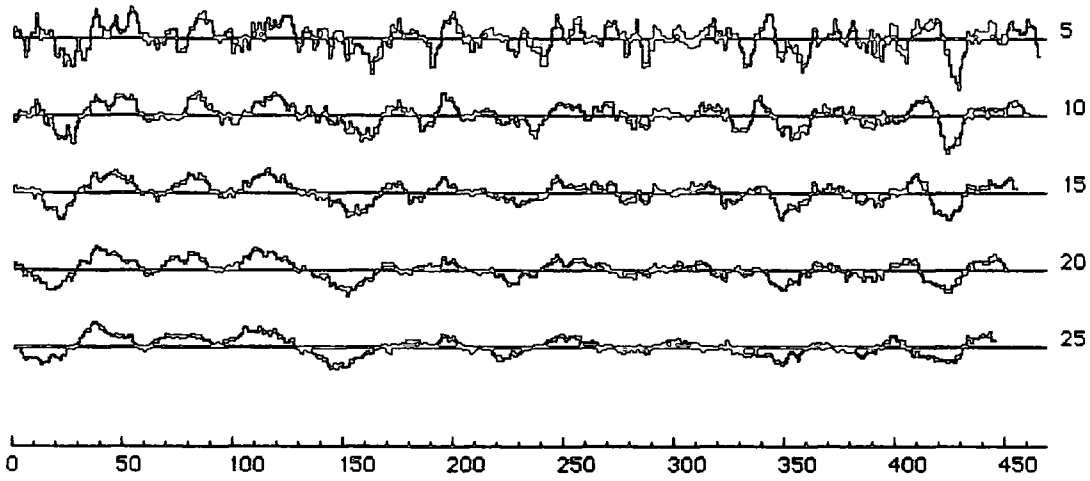


a

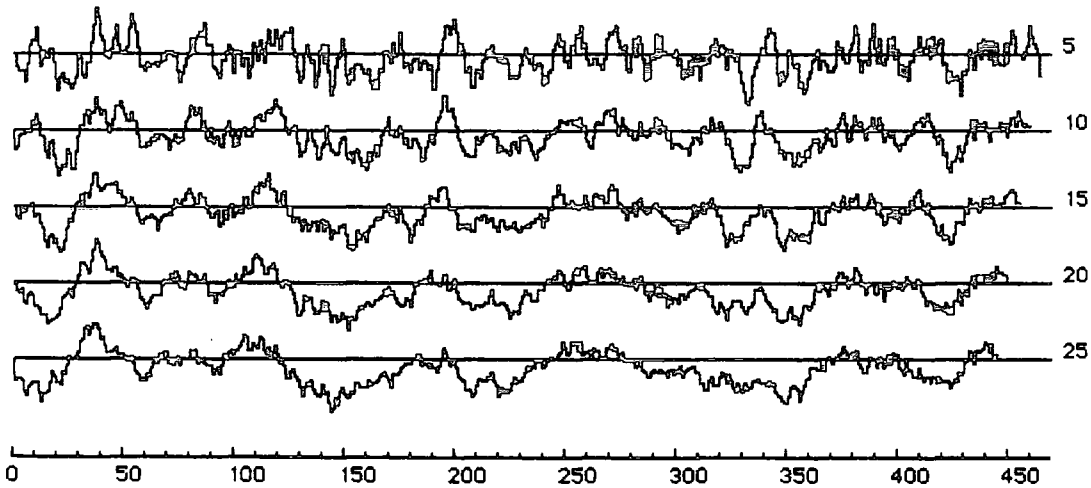


b

Figure 29: Hydropathy profiles of aerolysin *A. hydrophila*. Program PLOT.A/HYD5 was used with the hydropathy scales of Eisenberg (**a**) (Eisenberg *et al.*, 1984) and Argos (**b**) (Argos *et al.*, 1982). The numbers at the right side represent the moving average windows along the sequences. The regions above the horizontal lines represent the hydrophobic stretches, and below the lines, the hydrophilic ones.

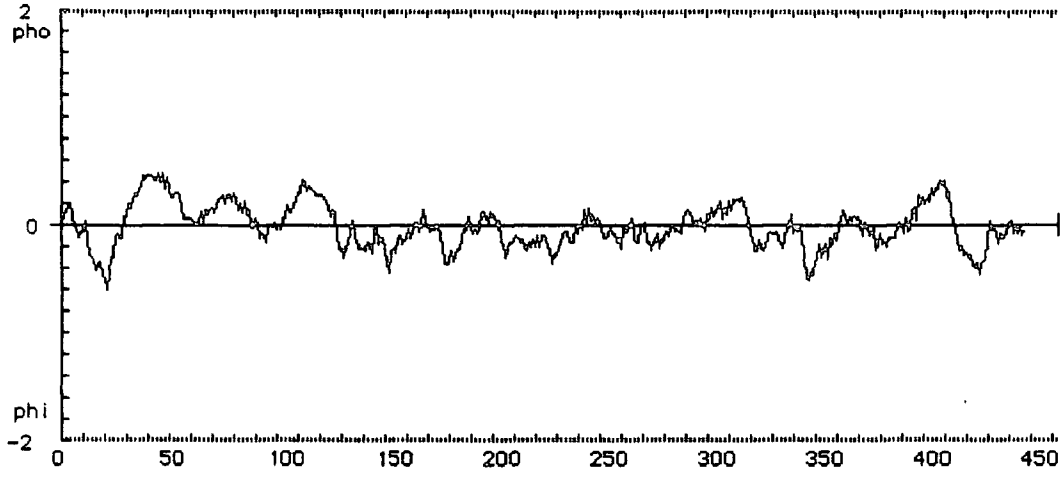


a

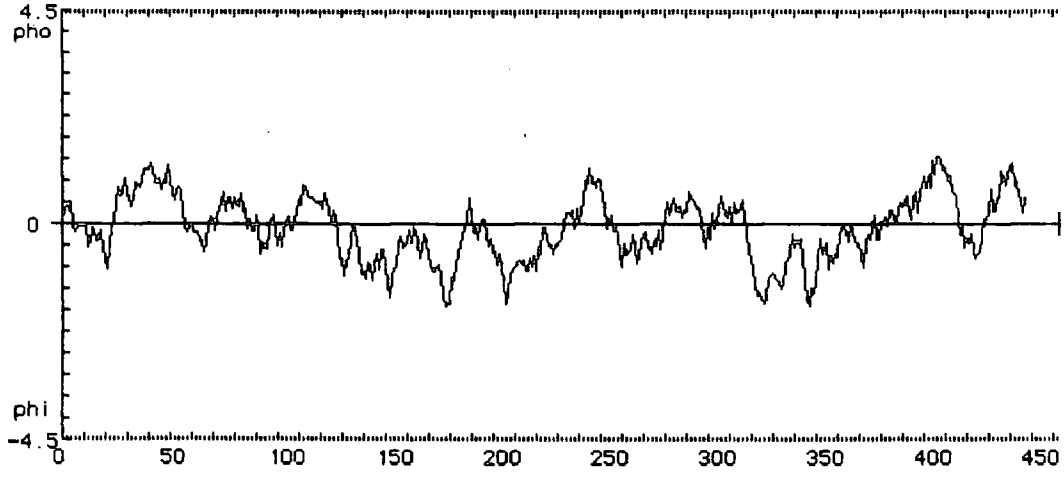


b

Figure 30: Hydropathy profiles of aerolysin *A. sobria*. Program PLOT.A/HYD5 was used with the hydropathy scales of Eisenberg (**a**) (Eisenberg *et al.*, 1984) and Argos (**b**) (Argos *et al.*, 1982). The numbers at the right side represent the moving average windows along the sequences. The regions above the horizontal lines represent the hydrophobic stretches, and below the lines, the hydrophilic ones.

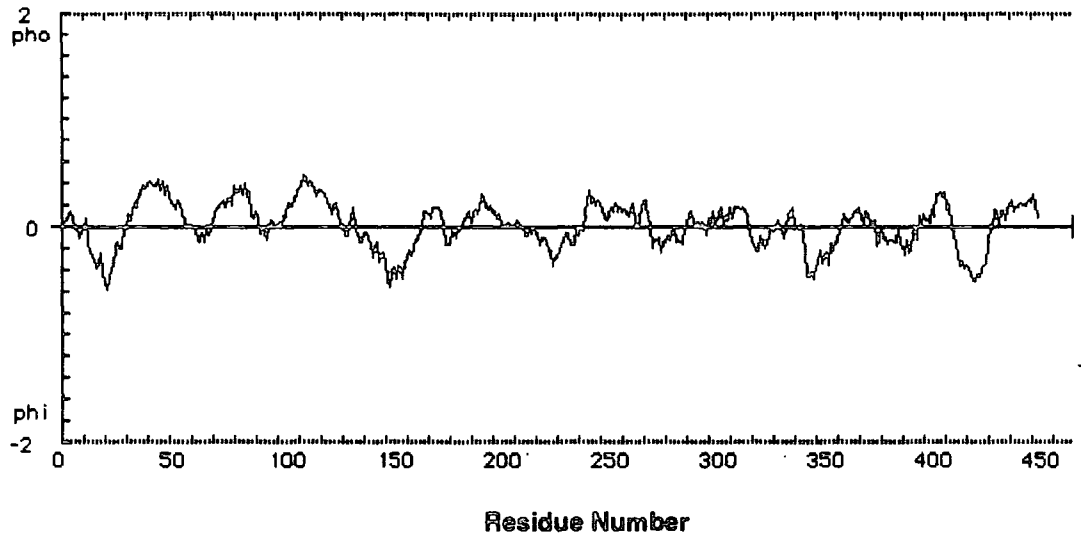


a

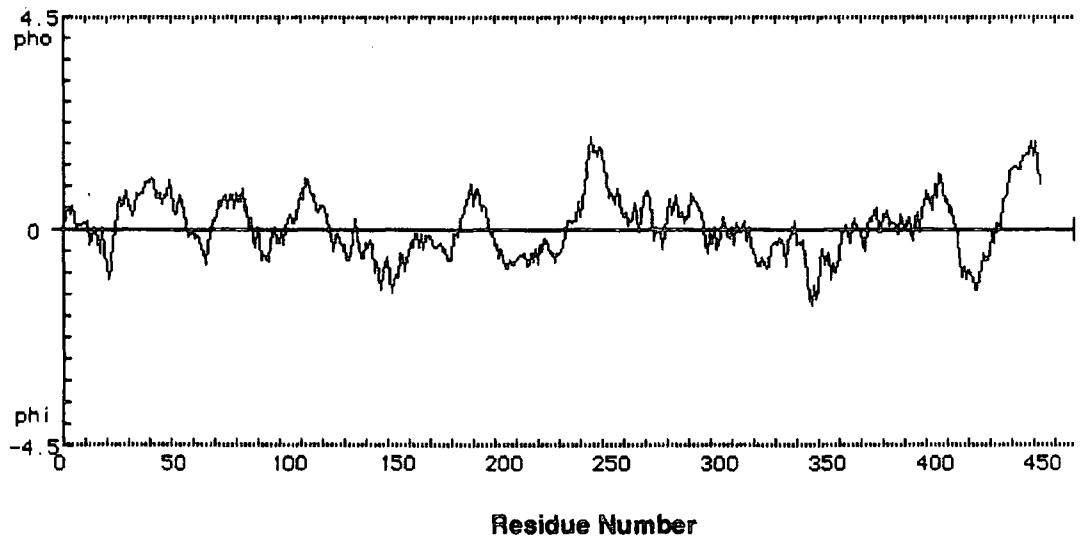


b

Figure 31: Detailed hydropathy profiles of aerolysin *A. hydrophila*. Program PLOT.A/HYD was used with the hydropathy scales of Eisenberg (**a**) (Eisenberg *et al.*, 1984) and Argos (**b**) (Argos *et al.*, 1982). A default moving average window value of 7 residues was used.

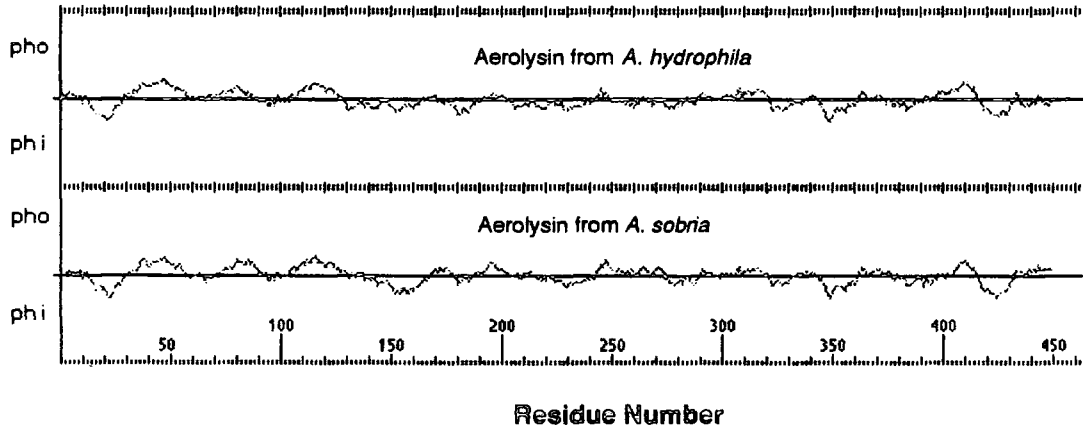


a

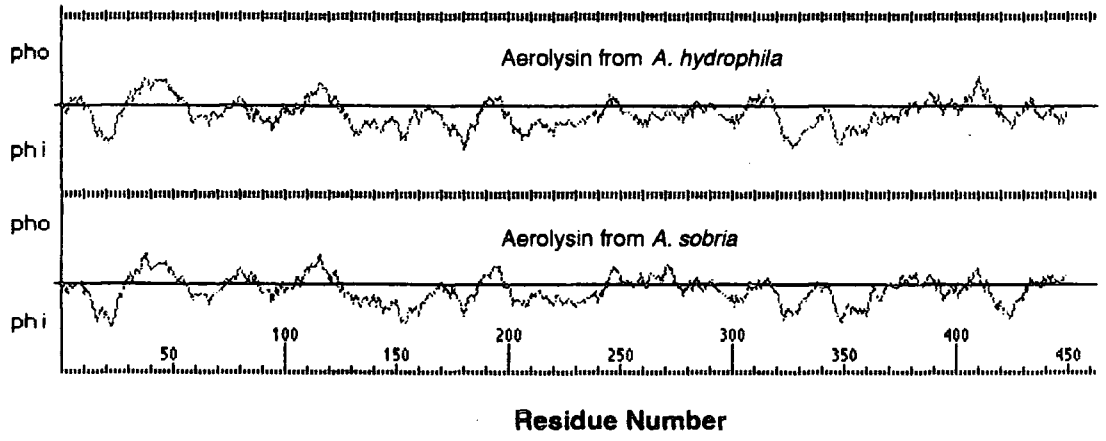


b

Figure 32: Detailed hydropathy profiles of aerolysin *A. sobria*. Program PLOT.A/HYD was used with the hydropathy scales of Eisenberg (**a**) (Eisenberg *et al.*, 1984) and Argos (**b**) (Argos *et al.*, 1982). A default moving average window value of 7 residues was used.



a



b

Figure 33: Comparative hydropathy profiles of aerolysins of *A. hydrophila* and *A. sobria*.. Program PLOT.A/H3 was used with the hydropathy scales of Eisenberg (**a**) (Eisenberg *et al.*, 1984) and Argos (**b**) (Argos *et al.*, 1982). A moving average window value of 15 residues was used.

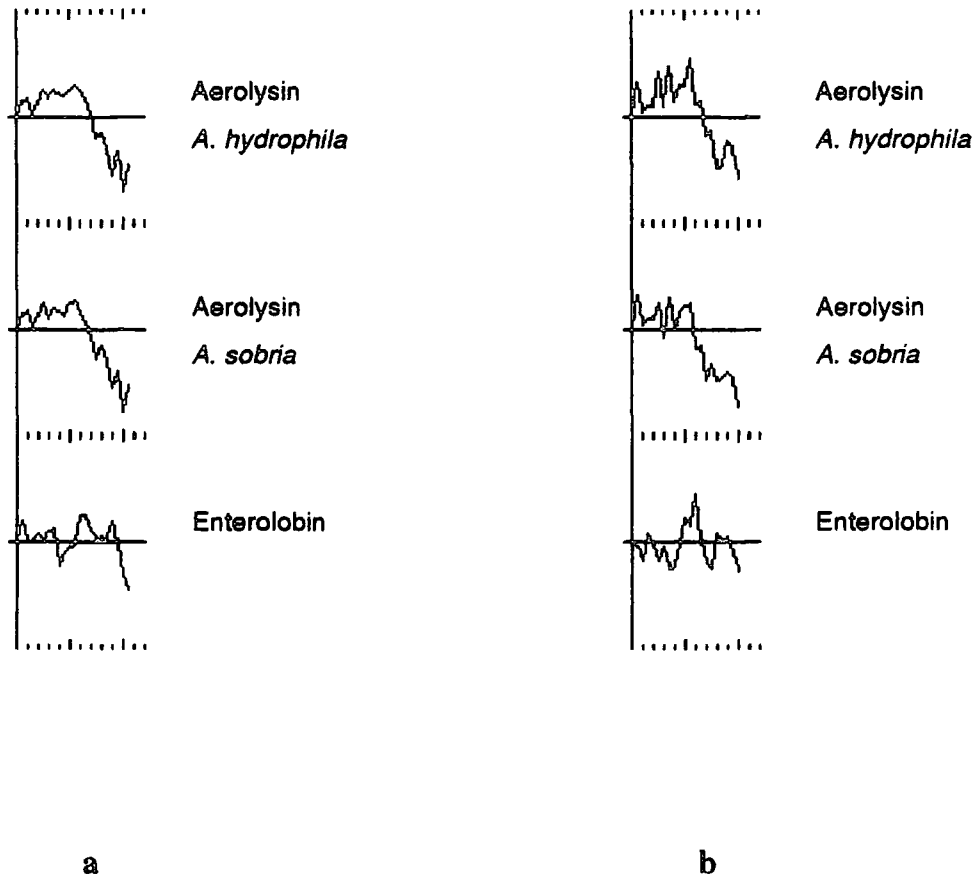


Figure 34: Comparative hydropathy profiles of the putative cytolytic sites of enterolobin and aerolysins. Program PLOT.A/H3 was used with the hydropathy scales of Eisenberg (**a**) (Eisenberg *et al.*, 1984) and Argos (**b**) (Argos *et al.*, 1982). A default moving average window value of 7 residues was used.

3.7 Kinetics of Haemolysis

The determination of the kinetic behaviour of haemolysis caused by enterolobin was performed by means of a light scattering continuous assay. The method was based on the fact that the transmittance of a suspension of erythrocytes increased during the course of the haemolysis reaction. The increase of the transmittance could be conveniently recorded in a spectrophotometer, where the wavelength of 680 nm was chosen in order that the difference of transmittance before and after haemolysis was the highest possible to provide a maximum sensitivity. The method permitted the inhibitory or activating effects of various agents on the haemolytic profile to be readily observed.

From the results of the kinetic experiments, it is clear that enterolobin can lyse red cells to 100% haemolysis in periods of time usually around 15 min (Fig. 35). The release of haemoglobin always started after few minutes of incubation (5-6 min). The shape of the haemolysis curve was sigmoidal as it is for many other haemolytic agents like saponin (Fig. 36), free radicals from hydrogen peroxides (McFaul and Everse, 1986; Richards *et al.*, 1988), *Prymnesium parvum* toxin (Martin and Padilla, 1971; Martin *et al.*, 1973), equinatoxin from the sea anemone *Actinia equina* (Macek and Lebez, 1981), haemolytic glycoprotein from the Portuguese Man-of-War venom (Lin and Hessinger, 1979), *Bombina variegata* haemolytic toxin (Mar and Michl, 1976), crotin (Banerjee and Sen, 1983) and several other cytolytic micromolecules and peptides, proteins (Thelestan and , 1979; Bashford *et al.*, 1986; Menestrina *et al.*, 1990).

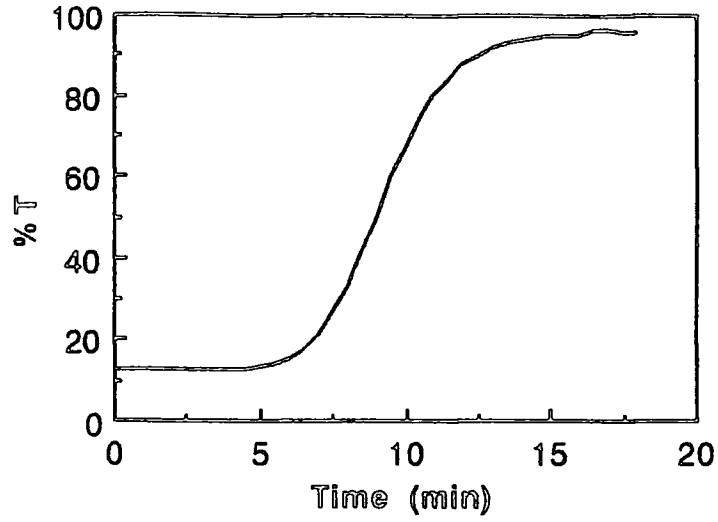


Figure 35: Curve of haemolysis by enterolobin. The concentration of enterolobin (fraction F-2) was 200 µg/ml in 10 mM tris-HCl pH 7.4. A 3% (v/v) suspension of human red cells (type O+) in the same buffer was used as target cells. The percentage of transmittance (%T) was recorded continuously at 680 nm at room temperature.

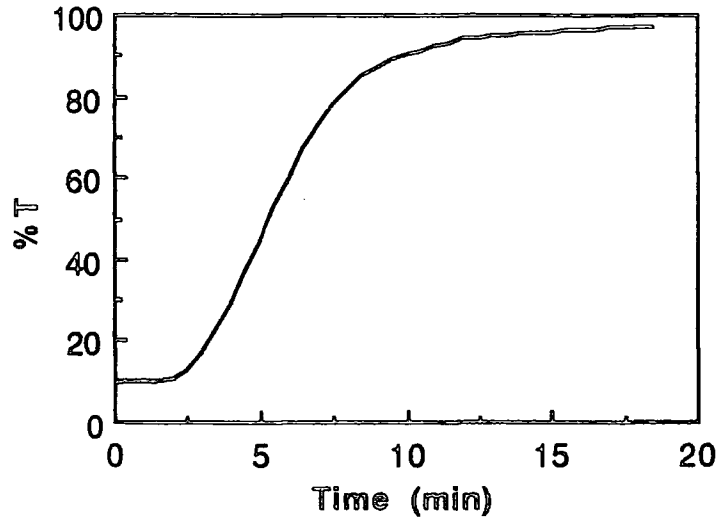


Figure 36: Curve of haemolysis by saponin. The concentration of saponin was 6 µg/ml in 10 mM tris-HCl pH 7.4. A 3% (v/v) suspension of human red cells (type O+) in the same buffer was used as target cells. The percentage of transmittance (%T) was recorded continuously at 680 nm at room temperature.

Enterolobin was non-specific for all the erythrocyte types tested (groups A, B, AB and O). Minor differences in the periods of time needed for the achievement of total haemolysis and shape of the curves (while always keeping their sigmoidal characteristic) occurred for different blood groups and individuals (figures not shown). It is known that factors such as diet can induce modifications on the molecular composition of the cell membrane (Kummerow, 1983; Holmes and Kummerow, 1985). Similarly, modification of components on the erythrocyte membrane can occur in function of age of individual. For example, band 3 protein degradation by the protease calpain is enhanced in erythrocytes of old people (Meir *et al.*, 1991). These and other factors might account for those small changes in the time-length of the reaction and the shape of the curve of haemolysis.

Ca^{2+} is a common inhibitor of haemolytic peptides and proteins (Cannicati, 1983; Bashford *et al.*, 1986; Osorio and Castro *et al.* 1989; Menestrina *et al.* 1990). On the other hand, this cation can be an activator or even a co-factor for some haemolytic agents (Mar and Michl, 1976; Louw and Visser, 1977; Macek and Lebeg, 1981; Young, 1989). The cation chelator EDTA generates the expected opposite effect of Ca^{2+}. Their mechanisms of inhibition and activation of haemolysins are still not completely clear. For enterolobin, in any way, Ca^{2+} and EDTA had no effect at all as shown in Fig. 37.

Cholesterol, phospholipids and proteins are well known as the main components of the red cell membrane. Gennis (1989) has summarized some data: phosphatidylcholine accounts for 25% of the total lipids of the erythrocyte membrane; cholesterol forms 25% as well; and band 3 and PAS-1 proteins have a lipid/protein (dry weight)

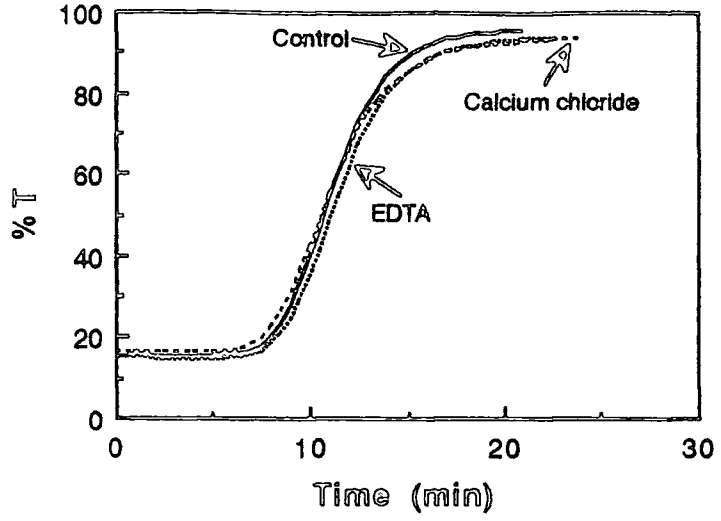


Figure 37: Curve of haemolysis by enterolobin in presence of Ca^{2+} and EDTA. The concentration of enterolobin (fraction F-2) was 200 µg/ml in 10 mM tris-HCl pH 7.4. A 3% (v/v) suspension of human red cells (type O+) in the same buffer was used as target cells. The concentrations of Ca^{2+} and EDTA were 10 mM. Control was enterolobin alone. The percentage of transmittance (%T) was recorded continuously at 680 nm at room temperature.

ratio of 0.75, being the principal proteins on the membrane. Band 3 and PAS-1 are common designations (from their positions and staining characteristics in electrophoresis gels) of the anion-exchanger protein and glycophorin respectively. These proteins have parts of their structures located at the external part of the membrane (Marchesi *et al.*, 1976). Also phosphatidylcholine and cholesterol are preferentially localized at the external layer of the membrane (Kamp, 1979).

Thelestan and Möllby (1979) classified most of the cytolysins tested in their work as belonging to a group of substances interacting with specific receptor molecules in the surface of the cell membrane and, then, giving rise to membrane lesions. Proteins like aerolysin, *S. aureus* δ-toxin, cereolysin, listeriolysin, streptolysin O,

vibriolysin, direct lytic factor (*Naja*), etc. fell in this group. Most of the cytolysins have either phosphatidylcholine or cholesterol as their binding site on the membrane (Bernheimer and Rudy, 1986). However, some of them possess glycoprotein receptors. Examples are the haemolytic toxin of *Physalia physalis* (Lin and Hessinger, 1979) and aerolysin (Howard and Buckley, 1982).

In this present work, an attempt was made to find out if enterolobin had any specific binding site on the red cell membrane, and, if so, what the chemical nature of this molecule was. The kinetic experiments in the presence of some possible effectors such as galactose, choline, phosphatidylcholine, cholesterol, ricin, and with the utilization of trypsinised erythrocytes produced results allowing some conclusions as discussed below.

Galactose is a monomeric carbohydrate with properties to inhibit erythrocyte agglutination by many lectins and hemi-lectins like RCA_I and RCA_{II} (ricin) from *Ricinus communis* for example (Lis and Sharon, 1986). It is not surprising since these proteins exhibit galactose-specific binding to the red cell membrane . This carbohydrate is a major constituent of the glycosidic moiety of many membrane glycoproteins such as the band 3 protein (Fukuda *et al.*, 1984). Galactose, however, was not seen to have any inhibitory effect upon haemolysis by enterolobin (Fig. 38). It is possible that a more complex carbohydrate or a glycopeptide isolated from the membrane red cell membrane would inhibit enterolobin. This is to be tested.

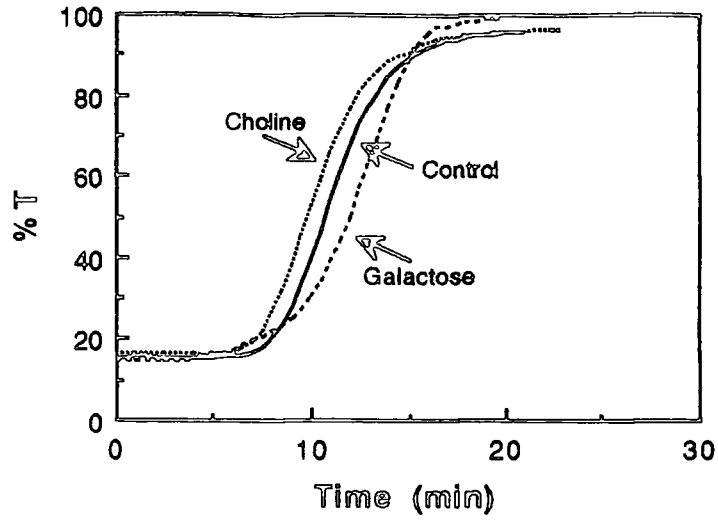


Figure 38: Curve of haemolysis by enterolobin in presence of galactose and choline. The concentration of enterolobin (fraction F-2) was 200 µg/ml in 10 mM tris-HCl pH 7.4. A 3% (v/v) suspension of human red cells (type O+) in the same buffer was used as target cells. The concentration of galactose was 100 mM and choline was 10 mM. Control was enterolobin alone. The percentage of transmittance (%T) was recorded continuously at 680 nm at room temperature.

The major lipids that compose biological membranes, phosphatidylcholine and cholesterol, are common interactive sites for a number of cytolysins (Bernheimer, 1983; Bernheimer and Rudy, 1986). However, they do not seem to be the receptors for enterolobin since they did not inhibit haemolysis by this protein (Figs. 39 and 40). The pattern of inhibition would have to be like the one for saponin, which was effectively inhibited by cholesterol (Fig. 41).

For phosphatidylcholine, an interesting phenomenon happened. When in presence of non-sonicated phosphatidylcholine, there was a partial decrease in the maximum percentage of haemolysis by enterolobin (Fig. 40). It could lead one to conclude prematurely that phosphatidylcholine was the site of interaction for the haemolysin.

However, sonicated phosphatidylcholine did not produce any inhibition (Fig. 40). Similarly, choline, the polar head of phospatidylcholine, exerted no effect on the haemolysis. A possible explanation for these results is that non-sonicated phosphatidylcholine might be encapsulated in micelles either normal or reverse ones. Reverse micelles are structures the reverse of normal micelles, with their polar heads turned to the core of the micelle and their apolar tails interacting outside with other reverse micelles. Reverse micelles have been shown to be hosts of a number of proteins (Luisi *et al.*, 1988).

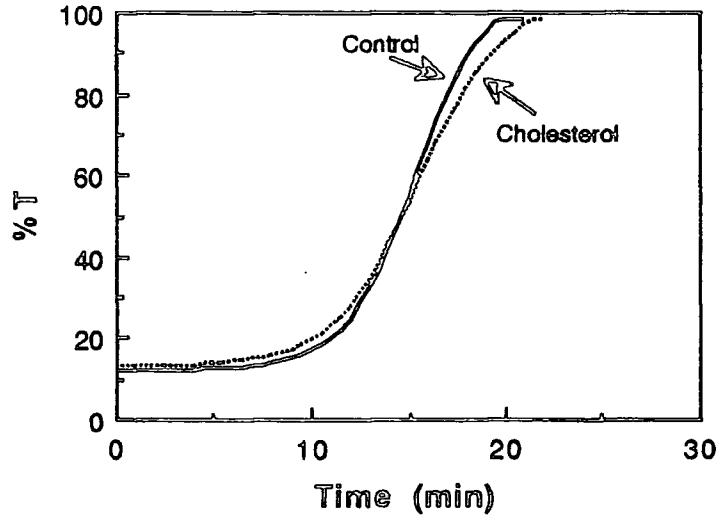


Figure 39: Curve of haemolysis by enterolobin in presence of cholesterol. The concentration of enterolobin (fraction F-2) was 200 µg/ml in 10 mM tris-HCl pH 7.4. A 3% (v/v) suspension of human red cells (type O+) in the same buffer was used as target cells. The concentration of cholesterol was 10 mM. Cholesterol was previously sonicated in a Bronwill Biosonik sonicator. Control was enterolobin alone. The percentage of transmittance (%T) was recorded continuously at 680 nm at room temperature.

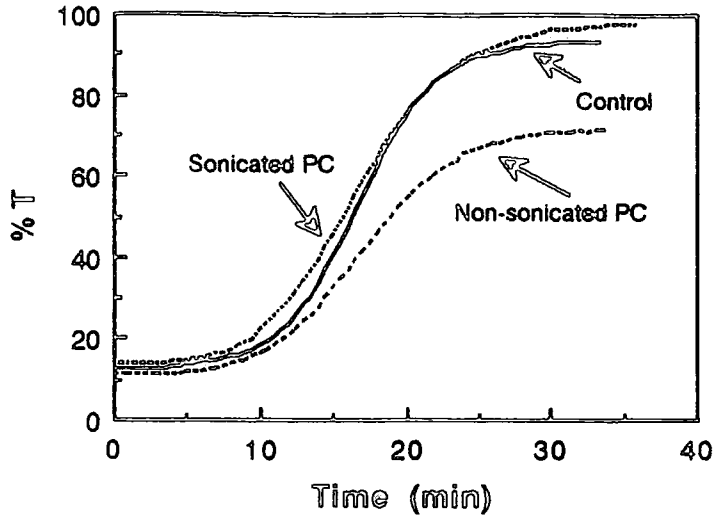


Figure 40: Curve of haemolysis by enterolobin in presence of sonicated and non-sonicated phosphatidylcholine. The concentration of enterolobin (fraction F-2) was 200 µg/ml in 10 mM tris-HCl pH 7.4. A 3% (v/v) suspension of human red cells (type O+) in the same buffer was used as.target cells. The concentration of phosphatidylcholine both sonicated and non-sonicated was 10 mM. The sonication of PC was done in a Bronwill Biosonik sonicator. Control was enterolobin alone. The percentage of transmittance (%T) was recorded continuously at 680 nm at room temperature.

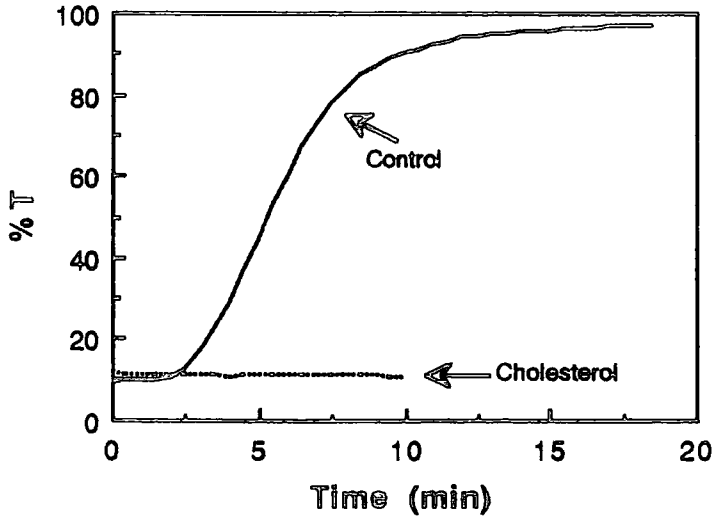


Figure 41: Curve of haemolysis by saponin in presence of cholesterol. The concentration of saponin was 6 µg/ml in 10 mM tris-HCl pH 7.4. A 3% (v/v) suspension of human red cells (type O+) in the same buffer was used as target cells. The concentrations of cholesterol was 10 mM. Cholesterol was sonicated with a Bronwill Biosonik sonicator. Control was saponin alone. The percentage of transmittance (%T) was recorded continuously at 680 nm at room temperature.

Trypsinised red cells were shown to be resistant to haemolysis by enterolobin (Fig. 42). There could be a doubt related to the possibility of any residual trypsin attacking and inactivating enterolobin during the incubation despite the cells having been washed several times. To remove this possibility, BTCI, a potent trypsin and chymotrypsin inhibitor from cowpea (Morhy and Ventura, 1987), was added in excess to the mixture, but the absence of haemolysis was also obtained here (Fig. 43). This set of results clearly indicated that a peptide or a protein was the receptor site for the haemolytic protein.

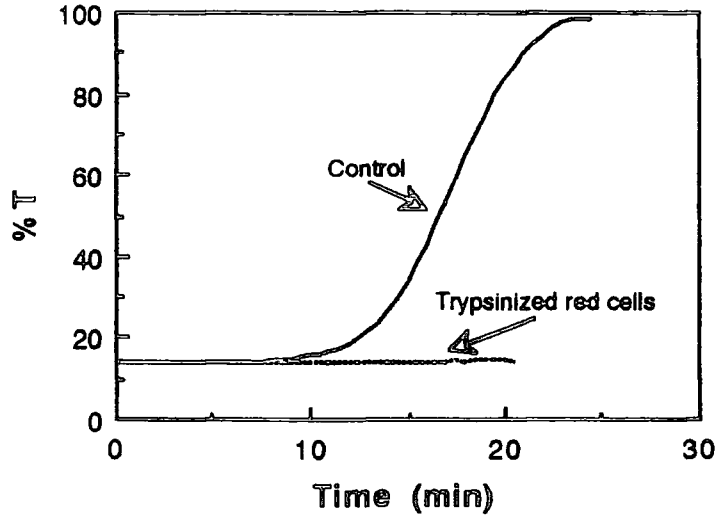


Figure 42: Curve of lysis of trypsinised red cells by enterolobin. The concentration of enterolobin (fraction F-2) was 200 µg/ml in 10 mM tris-HCl pH 7.4. A 3% (v/v) suspension of trypsinised human red cells (type O+) in the same buffer was used as target cells. Control was normal red cells with enterolobin. The percentage of transmittance (%T) was recorded continuously at 680 nm at room temperature.

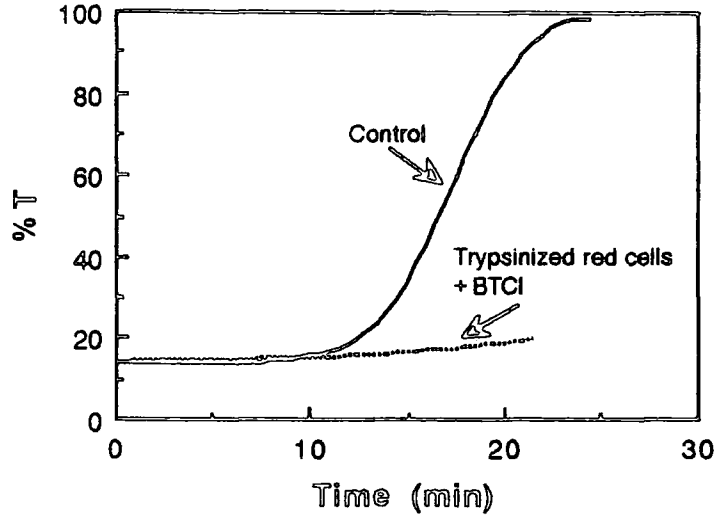


Figure 43: Curve of lysis of trypsinised red cells by enterolobin in presence of trypsin/chymotrypsin inhibitor. The concentration of enterolobin (fraction F-2) was 200 µg/ml in 10 mM tris-HCl pH 7.4. A 3% (v/v) suspension of trypsinised human red cells (type O+) in the same buffer was used as target cells. One mg of BTCI, black-eyed bean trypsin and chymotrypsin inhibitor (Morhy and Ventura, 1987), was added to the mixture. Control was normal red cells with enterolobin. The percentage of transmittance (%T) was recorded continuously at 680 nm at room temperature.

Haemolysis was also extensively inhibited by ricin (Fig. 44). Although ricin could just be causing steric obstruction for the approach of enterolobin to the membrane surface, these two proteins might actually be competing for the same binding site. In this case it would be a glycopeptide or a glycoprotein. There may be the hypothesis that band 3 protein is actually this binding site as explained below.

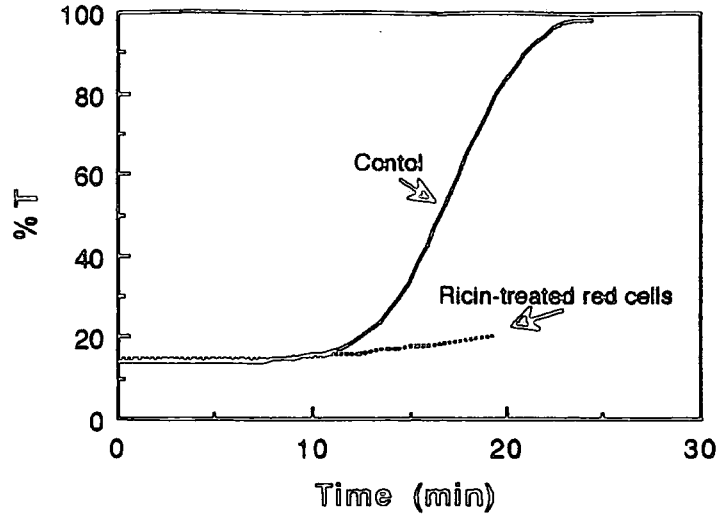


Figure 44: Curve of haemolysis by enterolobin in presence of ricin. The concentration of enterolobin (fraction F-2) was 200 µg/ml in 10 mM tris-HCl pH 7.4. A 3% (v/v) suspension of human red cells (type O+) in the same buffer was used as target cells. One mg of ricin was incubated with the mixture. Control was enterolobin alone. The percentage of transmittance (%T) was recorded continuously at 680 nm at room temperature.

It is known that band 3 is a binding site on the red cell membrane for ricins (Jenkins and Tanner, 1977; Chicken and Sharon, 1985). While their interactions are directed predominantly to galactose residues, the presence of sialic acid substituents on the residues markedly reduces or abolishes their association constants for glycopeptides (Baenziger and Fiete, 1979). This suggests that ricins do not bind to glycophorin, a protein rich in sialic acid groups (Fukuda *et al*, 1984). From these considerations, one can build the hypothesis that the binding site of enterolobin could be, as for ricins, the anion-exchanger band 3. Alternatively, as said above, ricin could just be impeding the approach to its real binding site by a steric blockage mode.

Some authors consider band 3 as a protein with no tryptic cleavage sites in its exofacial region, but only in its cytoplasmic portion (Jay and Cantley, 1986; Low, 1986). However, if the amino acid sequence of band 3 is examined, one can see several lysine and arginine residues along its outside region. In addition, Jenkins and Tanner (1977) have already reported the cleavage of external peptides of this protein. So, the hypothesis that band 3 could be the binding site for enterolobin is not destroyed by the fact that trypsinised red cells became resistant to haemolysis by this haemolysin.

As demonstrated earlier (Section 3.6), enterolobin seems to be similar to aerolysin. Howard and Buckley (1982) also found that trypsinised erythrocytes are more resistant to haemolysis by the bacterial toxin. They reached the conclusive hypothesis that the binding site for aerolysin on the red cell membrane would be glycophorin, although this is still to be fully proved.

Further work is necessary in order that the binding site for enterolobin might be precisely discovered. The use of other lectins such as ConA, WGA and PHA would help in this determination very much, since the first one is known to bind to band 3 and the latter ones bind to glycophorin (Grant and Peters, 1984). Likewise, DIDS, a specific inhibitor of ions and water transport by band 3 with a binding site on the external part of the protein (Jennings, 1985), could be useful, as well as antibodies against the anion-exchanger protein. Enterolobin could also be marked with colloidal gold or a fluorescent probe such as FITC, and used to detect its binding site after the separation of the red cell membrane proteins by gel electrophoresis. It could probably be done after the transference of the membrane proteins to a blotting paper.

3.8 Microscopic Observations of Haemolysis and Lysed Erythrocyte Membrane

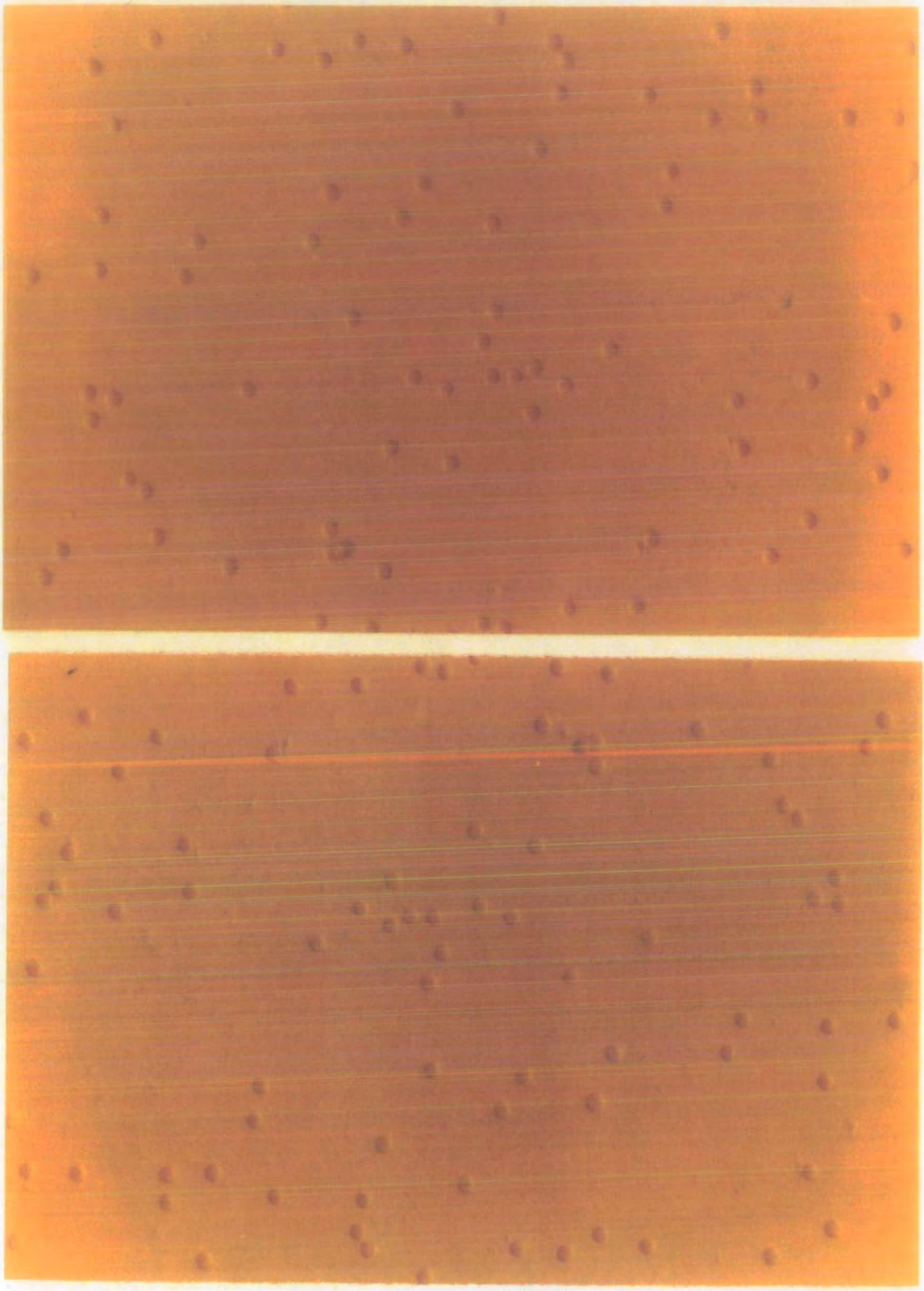
3.8.1 Haemolysis and Membranes Lysed by Enterolobin

The observation of haemolysis by enterolobin under optical microscopy was carried out in order to provide visual time-course information on the morphological transformation that red cells suffer during the lytic process. This study generated useful data that were photographed at given times and dynamically recorded on video casette.

It appears that there were three distinct phases. In the first one (0 to 2 min) no changes on the red cells could be noted (Figs. 45 a and b). Probably, this was the phase.when enterolobin established its binding to the cell membrane.

The second phase (2 to 4 min) clearly showed the swelling of the red cells (Fig. 46). Some few cells had already been lysed. It seems that water could somehow be entering into the cells during this phase, as if they were suffering an osmotic pressure difference caused by their placement in hypotonic solutions. Due to the high elasticity of the erythrocyte membranes (Hochmuth and Waugh, 1987; Mosior, 1988), they were able to resist to this deformation for some minutes.

The last phase (4 to 8 min) was the lysis of the erythrocytes, after which the lysed cells took on a translucid appearance due to the loss of the cytoplasmic content (Figs. 47 a and b). After 10 min of incubation, no cell was left intact (Fig. 48). Control cells did not undergo any modification after standing alone for 10 min (Fig 49).



a

b

Figure 45: Visualization of haemolysis by enterolobin under optical microscopy. One drop of enterolobin F-3 at 1 mg/ml was mixed with one drop of red cell suspension (3% v/v) in saline solution, and taken for observation under differential interference contrast microscopy at a magnification of 20 times. The incubation periods were of 0 min (**a**) and 2 min (**b**).

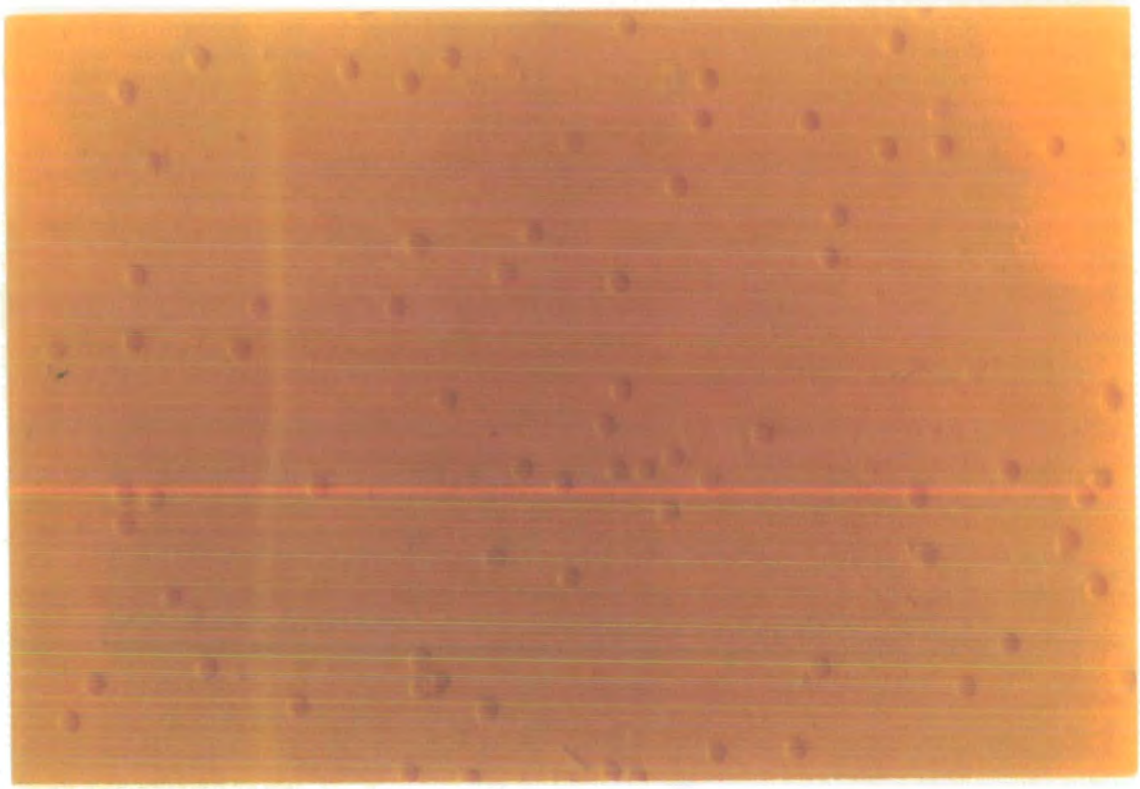


Figure 46: Visualization of haemolysis by enterolobin under optical microscopy. One drop of enterolobin F-3 at 1 mg/ml was mixed with one drop of red cell suspension (3% v/v) in saline solution, and taken for observation under differential interference contrast microscopy at a magnification of 20 times. The incubation period was of 4 min.

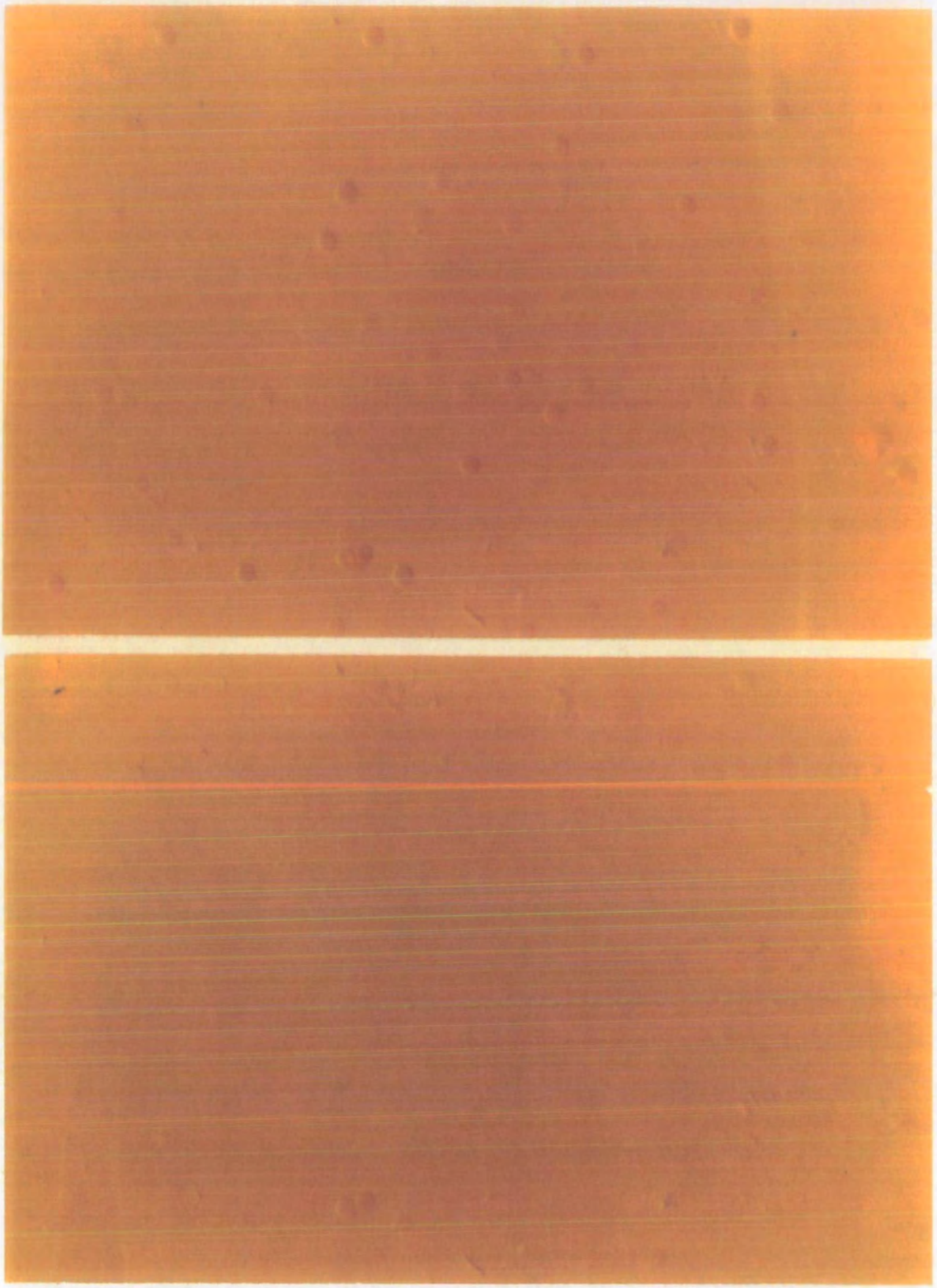


Figure 47: Visualization of haemolysis by enterolobin under optical microscopy. One drop of enterolobin F-3 at 1 mg/ml was mixed with one drop of red cell suspension (3% v/v) in saline solution, and taken for observation under differential interference contrast microscopy at a magnification of 20 times. The incubation periods were of 6 min (**a**) and 8 min (**b**).

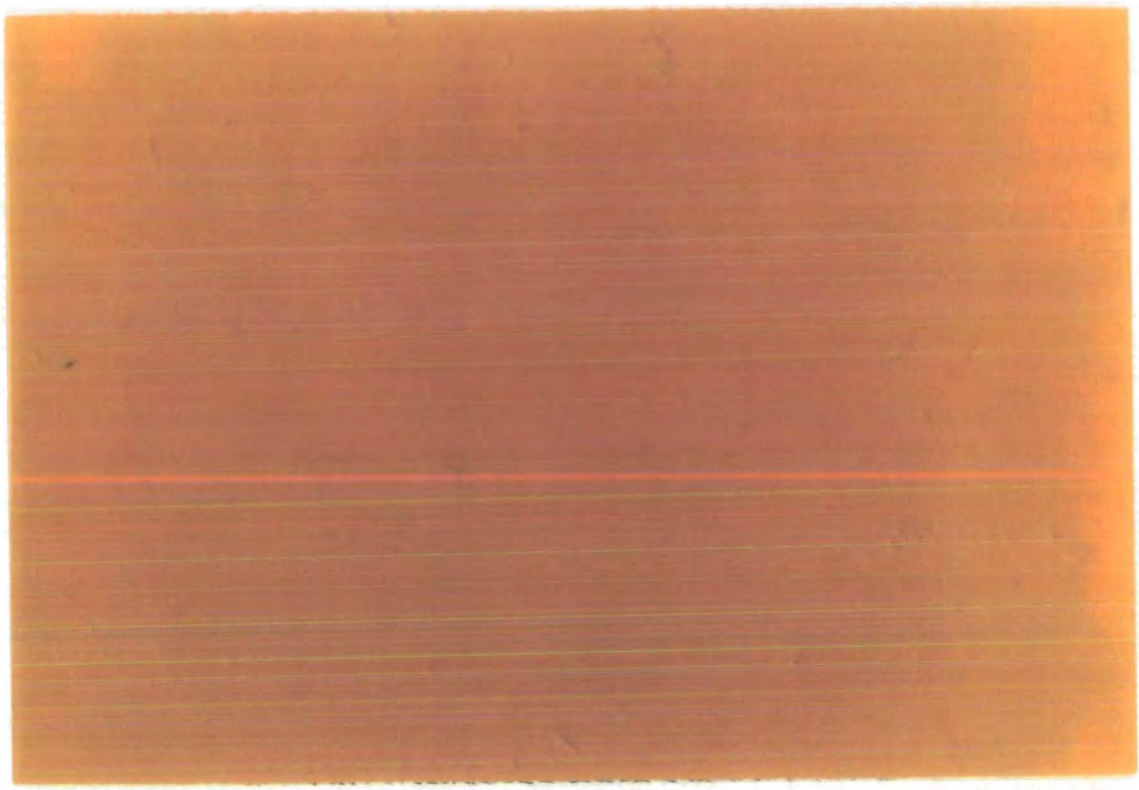


Figure 48: Visualization of haemolysis by enterolobin under optical microscopy. One drop of enterolobin F-3 at 1 mg/ml was mixed with one drop of red cell suspension (3% v/v) in saline solution, and taken for observation under differential interference contrast microscopy at a magnification of 20 times. The incubation period was of 10 min.

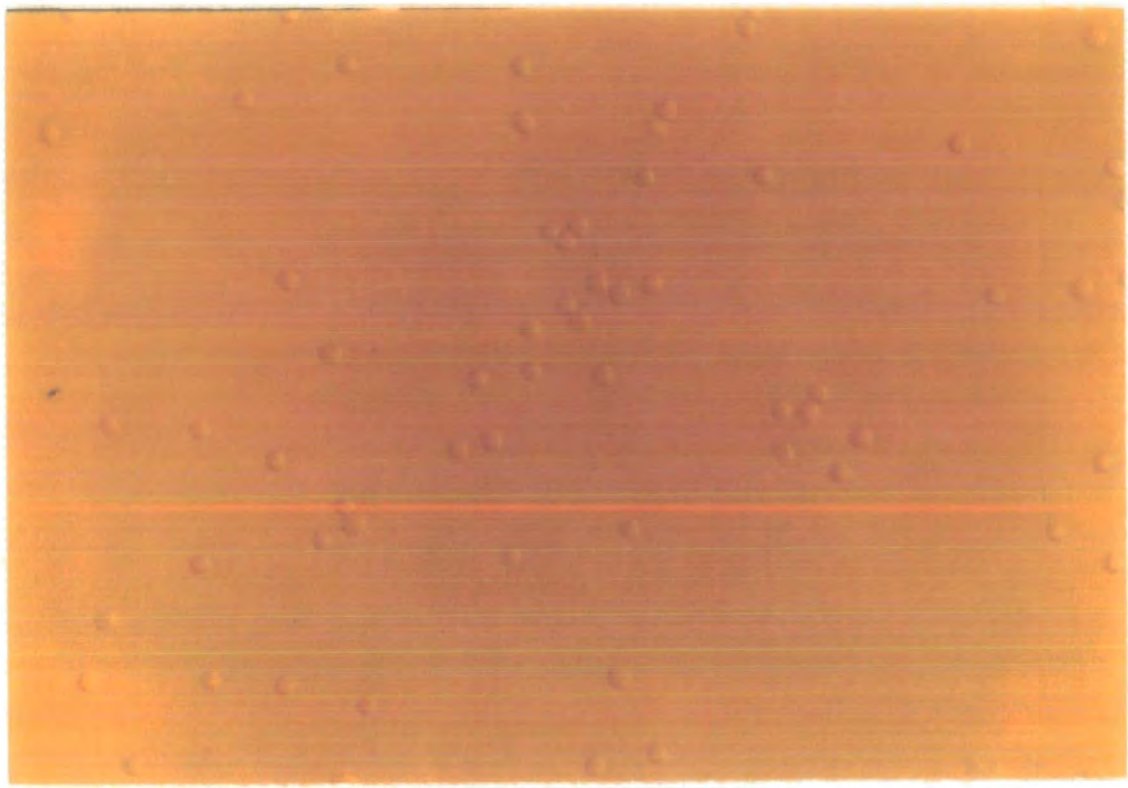


Figure 49: Control visualization of red cells under optical microscopy. One drop of red cell suspension (3% v/v) in saline solution was observed under differential interference contrast microscopy at a magnification of 20 times after standing undisturbed for 10 min .

When the red cell membranes were examined by electron microscopy, very few perforations could be seen by 2 min of incubation with enterolobin (Fig. 50). After 15 min, the membranes had been covered by holes of several sizes ranging from 40-50 nm to 300 nm or more (Figs 51 and 52). The control cells did not show any perforation on their membranes (Fig. 53).

Other cytolytic proteins like, for example, staphylococcal α-toxin, streptolysin O, perforin and complement produce pores of defined and constant sizes as measured by electron microscopy (Bhakdi and Tranum-Jensen, 1987). Their general mechanism of cytolysis is by means of the formation of oligomers with pore shape that transverse the membrane causing the leakage of intracellular material. From the results for enterolobin, this appears not to be the case since it coats the membrane with pores of several different sizes as if the cell had been burst.

The understanding of the molecular chemical and physical mechanisms that determines the stability/instability of biological membranes is fundamental for the comprehension of several important biological processes, including haemolysis.

In many of these biological phenomena, membranes can undergo two basic types of instability - (a) rupture, which leads to the formation of pores or/and fragmentation of the membrane; and (b) buckling, resulting in folding or bending of the membrane (Dimitrov and Jain, 1984).

One of the most typical and best studied examples of instability of biological membranes is the lysis of erythrocytes, which

can occur under the action of different physical and chemical factors. Basically, two kinds of membrane breakage can be observed - (a) rupture of local areas on the membrane leading to the formation of pores with the release of osmotically active substances; and (b) fragmentation of the membrane involving breakage of the supramolecular structure of the cytoskeleton, and leading to the loss of the membrane integrity (Dimitrov and Jain, 1984). In the first case, the rupture is usually reversible and the membrane returns to its original state after the cessation of the external stress. The integrity of the membrane is kept, with the formation of ghosts for example.

The results from the electron microscopy observation of the red cell membranes treated with enterolobin (Figs 51 and 52), showed clearly that there was fragmentation of the membrane, since its integrity was not maintained.

Dimitrov and Jain (1984) summarized the stages through which the red cell passes when in contact with lysins such as saponin. The three first stages are transformations of the shape of the red cell from a biconcave disc to a smooth, glistening sphere. Then the glistening of the prolytic sphere is replaced by a uniform duskiness. After some time (variable as a function of the lytic agent and physical-chemical conditions), the prolytic sphere begins to haemolyse, and fade from view. The membrane rupture (or fragmentation) occurs in this last stage.

The photographs of the optical microscopy observations of the haemolytic process by enterolobin (Figs 45 to 48 above) revealed apparently the same set of stages as those described by Dimitrov and Jain (1984). The video record of this process showed dynamically all

the phases, including the passage from the glistening prolytic sphere to the dusky one, and the fading of the cells.

The erythrocyte membrane possess certain elasticity that enables it to tolerate some increment of the cell volume. However, when a critical volume is reached during the swelling of the cells, haemolysis occurs.

A mathematical expression for the calculation of the critical cell volume, V_C, is available (Mosior, 1988):

$$V_C = (\varphi \cdot \eta / \pi_h) + b$$

where φ is the mean osmotic coefficient of internal solutes, η is the amount of internal solutes, π_h is the mean osmolarity of solutions in which haemolysis took place, and b is the osmotically non-active volume of the cell.

Assuming that φ, π_h and b remained constant in all the haemolytic assays with enterolobin, as they were supposed to be since the experiments were done in isoosmotic saline solution, the only variable that could change would be η. Consequently, it can be inferred that enterolobin induces some changes in the red cell that make it to overpass its critical volume, V_C, and haemolyse, and these changes are related to the variation of the amount of internal solutes.

3.8.2 Membranes Lysed by Gold-Enterolobin

Gold-enterolobin was successfully used in this work to show the pattern of distribution of the enterolobin on the red cell

membrane and the complexation of the protein at sites in the initial prolytic stage of the cytolytic process.

It was demonstrated that some black gold-enterolobin dots were already gathered surrounding a site at 2 min (Fig. 54), which would probably become a pore afterwards caused by the combined action of a number of enterolobin molecules.

By 15 min, many gold-enterolobin dots could be seen scattered around the holes (Fig.55). The pattern of distribution of the gold-enterolobin complexes on the membrane was rather more random than organized, thus suggesting that this cytolytic protein does not form organized pores by polymerization of oligomers as for staphylococcal α-toxin, streptolysin O, perforin and complement for example (Bhakdi and Tranum-Jensen, 1987). This must be further investigated to allow a undoubtable conclusion The control of gold-albumin showed no black dots characteristic of gold complexes (Fig. 56), indicating that there was no non-specific binding of the two moieties, albumin and gold, to the membrane surface.

Colloidal gold has been used frequently for the study of interactions of lectins and other proteins to the membrane (Horisberger, 1984). However, as far as it could be searched in the literature, its utilization with cytolytic peptides and proteins had never been exploited before. This present study was presumably one of the first ones in this direction. Much can be exploited from this technique for the study of cytolysin-membrane interactions.

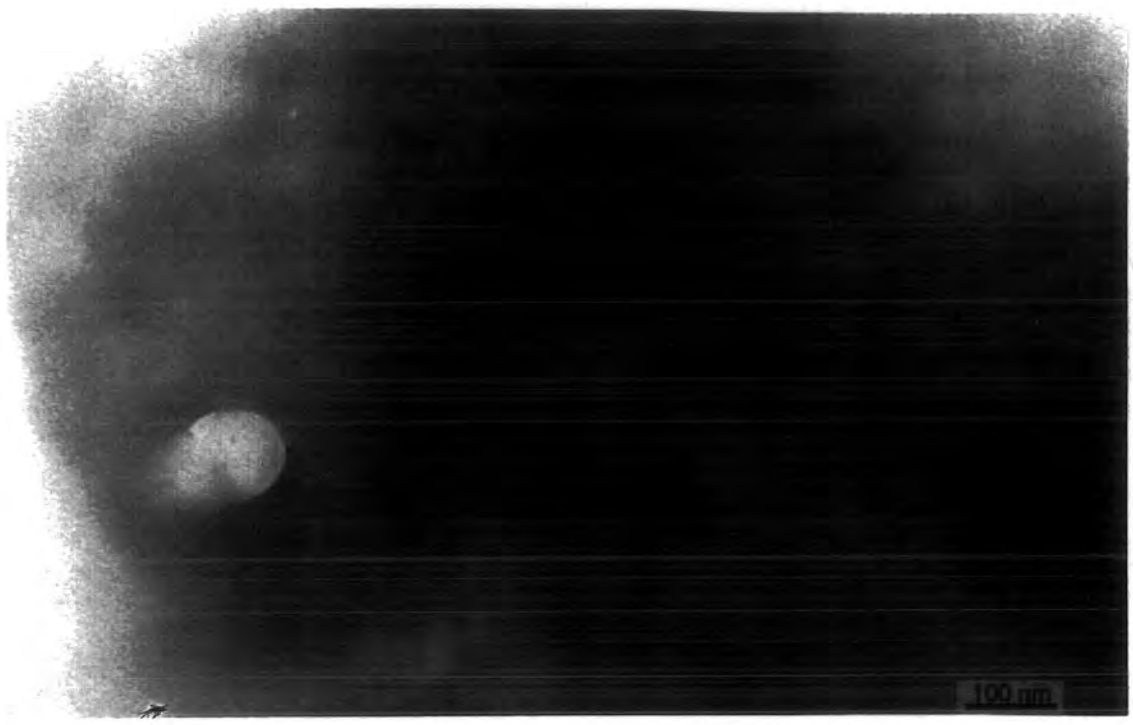


Figure 50: EM visualization of membranes of red cell after 2 min in contact with enterolobin F-3.The haemolysis reaction and preparation of the specimens were carried out as in Material and Methods (Section 2.2.5.3) A Phillips 400 electron microscope was used at a magnification of x46 000. The print magnification was of 2 times.

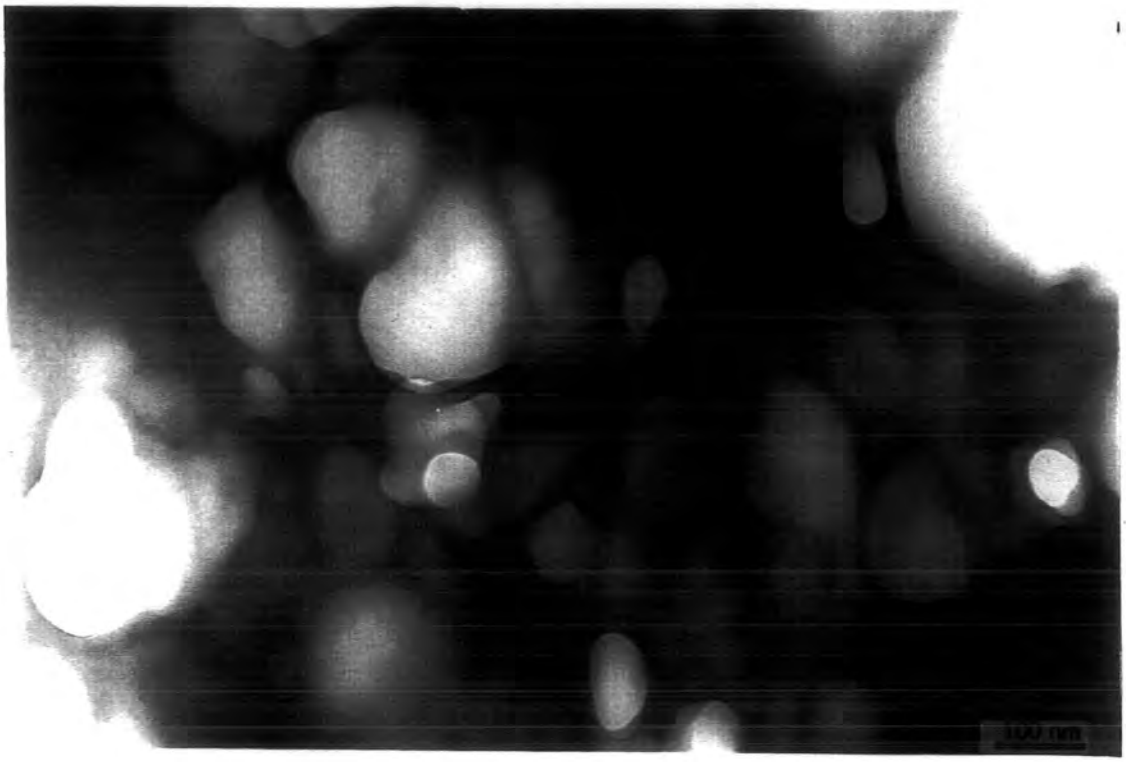


Figure 51: EM visualization of membranes of red cell after 15 min in contact with enterolobin F-3.The haemolysis reaction and preparation of the specimens were carried out as in Material and Methods (Section 2.2.5.3) A Phillips 400 electron microscope was used at a magnification of x46 000. The print magnification was of 2 times.

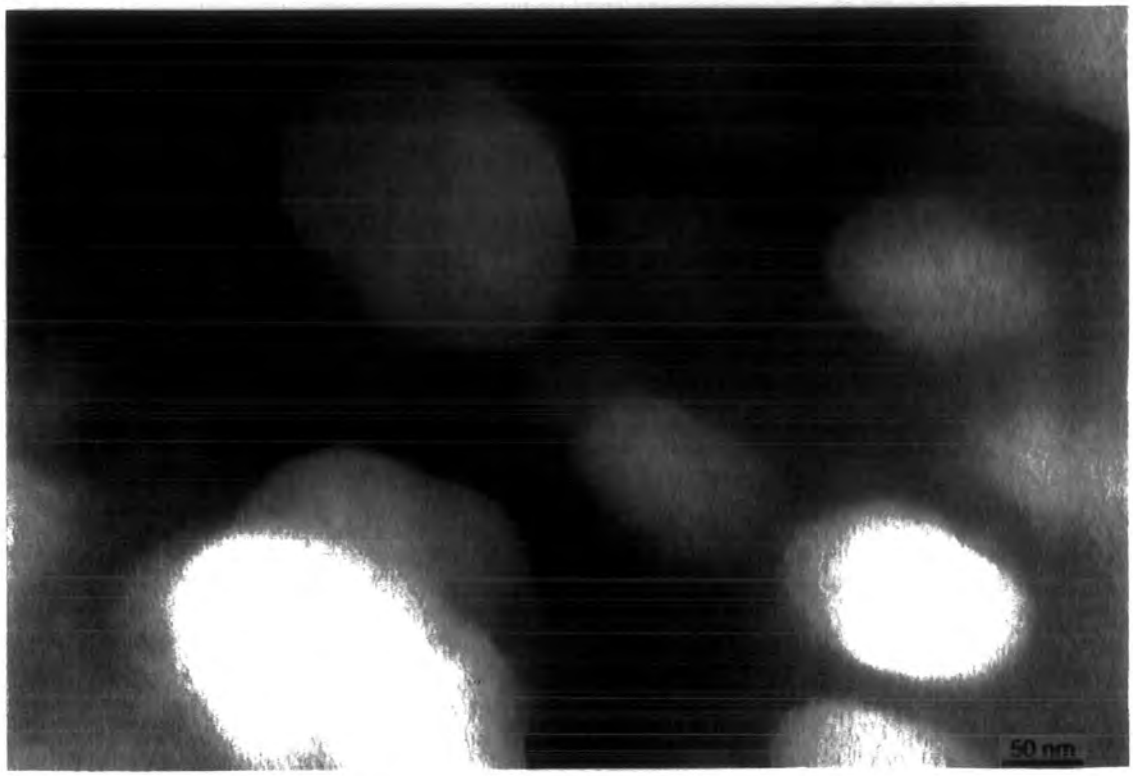


Figure 52: Detailed EM visualization of membranes of red cell after 15 min in contact with enterolobin F-3.The haemolysis reaction and preparation of the specimens were carried out as in Material and Methods (Section 2.2.5.3) A Phillips 400 electron microscope was used at a magnification of x100 000. The print magnification was of 2 times.

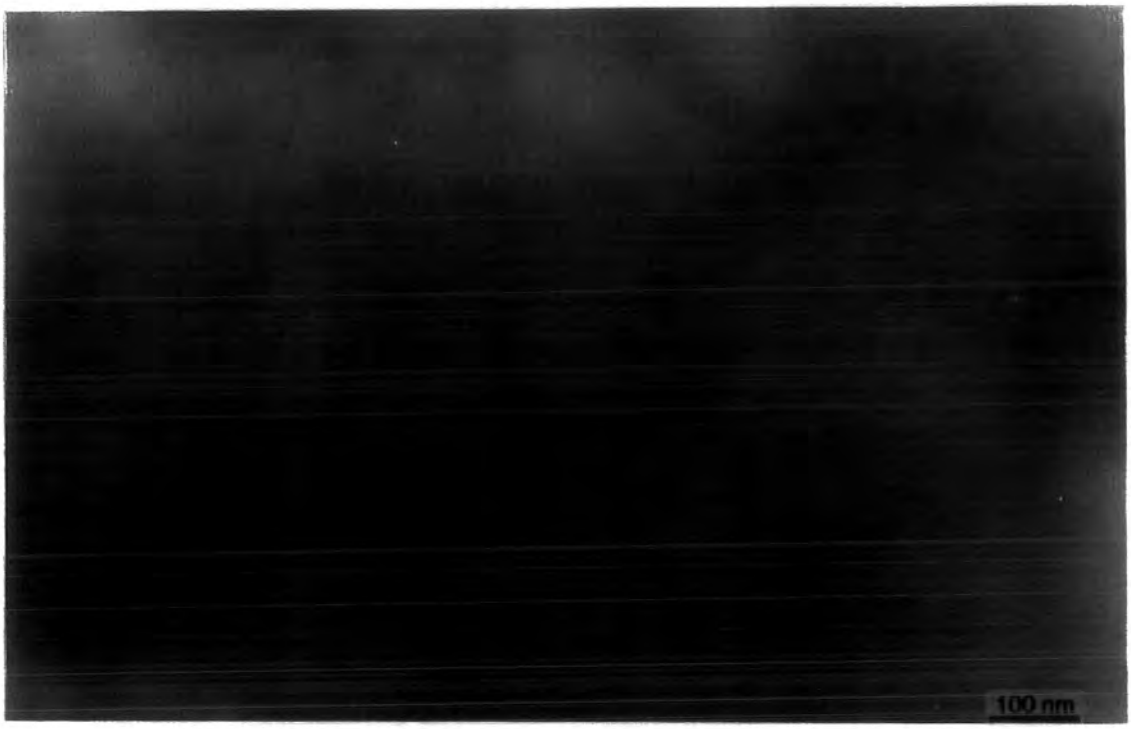


Figure 53: EM visualization of membranes of control red cell. The preparation of the specimens were carried out as in Material and Methods (Section 2.2.5.3) A Phillips 400 electron microscope was used at a magnification of x46 000 to observe the control cell membranes after 15 min of repose. The print magnification was of 2 times.

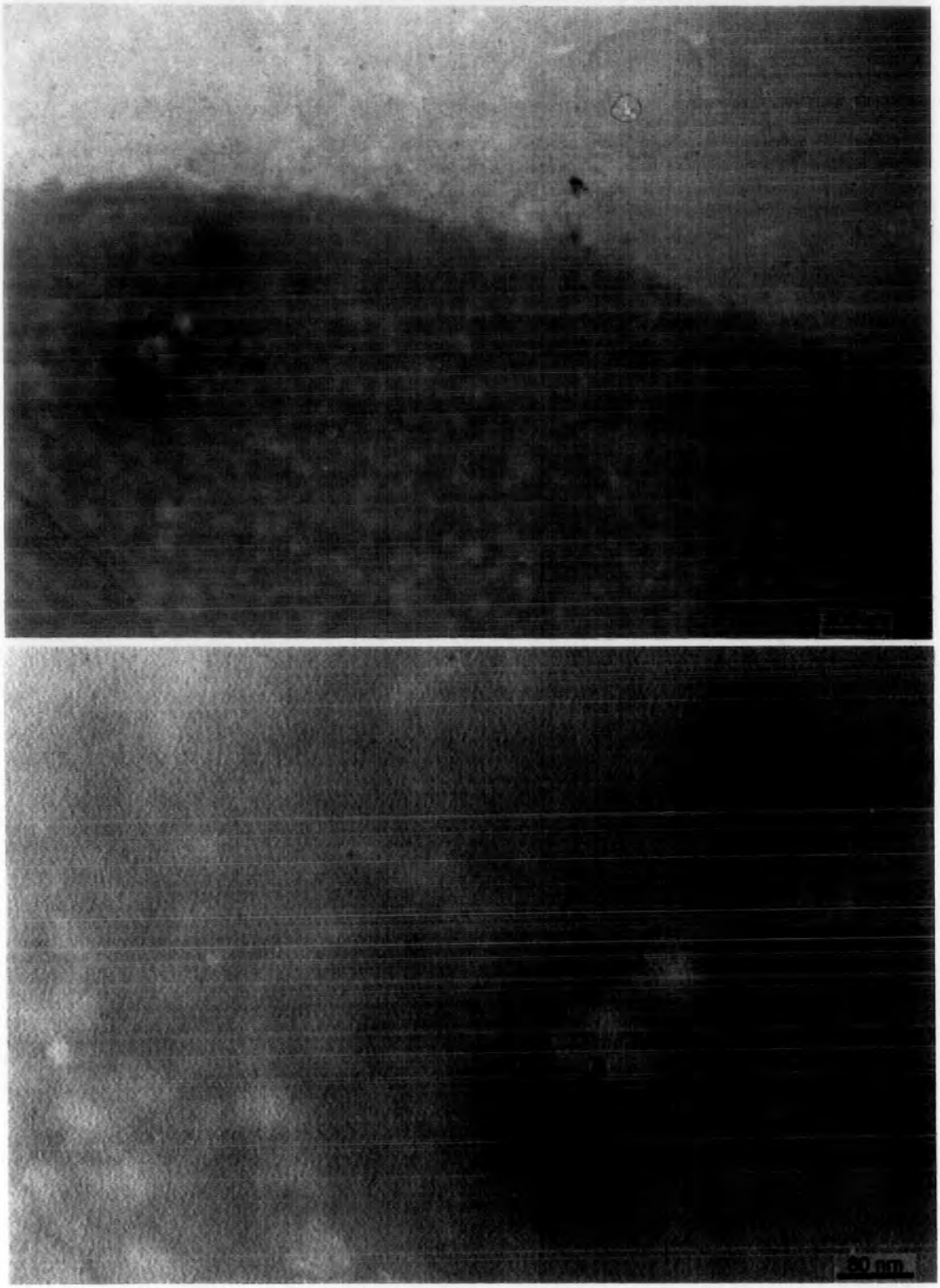


Figure 54: EM visualization of membranes of red cell haemolysed by gold-enterolobin. The preparation of gold-labelled enterolobin, haemolysis reaction and preparation of the specimens were carried out as in Material and Methods (Section 2.2.5.3) A Phillips 400 electron microscope was used at a magnification of x25 000 (**a**) and x70 000 (**b**). The incubation period of the cells with gold-enterolobin was of 2 min. The dense black dots are the gold-enterolobin complexes. The print magnification was of 2 times.

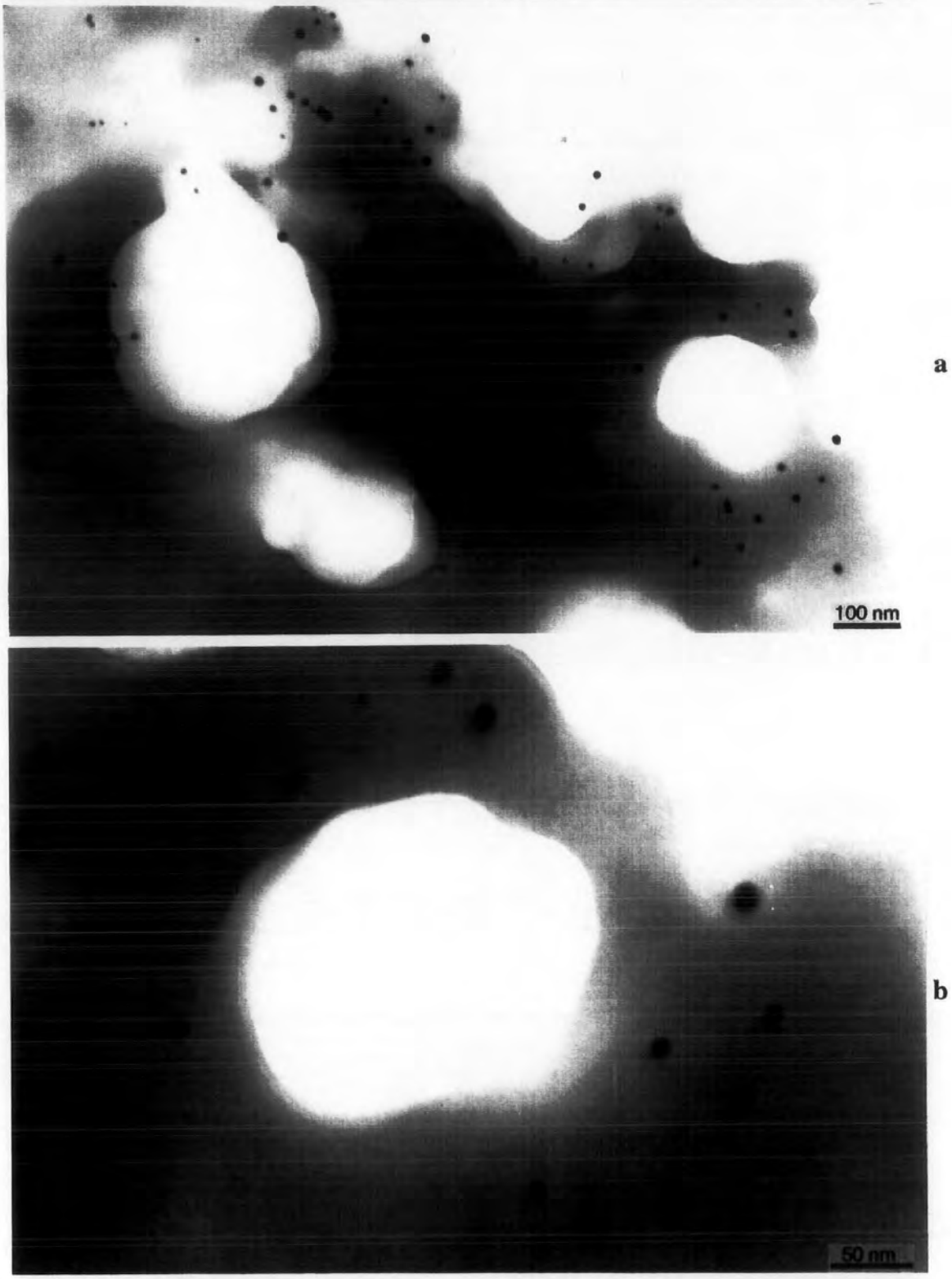


Figure 55: EM visualization of membranes of red cell haemolysed by gold-enterolobin. The preparation of gold-labelled enterolobin, haemolysis reaction and preparation of the specimens were carried out as in Material and Methods (Section 2.2.5.3) A Phillips 400 electron microscope was used at a magnification of x55 000 (**a**) and x120 000 (**b**). The incubation period of the cells with gold-enterolobin was of 15 min. The dense black dots are the gold-enterolobin complexes. The print magnification was of 2 times.

Figure 55: EM visualization of membranes of red cell treated with gold-ovalbumin. The preparation of gold-labelled ovalbumin, incubation with the cells and preparation of the specimens were carried out as in Material and Methods (Section 2.2.5.3) A Phillips 400 electron microscope was used at a magnification of x25 000 . The incubation period of the cells with gold-ovalbumin was of 15 min. The print magnification was of 2 times.

3.9 Effect of Enterolobin on Cancer Cells

It has been shown that many cytolytic agents, including several peptides and proteins can induce lysis on culture cells as MRC5 (Thelestam and Möllby, 1979). If this occurs for a number of cytolytic proteins, it could also be true for enterolobin.

In this study, enterolobin was tested against two cancer cell lines, L929 and MRC5, in a *in vitro* assay on a microtitration plate with TNF (for a review see Bonavida and Granger, 1990) as a positive control. The TNF- and enterolobin-treated cells were also observed under a differential interference contrast microscope in order to provide a visual comparison of the pattern of cytotoxicity caused by the two proteins.

Enterolobin proved to be cytotoxic to both cancer cell lines tested - L929 and MRC5. Both L929 (Fig. 57) and MRC5 (not shown) were killed at an enterolobin concentration as low as 250 ug/ml (titre = 2) in the microtitration plate assay. However, this concentration of enterolobin was much higher than the concentration of TNF that was required to kill the cells. TNF was able to cause death to the cells at concentrations as low as 1 U/ml (25 pg/ml).

No damage to the cells could be observed by differential interference contrast microscopy at 2 hours of incubation with enterolobin and TNF. After 24 h, however, TNF had caused death to many of the cells, as they became detached from the plastic surface of the petri dish and contracted a round shape (Fig. 58). Enterolobin also

had killed the cells by 24 h of incubation, but by inducing lysis to them, since cell debris could be seen in profusion in the supernatant (Fig. 59). No changes had occurred to the control cells after 24 h.

It is still early to suggest any mechanism of cytotoxicity of enterolobin on the cancer cells. However, it would be probably correct to assume that enterolobin and TNF produce the killing of the cells by different mechanisms given the above results and the knowledge of their physical-chemical characteristics.

The results demonstrated differences in the toxic concentrations and way of killing of these two proteins. It became clear that enterolobin caused cell death by a cytolytic mechanism, whereas TNF killed them with no apparent lysis. Enterolobin was needed in higher concentrations than TNF to induce its cytotoxic effect to the cells tested.

Furthermore, while TNF in its soluble form is a compact trimer with 17 KDa per protomer (Old, 1990) , enterolobin is a 55 KDa protein of only one polypeptide chain (Sousa and Morhy, 1989). TNF is relatively stable in acidic and apolar conditions (Aggarwal,1990), while enterolobin is unstable under these conditions. Further investigation must be done in order to clarify the mechanism of action of enterolobin on cancer cells. An initial line would be to question if there is a similar binding site molecule for enterolobin on the red cell and cancer cell membranes, and then if the mechanisms of cytolysis are the same.

It is worth noting another characteristic in common between enterolobin and aerolysin. Aerolysin induces lysis of MRC5 cells as well (Thelestam and Möllby, 1979). In their study, Thelestam and Möllby (1979) suggested that there may be a specific receptor

involved in the interaction of aerolysin with the cancer cell, although the nature of this receptor could not be deduced from their results.

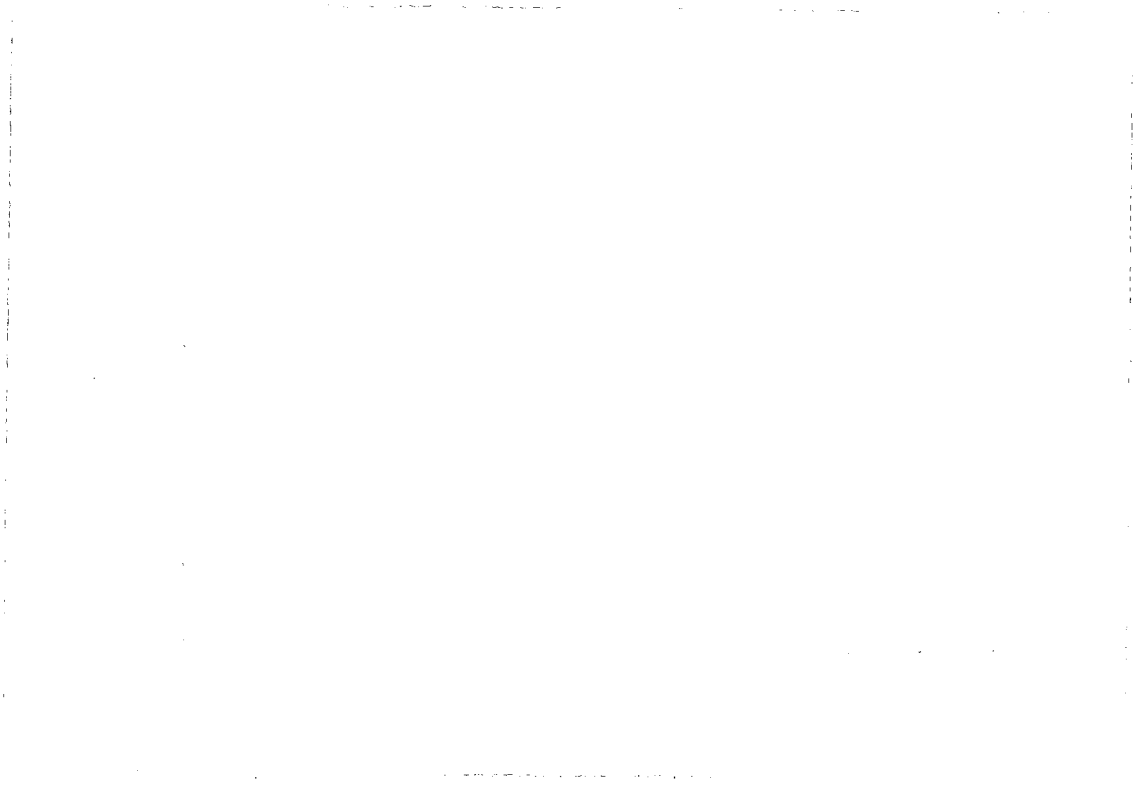


Figure 57: Cytotoxic effect of enterolobin on cancer cells. Enterolobin was two-fold serially diluted on microtitration plates and incubated with L929 cancer cells for 3 days. Experimental details are given in Material and Methods (Section 2.2.6). Rows B2 to D9 - enterolobin F-3 (initial concentration of 500 µg/ml). Rows E2 to G9 - enterolobin F-2 (initial concentration of 2 mg/ml). Rows D10/11 and G10/11 - control cells with no enterolobin. Rows A and H plus columns 1 and 12 were left empty. The assays were done in duplicate; e.g. wells A2/3 are duplicates and so on. The clearer wells (A2 to A5 and E2/3) contain dead cells.

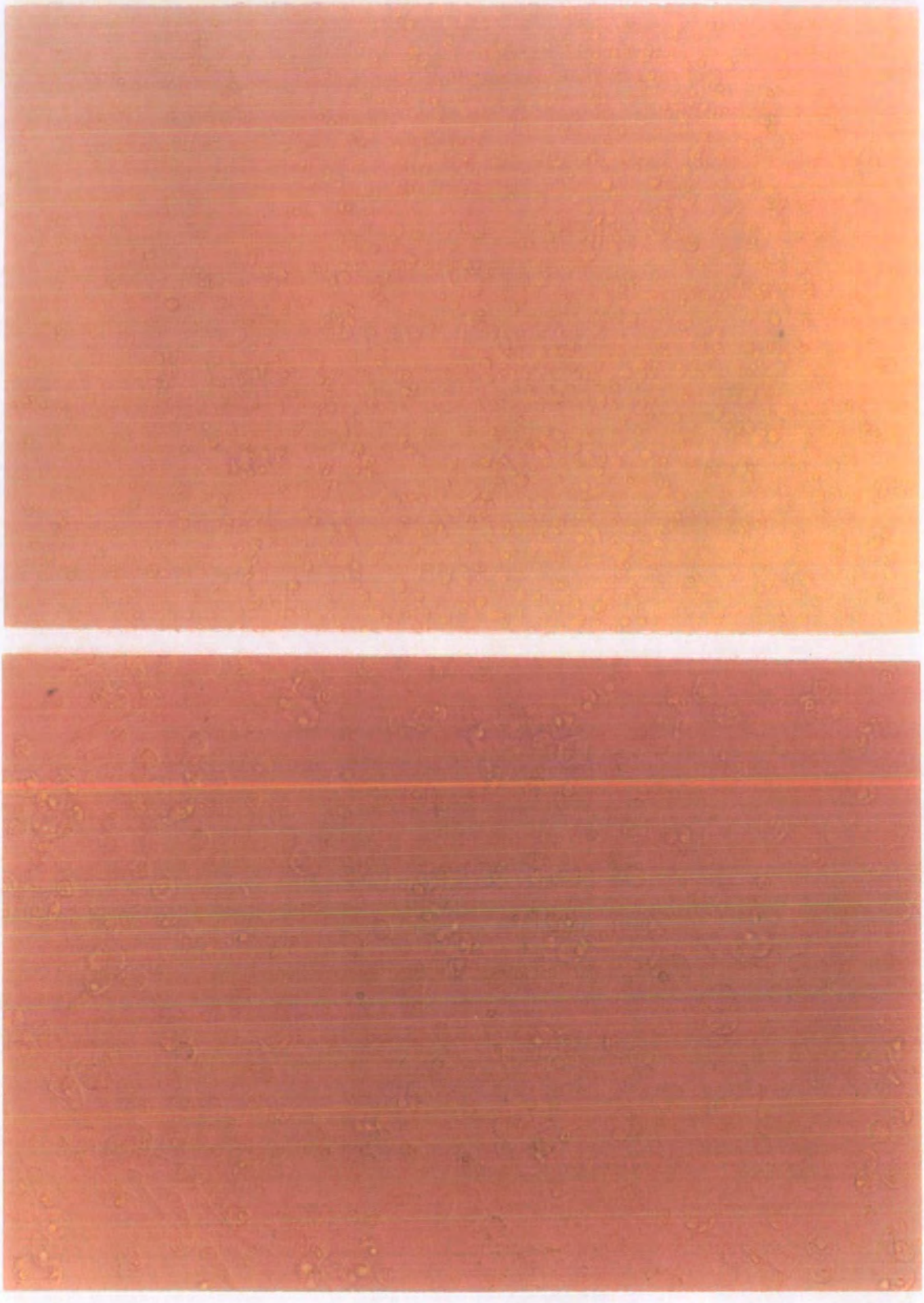
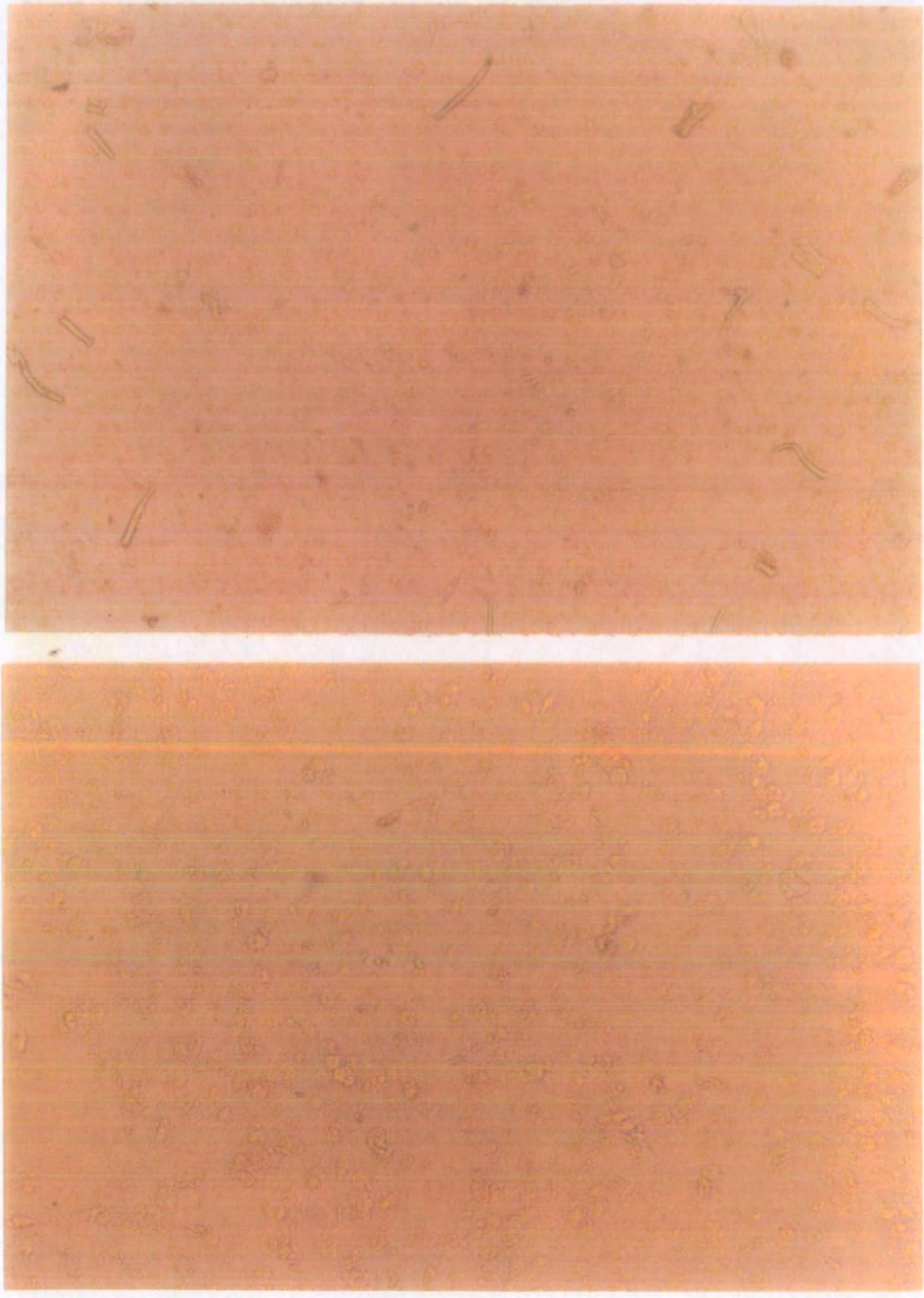


Figure 58: Visualization of the cytotoxic effect of TNF-α on cancer cells. TNF at 100 U/ml was incubated with L929 cancer cells for 24 h, and then taken for observation under differential interference contrast microscopy at a magnification of x10 (**a**) and x20 (**b**).



a

b

Figure 59: Visualization of the cytotoxic effect of enterolobin on cancer cells. Enterolobin F-3 at 500 μg/ml was incubated for 24 h with L929 cancer cells, and then taken for observation under differential interference contrast microscopy at a magnification of x10 (**a**) and x20 (**b**).

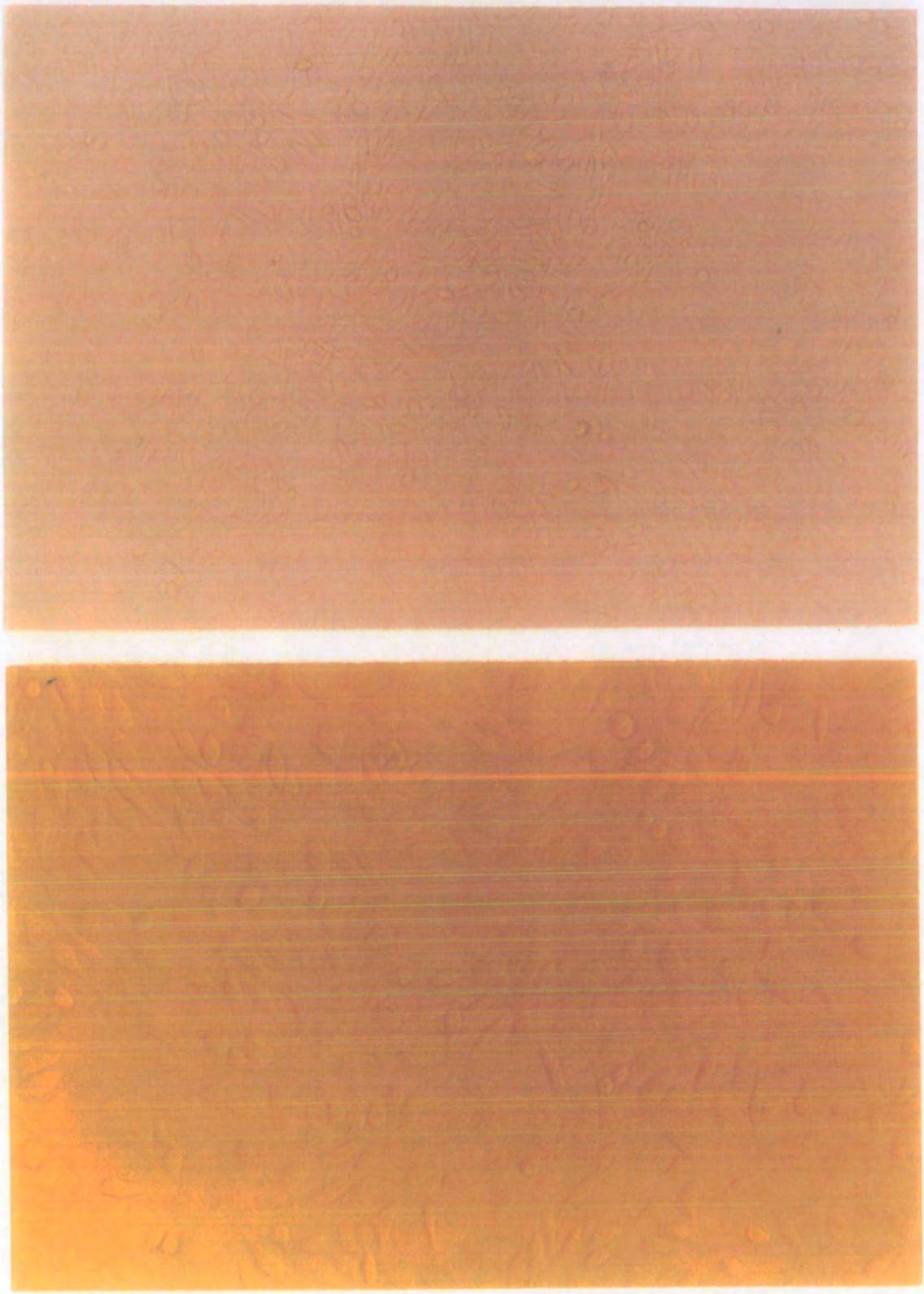


Figure 59: Control visualization of the cancer cells. L929 cancer cells were left standing for 24 h, and then taken for observation under differential interference contrast microscopy at a magnification of x10 (**a**) and x20 (**b**).

3.10 Effect of Enterolobin on Insect Larvae

Many compounds have been found in plants, especially in legume seeds, which exhibit some toxicity to insects. These compounds range from micromolecules to proteins (reviewed by Gatehouse *et al.*, 1990). Within the group of seed proteins, protease inhibitors (Gatehouse *et al.*, 1979; Gatehouse and Boulter, 1983), lectins (Gatehouse *et al.*, 1984; Pratt *et al.*, 1990; Chrispeels and Raikhel, 1991), lectin-like proteins (Osborn *et al.*, 1988a and b) and ribosome inactivating proteins (RIPs) (Gatehouse *et al.*, 1990) have been shown to possess antimetabolic effects against insects.

Some cytolytic crystal proteins of the soil bacterium *Bacillus thuringiensis* also contain insecticidal activity against a broad spectra of insect pests (review by Höfte and Whiteley, 1989). It is believed that these crystal proteins are dissolved in the midgut of the insects forming pores on the epithelium cells, and that they may interfere in the amino acid transport driven by a K^+ gradient. However, complete understanding of their mode of action is still to be elucidated.

Enterolobin, which is both a seed and a cytolytic protein, was tested in order to determine whether it had any toxic effects against insect larvae of the coleopteran *Callosobruchus maculatus* and the lepidopteran *Spodoptera litorallis*. The study also attempted to provide a possible explanation for the results obtained *in vivo*.

Initially, the effects of a partially purified preparation of enterolobin (fraction F-1) on the development and survival of a

coleopteran, *Callosobruchus maculatus* (cowpea seed weevil), and a lepidopteran, *Spodoptera littoralis* (armyworm) were investigated. Both are pests of major economic importance especially in tropical countries.

The partially purified enterolobin fraction (F-1) was added in a range of concentrations to artificial seeds in order to test its effects on *C. maculatus* larvae. The results for survival of the insect to adult are shown in Fig. 61 This shows that at a concentration of 0.25% (w/w), adult survival was reduced by 95% relative to the controls, and at a concentration of 0.5% (w/w), complete mortality was observed.

Since the partially purified preparation was proved to be toxic to *C. maculatus*, it was further purified (fraction F-2) and shown by FPLC to be essentially homogeneous (Fig. 62). This purer sample (F-2) was incorporated to the artificial seeds at a range of up to 0.25% (w/w). At a concentration of 0.01%, this sample reduced adult survival to 30% (Fig. 63); in this instance, an enterolobin concentration of 0.025% caused 100% mortality.

The effects of the partially purified (F-1) and purified (F-2) enterolobin were also tested against *S. littoralis*. Both samples failed to show any deleterious effects, despite being offered to the larvae at concentrations of 5% (F-1) and 0.1% (F-2). After 6 days of feeding, the percentage of survival was 83% for both the insects fed with enterolobin and the control insects (Fig. 64).

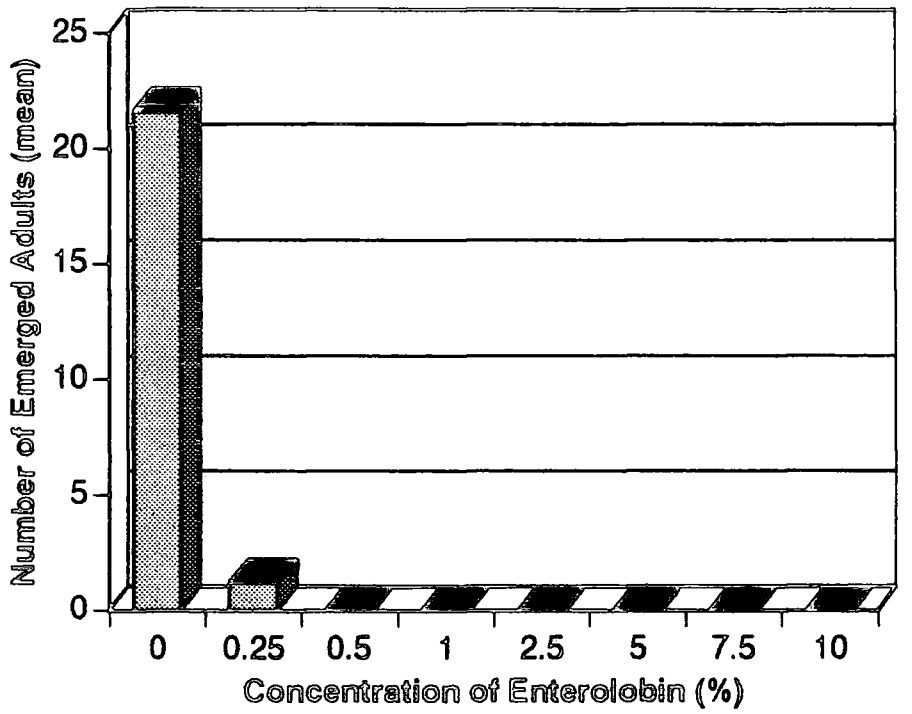


Figure 61: Effect of enterolobin F-1 on the survival of *C. maculatus* larvae. Enterolobin F-1 was incorporated in artificial seeds and disposed to the insects for oviposition. After 42 days, the assay was terminated, and the number of emerged adults recorded. The concentration of enterolobin is given as a percentage (per dry weight) of each artificial seed. Six replicas were used for the calculation of the mean of the number of emerged adults.

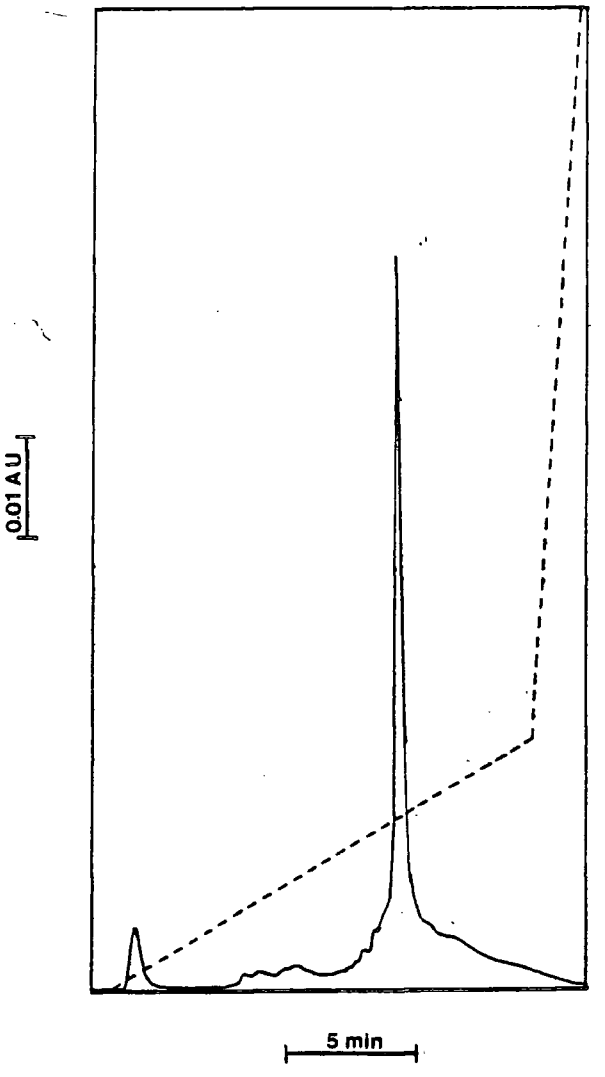


Figure 62: FPLC chromatography of enterolobin F-2. An aliquot (500 µl) from a solution of enterolobin F-2 (1 mg/ml) was applied to a Mono Q HR 5/5 column equilibrated with 20 mM tris-HCl pH 8.7, and eluted with a gradient (- - -) of NaCl (0-0.1 M in 18 min and 0.1-0.35 in 1 min). The flow rate was 1.0 ml/min, and the absorbance (——) was recorded continuously at 280 nm at AUS 0.1.

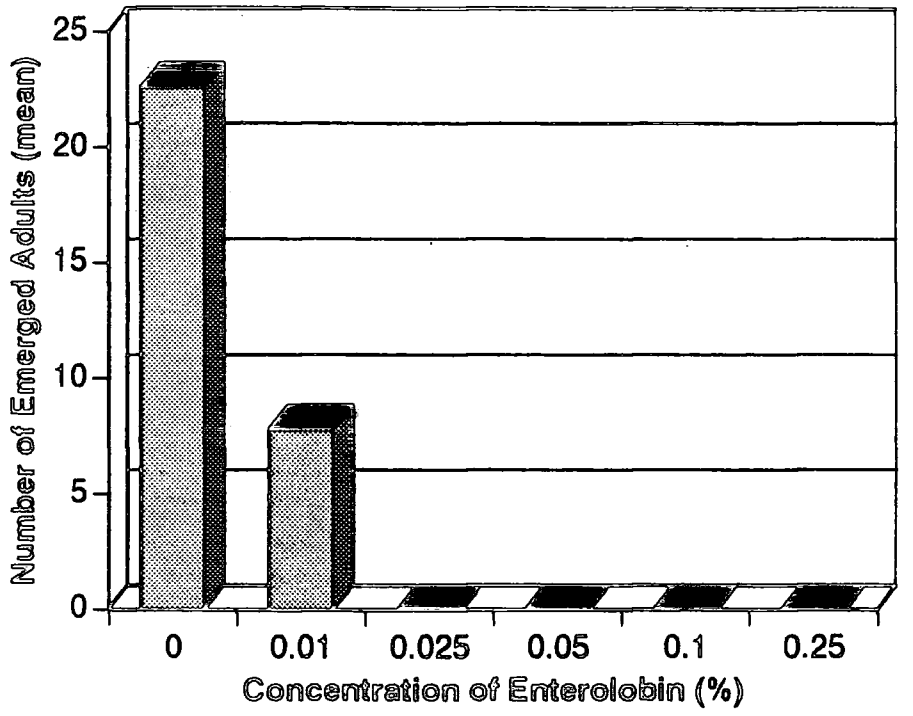


Figure 63: Effect of enterolobin F-2 on the survival of *C. maculatus* larvae. Enterolobin F-2 was incorporated in artificial seeds and disposed to the insects for oviposition. After 42 days, the assay was terminated, and the number of emerged adults recorded. The concentration of enterolobin is given as a percentage (per dry weight) of each artificial seed. Six replicas were used for the calculation of the mean of the number of emerged adults.

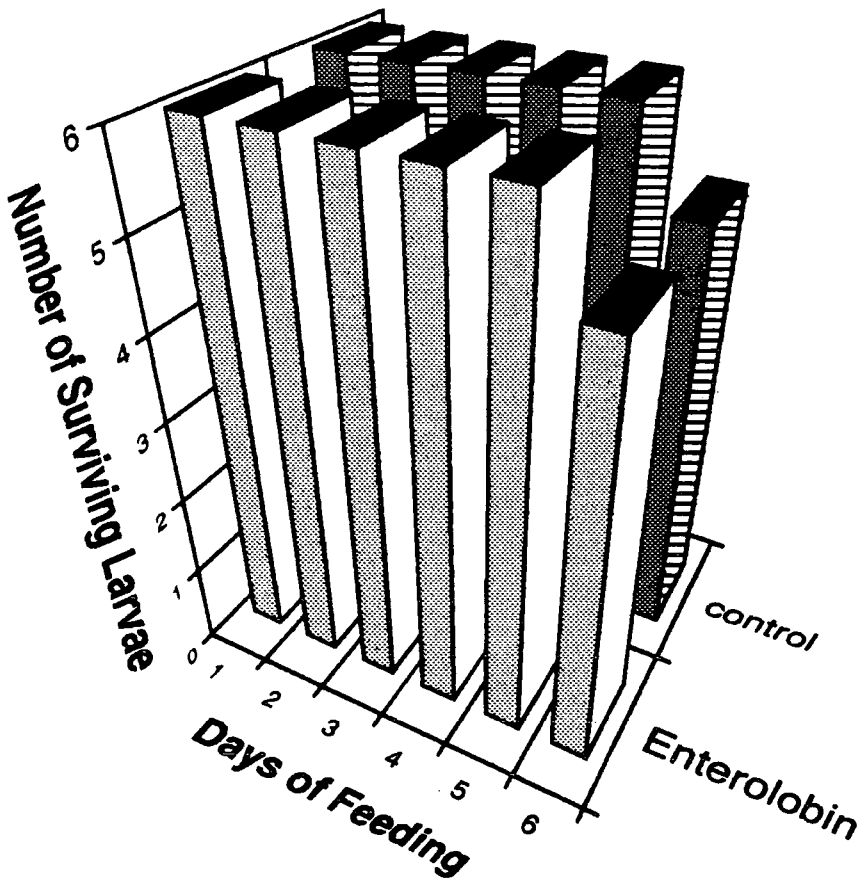


Figure 64: Effect of enterolobin F-2 on the survival of *S. littoralis* larvae. Enterolobin F-2 was incorporated in artificial diet at 0.1 % (w/w/), and presented to the larvae for feeding. The number of surviving larvae was recorded daily for six days.

From the results of the feeding trials (Figs. 61, 63 and 64), it is evident that enterolobin, the cytolytic protein, has a toxic effect against *C. maculatus* but not against *S. littoralis*. Similar results have also been obtained for the RIPs ricin and saporin (Gatehouse et al., 1990). Ricin, with a molecular weight of 60 KDa, was found to cause 100% mortality at a concentration of 0.005% (w/w). Saporin (30 KDa) resulted in complete larval mortality at 0.01% (w/w). Enterolobin (55 KDa) appears to be slightly less toxic than ricin when compared either on a weight or on a molar basis. However, enterolobin has approximately the same degree of toxicity as saporin when examined on a percentage basis, but it is more toxic than this toxin if a molar relation is considered.

In order to determine how enterolobin was highly toxic to the coleopteran but innocuous to the lepidopteran, the stability of the protein to digestive proteolysis was investigated *in vitro*. For each insect, a larval gut preparation was incubated with the purer enterolobin sample (F-2) for given time intervals up to 48 h, either at pH 5.4 in the case of *C. maculatus* or pH 9.5 for *S. littoralis*. Control incubations in the absence of larval gut extracts were run concurrently at the two different pH values in order to detect any autolysis of the protein. The hydrolysis products were then analysed by SDS-PAGE.The results clearly show that after an incubation period of only 2 h under optimal conditions, the *S. littoralis* gut enzymes had completely hydrolysed enterolobin (Fig. 65). The *C. maculatus* enzymes, on the other hand, were unable to hydrolyse the cytolytic protein *in vitro* even after 48 h of incubation (Fig. 66).

Gatehouse *et al.* (1990) demonstrated, by *in vitro* digestion, that the gut enzymes of *S. littoralis* were able to digest both ricin and saporin, while the enzymes from *C. maculatus* were unable to cleave the proteins. This could explain the lack of toxicity of these proteins against *S. littoralis*. Likewise, enterolobin was susceptible to hydrolysis by the enzymes of the lepidopteran, but was resistant to those of the coleopteran (Figs. 65 and 66). The same explanation for the differential toxicity of the RIPs to the two species of insects appears to apply to enterolobin as well. *S. litorallis* gut enzymes can digest the toxic proteins, and thus rendering them inactive, before they are able to exercise their deleterious effects, while the *C. maculatus* enzymes do not possess this capacity.

The question as to how enterolobin exerts its toxicity to *C. maculatus* still remains to be elucidated. It is well known that this protein has haemolytic (Sousa and Morhy, 1989) and other activities, such as cytolysis to cancer cells (discussed above) and inflammation to rats (Castro-Faria-Neto *et al.*, in the press; Cordeiro *et al.*, in the press). Since enterolobin is such a cytolytic and toxic protein, the ultrastructure of the epithelial cells of the enterolobin-treated gut of *C. maculatus* larva was worth investigating by electron microscopy.

There appeared to be no evidence that, between control and experimental insects, there were morphological differences caused by structural damage in the microvillae membrane of the epithelial cells (Fig. 67). Futhermore, there were no apparent differences between the internal organelles of the gut epithelial cells from either experimental or control insects. Then, the hypothesis that enterolobin might exert its toxic effects by causing lysis to the membrane of the epithelium cells had to be discarded.

Figure 65: Analysis by PAGE-SDS of the proteolytic effect of *S.littoralis* larval gut enzymes on enterolobin. Enterolobin F-2 (**ent.**) was incubated with gut enzymes from *S.littoralis* for 0 (**a**), 1 (**b**), 2 (**c**), 4 (**d**), 8 (**e**), 24 (**f**) and 48 h (**g**). At each of these times, aliquots were taken for analysis by PAGE-SDS slab mini-gel at an acrylamide concentration of 5% . Myoglobin (**myo.**) was treated in the same manner, and samples at 0 (**h**) and 48 h (**i**) were submitted to SDS-PAGE at an acrylamide concentration of 17%. The runs was carried out at constant voltage (100 V) for 1 h.

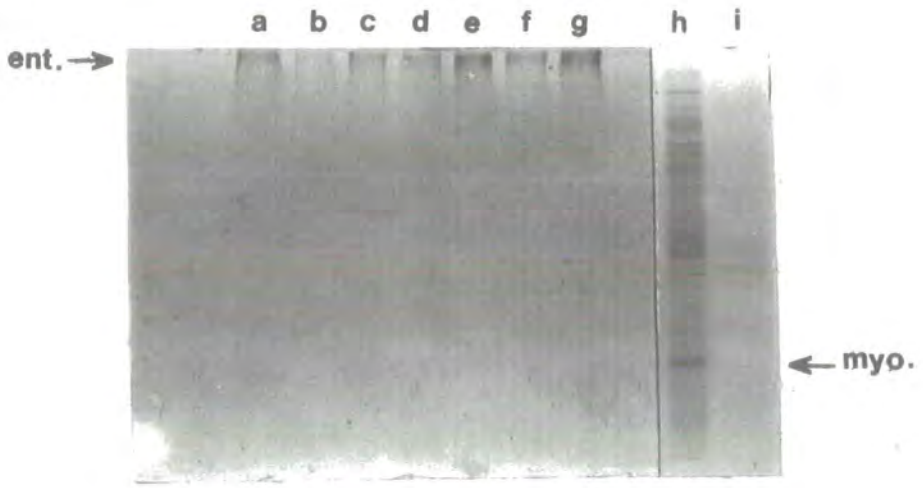


Figure 66: Analysis by PAGE-SDS of the effects of *C. maculatus* larval gut enzymes on enterolobin. Enterolobin F-2 (**ent.**) was incubated with gut enzymes from *C. maculatus* for 0 (**a**), 1 (**b**), 2 (**c**), 4 (**d**), 8 (**e**), 24 (**f**) and 48 h (**g**). At each of these times, aliquots were taken for analysis by PAGE-SDS slab mini-gel at an acrylamide concentration of 5% . Myoglobin (**myo.**) was treated in the same manner, and samples at 0 (**h**) and 48 h (**i**) were submitted to SDS-PAGE at an acrylamide concentration of 17%. The runs was carried out at constant voltage (100 V) for 1 h.

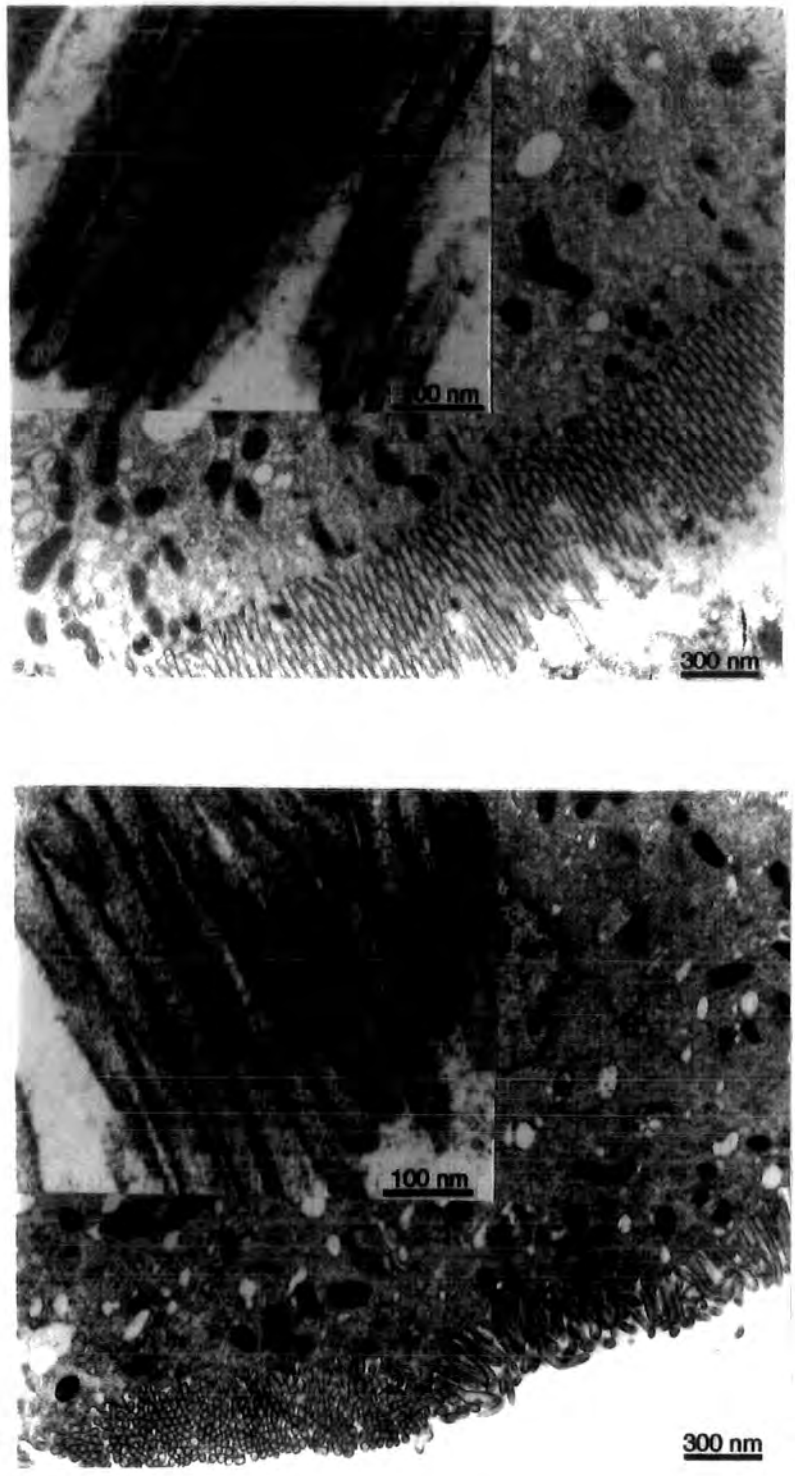


Figure 67: Visualization by EM of gut epithelial cells of *C. maculatus* fed with enterolobin. Section of a cell treated with enterolobin (**a**) and control cell (**b**) showing internal organelles and microvilae at magnifications of x17 000 and x46 000 (insert).

The basis of toxicity may reside at the biochemical level. *C. maculatus* larvae do not contain serine proteases as their main proteolytic digestive enzymes (Gatehouse *et al.*, 1985) as do *S. littoralis* and other lepidopterans (Ahamad *et al.*, 1980). Instead, they possess thiol proteases (Gatehouse *et al.*,1985). *Enterolobium contortisiliquum* contains a thiol protease inhibitor (Oliva *et al.*, 1988) which molecular weight (60 KDa) was found to be very close to that of enterolobin (55 KDa). Preliminary results (data not shown) suggested some slight inhibition of papain, a thiol protease, by enterolobin. Currently, we are investigating whether enterolobin and the thiol protease inhibitor from *E. contortisiliquum* are in fact the same protein.species. If this were to be the case, the toxicity of this protein towards *C. maculatus* could be explained by the inhibition of thiol proteases within the guts of these insects.

On the other hand, the possibility of the presence of a minor contamination of a thiol-protease inhibitor in the enterolobin preparation cannot be ruled out. However, it would have to be an exceptionally potent inhibitor to cause such response at so low a concentration since enterolobin was demonstrated to be in a high level of purity by analytical FPLC (Fig. 62). The automatic integrator of the FPLC apparatus showed that the area of the enterolobin F-2 peak accounted for 87% of the total area of the chromatogram. This class of inhibitor characteristically does not exhibit such levels of toxicity (Gatehouse *et al.*, 1979).

Another speculative hypothesis is currently being investigated. Enterolobin appears to have a glycoprotein binding site on the membrane of erythrocytes (Sousa *et al.*, work in progress). There is

evidence that this protein is the anion exchanger protein band 3 (discussed above, Section 3.7). This protein regulates the transport of ions (especially Cl^-), small nonelectrolytes and water through the membrane of erythrocytes (Solomon *et al.*, 1983; Jennings, 1985; Low, 1986). Enterolobin could also be binding to a similar carrier protein on the membrane of the insect gut epithelial cells thus deregulating its transport of anions and water. Interestingly, Höfte and Whiteley (1989), in a review on the insecticidal cytolytic proteins of *Bacillus thuringiensis*, observed that one of the proposed mode of actions of these toxins is the inhibition of a K^+-amino acid co-transporter protein.

The above results and considerations suggested that enterolobin *in vivo* could well be one of the natural defensive proteins for *E. contortisiliquum*. The concentration of the toxic protein that began to cause death to the larvae was 0.01 % (w/w), and this is the supposed concentration of the protein in the seeds, as calculated from typical purification yelds. This plant also contains other proteins and small compounds toxic to insects such as, for example, a set of protease inhibitors in the seeds (Oliva *et al.*, 1988; and personal communication) and saponins in the fruit pod (unpublished results).

Further work has yet to be carried out in order to understand fully the mechanism by which enterolobin kills *C. maculatus*. However, a potential application of this cytolytic and toxic protein in plant genetic engineering for the control of crop pests can be already envisaged.

3.11 Concluding Remarks

In 1989, Sousa and Morhy published a paper on the purification and initial characterization of enterolobin, the first haemolytic/cytolytic protein founds in seeds. They showed that this large new protein was able to cause haemolysis by a mechanism that did not involve any phospholipase D activity. Apart from characteristics such as red cell specificities, stability to different pHs and temperature, molecular weight, isoelectric point, amino acid composition and some spectroscopic properties, nothing else was known about the protein. Since that time, more data has been accumulated on this phytocytolysin.

During the present work, the digestion of enterolobin with chymotrypsin, elastase and trypsin generated hundreds of peptides. The separation of these peptides by a combination of gel filtration and RP-HPLC proved to be a lengthy process, and, in some cases, was ineffective in providing them in a suitable level of purity for the subsequent sequence determination.

A further obstacle was the finding that the N-terminal residue of the protein was chemically blocked. This blockage was refractory to two unblocking methods. In addition, during the early stages of this study, no other amino acid sequence similar to enterolobin was known that could help in the alignment of the peptides. Also, since no automated sequencing facilities were available until the later stages of this work, only the manual method was extensively employed for the sequencing of the peptides. This meant that large amounts of protein were always necessary for the hydrolytic digestions. However, despite these drawbacks, a considerable amount of the sequence was resolved.

The alignment of this partial sequence indicated that enterolobin is similar (if not homologous, given their functional relationship) to the aerolysins of *A. hydrophila* and *A. sobria.* The use of pattern searching revealed that enterolobin and aerolysins could be related to other pore- and channel-forming proteins as well as transport proteins from membranes of bacteria. This could be true not only for proteins from lower organisms, but also from higher species, as is suggested by the finding of the same pattern in α-macroglobulin.

The results of this investigation pose many new questions. Is there an evolutionary line from the transport proteins, porins, colicins, and cytolysins from bacteria to the cytolytic proteins of higher organisms in which enterolobin could fit as a link?

To respond to this and other questions, enterolobin needs to have its amino acid sequence completely determined. Although manual sequencing alone can be useful for cheaply sequencing small and abundant peptides and proteins, it is not sufficient for carrying out the determination of primary structure of large and complex proteins such as enterolobin. For the accomplishment of this objective, new methods and strategies must be employed.

Needless to say, automatic sequencing would have to be available throughout the work. It provides a much higher sensitivity and efficiency than the manual method, and frees the researcher for other tasks. To exploit the automatic method to its full, some changes in strategy should be envisaged. For example, the use of very specific enzymes, like the Arg-specific enzyme from mouse sub-maxillary gland and the Lys-specific enzyme from *Armillaria mellea*, would be indicated for generating a smaller number of larger peptides. A parallel

monitoring of the cleavages by mini-electrophoresis, capillary electrophoresis and/or micro-bore HPLC in order to optimize the conditions of the reactions would be advisable. The aim would be to reach a situation where one would obtain cleavages of small amounts of the protein (pmol level) that should produce mainly only long peptides ideal for the sequencing machine.

Likewise, the use of micro-bore HPLC columns and/or two dimensional electrophoresis combined with blotting would permit the purification of peptides from very low quantities of protein. Also new methods and equipment for the separation of peptides and proteins are being introduced in the literature and in the market. As an example, a high performance preparative column electrophoresis device from Applied Biosystems has joined the arsenal of machinery that facilitates the work of the protein chemist and will be available for the continuation of the work.

Similarly, amino acid analysis is essential as a complement to the determination of amino acid sequences. It can be decisive in cases where there are some doubts remaining in the sequence. Another potentially useful technique is mass spectrometry for the elucidation of the structure of chemical blockages at the N-terminus, as well as for determining peptide sequences. The use of molecular biology as an auxiliary way of indirectly determining protein sequences has to be acknowledged. Some new techniques, such as the polymerase chain reaction (PCR), have made remarkable progress, and are becoming still more important as a tool for the protein investigator.

When the complete sequence of enterolobin becomes available, the application of computational analytical methods will be

utilized, this time with more appropriate software and hardware. Even though useful data can be produced with a microcomputer, it lacks raw power to run programs at high processing speeds. For these programs, workstations are indicated, since some of them combine ease of use, graphical interface, large memories and high processing speeds. As far as possible, workstations running sensitive programs and holding non-redundant sequence databases will be employed for the comparative analyses of the sequence of enterolobin and other related and apparently non-related proteins, and for other types of searches and analyses.

The experiments on the kinetics of haemolysis by enterolobin indicated the chemical nature of its binding site on the erythrocyte membrane. It is known that many cytolysins have lipids as their binding molecules, but enterolobin is not included in this group. It was shown that this phytocytolysin could have a protein receptor, with the suggestion that it could be perhaps the band 3 anion exchanger protein. If this is the case, enterolobin could maybe be interfering somehow with the functions of transport of ions and water by the exchanger. This could lead to a change in the concentration of internal solutes, and cause lysis of erythrocytes by exceeding their critical cell volume. However, aerolysin, in its turn, appears to bind to glycophorin, which is the most abundant protein on the red cell membrane after band 3. Further studies will confirm the identity of the receptors for these two similar proteins. The extension of the kinetic analysis to red cells treated with other enzymes (e.g. chymotrypsin, papain, neuraminidase, phospholipases, etc.), agglutinins (e.g. WGA, ConA, PHA, etc.), and chemicals (e.g. DIDS) should be further tried, as they might yield clues. Methods exploring the labelling of the protein with fluorescent probes

or dense markers as colloidal gold could be used to detect the receptor after its separation by electrophoresis and blotting.

The utilization of optical and electron microscopic techniques in this work proved to be very helpful in giving a visual idea of what occurs during the process of haemolysis by enterolobin. This kind of study could be repeated for every type of cell shown to be lysed by the protein. This would reveal whether the pattern is similar to that which occurs with cancer and red cells (damage to the membrane) or to that of insect larvae gut cells (apparent intactness).

It was demonstrated that enterolobin is toxic against two cancer cell lines and one insect species. It would be interesting if other species of insect and animals and other cell types could be also tested. Besides increasing the volume of information about this protein, it could suggest some interesting applications for the phytocytolysin. For example, its gene could be cloned and expressed in plants attacked by insect pests, possibly confering resistance to the plant. Before that however, it is essential to test the degree of toxicity of enterolobin when ingested by animals. Currently, experiments are under way to assay its effects in the diet of rats.

However, some basic questions remain. It would be interesting to know if the haemolysis, lysis of cancer cells, toxicity to insects and inflammatory activity are all related to the same mechanism of action, or are exerted through different pathways. An initial point for investigation would be to determine whether the receptors in these different cells are similar proteins or not. This would open another line of research on the recptor(s) for enterolobin.

The physiological role(s) of enterolobin in the seeds of *Enterolobium contortisiliquum* remains an intriguing question. Enterolobin could be one of the seed storage protein or a natural defensive protein against microorganisms, insect pests and animal predators. Another hypothesis is that this cytolytic protein (*in vitro*) could be an endogenous porin-like, colicin-like or transport protein in the plant cell membranes. When extracted and isolated, this pore-forming ability would be preserved in the protein.

The answers are dependent not only on experiments in plant physiology, but also on a great effort in membrane biochemistry and protein chemistry, and will involve methods from other auxiliary areas, such as molecular genetics, immunology, protein crystallography and protein modelling.

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