INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

I. THE APPLICATION OF PHAGE DISPLAY TECHNOLOGY TO TRYPANOSOME SURFACE PROTEINS.

II. STUDIES OF A THROMBIN INHIBITOR FROM GREEN TEA.

by

JUN LU

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

2000

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

3/10/00
Date

Chair of Examining Committee

March 10, 2000
Date

Executive Officer

Supervisory Committee

The City University of New York

ABSTRACT

I. THE APPLICATION OF PHAGE DISPLAY TECHNOLOGY TO TRYPANOSOME SURFACE PROTEINS.

II. STUDIES OF A THROMBIN INHIBITOR FROM GREEN TEA.

By

Jun Lu

Adviser: Professor Manfred Philipp

In part I of this study, living trypanosome cells, which cause African trypanosomiasis in human and in wild and domestic animals, have been used as a target to screen a phage display peptide library. Phage display technology is a method for the generation of molecular diversity and the screening for binding species of interest. This study demonstrates the first application of phage display technology to living African trypanosomes.

One strong binder (peptide 307) with an IC_{50} of 1 μM has been identified. Another peptide (peptide 608) has shown trypanolytic activity. The observed similar activities of both L- and D- type peptide 608 suggest that peptide 608 may attack the non-chiral lipid layers of cell membranes. The lytic activity of peptide 608 inhibited by wheat germ agglutinin indicates the presence of an interaction between peptide 608 and the flagellar pocket of trypanosomes. Peptide 307 also protected trypanosome cells from lysis by peptide 608, suggesting that these peptides bind at related sites on the trypanosomes. The predicted α-helix content in peptide 608 is 63%. The low hemolytic activity of peptide 608 also suggests that it may be possible to develop this peptide or others like it as anti-trypanosome drug candidates.

In part II of this study, extracts of green tea (*Camellia sinensis*) were tested for their ability to inhibit thrombin, an enzyme important in blood coagulation. An active fraction was proven to be the major polyphenol of green tea, (-)-epigallocatechin gallate (EGCG). The chemical identity of the active fraction was determined by proton NMR, mass spectrometry, and by its chromatographic identity with an authentic sample. EGCG is a noncompetitive (K_i 80 μM) thrombin inhibitor that inhibits blood coagulation when measured by the Activated Partial Thromboplastin Time test, with an IC_{50} of 600 μM.

Green tea is not likely to provide enough EGCG to cause a noticeable inhibition of blood coagulation. However, epidemiological studies have reported a reduced risk of coronary heart disease in subjects with a high intake of tea components. These results suggest that the anticoagulant activity of tea components should also be considered in interpreting such studies.

ACKNOWLEDGEMENTS

I would like to express my sincerely thanks to Dr. Manfred Philipp, my thesis adviser, for his advice, support, and encouragement throughout the course of this study. Thanks are due to Drs. John Davis, Edward Kennelly, Susan Rotenberg, and Horst Schulz for serving on my thesis committee and for their helpful suggestions.

I thank Dr. Mary Lee at New York University for providing me with trypanosome strains and giving me valuable suggestions in the study of trypanosomes and Dr. Hanna Shear at Montefiore Hospital for helping me in the malaria project. I thank Drs. James Ciaccio and Donald Clarke at Fordham University for their help in the NMR studies and Dr. Dixie Goss at Hunter College for access to the CD spectrometer. I thank Dr. Thomas Jensen and Mr. Mike Baxter at Department of Biological Sciences for their assistance in the study of electron microscopy. I also thank Drs. Reuben Baumgarten, John Richards, and Marc Lazarus at Chemistry Department of Lehman College for their support. My thanks are extended to Ms. Donna Barrington, Ms. Raji Thamarathu and Ms. Mini Thankachan for their kind assistance.

Finally, I thank my wife, Suilan Rong, for her support and encouragement throughout the duration of this study.

LIST OF ABBREVIATIONS

Part I:

apoA-I	=	Apolipoprotein A-I
CD	=	Circular dichroism
DCM	=	Dichloromethane
DIC	=	N,N-diisopropylcarbodiimide
DMF	=	N,N-dimethylformamide
FBS	=	Fetal bovine serum
Fmoc	=	9-Fluorenylmethyloxycarbonyl
GPI	=	Glycosylphosphatidylinositol
HDL	=	High density lipoproteins
HOBT	=	N-hydroxybenzotriazole
Hpr	=	Haptoglobin-related protein
HRP	=	Horseradish eroxidase
IPTG	=	Isopropyl β-D-thiogalactoside
LDL	=	Low density lipoproteins
MMP	=	Matrix metalloproteinase
PEG	=	Polyethylene glycol-8000
pfu	=	Plaque forming units
SEM	=	Scanning electron microscopy
SPCL	=	Synthetic peptide combinatorial library
T. b. b.	=	*Trypanosoma. brucei brucei*

Tf = Transferrin

TFA = Trifluoroacetic acid

TLF = Trypanosome lytic factor

TNF = Tumor necrosis factor

VSG = Variant surface glycoproteins

WGA = Wheat germ agglutinin

Xgal = 5-Bromo-4-chloro-3indolyl-β-D-galactoside

Part II:

APTT = Activated partial thromboplastin time

EGCG = (-)-Epigallocatechin gallate

ESI = Electrospray ionization

PT = Prothrombin time

TFA = Trifluoroacetic acid

TLC = Thin layer chromatography

t-PA = Tissue-type plasminogen activator

TT = Thrombin time

TABLE OF CONTENTS

LIST OF TABLES

Part I

Part II

LIST OF FIGURES

Part I

Part II

PART I

IDENTIFICATION AND CHARACTERIZATION OF STRONG TRYPANOSOME BINDERS AND ANTI-TRYPANOSOME PEPTIDES FROM A PHAGE DISPLAY PEPTIDE LIBRARY

INTRODUCTION

African trypanosomes are uniflagellated, unicellular eukaryotic protozoan parasites. *Trypanosoma* subspecies *T. brucei brucei* (*T. b. b.*) causes the bovine disease nagana. *T. b. gambiense* and *T. b. rhodesiense* are the causative agents for human sleeping sickness. There are about 300,000 newly infected cases per year in humans (1). Currently, the recommended drugs, nifurtimox and benznidozole, are only approximately 60% effective in the acute phase (2).

Variant Surface Glycoproteins of African Trypanosomes

African trypanosomes are transmitted by tsetse flies (*Glossina* spp.). In mammals, the trypanosomes are present in the bloodstream of their hosts. Bloodstream-form trypanosomes are completely covered by a 10-15 nm thick coat of highly antigenic variant surface glycoproteins (VSG) (3), which may have variable amounts of carbohydrate attached. Reported molecular weights of VSG molecules range from 53,000 to 63,000 (4). Much of these molecular weight differences may be due to differences in carbohydrate content. About 10,000,000 VSG molecules in the coat of a single cell are glycosylphosphatidylinositol (GPI)-anchored by their C termini to the trypanosome membrane (5). VSG proteins are present as dimers that are tightly packed on the cell surface (3). The coat functions as a highly dynamic molecular filter that allows penetration of small proteins such as trypsin, but effectively restricts the access of much larger antibody molecules to buried epitopes (6). VSG molecules themselves can stimulate the formation of antibodies by the host. However, to evade the host's immune

response, trypanosomes vary their VSG proteins by selectively rearranging the 10^3 VSG genes to allow the expression of only one VSG at a time. The switching frequency from one variant to the next was estimated in the range of 10^{-2} to 10^{-7}/cell generation (7, 8). Because the parasite population always contains variants covered with VSG coats before the host produces specific antibodies, the infection by trypanosomes becomes chronic.

Receptors in the Flagellar Pocket of *T. brucei*

Communication between the trypanosome and its environment is confined to the flagellar pocket (reviewed in ref. 9). This is a deep invagination of the plasma membrane where the flagellum extends from the cell (see ultrastructure of *T. brucei* in Figure 1) (10). The flagellar pocket is a small lumenal volume bounded by an invaginated portion of the surface membrane called the flagellar pocket membrane and the flagellar membrane. The flagellar pocket membrane is the only part of the cell surface that supports exocytosis and endocytosis, explaining the exclusiveness of this membrane that lacks attachment of a microtubular array, which are present in the rest of cell membranes (3).

To acquire the nutrients required by cells in the bloodstream of their host, trypanosomes use receptor-mediated endocytosis to absorb macromolecules. This is mainly done in the flagellar pocket (10). Uptake of albumin, ferritin, and some other macromolecules is not saturable and they are internalized by fluid-phase endocytosis (11). Transferrin (Tf) and low-density lipoproteins (LDL) both are required for growth of *T. brucei* (12, 13, 14). They bind specifically and saturably to trypanosomes. The rates of

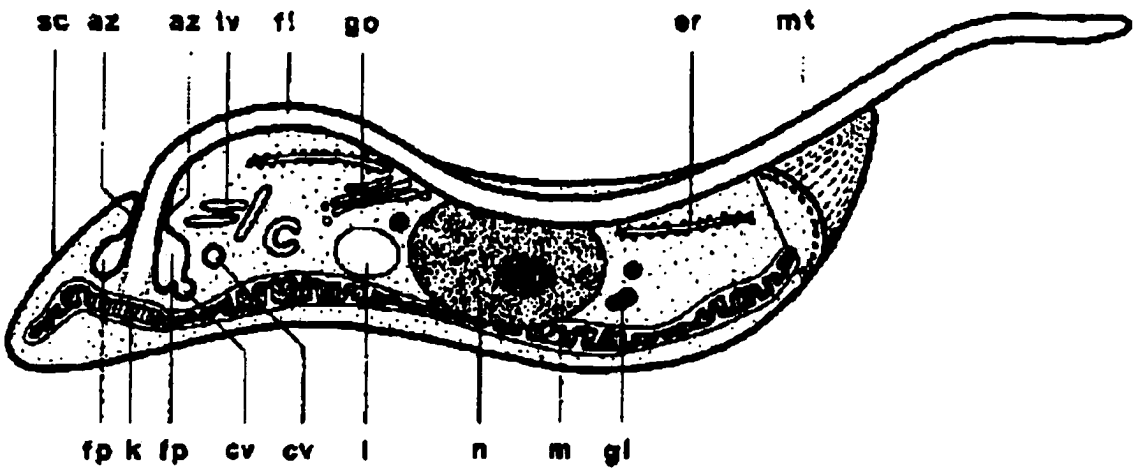


Figure 1. Ultrastructure of *Trypanosoma brucei* (bloodstream form, length 20 μm, width 2-3 μm) (10).

az: adhesion zone at the entrance of the pocket; cv: coated vesicle; er: endoplasmic reticulum; fl: flagellum; fp: flagellar pocket; gl: glycosome; go: Golgi; k: kineplast; l: lysosome; m: mitochondrion; mt: subpellicular microtubules; n: nucleus; sc: surface coat; tv: tubovesicular structures.

uptake measured for Tf and LDL are two-to-three orders of magnitude higher than those molecules taken up by fluid phase endocytosis (15). Electron microscopic studies have identified the flagellar pocket as the location of Tf and LDL uptake (14). These observations suggest that endocytosis of transferrin and LDL is receptor-mediated.

The Tf receptor of *T. brucei* is a heterodimer with two proteins identified as pESAG6 and pESAG7 (16, 17). The stoichiometry of these two proteins in the complex is 1:1. pESAG6 has a glycosylphosphatidylinositol anchor, like VSG, but unlike VSG, it does not spread over the entire surface. It is only concentrated in the flagellar pocket. pESAG7 does not directly interact with the membrane. pESAG7 remains in the complex by binding to pESAG6. However, both pESAG6 and pESAG7 proteins are required to bind to Tf (18, 19). Tf uptake is 90% inhibited by Fab fragments from antibodies against the Tf receptor (18).

The structure diversity of Tf receptors in the trypanosome solves the problem that *T. brucei* can infect a very large number of mammalian hosts, whose Tf sequences differ by as much as 28% (20). This diversity is reflected in the variable binding constants for bovine Tf from 2.1 nM to 131 nM to the receptors from different *T. brucei* clones (18, 19). This variation is not thought to affect the rate of uptake in view of the very high concentration of Tf present in mammalian blood (25-50 μM) (21)

The LDL receptor is a 145-kDa membrane protein isolated by affinity chromatography. It is located in the flagellar pocket membrane and in the membrane covering the flagellum (14, 22). Binding data suggest that two types of the receptor are present: a high-affinity receptor with a dissociation constant of 6 nM and a low-affinity receptor with a K_{diss} of 250 nM. LDL binding was inhibited at 4°C by antibodies against

the 86-kDa fragment from the LDL-receptor. These antibodies significantly slowed *in vitro* growth of *T. brucei* (14).

Trypanosome Lytic Factors

Trypanosome lytic factors present in human serum prevent humans from being infected by *T. brucei brucei* (23). A lytic factor (called TLF1) from human serum that lyses *T. b. brucei* was characterized as a subset of high-density lipoprotein (HDL) (24, 25). The early work showed that the lytic factor had a density between 1.063-1.216 g/ml and an approximate molecular weight of 500,000. The HDL nature of the lytic activity was confirmed by the presence of the 28-kDa apolipoprotein A-I (apoA-I) (24). Haptoglobin-related protein (Hrp), which was not found in nontrypanolytic HDL, was identified as another component in TLF1 (26). Haptoglobin is an abundant serum protein that binds free hemoglobin and facilitates its clearance via receptors in the liver (27). A second lytic factor, called TLF2, which was not associated with HDL in human serum, was later reported (28, 29). TLF2 is a 100-kDa protein complex containing immunoglobulin M, apoA-I, and Hrp. It has less than 1% detectable lipid (30).

Haptoglobin has been found to inhibit TLF1-mediated lysis but not to inhibit lysis mediated by TLF2 in normal human serum. This raises questions about the physiological role of TLF1 (28, 31). Both apoA-I and Hrp are present in TLF1 and TLF2, suggesting both components play a role in trypanolysis. The physiological role and biological properties of Hrp are unknown. It is unclear how the complexes are arranged and held together in the TLF1 or TLF2 complex.

The mechanism of human serum-mediated lysis of *T. b. brucei* is not clear. Most work has focused on TLF1. TLF1 has been characterized as a subset of human HDL, suggesting that HDL is involved in the lysis of trypanosome. Rifkin (32) hypothesized that the insertion of HDL-specific lipids into the trypanosome plasma membrane caused alteration of the properties of their membrane and leads to cell death. Another possible mechanism addresses the role of apoA-I, the major apolipoprotein of HDL (33). The presence of amphipathic helices in apoA-I might be responsible for the interaction of TLF1 with lipids in the trypanosome membranes. The helical arrangement of amino acids with opposing polar and nonpolar surfaces has been suggested to increase association of apoA-I with the trypanosome membrane and result in membrane disruption (33).

The third hypothesis of cell lysis by TLF1 is that multiple processes are involved, including binding of TLF1with a receptor in the trypanosome flagellar pocket membrane, endocytosis via coated vesicles, and delivery to lysosomes (34). At low pH in the lysosome, the haptoglobin-related protein (Hrp) present in TFL1 induces a peroxidase activity that results in lipid peroxidation, lysis of lysosomal membrane, and autodigestion of the parasite (26). This mechanism is supported by the following observations (34): (a) TLF1-mediated lysis of trypanosome cells only starts after a lag of 20-30 minutes. (b) TLF1-mediated lysis is temperature sensitive. Lysis of cells is completely blocked at 17°C or below. After incubation of TLF1 with trypanosome cells at 17°C, cells were extensively washed to remove TLF1, and subsequently shifted to 37°C. Cell lysis occurred. (c) Electron microscopy of trypanosomes incubated with gold-labeled TLF1 at 37°C shows that TLF1 is localized in the cytoplasm or within degenerating large vesicles,

which might be lysosomes. The receptor of TLF1 in the flagellar pocket has not been identified and characterized.

Tumor Necrosis Factor-α as a Trypanolytic Factor

During trypanosome infection, a cytokine mainly produced by activated macrophages is induced. This cytokine is called tumor necrosis factor-α (TNF). TNF was initially isolated from the serum of *T. brucei*-infected rabbits as a factor responsible for systemic suppression of lipoprotein lipase activity (35). It has been reported that both TNF and peptides derived from it are directly trypanolytic for trypanosomes (36, 37). The lectin-like activity of TNF is responsible for this lytic activity, which can be inhibited by N,N'-diacetylchitobiose, an oligosaccharide that binds TNF, and wheat germ agglutinin, a lectin displaying a specificity for N,N'-diacetylchitobiose. With further investigation of the mechanism underlying TNF-mediated trypanolysis (37), it was found that the lytic activity is temperature and pH dependent. Binding and endocytosis of TNF can occur at 17, 21, and 30°C, but for cell lysis by TNF, the temperature has to be over 25°C. Thus, at this stage TNF follows the same mode as TLF1 mentioned before.

However, a different mechanism of TNF-mediated lysis was proposed (37) Incubation with ammonium chloride strongly inhibits the lytic activity, suggesting an acidic pH is required to exert its lytic activity. At low pH, TNF can form ion-permeable channels in mammalian membranes (38). It was proposed that this pore-forming capacity of TNF could account for trypanosome lysis (37).

Phage Display Technology

Phage display technology is a method developed for the generation of molecular diversity and the screening for binding species of interest. This technology was developed by Smith in 1985 (39). He cloned a restriction enzyme digest of plasmid DNA into the gene III insertion site of filamentous phage f1. A fusion protein with the foreign sequence in the middle of gene III product was created. It was displayed in an immunologically accessible form on the virion surface. This technology was then expanded to create a library of random peptides in the phage that result from the insert of a random oligonucleotide in the phage genome (40). Each display variant in the library is physically linked, via fusion to the coat protein encapsidating the phage DNA, to its own encoding display gene. The selection of binding variants and the ready determination of the predicted amino acid sequence of the variants from DNA analysis enable rapid and effective screening of a library against a number of targets. The filamentous bacteriophage M13 is the most commonly used library vehicle.

The wild type M13 virus particle is about one micron in length (41). The single stranded, circular DNA genome is stretched along the entire particle length and is coated by thousands of copies of pVIII (41). Five copies each of pIII and pVI are located at one end of the virus particles and are involved in host-cell binding and the termination of the assembly process. Five copies each of pVII and pIX are required for the initiation of assembly and for the maintenance of virion stability (41). The most commonly used structural protein as a vehicle for phage display is pIII, followed by pVIII and rarely pVI. The amino terminus of pIII is the most common site used for display.

One application of the display of proteins or random peptides is to identify any phage clone that binds to a target in a highly specific manner. Random phage display screening has been used to produce specific inhibitors to naturally occurring enzymes or receptors (42, 43, 44). Koivunen *et al.* (45) reported that cyclic peptides from phage display peptide libraries containing the sequence His-Trp-Gly-Phe are potent and selective inhibitors of matrix metalloproteinase-2 (MMP-2) and MMP-9, which play major roles in tissue remodeling and cell migration during morphogenesis and wound healing. The His-Trp-Gly-Phe peptide inhibited the migration of human endothelial cells and tumor cells. It also prevented tumor growth and invasion in animal models and improved survival of mice bearing human tumors. The peptide or protein displayed on the phage surface need not imitate a naturally occurring protein or protein domain. Pasqualini and Ruoslahti (46) have identified peptides capable of mediating selective localization of phage to brain and kidney blood vessels upon *in vivo* administration of phage libraries. Similar work has been done on targeting cell-surface antigens specific to the tumor (47, 48).

Phage display technology can also be applied to display a real protein or protein fragment or one, which has purposely been engineered to carry variants. Then, those displayed proteins or peptides can be used to conduct a rapid functional assay such as binding affinity. Directed molecular evolution studies on a wide variety of proteins, including hormones (49, 50) and growth factors (51), have been carried out. Other than binding affinity, the technology of phage display has been used to select for proteins with improved biophysical properties (i.e. thermodynamic stability and folding) (52, 53, 54)

and for proteins with novel enzymatic activities. Such phage-displayed proteins include β-lactamase (55), glutathione-S-transferase (56), and catalytic antibodies (57, 58, 59).

Random peptides or gene fragments display libraries have been constructed to map contact points in protein-protein interactions in the absence of a three dimensional structure. A large number of protein-antibody, virus-antibody and ligand-receptor interfaces have been mapped (60, 61, 62, 63).

The use of combinatorial libraries offers an efficient way to identify novel peptides that display antimicrobial activity. There are two commonly used ways to generate peptide libraries: phage display and chemical synthesis on a solid phase. Houghten *et al.* (64) developed synthetic peptide combinatorial libraries (SPCLs) to discover bioactive peptide sequences. Several other studies have used an iterative process to screen SPCLs to identify peptide sequences that bind and lyse pathogenic bacteria, fungi, or yeast (65, 66). The advantage of phage display libraries is that a single peptide selected by an appropriate screening technique can be readily identified by large-scale growth of that viral particle and sequencing of that viral particle's nucleic acid.

Since some diseases caused by trypanosomes are still not curable (2), there is a clear need for developing new medications to treat these diseases. Screening phage display peptide libraries with living trypanosome cells provides an efficient and low-cost way to identify peptides that either bind with high affinity and specificity with trypanosomes or directly kill trypanosomes. By selecting the phage library against three *T. brucei* strains, each of which carries different VSG, the selection procedure is expected to favor only those members of the library that bind to invariant and common epitopes that may not be visible to the host immune system. The similar strategy has been

successfully used to screen combinatorial nucleic acid libraries with trypanosome cells (67). Short RNA ligands (aptamers) for constant surface components on the trypanosome cells were isolated.

In this study, three strains of trypanosomes are used to screen a 12-mer peptide library. A peptide motif with high affinity with trypanosome cells has been found. A peptide sequence from the library shows high trypanolytic activity. Both D- and L-isomers of this peptide display the same lytic activity. The high trypanolytic activity and low hemolytic activity of these peptides suggest that this technique may be able to identify anti-trypanosome drug candidates.

MATERIALS AND METHODS

Trypanosoma brucei brucei

Three strains of *T. brucei brucei*, 118, 221, and Bleo36, were provided by Dr. Mary Lee from New York University. The bloodstream form of *T. brucei brucei* was grown in HMI-9 (Appendix I) (68). For strain Bleo36, the medium contained two antibiotics: 2 μg/ml hygromycin B and 1 μg/ml phlemycin. Cells were incubated at 37°C in 5% CO_2.

Scanning Electron Microscopy (SEM)

Cells of Strain 118 were washed three times with washing medium (growth medium without FBS, Serum Plus, and penicillin) and one more time with PBS. Cells were fixed by adding 200 μl 0.5% glutaraldehyde in PBS. After 10 minutes incubation on ice, 200 μl 0.02 M Gly in PBS was added to the cell suspension. Five minutes later, cells were washed three times with PBS and three more times with deionized water. A drop of the cell suspension was placed on a slide. After air drying, the slide was directly observed using SEM (Hitachi S-2700). An SEM picture of *T. brucei brucei* is shown in Figure 2.

Phage Display Peptide Libraries

Ph.D.-12 Phage Display Peptide Library Kits were purchased from New England BioLabs, Inc.. The kit is based on a combinatorial library of random peptide 12-mers fused to a minor coat protein (pIII) of M13 phage. The displayed peptide 12-mers are

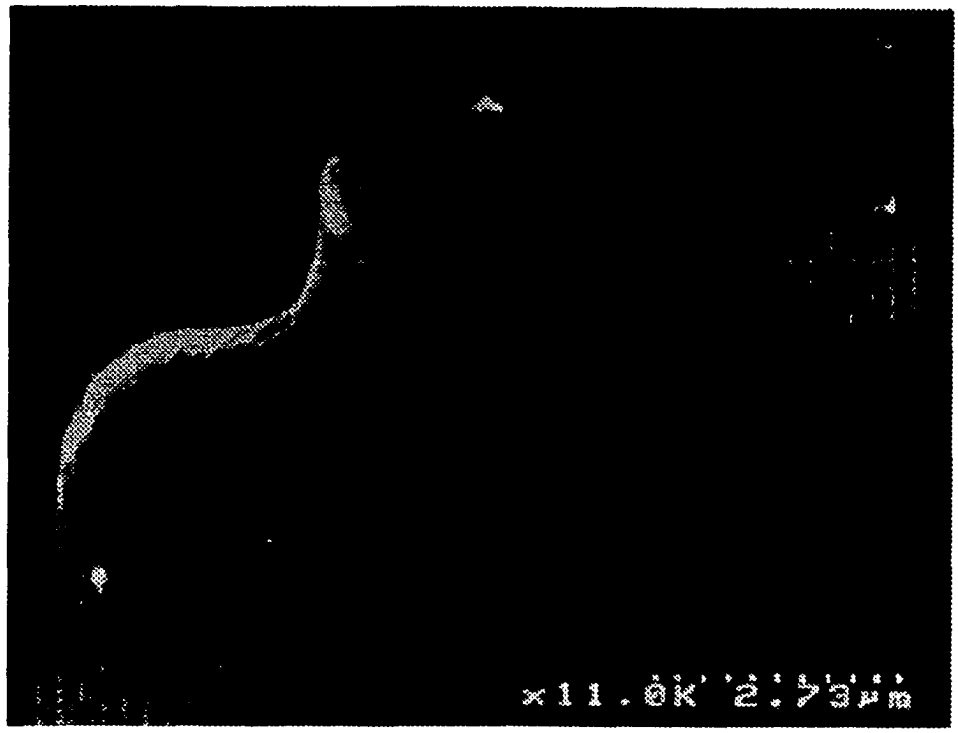


Figure 2. SEM picture of strain 118 of *T. brucei brucei*.

See Materials and Methods for the experimental procedure.

expressed at the N-terminus of pIII, followed by a short spacer (Gly-Gly-Gly-Ser) and then the wild type pIII sequence. The library phage carries the inducible *lacZ* gene, which codes for β-galactosidase. Thus, phage plaques appear blue (as shown in Figure 3) when plated on medium containing 5-bromo-4-chloro-3indolyl-β-D-galactoside (Xgal) substrate and isopropyl β-D-thiogalactoside (IPTG) inducer.

Phage Titering

The host strain *E. coli* ER2537 provided in the kit was streaked out from the glycerol culture onto a minimal plate (see Appendix II). The plate was incubated at 37°C for 24 hours and stored at 4°C. A single colony of ER2537 was inoculated into 10 ml of LB medium (Appendix III) and incubated with shaking until mid-log phase (about 0.5 O.D. at 600 nm). Ten-fold serial dilutions of phage were prepared in LB medium. *E. coli* cell culture (200μl) was mixed with 10 μl of each dilution of phage, vortexed, and incubated at room temperature for 1-5 minutes. The mixture containing infected cells was transferred to a 45°C pre-warmed Agarose Top tube (Appendix IV). After quick vortexing, the tube was immediately poured onto a pre-warmed LB/IPTG/Xgal plate (Appendix V). Agarose Top was spread evenly by tilting the plate. Plates were cooled for 5 minutes and incubated overnight at 37°C. Plaques were counted on plates and the number of plaque forming units (pfu) per 10 μl of phage solution was calculated.

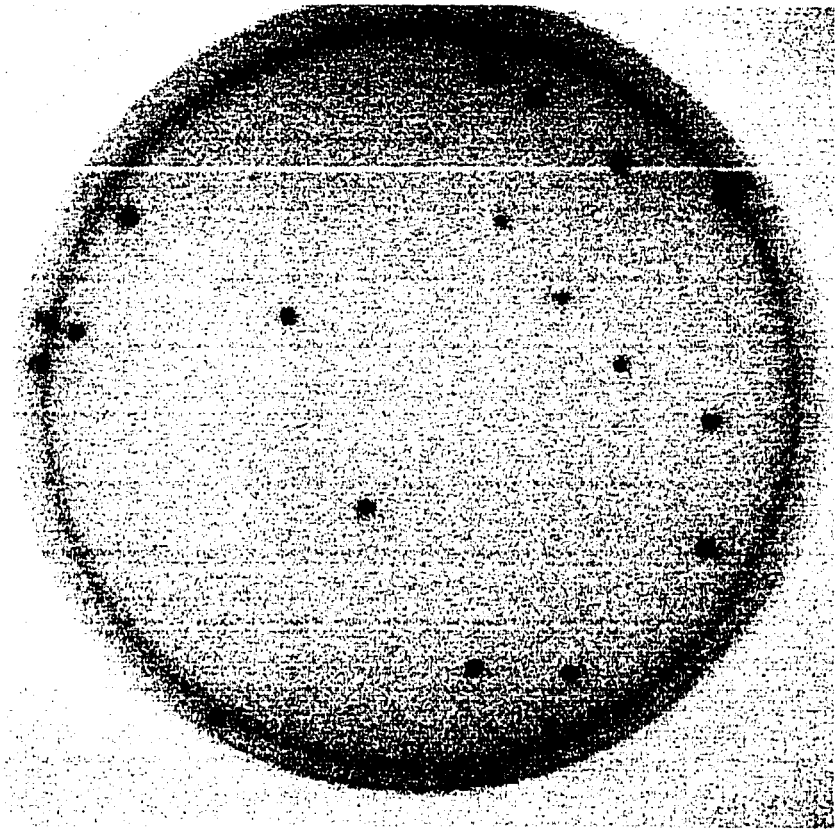


Figure 3. Phage clones grown on an IPTG/Xgal plate.

Phage Selection

T. brucei brucei (strain 221) were harvested at a cell density of around $1x10^7$ cells/ml and washed three times with the washing medium. One milliliter cell suspensions of strain 221 ($5x10^7$ cells) were mixed with 10 µl of the original phage library ($4x10^{10}$ phage particles). After 30 minutes incubation at room temperature with gentle shaking, unbound and weakly associated phage were washed off by four consecutive washes with 5 ml washing medium. To avoid collecting phage that bound to the test tube, trypanosomes and bound phage were transferred to a fresh test tube. To recover bound phage, 1 ml 0.2 M Glycine-HCl (pH 2.2) was added for five minutes. The mixture was then neutralized with 150 µl 1M Tris-HCl (pH 9.1). Five microliter of this phage solution was used to titer the number of bound phage and the rest were amplified in *E. coli*. Second and third rounds of biopanning with the other two trypanosome strains (strain 118 and Bleo 36) were conducted by using about $2x10^{11}$ pfu of the proceeding amplified phage. After the third biopanning, the recovered phage were titered on LB/IPTG/Xgal plates. With 12-18 hours incubation, the plates were used to pick up individual blue plaques. Those clones were amplified individually.

Phage Amplification and Purification

Phage collected from biopanning experiments were transferred to a 20 ml early-log *E. coli* culture and incubated with vigorous shaking for 4.5 hours. The culture was then transferred to a centrifuge tube and spun 10 minutes at 10,000 rpm at 4°C. 1/6 volume of aqueous solution containing 20% (w/v) polyethylene glycol-8000 and 2.5 M NaCl (PEG/NaCl) was added to the supernatant. Phage were precipitated at 4°C overnight. The PEG precipitate was spun for 15 minutes at 10,000 rpm at 4°C and the precipitate was suspended in 1ml TBS. The suspension was re-precipitated with 1/6 volume of PEG/NaCl. After incubation on ice for 60 minutes, the precipitate was collected and re-suspended in 200 µl TBS. This purified phage is ready for the next biopanning.

Purification of Sequencing Templates

After amplification, phage clones with a single DNA sequence were precipitated with 1/6 volume of PEG/NaCl in a refrigerator overnight. Pellets were collected by centrifugation and suspended in 100 µl iodide buffer (Appendix VI) and 250 µl ethanol. After 10 minutes incubation at room temperature, the tube was spun and the pellet was collected. The pellet was washed with 70% ethanol and dried under vacuum. The pellet was suspended in 30 µl water. The pellet, containing the purified DNA, was used to sequence the DNA templates.

DNA Sequencing

All templates for sequencing were sent to DNA Synthesis and Sequencing Laboratory at Robert Wood Johnson Medical School of University of Medicine and Density of New Jersey. The amino acid sequence was determined using reduced genetic codes (69) (see Table 1). A diagram of the N-terminal sequence of random 12-mer peptide-gIII fusion is shown as follows (69):

pIII leader sequence

5'- ...TTA TTC GCA ATT CCT TTA GTG GTA CCT TTC TAT TCT CAC
3'- ...AAT AAG CGT TAA GGA AAT CAC CAT GGA AAG ATA AGA GTG

Start of mature 12-mer peptide-gIII fusion

↓

TCT **NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK** GGT
AGA **NNM NNM NNM NNM NNM NNM NNM NNM NNM NNM NNM** NNMCCA

GGA GGT TCG GCC GAA ACT GTT GAA AGT TGT TTA GCA AAA TCC CAT
CCT CCA AGC CGG CTT TGA CAA CTT TCA ACA AAT CGT TTT AGG GTA

ACA GAA AAT TCA TTT ACT AAC GTC TGG AAA GAC GAC AAA ACT TTA
TGT CTT TTA AGT AAA TGA TTG CAG ACC TTT CTG CTG TTT TGA AAT

GAT CGT TAC GCT AAC TAT GAG GGC...-3'
CTA GCA ATG CGA TTG ATA CTC CCG....-5'

◄—— *sequencing primer*

The pIII coat protein is expressed with a leader sequence that is removed upon secretion at the position indicated by the arrow. This results in the peptide positioned directly at the N-terminus of the mature protein. The hybridization position of the sequencing primers is indicated by an underscore. Codons for random peptide sequences are printed in bold (N stands for G, T, A, or C; K stands for G or T, and M stands for A or C).

Table 1. Reduced genetic code (69).

First Position	Second Position					Third Position
	T	C	A	G		
T	Phe	Ser	Tyr	Cys	T	
	Leu	Ser	Gln	Trp	G	
C	Leu	Pro	His	Arg	T	
	Leu	Pro	Gln	Arg	G	
A	Ile	Thr	Asn	Ser	T	
	Met	Thr	Lys	Arg	T	
G	Val	Ala	Asp	Gly	T	
	Val	Ala	Glu	Gly	G	

ELISA Assay

ELISA assays were carried out in 96-well microplates, which were pre-coated with 0.5% BSA solution in 0.05M phosphate buffer (pH 7.0) at 4°C overnight. Approximately $5x10^6$ trypanosome cells were added to each well. After washing three times with the washing medium, cells were mixed with 10^{12} pfu phage from amplified individual phage clones and incubated at room temperature for 30 minutes. The trypanosomes were washed four times to remove unbound phage. Horseradish peroxidase (HRP)-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) was diluted 1:5000 by using 0.5% BSA in the washing medium. Diluted antibody (200 μl) was added to each microplate well. After incubation for 1 hour at room temperature with agitation, cells were washed four times. 40 mM HPR substrate (200 μl) (2,2'-Azino-bis(3-Ethylbenz-Thiazoline-6-Sulfonic Acid) Diamonium Salt) dissolved in 50 mM sodium citrate buffer (pH 4.0) with 100 μM H_2O_2 was added to cells. After 10 minutes incubation, the microplates were read at 405 nm using a microplate reader (from Molecular Devices). Wild type M13 phage and a recombinant phage with an unrelated sequence were used as controls.

Peptide Synthesis

The Rink resin (Advanced ChemTech) (70) was used as a solid phase. As puchased, the amino ends of this resin are protected by 9-fluorenylmethyloxycarbonyl (Fmoc) groups. The Fmoc groups were removed by 20% piperidine in N,N-dimethylformamide (DMF) for 20 minutes. Amino groups of L- or D- type amino acids used were protected by Fmoc groups. All amino acids were purchased from Bachem

California. The coupling solution contained 0.3 M modified amino acid, 0.3M N-hydroxybenzotriazole (HOBt), and 0.3 M N,N-diisopropylcarbodiimide (DIC) in DMF. The coupling time was 1.5 hour. After each coupling reaction, the resin was washed with DMF six times and rinsed with dichloromethane (DCM) three times. After removing the Fmoc group, the resin was washed and rinsed again for the next coupling reaction. The completed sequence was cleaved from the resin and side chain protecting groups were removed simultaneously by using a trifluoroacetic acid/tri-isobutylsilane/water/phenol (90/5/2.5/2.5) (v/v) mixture. After two hours incubation with this mixture, the resin was removed by filtration. The filtrate was mixed with cold ether, which results in peptide precipitation. The precipitate was washed twice with cold ether and was then dissolved in water. After lyophilization, peptides were purified by using HPLC, and the molecular weights of purified peptides were confirmed by mass spectrometry.

Mass Spectral Analysis of Peptides

Mass spectra were taken in a Finnigan LCQ quadrupole ion trap instrument equipped with electrospray ionization (ESI). HPLC purified peptides at a concentration of 10 μM were dissolved in 1% acetic acid in methanol/water (1:1). They were directly injected by a syringe pump at the flow rate of 3 μl/minute.

Competitive Assay of Peptide 307 with *T. brucei brucei*

Trypanosome cells ($5x10^6$ – $1x10^7$, Strain 118 or 221) were mixed with $1x10^{14}$ pfu of phage clone 307. Varied concentrations of peptide 307 were then added. After 30 minutes incubation with gentle rocking at room temperature, the mixture was washed

four times with the washing medium. The cells were then transferred to a new test tube and treated with 1ml 0.2 M Glycine-HCl buffer (pH 2.2) for 5 minutes. After neutralization of with 150 μl 1M Tris-HCl buffer (pH 9.1), the mixture was titered and the amount of phage recovered was recorded.

***In vitro* Trypanolysis Assay**

Trypanosomes (strain 118, 221, or Bleo36) were harvested from cell culture medium at approximately $5x10^7$ cells/ml. After washing three times with the washing medium, $1\text{-}5x10^6$ cells were incubated for two hours with different concentration of peptides (0.1 μM to 100 μM) in 96-well microplates in a high humidity cell culture incubator at 37°C and a 5% CO_2 atmosphere. Living cells in each well were counted by using a hemocytometer (Hausser Scientific Company). For assays of trypanolysis affected by wheat germ agglutinin (WGA) or peptide 307, cells were preincubated with 100 μg/ml WGA or 50 μM peptide 307 at 4°C for one hour. After two hours incubation of cells with tested peptides, cells were counted. Each experiment was performed in triplicate.

Circular Dichroism Studies

All spectra were taken on a J-710 spectropolarimeter (JASCO, Japan). D-peptide 608 (0.6mg/ml) was dissolved in 0.05 M phosphate buffer (pH 7.0) and measured in a quartz cell with a 0.01 cm path length. The scanning range was 180 - 260 nm.

Hemolytic Activity of D-608

This assay was conducted by using human red blood cells. Red blood cells were isolated by centrifugation at 2,000 rpm for 10 minutes at 5°C. The cells were washed three times in phosphate buffered saline (PBS). Approximately $1x10^6$ cells were incubated with the peptide (concentrations ranged from 0.1 μM to 100μM). After two hours incubation at 37°C, the blood cells were counted with the use of a hemacytometer. Each experiment was performed in triplicate.

RESULTS

Phage Selection

Three strains of *T. brucei brucei* (221, 118, and Bleo36), each with different variant surface glycoproteins (VSG), were used to select potential binders from a phage display peptide library. The starting phage library contains about $2.7x10^9$ particles, a significant subset of the $4.1x10^{15}$ (20^{12}) possible 12-mer sequences. After the first biopanning with strain 221, the recovered phage were amplified and biopanned with strain 118, followed by the last biopanning with strain Bleo36. The enrichment for phage binding by those three strains can be seen in Figure 4. 10- and 500-fold more phage was recovered in the second and third rounds of selection, respectively.

The eluate from the third round of biopannings was plated out to yield single colonies and 18 single clones were chosen for amplification. ELISA assays were performed to determine the binding ability of the individual clones for three strains. To do this, an anti-M13 antibody conjugated with horseradish peroxidase (HRP) was mixed with the complex of cells and an individual clone. A microplate reader was used to take readings at a wavelength of 405 nm 10 minutes after the addition of HPR substrate. The wild type phage and a phage carrying an unrelated sequence gave very low background readings in this assay. As shown in Table 2, most clones showed binding with all three strains. Clone 307 displayed the highest binding signal. All of these 18 clones were sequenced (Table 2). A comparison of the sequences in Table 2 suggests a short consensus sequence (amino acids in bold in the table):

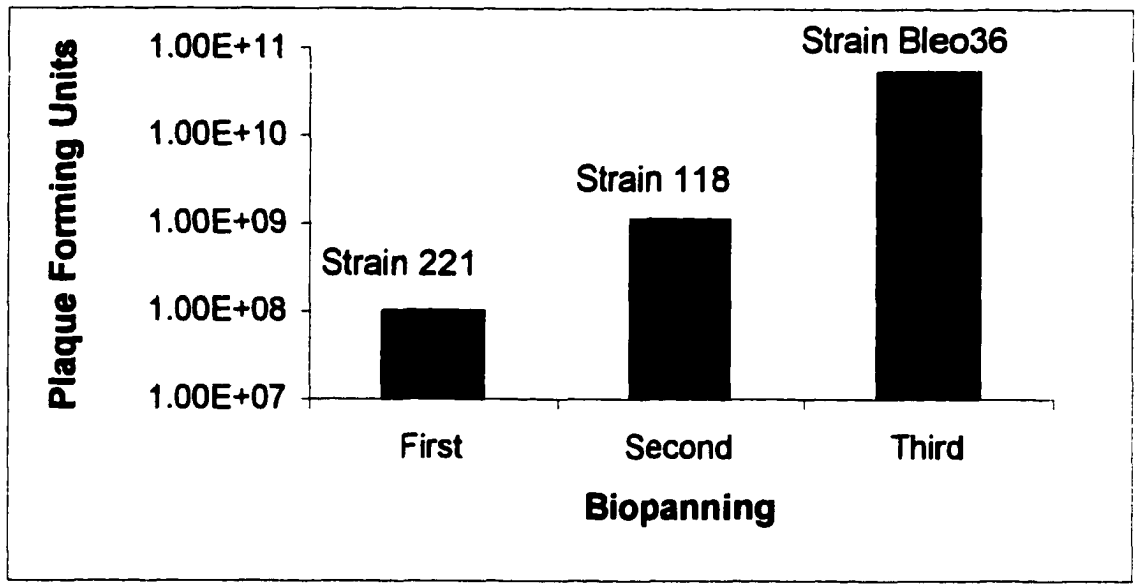


Figure 4. Selective enrichment of phage that interacts with trypanosome cells of three strains.

One milliliter of $5x10^7$ cells of strain 221 was mixed with 10 µl original phage library ($4x10^{10}$ phage particles). After 30 minutes incubation at room temperature with gentle shaking, unbound and weakly associated phage were washed off by four consecutive washes with 5 ml washing medium. Bound phage were recovered by addition of 1 ml 0.2 M Glycine-HCl (pH 2.2) with five minutes incubation followed by neutralization with 150 µl 1M Tris-HCl (pH 9.1). A second and third round of biopanning with strains 118 and Bleo36 were conducted by using the proceeding amplified phage with about $2x10^{11}$ pfu. Pfu of recovered phage were obtained from titering phage.

Table 2. Peptide sequences and their relative binding efficiencies with trypanosome cells.

Clone	Sequence												Relative binding*		
													Bleo36	221	118
12T-605	Asp	Ala	Arg	Ser	Ser	Leu	Ala	Ile	Ser	Leu	Pro	Thr	+	+	+
12T-606	His	Thr	Gly	Leu	Ile	Ser	Ile	Ser	Leu	Leu	Arg	Ser	++	+	-
12T-607	Thr	Leu	Pro	Ser	Pro	Leu	Ala	Leu	Leu	Thr	Val	His	++	++	-
12T-608	Leu	Pro	Tyr	Thr	Ala	Thr	Leu	Ser	Leu	Ser	Ile	Lys	++	+	+
12T-609	Ser	Leu	Trp	Ser	Leu	Pro	Ala	Ser	Pro	Leu	Ser	Lys	-	-	-
12T-610	Trp	Pro	Leu	Gln	Phe	Gly	Pro	Leu	Tyr	Leu	Thr	Pro	+	+	+
12T-612	Val	Leu	Pro	Asn	Ile	Tyr	Met	Thr	Leu	Ser	Ala	Gly	+++	++	++
12T-614	Pro	Ala	Ser	Leu	Phe	Pro	Leu	Ile	Pro	Leu	Arg	Thr	+++	+	++
12T-615	Ser	Thr	Trp	Pro	Ala	Phe	Arg	Leu	Phe	Thr	Asn	Ile	+++	+	++
12T-616	Val	Leu	Pro	Asn	Ile	Tyr	Met	Thr	Leu	Ser	Ala	Gly	+++	++	++
12T-301	Ala	Asn	Tyr	Phe	Leu	Pro	Pro	Val	Leu	Ser	Ser	Ser	++	+	+
12T-304	Val	Met	Gln	His	Gln	Val	Ile	Leu	Glu	Pro	Leu	Tyr	+	-	-
12T-307	Phe	Thr	Leu	Pro	Leu	Tyr	Ala	Thr	Thr	Arg	Leu	Met	++++	+++	+++
12T-308	Gly	Pro	Ser	Ile	Ile	Pro	Gly	Tyr	Leu	Ser	Ile	Thr	++	-	+
12T-309	His	Ser	Leu	Gly	Leu	Ile	Asp	Val	Ser	Ile	His	Arg	++	+	++
12T-311	Val	Pro	Gln	Ala	Pro	Leu	Pro	Val	Arg	Tyr	Pro	Met	-	-	+
12T-313	His	Ser	Ala	Leu	Ser	Asp	Gly	His	Tyr	Ala	Ser	Phe	-	+	+
12T-315	Leu	Pro	Ser	Lys	His	Gln	Asp	Leu	Leu	Thr	Pro	Lys	-	-	-

*: Extent of binding to *T. brucei brucei* as measured by ELISA assays. See Materials and Methods for the experimental procedure.

Leu (Ile or Val)-Ser (Thr or Tyr)-Ile (Leu, Ala, or Val). A hydrophilic amino acid with a hydroxyl group is flanked with two very strong hydrophobic amino acids with short side chains. All clones with this motif showed binding affinity with trypanosomes. Clones such as 609, 304, 311, and 315 without this motif had very weak binding signals. Clones 612 and 616, which show a strong binding activity, carry the same sequence. Although clones 615, 301 contained no clear motif, their ability to bind cells is still strong, suggesting the binding sites for these two phage with cells may be different from others listed in Table 2.

Competitive Assay of Peptide 307 with *T. brucei brucei*

Phage 307 was selected for further investigation due to its highest binding signal from ELISA assay. The peptide sequence expressed by clone 307 (called peptide 307) Phe-Thr-Leu-Pro-Leu-Tyr-Ala-Thr-Thr-Arg-Leu-Met was synthesized using standard chemical peptide synthesis. Trypanosome cells were mixed with excess phage 307, followed by addition of varied concentrations of peptide 307. After 30 minutes incubation at room temperature, the mixture was then washed to remove unbound phage. The trypanosomes were transferred to a new test tube and treated with mild acid. Bound phage were titered on Xgal plates. The number of plaque forming units (pfu) from titering was used to assess the binding affinity of the peptide with trypanosome cells. Figure 5 shows that peptide 307 inhibited the binding of the phage 307 with an IC_{50} of about 1μM. The same binding pattern, obtained from both strain 118 and 221, suggests that this high affinity peptide binds to the invariant epitopes on the surface of the trypanosome cells.

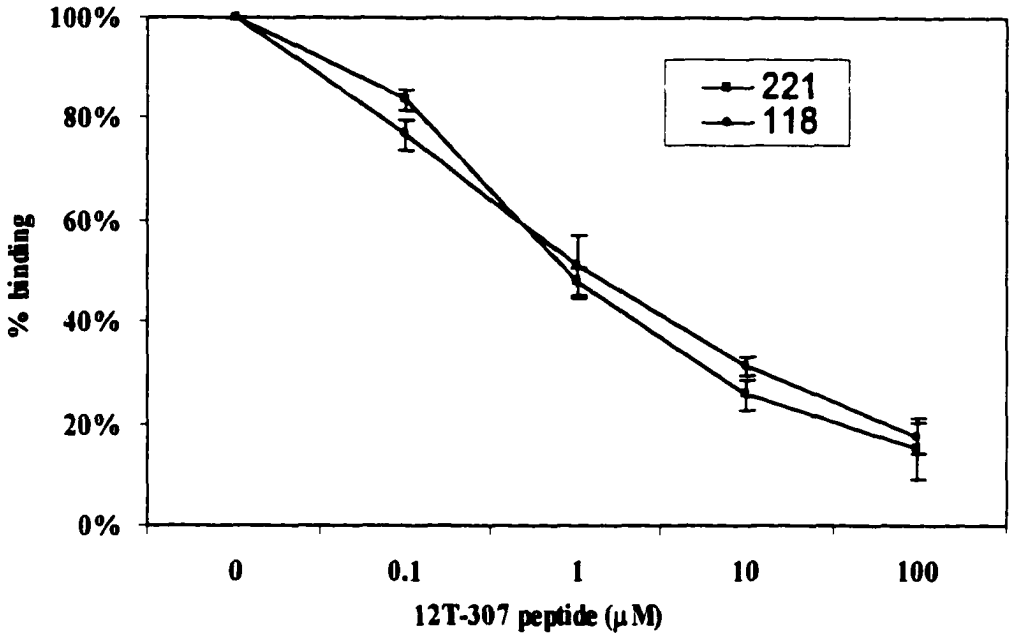


Figure 5. Effect of peptide 307 on the interaction of trypanosome cells with clone 307.

Trypanosome cells ($5x10^6$ – $1x10^7$, Strain 118 or 221) were mixed with $1x10^{14}$ phage clone 307 at varied concentrations of peptide 307. After 30 minutes incubation, at room temperature, the mixture was washed with washing medium 4 times. The cells were then transferred to a new tube and treated with 0.2M glycine-HCl buffer (pH 2.2) for 5 minutes. After neutralization with 1M Tris-HCl buffer (pH9.1), the mixture was titered and the phage recovery was recorded. All values represent the mean of duplicates (error bars indicate ranges of measurements).

Lytic Activity of Peptide 608

The common sequence from phage 612 and 616 was synthesized and it was found that its solubility in water was very low. Further investigation of this peptide could not be done even though it contains the Leu-Ser-Ala motif and the number of its occurrence from phage selection was two, higher than that of all other sequences. The sequence from phage 608 (called peptide 608) with the Leu-Ser-Ile motif was then selected for synthesis using standard Fmoc peptide chemistry. After HPLC purification, the peptide structure was confirmed by mass spectrometry (Figure 6). Incubation of 100μM of peptide 608 with cells dramatically lowered the turbidity of the cell suspension (Figure 7), suggesting that cell lysis took place. With different concentrations of the peptide mixed with cells in the growth medium at 37°C for two hours, the number of motile trypanosomes was assessed with the use of a hemacytometer. The percentage of cell lysis depended on the concentration of peptide 608 (Figure 8). All three strains (221, 118 and Bleo36) were totally lysed in 100μM of peptide 608. The IC_{50} of the lytic activity of peptide 608 is approximately 10μM.

To investigate the mechanism involved in the lytic activity of peptide 608, a lectin was used to compete with the peptide in the binding of trypanosome cells. Several lectins have been reported to bind exclusively to the flagellar pocket of trypanosome cells through protein–carbohydrate interactions (70). Wheat germ agglutinin (WGA) displays a specificity for N,N' – diacetylchitobiose. It specifically binds to carbohydrates at the flagellar pocket (70). Another group has reported that tumor necrosis factor-α (TNF) displays trypanolytic activity and the activity can be inhibited by preincubation of

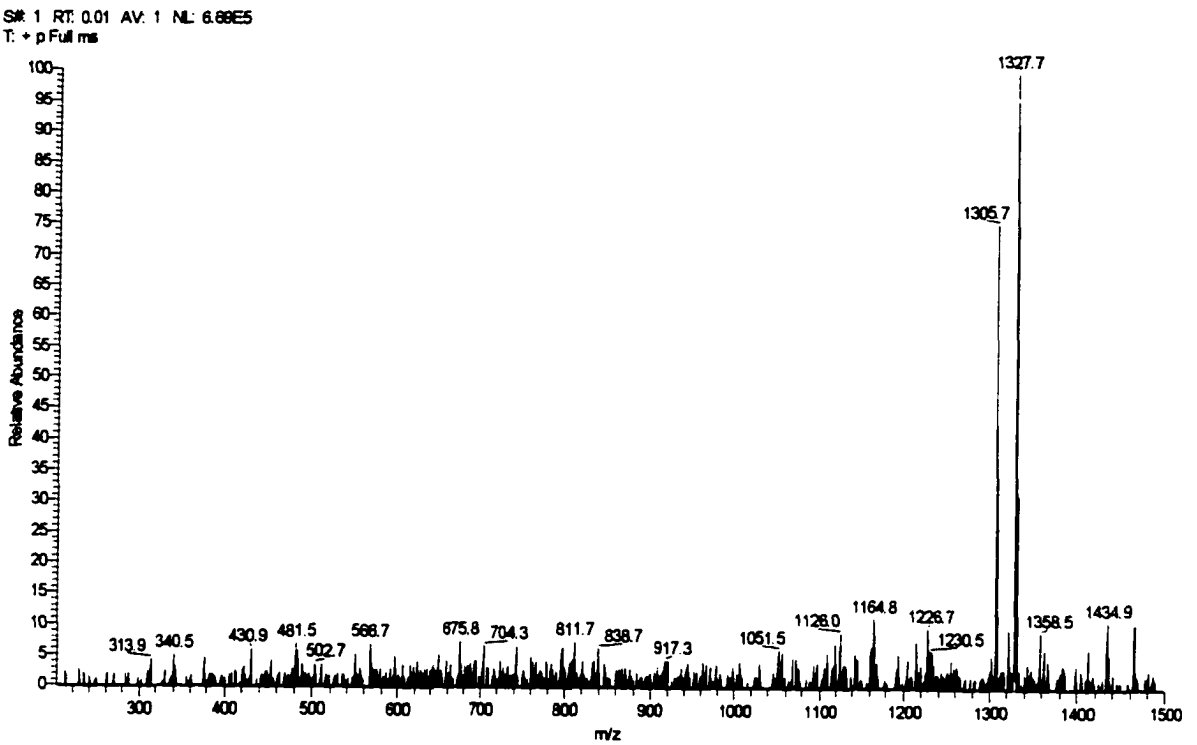


Figure 6. ESI-MS spectrum of peptide 608.

See Materials and Methods for the experimental procedure.

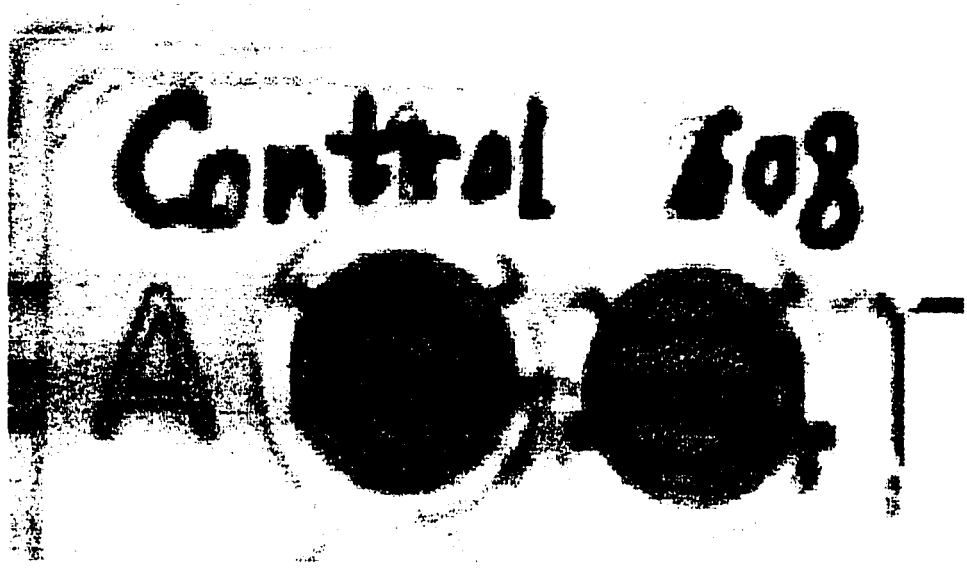


Figure 7. Turbidity change of the trypanosome cell suspension upon addition of 100 μM peptide 608.

Cells of strain 118 ($5x10^6$) were mixed with 100 μM peptide 608. Pictures were taken after incubation of 30 minutes at room temperature.

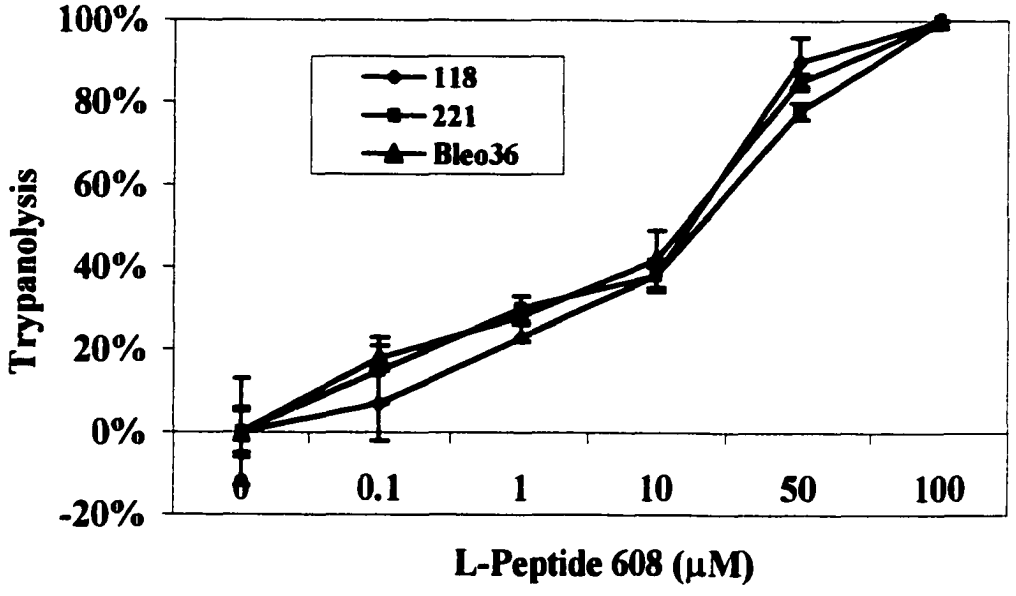


Figure 8. Trypanolytic activity of the L type peptide 608 to strains 118, 221 and Bleo36.

Trypanosome cells ($1x10^6$ –$1x10^7$) washed twice with growth medium were mixed with varied concentration of the peptide 608 at 37°C for 2 hours. Cells were counted by using hemacytometer. All values represent the means of triplicates (error bars indicate ± SD).

trypanosome cells with WGA due to the lectin-like activity of TNF (36). Preincubation of 100μg/ml WGA with trypanosome cells inhibited the lytic activity of peptide 608 (Figure 9). This inhibition can be overcome with excess peptide 608 (50μM and 100μM). Incubation of trypanosome cells with WGA alone had no effect on cell population.

This experiment clearly indicates that peptide 608 acts on the trypanosomal flagellar pocket, an invagination of the plasma membrane arround the base of the flagellum which function as an exo- and endocytosis site. From the flagellar pocket, where the membrane is not coated with the layer of VSG, the peptide can specifically bind with the membrane and result in membrane disruption. This result is consistent with the selection strategy by which the phage selected only binds with the invariant epitopes on the surface of the trypanosome cells, most of which are located in the flagellar pocket.

A very well known trypanosome lytic factor from human serum, which contributes to the human resistance to *T. brucei brucei* infections, has been characterized as a subset of high-density lipoprotein now called trypanosome lytic factor 1 (TLF1). The mechanism of cell lysis mediated by the TLF1 is not clear. A multistep process of this trypanolysis was hypothesized on the basis of observations that the lysis *of T. brucei brucei* only commenced after a lag of 20-30 minutes and TLF-mediated lysis is temperature sensitive (34). To investigate if the multistep process was involved in the lytic activity of peptide 608, a time course study of 100 μM peptide mixed with cells was carried out (Figure 10). There was no time lag found during cell lysis. About 50% of the cells were lysed after two minutes incubation of the peptide and cells. At 20 minutes, no living cells could be observed under the microscope, suggesting that the peptide 608 directly attacked the cell membrane and no multistep process is involved in the lysis.

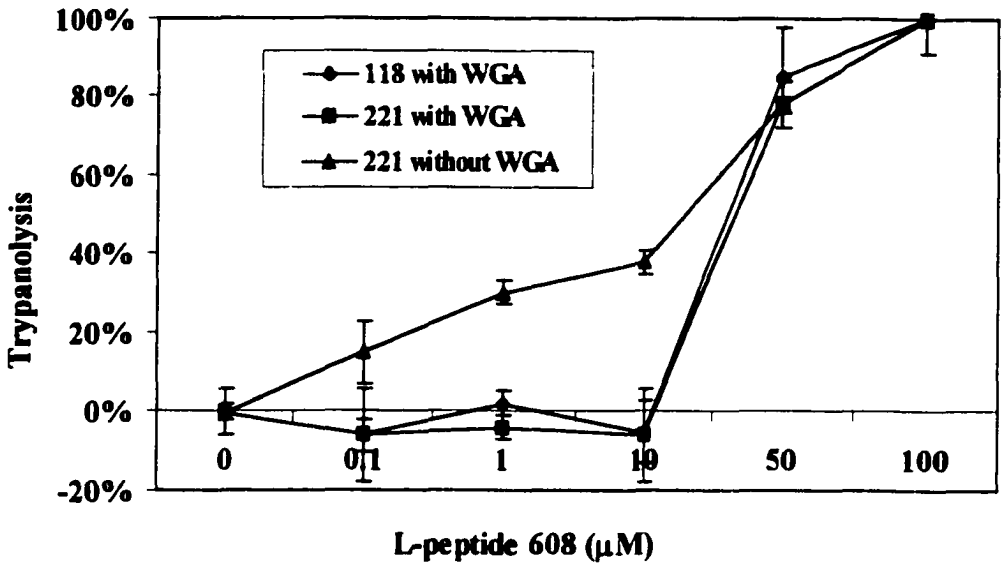


Figure 9. Effect of 100 μg/ml wheat germ agglutinin (WGA) on binding of the L-peptide 608 to strains 221 and 118.

Trypanosome cells ($1x10^6$ – $1x10^7$) were preincubated with 100μg/ml WGA at 4°C for 1 hour. After addition of different concentration of the peptide 608, cells were incubated at 37°C for 2 hours. Cells were counted by using hemacytometer. All values represent the means of triplicates (error bars indicate ± SD).

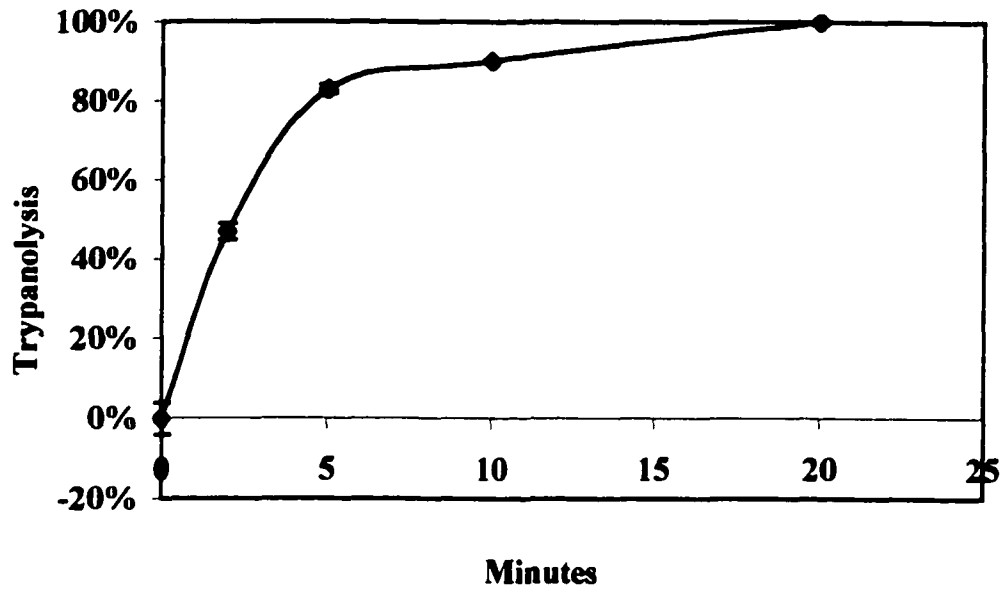


Figure 10. Time course of trypanolysis by L-peptide 608.

Trypanosome cells ($5x10^6$) were mixed with 100 μM peptide 608 at room temperature. Cells were accounted at different time interval. All values represent the means of triplicates (error bars indicate ± SD).

At 0°C, cell lysis by the peptide 608 was not blocked, providing another evidence of direct attack of the peptide to cells.

Trypanolysis with the Reversed Sequence of L-peptide 608

To study the structure-activity relationship, the reversed sequence of peptide 608 (called R-608, NH_2-Lys-Ile-Ser-Leu-Ser-Leu-Thr-Ala-Thr-Tyr-Pro-Leu-COOH) was synthesized and its trypanolytic activity with strain 118 was determined. R-608 shows the same lytic activity as peptide 608 (Figure 11).

Trypanolysis with the D-608 peptide

To further examine the lytic mechanism of peptide 608, and to determine if chiral molecules such as proteins or saccharides at the flagellar pocket or non-chiral ones like lipids in membranes are involved in the lytic process, D-type peptide 608-mediated lysis was tested. The D-608 peptide showed the same lytic activity as the L-608 peptide (Figure 12), indicting that chiral proteins, receptors, or saccharides on the cell surface are not likely to be involved in the interaction between the peptide and cells. The equal activities of D- and L-peptides indicate that they bind to a non-chiral target. The only known non-chiral target in the membrane is the interior of lipid bilayer. Direct disruption of lipid layers of the membrane by 608 peptide is likely to be the cause of cell lysis. 100 μg/ml WGA showed the same inhibition on cell lysis by D-608 as it showed when using L-608 (Figure 13). Binding of the flagellar pocket by WGA blocked cell lysis by D-608.

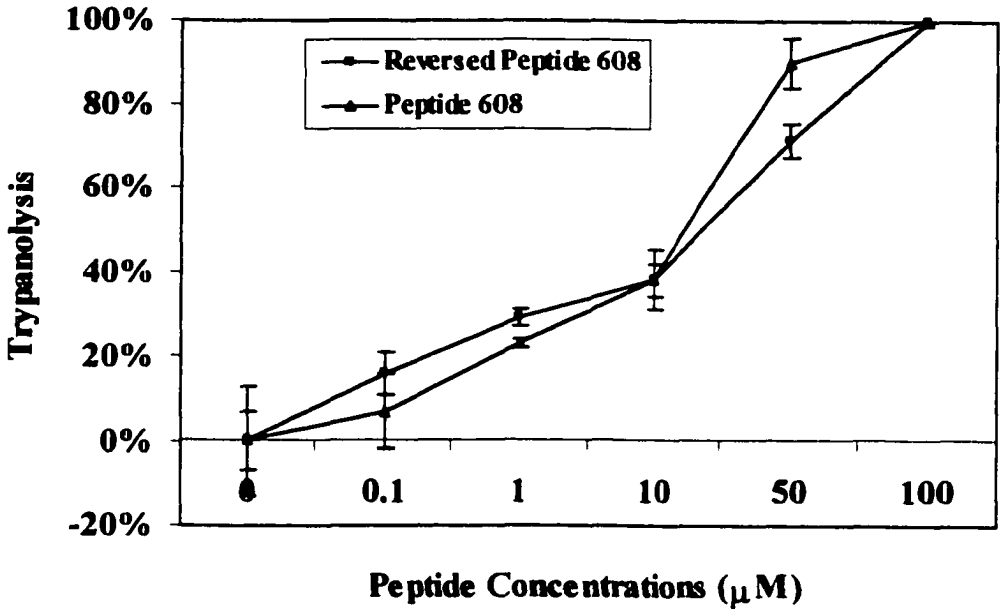


Figure 11. Trypanolytic activity of the reversed sequence of peptide 608 to strain 118.

Trypanosome cells ($1x10^6$ –$1x10^7$) washed twice with growth medium were mixed with varied concentration of the reversed sequence of peptide 608 at 37°C for 2 hours. Cells were counted by using hemacytometer. All values represent the means of triplicates (error bars indicate ± SD).

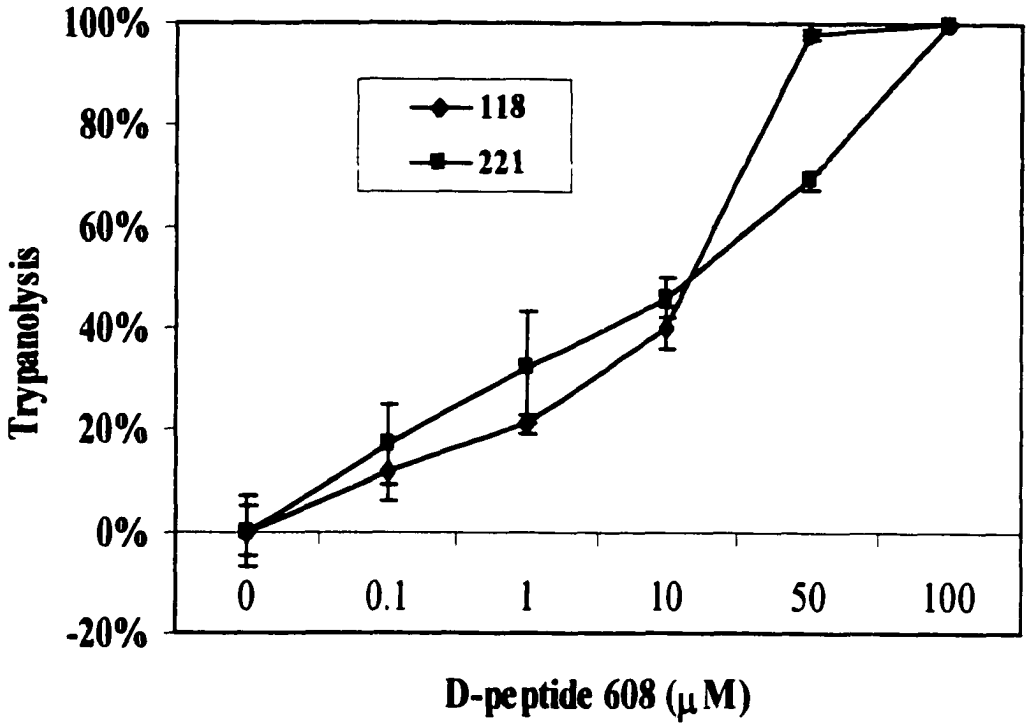


Figure 12. Trypanolytic activity of the D type peptide 608 to strains 118 and 221.

Trypanosome cells ($1x10^6 - 1x10^7$) washed twice with growth medium were mixed with varied concentrations of the peptide 608 at 37°C for 2 hours. Cells were counted by using hemacytometer. All values represent the means of triplicates (error bars indicate ± SD).

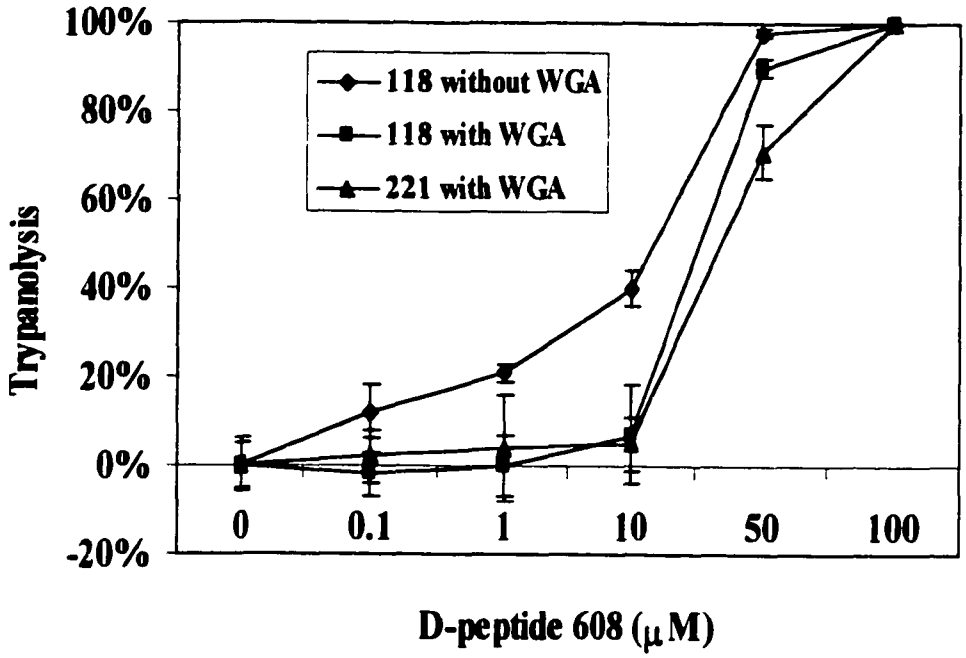


Figure 13. Effect of 100 μg/ml wheat germ agglutinin (WGA) upon binding of the D-Peptide 608 to strains 118 and 221.

Trypanosome cells ($1x10^6$ – to $1x10^7$) were preincubated with 100μg/ml WGA at 4°C for 1 hour. After addition of different concentrations of the peptide 608, cells were incubated at 37°C for 2 hours. Cells were counted by using hemacytometer. All values represent the means of triplicates (error bars indicate ± SD).

Mechanism of Lysis by Peptide 608 as Compared with Other Lytic Agents

Naturally occurring antimicrobial peptides are produced by plants and animals to resist infection by pathogenic fungi and bacterial (72, 73). The composition and structure of naturally occurring peptides have been extensively studied and their modes of action have been examined through the use of synthetic peptides (74, 75, 76). One class of these peptides are cationic and amphipathic with an α-helix structure (73). Magainin-2 is one of such peptide. It is a 23-amino acid peptide isolated from the skin of the clawed African toad *Xenopus laevis* (77). The peptide displays a wide range of potent anti-bacterial activity (77, 78). It can also inhibit the growth of fungi and viruses and induce osmotic lysis in protozoa (77, 78). The mode of magainin-induced membrane permeabilization has been proposed by Matsuzaki (79). Magainin forms an amphipathic helix in lipid bilayers, which essentially lies parallel to the membrane surface. Five helices on average, together with several surrounding lipids, form a membrane-spanning pore comprising a dynamic, peptide-lipid supramolecular complex, which allows not only ion transport but also a rapid flip-flop of the membrane lipids. Upon disintegration of the pore, a fraction of the peptide molecules stochastically translocates into the inner leaflet.

It has been known that one protozoa, *Paramecium caudatum*, can be lysed by magainin-2 (77, 78). There are no prior reports about the trypanolytic activity of magainin-2. The trypanolytic activity of magainin-2 was determined here. Magainin-2 shows a dose-dependent anti-trypanosome activity as peptide 608 does (Figure 14). However, in the presence of 100 μM of magainin-2, many dead cells were observed under the microscope. Dead cells with complete cell membranes can explain a possible

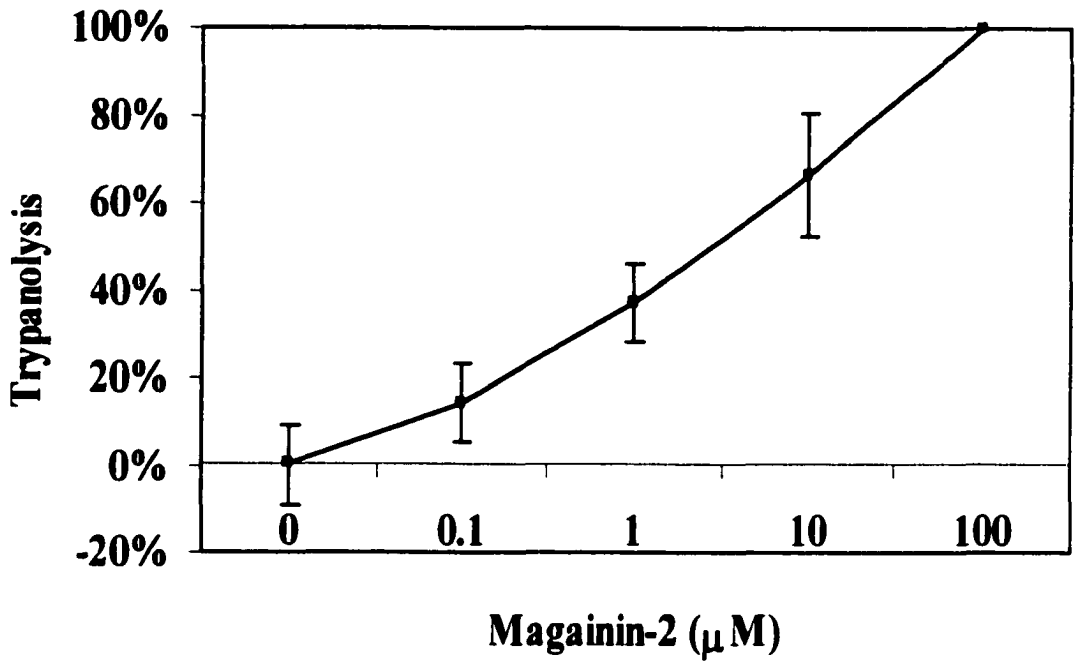


Figure 14. Trypanolytic activity of magainin-2 to strain 118.

About $2x10^7$ trypanosome cells washed twice with growth medium were mixed with varied concentrations of magainin at 37°C for 2 hours. Cells were counted by using hemacytometer. All values represent the means of triplicates (error bars indicate ± SD).

lytic mechanism that magainin-2 forms a pore structure in cell membranes and results in a leakage of cell contents. At the same concentration of peptide 608, very few dead cells were present, which suggests that cell membranes are disrupted directly by peptide 608. This observation suggests that lytic modes of peptide 608 and magainin-2 may be different.

Effect of Peptide 307 on the Trypanolytic Activity of D-608

Peptide 307 (50μM) was first incubated with trypanosome cells at 0°C for one hour. Different concentrations of peptide D-608 were added and cells were incubated at 37°C for two hours. Cells were then counted. Figure 15 shows that 50 μM peptide 307 protects cells from lysis by peptide 608. At 50 μM peptide 608, in the presence of peptide 307, about 60% of cells were lysed. The percentage of cell lysis was 98% in the absence of peptide 307. The inhibition of cell lysis by peptide 307 could be overcome by adding 100 μM of peptide 608. Combined with the results from WGA inhibition experiments, peptide 307 may bind to the flagellar pocket of trypanosomes, which is consistent with the selection strategy.

Secondary Structure of Peptide 608

The mechanism of HDL's trypanolytic activity has been suggested to be due to the presence of amphipathic helices in apolipoprotein A-1 (apoA-I) and apoA-II, which are major apolipoproteins of HDL (33). The helical arrangement of amino acids with opposing polar and nonpolar surface is likely to be responsible for the interaction of these apoA-I and apoA-II apolipoproteins with lipids of trypanosome cells. To further study the

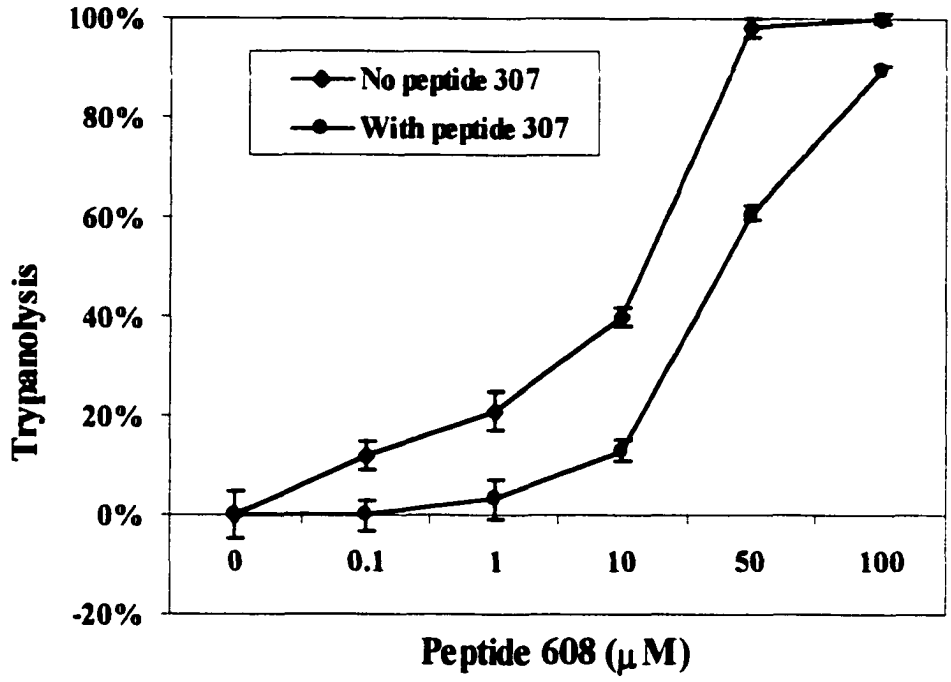


Figure 15. Effect of 50 μM peptide 307 on binding of the D-Peptide 608 to strain 118.

Trypanosome cells ($1x10^6$ – to $1x10^7$) were preincubated with 100μg/ml WGA at 4°C for 1 hour. After addition of different concentrations of the peptide 608, cells were incubated at 37°C for 2 hours. Cells were counted by using hemacytometer. All values represent the means of triplicates (error bars indicate ± SD).

mechanism of the lytic activity of peptide 608, the secondary structure of the D-type peptide was investigated. The circular dichroism (CD) spectrum of peptide 608 was measured in 0.01M sodium phosphate buffer (pH 7.0). The concentration of peptide 608 was 0.6 mg/ml. The spectrum of the peptide showed the typical appearance of α-helix rich structure, with negative elipticities at 200 and 220 nm (Figure 16). An estimate of percentage of α-helicity was obtained from CDNN program (Version 2.1) (Table 3). This program predicted 63% α-helices present in the secondary structure.

Hemolytic Activity of Peptide 608

The hemolytic activity of peptide 608 was determined by using human red blood cells. The peptide showed almost no hemolytic activity at 10 μM (Figure 17), at which about 50% of the trypanosomes were lysed. At a peptide concentration with 100% lytic activity (100 μM), the percentage of hemolytic activity was 17%. This result, combined with results from the lytic activity inhibited by WGA, strongly suggests that there is a specific interaction between peptide 608 and trypanosome cells This specificity suggests that it may be possible to develop such peptides as therapeutic agents against trypanosome infections.

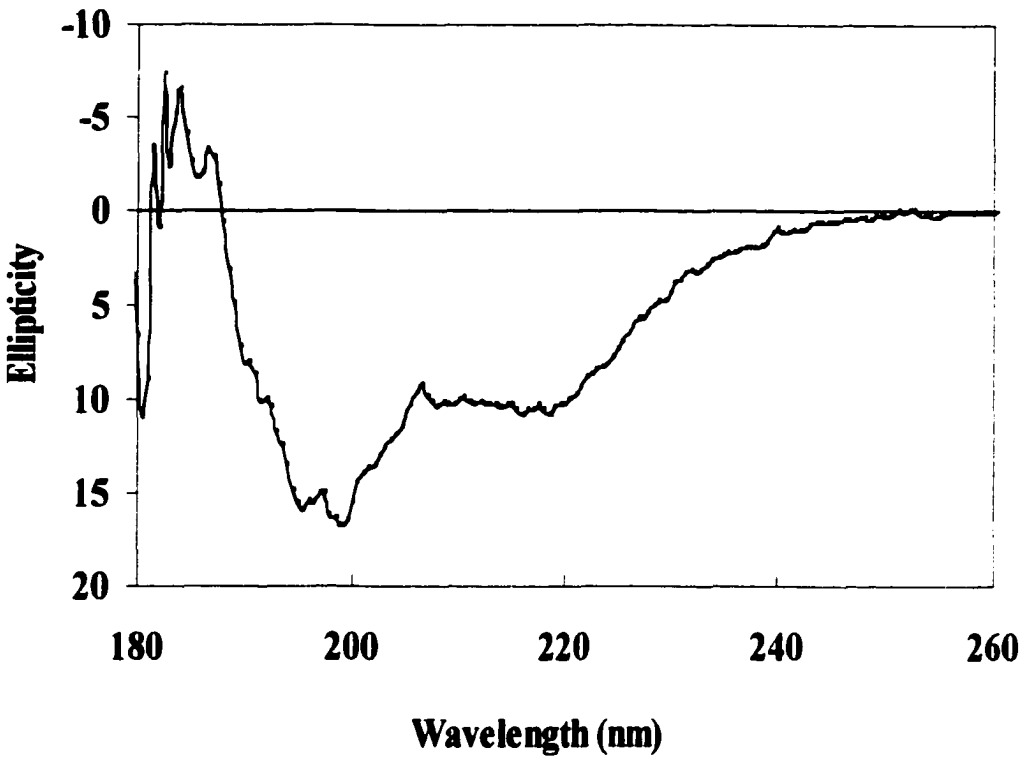


Figure 16. Circular dichroism spectrum of the D-Peptide 608.

See Materials and Methods for the experimental procedure.

Table 3. Predicted Secondary Structure Elements of the D-Peptide 608.

Type	Percentage
α-Helix	62.6
Antiparallel	3.7
Parallel	3.7
β-Turn	12.9
Random coil	17.1

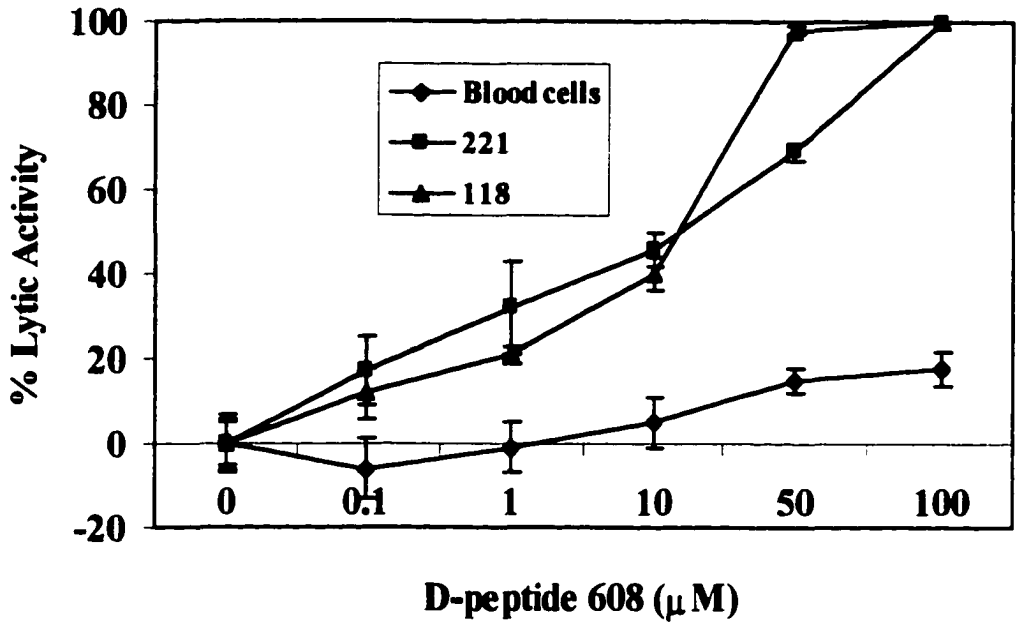


Figure 17. Lytic activity of the D-Peptide 608.

Red blood cells from human were incubated with different concentration of the D-608 at 37°C for 2 hours. Cells were counted by using hemacytometer. All values represent the means of triplicates (error bars indicate ± SD).

DISCUSSION

A peptide with strong binding affinity to trypanosomes and one with trypanolytic activity have been identified from a 12-mer phage display peptide library. The strategy of this selection was based on one important feature of trypanosome cells whose surface are coated with a 10 – 15 nm thick layer of VSGs (3). By screening 3 strains of trypanosomes, which carry their own specific VSGs, the strategy favored the selection of phage sequences that only bind to invariant, common epitopes on the surface of trypanosome cells. Eighteen clones were selected and sequenced (Table 2). Eleven out of eighteen clones (60%) showed a Leu (Ile or Val)-Ser (Thr or Tyr)-Ile (Leu, Ala, or Val) motif. All these peptides showed high affinity to trypanosome cells based on ELISA assays. Some of these clones without this motif (609, 304, 311, and 315) were weak binders. Other clones without this motif, like 615 and 301, showed high binding signals. The target sites are not clear. However, according to the selection strategy, all clones showing strong binding signals to all three strains of trypanosomes should target the universal structures of cells, which are located in the flagellar pocket.

Another group has used a similar strategy to search for nucleic acid ligands (aptamers) that bind to trypanosome cells (67). Three classes of RNA aptamers were shown to crosslink to a single 42 kDa protein located within the flagellar pocket. One RNA ligand showed a dissociation constant of about 60 nM. In the work described here, peptide 307 selected from the peptide library in this study showed the highest binding signal from ELISA assay. The dissociation constant of peptide 307 to trypanosomes derived from the competitive assay was approximately 1 μM (Figure 5). Since the

selection of single clones after the 3rd biopanning was random, it is possible that sequences with higher binding affinities than peptide 307 can be found if a larger population of phage is analyzed. Also, the library used in this study is not a complete representation of all dodecapeptide sequences ($2.7x10^9$ vs $4.1x10^{15}$).

Peptide 608 showed lytic activity to all three strains of trypanosomes with an IC_{50} of 10 μM (Figure 8). Peptide 608 has the Leu-Ser-Ile motif and also shows moderate affinity with trypanosome cells (Table 2). The WGA competitive experiments (Figures 9 and 13) suggested that the peptide must interact with cells through the flagellar pocket, which is a common structure of all three strains and functions as an exo- and endocytosis site. WGA is a lectin and it has been shown that WGA exclusively binds at the flagellar pocket (71). WGA blocked the interaction between peptide 608 and the flagellar pocket and resulted in the disappearance of lytic activity of the peptide at the low concentration. At high concentrations such as 50 and 100 μM, the peptide was able to competitively overcome the effect of WGA on cell lysis (Figures 9 and 13)

Both peptide 307 and WGA protect trypanosome cells from lysis by peptide 608 (Figures 9, 13, and 15). This suggests that peptide 307 also binds at the flagellar pocket of trypanosomes. This experiment provides additional evidence to support the strategy of phage selection. Since both peptide 608 and 307 contain the Leu-Ser (Tyr)-Ile (Ala) motif, it is not surprising that these two peptides seem to compete for a common binding location.

Both tumor necrosis factor (TNF), which is a host cytokine and can be induced by the parasite itself, and synthetic peptide sequences derived from it, were reported to have trypanolytic activity (36, 37). There is no structural similarity between peptide 608 and

TNF-derived sequences, suggesting that different mechanisms may be involved. However, WGA can block cell lysis in both cases. The lectin-like activity of TNF was proposed to play the role in its trypanolytic activity (36). Both peptide 608 and TNF need to bind to the flagellar pocket before they exert lytic activity with trypanosomes.

The exact mechanism underlying peptide 608-mediated trypanolysis is not known. Several observations provided clues to explain the process of cell lysis by this peptide. The time course study (Figure 10) showed that cell lysis started immediately after the addition of the peptide to cells. This suggests that a multiple intracellular process is not involved. This can also be supported by the fact that cell lysis by peptide 608 was not temperature sensitive. For two other reported trypanolytic factors, TLF1 and TNF-mediated cell lysis is strongly temperature dependent (34, 37). TNF can be adsorbed at 30°C as well as at 21°C and 17°C and its lytic activity requires a temperature > 25°C. Lysis of trypanosomes by TLF1 is completely inhibited at 17°C or below. If cells are incubated with TLF1 at 17°C, then washed extensively to remove TLF1 from the medium and subsequently shifted to 37°C, lysis still occurs (34). These observations strongly suggest that binding and endocytosis of TLF1 and TNF are necessary but not sufficient to induce trypanolysis. So far, no receptors for TNF and TLF1 have been reported. In contrast, peptide 608 does not need a multiple process to exert its lytic activity.

To investigate the interaction of peptide 608 with trypanosomes, D-type peptide 608 was synthesized. The trypanolytic activity of D-608 was very similar to that of L-type (Figure 12), strongly suggesting that the target of this peptide on the trypanosomes is non-chiral. The only possible non-chiral molecule is the lipid layer of cell membranes. WGA also inhibited the trypanolytic activity of D-608 (Figure 13) as it did in L-608. This

indicates that D-608 may exert it trypanolytic activity through the site of flagellar pocket. This experiment also excludes the possibility that the interaction between WGA and the peptide itself is the cause of WGA's effects on peptide 608-mediated lysis, since WGA is chiral and it blocks the trypanolytic activities of both D- and L- peptides.

There is no direct evidence to support that trypanolytic activity of peptide 608 is due to channel formation in the lipid layer of membrane. However, the presence of α-helices (63%) (Table 3) in peptide 608 suggests possible pore formation in the membrane by peptide 608. The thickness of the hydrophobic part of the lipid bilayer (approximately 3 nm) requires that the channel be at least 3 nm long and 20 amino acids are required (80). Peptide 608 is too small to allow the membrane to be spanned with one molecule. However, studies show that short, cationic peptides (8 – 12 peptides in length) can form ion channels (81). It was postulated that oligomeric bundles of head-to-tail dimers of short peptides as a potential explanation for the bioactivity of those peptides. It is possible that peptide 608 forms the channels with oligomeric bundles of head-to-tail dimers of α-helices. This kind of structure requires many peptide molecules (for example 12 molecules for a hexameric bundle), which would explain why a high concentration of peptide 608 is needed to lyse trypanosome cells.

Pore formation of TNF has been proposed as a possible mechanism involved in TNF-mediated trypanolysis (37). The sequence Cys-^{1}Thr-^{2}Pro-^{3}Glu-^{4}Gly-^{5}Ala-^{6}Glu-Cys derived from TNF also showed high trypanolytic activity (36), but unlike peptide 608, it lacks secondary structure. Since there is no way for this short peptide to form a channel in the absence of secondary structure like an α-helix, the lytic mechanism of TNF and its derived peptide is different. An all-D-form of this peptide was prepared and its lytic

activity was examined. *The D-TNF peptide has no activity at all* (data not shown). Thus, it is clear that peptide 608 and the TNF peptide differ in their mode of action.

The L-sequence-reverse isomer of peptide 608 was also prepared. Like peptide 608, this peptide is also trypanolytic (Figure 11). The sequences of peptide 608 and its reverse sequence are listed as follows:

Sequence of Peptide 608: L P Y T A T L S L S I K

Reverse Sequence of Peptide 608: K I S L S L T S T Y L P

Comparing these two sequences, it is found that the motif Leu (or Ile)-Ser-Leu (or Ile) in peptide 608 is still present in the reverse sequence. In the reverse sequence, the nature of secondary structures of the α-helix should not change. Since the key motif and the α-helices are still present, it is not surprising that both peptides share a lytic activity. Unlike peptide 608, the trypanolytic activity of the TNF-derived sequence is very sensitive to amino acid substitutions (36). The amino acid at each position of Cys-^{1}Thr-^{2}Pro-^{3}Glu-^{4}Gly-^{5}Ala-^{6}Glu-Cys sequence has been substituted by Ala. A replacement of ^{1}Thr, ^{3}Glu, or ^{6}Glu by an Ala residue resulted in a total loss of trypanolytic activity, while the replacement of ^{2}Pro or ^{4}Gly by Ala did not influence the activity of this peptide (36). This again indicates that the nature of trypanolysis of these two peptides is different.

During the development of new anti-trypanosome peptides, it is important to determine if a peptide possesses hemolytic activity. Peptide 307 showed almost no hemolytic activity at the concentration of 10 μM, at which about 50% of trypanosome cells were lysed (Figure 17). In a study of evaluation of amphipathic peptides with antimicrobial activity and hemolytic activity, Blondelle and Houghten (82) found that hemolytic activity was more sequence-dependent than lysis of bacterial cells and

suggested that two different mechanisms of action were involved in lysis of bacterial and red blood cells. Since the presence of the consensus motif is important to bind trypanosome cells, it may be true that the mechanisms involved in lysis of trypanosomes and red blood cells are different. This can explain the low hemolytic activity of peptide 608 at 100 μM, when all trypanosome cells were lysed.

CONCLUSIONS

This is the first time that phage display technology has been applied to search for peptides with high affinity with African trypanosomes or anti-trypanosome activity. Three strains of *Trypanosoma brucei brcei*, each with different variant surface glycoproteins, have been used to screen a phage display peptide library. This library contains about $2.7x10^9$ 12-mer peptide sequences. The selection process was set up to favor only those members of the library that bind to invariant, common epitopes. Sixty percent of selected clones share a consensus sequence Leu (Ile or Val)-Ser (Thr or Tyr)-Ile (Leu, Ala, or Val). All of these clones show high affinity with trypanosome cells based on ELISA assays. The peptide expressed by clone 307, which has the strongest binding signal, has been synthesized and its binding constant was determined. The IC_{50} of peptide 307 with trypanosome cells is 1 μM on the basis of competitive assays. Peptide 608 from clone 608 shows trypanolytic activity, which can be inhibited by WGA, a lectin that only binds to flagellar pockets of trpanosomes. Peptide 307 can also protect trypanosome cells to be lysed by peptide 608. Both L- and D- peptide 608 have a similar lytic activity. This observation suggests that peptide 608 attacks a non-chiral target such as the lipid layers of trypanosome membranes, thereby resulting in cell lysis. Cell lysis by peptide 608 takes place without a time lag and is temperature independent, suggesting that there are no multiple processes involved in trypanolysis. The potential pore-forming capacity of peptide 608 is evident by the presence of 63% α-helix interpreted from the CD spectrum of the peptide. This may account for trypanosome lysis. The low hemolytic

activity of peptide 608 suggests that this method has the potential to be used to develop anti-trypanosome agents.

APPENDIX I

HMI-9 medium for bloodstream form trypanosomes (68)

Ingredient	**Amount/Liter**
Iscove's Modified Dulbecco's Medium	17.67 g
Sodium bicarbonate	3.024 g
L-cysteine	236 mg
Thymidine	39 mg
Sodium Pyruvate	110 mg
Hypoxanthine	136 mg
Bathocuproine Disulfonic Acid	28 mg
2-Mercaptoethanol	14 µl
10% Fetal Bovine Serum (heat activated)	100 ml
10% Serum Plus	100 ml
Penicillin (60 mg/ml)	1 ml

Dissolve above ingredients in 800 ml deionized water and bring the volume to 1 Liter. Rinse media bottles with deionized water and autoclave. Filter sterilize the media. The medium is only known to be stable for 10 days.

APPENDIX II

Minimal Plates (69)

Ingredient	**Amount/Liter**
2X M9 salts*	500 ml
Agar (3%)	500 ml
Glucose (20%)	20 ml
$MgSO_4$ (1 M)	2 ml
$CaCl_2$ (1 M)	0.1 ml
Thiamine (10 mg/ml)	1 ml

Autoclave all components seperately and cool to < 70°C (filter sterilize glucose and thiamine) before combining. Store plates at 4°C.

*: 2X M9 salts/per liter: 12 g Na_2HPO_4, 6 g KH_2PO_4, 1 g NaCl, 2 g NH_4Cl.

APPENDIX III

LB Medium (69)

Ingredient	**Amount/Liter**
Bacto-Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

Autoclave and store at room temperature.

APPENDIX IV

Agarose Tope (69)

Ingredient	**Amount/Liter**
Bacto-Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
$MgCl_2 \bullet 6H_2O$	1 g
Agarose	7 g

Autoclave and dispense into 50 ml aliquots. Store solid at room temperature and melt in microwave as needed.

APPENDIX V

LB/IPTG/Xgal Plates (69)

Ingredient	**Amount/Liter**
Bacto-Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g

Autoclave and cool to < 70°C. Add 1 ml IPTG/Xgal* mixture and pour. Store plates at 4°C in the dark.

*: IPTG/Xgal mixture: Mix 1.25 g IPTG and 1 g Xgal in 25 ml Dimethyl formamide. Solution can be stored at –20°C in the dark.

APPENDIX VI

Iodide Buffer (69)

10 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 4 M NaI. Store at room temperature in the dark.

REFERENCES

1 Smith, D.H., Pepin, J. and Stich, A.H.R. (1998) *Br. Med. Bull.*, **54**, 341.

2 Van Gompel, A. and Vervoort, T. (1997) *Curr. Opin. Infect. Dis.*, **10**, 469.

3 Vickerman, K. (1969) *J. Cell Sci.* **5**, 163.

4 Borst, P. and Cross, G.A.M. (1982) *Cell*, **29**, 291.

5 Ferguson, M.A., Homans, S.W., Dwek, R.A., and Rademacher, T.W. (1988) *Science*, **239**, 753.

6 Hall, T. and Esser, K. (1984) *J. Immunol.*, **132** 2059.

7 Lamont, G.S., Tucker, R.S., and Cross, G.A.M. (1986) *Parasitology*, **92**, 355.

8 Turner, C.M. and Barry, J.D. (1989) *Parasitology*, **99**, 67.

9 Balber, A.E. (1990) *Crit. Rew. Immunol.* **10**, 177.

10 Overath, P., Y. Stierhof, and M. Wiese (1997) *Trends in Cell Biol.* 7: 27.

11 Webster, P. and Fish, W.R. (1989) *Eur. J. Cell. Biol.* **49**, 303

12 Black, S. and Vandeweerd, V. (1989) *Mol. Biochem. Parasitol.* **37**, 65

13 Schell, D., Borowy, N.K. and Overath, P. (1991) *Parasitol. Res.* 77, 558

14 Coppens, I., Baudhuin, P., Opperdooes, F.R., and Courtoy, P.J.(1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 6753.

15 Coppens, I., Opperdooes, F.R., and Baudhuin, P. (1987) *J. Protozool.* **34**, 465

16 Schell, D., Evers, R., Ziegelbauer, K., Kiefer, H. et al. 1991 *EMBO J.* **10**, 1061

17 Schell, D., Evers, R., Ziegelbauer, K., Kiefer, H. et al. 1993 *EMBO J.* **12**, 2990

18 Steverding, D., Stierhof, Y., Fuchs, H., Tauber, R., and Overath, P. (1995) *J. Cell Biol.* **131**, 1173.

19 Salmon, D., Geuskens, M., Hanocq, F., Hanocq-Quertier, J., Nolan, D., et al. (1994) *Cell* **78**, 75

20 Borst, P. (1991) *Trends Genet.* **7**, 307.

21 Borst. P. and Fairlamb, A.H. (1998) *Annu. Rew. Microbiol.* **52**: 745.

22 Coppens, I., Bastin, P., Courtoy, P.J., Baudhuin, P., and Opperdoes, F.R. (1991) *Biochem. Biophys. Res. Commun.* **178**, 185.

23 Hawking, F. (1973) *Trans. R. Soc. Trop. Med. Hyg.* **67**, 517.

24 Rifkin, M.R. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3450.

25 Hajduk, S.L., Moore, D.R., Vasudevacharya, J., Siqueira, H., Torri, A.F., et al. (1989) *J. Biol. Chem.* **264**, 5210.

26 Smith, A.B., Esko, J.D., and Hajduk, S.L. (1995) *Science* **268**, 284.

27 Bowman, B.H. (1993) in: Hepatic Plasma Proteins: Mechanism of Function and Regulation. Academic Press, Inc., San Diego, CA. p 159.

28 Raper, J., Nussenzweig, V., and Tomlinson, S. (1996) *J. Exp. Med.* **183**, 1023

29 Tomlinson, S., Janson, A.M., Koudinov, J.A., Ghiso, N.H., Chio-Miura, M.R., et al. (1995) *Mol. Biochem. Parasitol.* **70**, 131.

30 Raper, J., Fung, R., Ghiso, J., Nussenzweig, and Tomlinson, S. (1999) *Infect. Immun.* **67**, 1910.

31 Smith, A.B. and Hajduk, S.L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 10262.

32 Rifkin, M.R. (1991) *J. Lipid Res.* **32**, 639.

33 Gillett, M.P.T. and Owen, J.S. (1992) *J. Lipid Res.* **33**, 513.

34 Hagar, K.M., Pierce, M.A., Moore, D.R., Tytler, E.M., Esko, J.D., et al. (1994) *J. Cell Biol.* **126**, 155.

35 Vassalli, P. (1992) *Annu. Rev. Immunol.* 10: 411.

36 Lucas, R., S. Magez, R. De Leys, L. Fransen, J.P. Scheerlinck, et al. (1994) *Science* 263: 814.

37 Magez, S., M. Geuskens, A. Beschin, H. Favero, H. Verschueren, et al. (1997) *J. Cell Biol.* **137**, 715-727.

38 Kagan, B.L., R.L. Baldwin, D. Munoz, and B.J. Wisnieski. (1992). *Science* 255: 1427-1430.

39 Smith, G.P. (1985) *Science* 228: 1315.

40 Cwirla, S.E., E.A. Peters, R.W. Barrett, and W.J. Dower (1990) *Proc. Natl. Acad. Sci. USA* 87: 6378.

41 Makowski, L., M. Russel (1997) In *Structural Biology of Viruses.* Edited by Chiu W, Burnnet RM and Garcia R., New York: Oxford University Press, p352.

42 Eichler, J., A.W. Lucka, C. Pinilla, and R.A. Houghten (1995) *Mol Diversity* 1: 233.

43 Ley, A.C., W. Markland, and R.C. Ladner (1996) *Mol Diversity* 2: 119.

44 Sato, A., N. Ida, M. Fukuyama, K. Miwa, J. Kazami, and H. Nakamura (1996) *Biochemistry* 35: 10441.

45 Koivunen, E., W. Arap, H. Valtanen, A. Rainisolo, O, Penate, and et al. (1999) *Nature Biotechnology* 17: 768.

46 Pasqualini, R. and E. Ruoslahti (1996) *Nature* 380: 364–366

47 Pasqualini, R., E. Koivunen, and E. Ruoslahti (1997) *Nat Biotechnol* 15: 542.

48 Rodenburg, C.M., R. Mernaugh, G. Bilbao, and M. Khazaeli (1998) *Hybridoma* 17: 1.

49 Lowman, H.B., and J.A. Wells (1993) *J Mol Biol* 234: 564.

50 Li, B, J.Y.K. Tom, D. Oare, R. Yen, W.J. Fairbrother, and et al. (1995) *Science* 270: 1657.

51 Ballinger, M.D., J.T. Jones, J.A. Lofgren, W.J. Fairbrother, R.W. Akita, and et al. (1998) *J Biol Chem* 273: 11675.

52 Ruan, B., J. Hoskins, L. Wang, and P.N. Bryan (1998) *Protein Sci* 7: 2345.

53 Kim, D.E., H. Gu, and D. Baker (1998) *Proc Natl Acad Sci USA*, 95: 4982.

54 Proba, K., A. Wörn, A. Honegger, and A. Plückthun (1998) *J Mol Biol* 275: 245.

55 Soumillion, P., L. Jespers, M. Bouchet, J. Marchand-Brynaert, G. Winter, and et al. (1994) *J Mol Biol*, 237: 415.

56 Hansson, L.O., M. Widersten, and B. Mannervik (1997) *Biochemistry* 36: 11252.

57 Fujii, I., S. Fukuyama, Y. Iwabuchi, and R. Tanimura (1998) *Nat Biotechnol* 16: 463–467.

58 Baca, M., T.S. Scanlan, R.C. Stephenson, and J.A. Wells (1997) *Proc Natl Acad Sci USA* 94: 10063.

59 Gao, C.S., C.H. Lin, C.L. Lo, S. Mao, P. Wirsching, and et al. (1997) *Proc Natl Acad Sci USA* 97: 11777.

60 Wright, R.M., H. Gram, A. Vattay, S. Byme, P. Lake, and D. Dottavio (1995) *Biotechnol.* (NY) 13: 165.

61 Burks, E.A., G. Chen, G. Georgiou, and B.L. Iverson (1997)) *Proc Natl Acad Sci USA 94: 412.*

62 Ryan, S.T., G. Chi-Rosso, L.L. Bonnycastle, J.K. Scott, V. Koteliansky, and et. al (1998) *Cell Adhes. Commun.* 5: 75.

63 Stricker, N.L., K.S. Christopherson, B.A. Yi, P.J. Schatz, R.W. Raab, and et. al (1997) *Nat. Biotechnol.* 15: 336.

64 Houghten, R.A., J.R. Appel, S.E. Blondelles, J.H. Cuervo, C.T. Dooley, and et al. (1992) *BioTechniques* 13: 412

65 Blondelle, S.E., E. Takahashi, P.A. Weber, and R.A. Houghten (1994) *AntiMicrob. Agents Chemoth.* 38: 2280.

66 Blondelle, S.E., E. Takahashi, P.A. K.T. Dink, and R.A. Houghten (1995) *J. App. Bacteriol.* 78: 39.

67 Homann, M. and H.U. Goringer (1999) *Nucleic Acid Res.* 27: 2006

68 Hirumi, H. and K. Hirumi (1989) *J. Parasitol.* 75: 985.

69 Ph.D.-12 Phage Display Peptide Library Kit, New England Biolabs, (1997).

70 Rink, H. (1987) *Tetrahedron Lett.* 28: 3787.

71 Brickman, M.J. and A.E. Bailber (1990) *J. Protozool.* 37: 219.

72 Zasloff, M. (1987) *Proc Natl Acad Sci USA* 84: 5449.

73 Hancock, R.E.W., T. Falla, and M. Brown (1980) *Adv. Microb. Physiol.* 37: 136.

74 Bessalle, R., A. Kapitkovsky, A. Gorea, I. Shalit, and M. Fridkin (1990) *FEBS Lett.* 274: 151.

75 Chikindas, M.L., M.J. Garcia-Garcera, A.J.M. Driessen, A.T. Ledeboer, J. Nissen-Meyer, and et al. (1993) *Appl. Environ. Microbiol.* 59: 3577.

76 Marcos, J.F., R.N. Beachy, R.A., Houghten, S.E. Blondelle, and E. Perez-Paya (1995) *Proc Natl Acad Sci USA 92: 12466.*

77 Zasloff, M. (1987) *Proc. Natl. Acad. Sci. USA 84: 5449.*

78 Zasloft, M., B. Martin, and H.C. Chen (1988) *Proc. Natl. Acad. Sci. USA* 85: 910.

79. Matsuzaki, K. (1988) *Biochim Biophys Acta*. 1376:391.

80. Lear, J.P., Z.R. Wasserman, and W.F. Degrado (1988) *Science* 246: 1177.

81. Anzai, K., M. Hamasuna, H. Kadono, S. Lee, H. Aoyagi, and et. al (1991) *Biochim Biophys Acta 1064: 256.*

82. Blondelle, S.E., and R.A. Houghten (1992) *Biochemistry* 31: 12688.

PART II

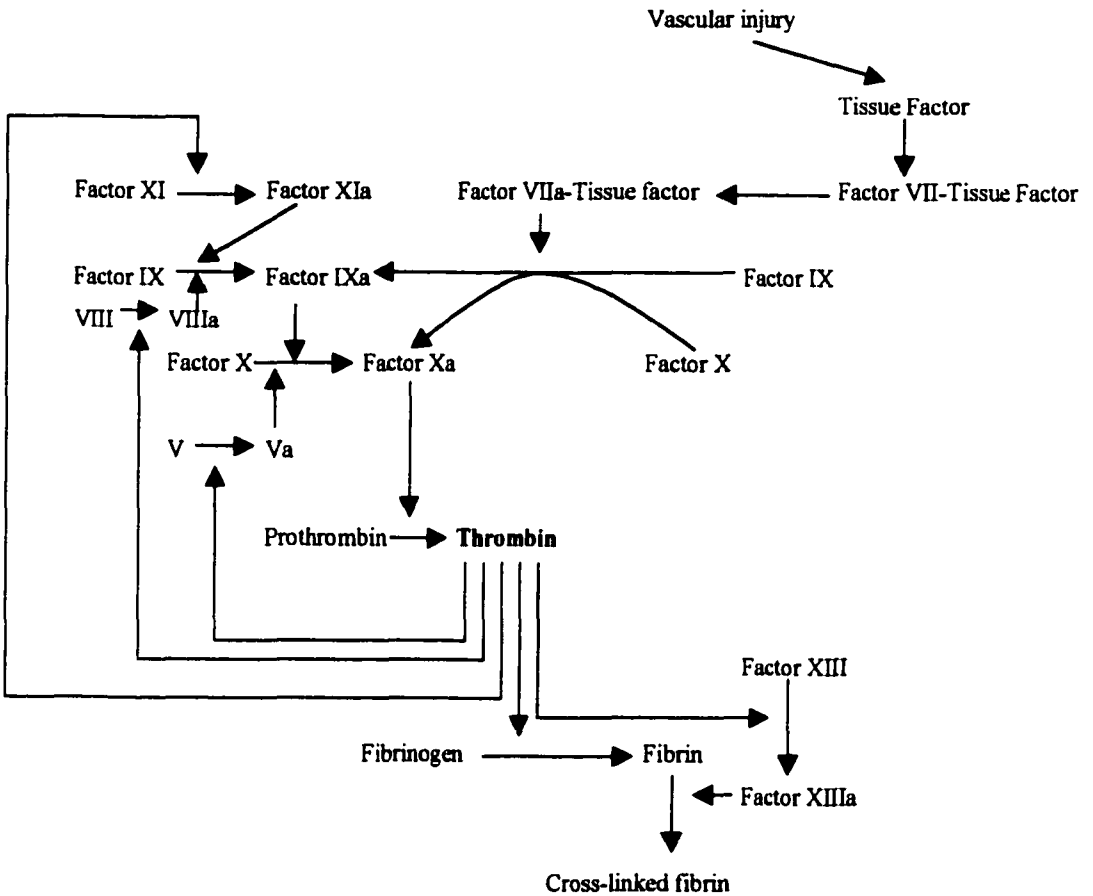
(-)-EPIGALLOCATECHIN GALLATE FROM GREEN TEA IS A THROMBIN INHIBITOR

INTRODUCTION

The control of blood coagulation is one of the most important issues in modern medicine, since two diseases related to coagulation, heart disease and stroke, are the major cause of death in many countries. Blood coagulation is most often controlled by the use of injected heparin (a polymeric glycosaminoglycan), which activates antithrombin III inhibition of coagulation factors (1), or by oral administration of coumarins, which inhibit vitamin K-mediated post-translational carboxylation of glutamate residues in coagulation factors (2). Both of these methods have drawbacks, which account for the considerable effort in the development of oral antithrombotics. Most effort has gone into the discovery and development of new orally active thrombin inhibitors (3). With possible oral antithrombotics in mind, we have studied the agents present in complex natural beverages. One such beverage is green tea, which is known to contain a polyphenol, (-)-epigallochatechin gallate (EGCG), with a number of interesting biological activities.

Thrombin and Its Inhibitors

Thrombin is a trypsin-like serine protease, which plays a crucial role at the end of blood coagulation cascade (Scheme 1) (4). Due to thrombin's central role in hemostatsis, and to the importance of thrombus formation in the pathogenesis of thrombotic diseases, inhibitors of thrombin are potential targets for drug design as antithrombotic and anti-coagulant agents. The search for clinically important natural and synthetic thrombin



Scheme 1. The central role of thrombin in the blood coagulation cascade (4).

inhibitors is progressing in numerous laboratories (3). Substrate-like analogs, for example, those containing the D-Phe-Pro-Arg sequence (5), exhibit high affinity for thrombin. The specificity of thrombin inhibitors requires that the inhibitors control thrombin's activity without interfering with proteases involved in the fibrinolytic system (6). In this system, tissue-type plasminogen activator (t-PA) activates plasminogen to form plasmin, which converts fibrin to soluble products. Both t-PA and plasmin are proteases. Limitations associated with currently employed anticoagulants highlight the need for more specifically-acting agents.

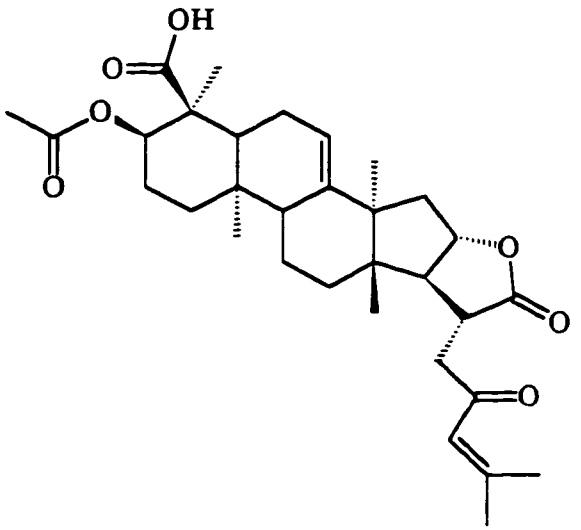
Thrombin catalyzes the cleavage of four Arg-Gly bonds in the natural substrate fibrinogen in order to form fibrin (7, 8, 9). In the blood, thrombin is produced by the action of prothrombinase complex (Factors Xa, Va, Ca^{2+} and phospholipid). Thrombin stimulates its own production by activating Factors V, VIII, and platelets. Clot formation is limited by the fibrinolytic system (6). Thus, hemostasis depends on a balance between the activity of thrombin, which promotes clot formation, and of t-PA and plasmin, which dissolve the clot.

Human thrombin consists of two peptide chains, A and B, connected through a single disulfide bond. The A chain has 36 residues and the B chain has 259 residues. The B chain contains active residues His-57, Asn-102, and Ser-195, which are flanked by the specificity pocket and an extended hydrophobic pocket. Residues of substrates of proteases are usually numbered from the cleaved bond as follows: P_n-...-P_3-P_2-P_1-P_1'-P_2'-P_3'-...P_n' where cleavage occurs at the P_1-P_1' bond. The corresponding binding sites for these residues on thrombin are designated as S or S' sites (10).

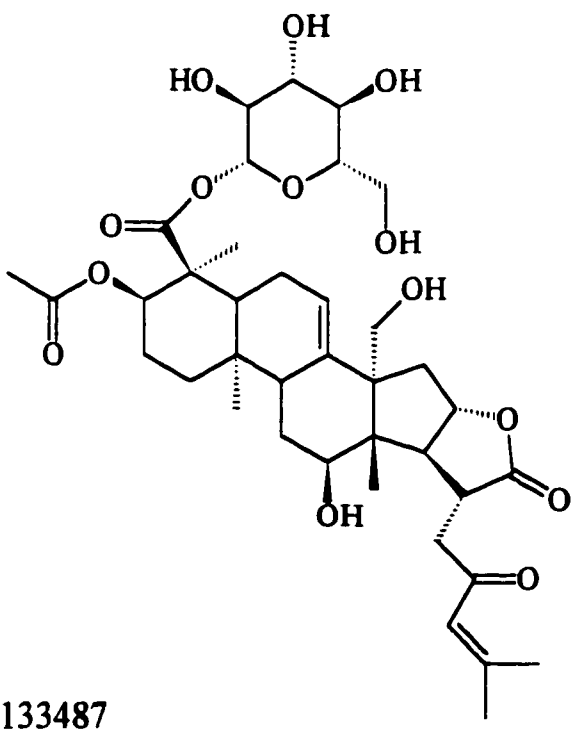
Hirudins are the most potent family of natural thrombin inhibitors. Hirudin is found in the saliva of the leech *Hurido medicinalis* (11). It is a single polypeptide chain with 65 amino acid residues, stabilized in a characteristic conformation by 3 disulfide bridges. Hirudin forms a noncovalent, high affinity (K_i = 25 fm) complex in which the N-terminal blocks the active site of the enzyme, whereas the C-terminus binds to an anion-binding exosite distant from the site of proteolysis (12, 13). The first and third residues of hirudin occupy the S_2 and S_3 pocket, but the basic side chain that binds in the Asp-189 specificity pocket is missing. Coupling of peptides that mimic the carboxyl terminal of hirudin to peptides that are specific for inhibition of the catalytic site of thrombin (D-Phe-Pro-Arg) has led to the development of a series of chimeric molecules termed hirulogs (14). Hirulogs inhibit thrombin by binding to both its catalytic site and its anion binding exosite.

A thrombin inhibitor from a plant *Lantana camara* has been identified as 5,5-*trans*-fused cyclic lactone euphane triterpenes (15). Two compounds named as GR133487 and GR133686 (see structures in Figure 1) showed tight-binding inhibition based on kinetic analysis. Electrospray ionization mass spectrometry of thrombin/inhibitor complex showed the tight-bound species to be covalently attached, suggesting acyl-enzyme formation by reaction of the active site Ser195 with the *trans*-lactone carbonyl.

The formation of a covalent intermediate which mimics the postulated tetrahedral species in the transition state during substrate proteolysis provides a way to design thrombin inhibitors. Boronate, trifluoromethyl ketone, and aldehyde inhibitors have been reported to form reversible covalent adducts to Ser195 that mimic the catalytic tetrahedral intermediates. Aliphatic and aromatic boronic acids have shown inhibitory activity on



GR133686



GR133487

Figure 1. Structures of GR133487 and GR133686 from a plant *Lantana camara* (15).

chymotrypsin and subtilisin (16). The current model for inhibition by boronic acids is that the trigonal boronic acids are esterified to the active site hydroxyl of thrombin (17). This results in formation of a tetrahedral boronic acid-enzyme complex. To develop a compound that would be specific for thrombin, the sequence D-Phe-Pro-Arg has been used. (5). Ac-(D)-Phe-Pro-boroArg-OH (Dup714) has been shown to be a potent and selective inhibitor of thrombin (K_i = 41 pM) (18, 19). Another group of boronic acid inhibitors contains a neutral side chain at the P_1 site, which is the peptide benzyloxycarbonyl-D-Phe-Pro-methoxypropylboroglycine (20). This is a potent inhibitor of thrombin (K_i = 7nM).

Electrophilic carbonyl groups such as an aldehyde or keto group that are placed at the site of a scissile bond in substrate analogs can undergo nucleophilic attack by the oxygen of Ser195 to form a hemiacetal or hemiketal that resembles the putative transition state for amide bond hydrolysis (21). An alpha-keto ester-containing derivative of H-D-Phe-Pro-Lys has been reported to be a potent thrombin inhibitor (22). A drawback of keto esters is that they are relatively unstable in buffers at physiological pH. Alpha-keto amides have shown stability and good inhibition (K_i = 0.25 nM) (21).

Polyphenols in Green Tea

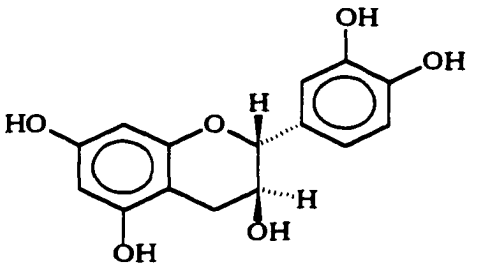
Tea is composed of the leaves of *Camellia sinensis*. Green tea refers to the product manufactured from fresh leaves under conditions that prevent oxidation of the polyphenolic components. Black tea is manufactured to ensure a high degree of

enzymatically catalyzed aerobic oxidation of the leaf polyphenols followed by a series of chemical condensations. Polyphenols constitute the best studied group of tea leaf components, especially the catechin group of the flavanols (23). Six of these occur in considerable quantities in fresh leaf (see structures in Figure 2). Table 1 shows the principal catechins in young tea leaf (23). Catechins are the predominant group of substances in green tea. In the manufacturing of black tea, most catechins are oxidized and polymerized to form other compounds, which are not well characterized.

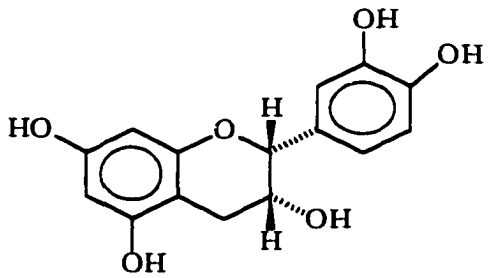
Biological Effects of EGCG

The biological properties of the major polyphenol in green tea, (-)-epigallocatechin gallate (EGCG) have been the object of hundreds of studies. These studies have shown that EGCG can exert a remarkably wide range of effects. While it is impossible to reasonably summarize them here, it may be useful to note that reported effects of EGCG include numerous reports on the inhibition of initiation (24) and growth (25) of human tumors. In addition, there are reports that it protects against radiation damage (26) and has antiprotozoal (27) and antibacterial (28, 29) activity as well as antimutagenic (30) and immunoregulatory (31) activity. It has been reported that drinking green tea daily would contribute to maintaining plasma catechin levels sufficient to exert antioxidant activity against oxidative modification of lipoproteins in blood circulation systems (32).

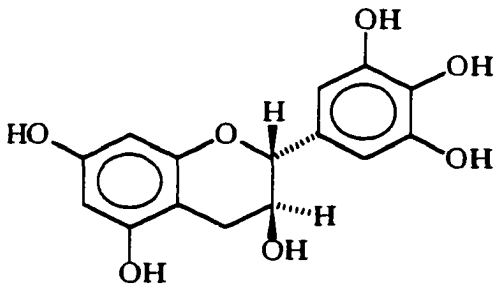
EGCG has been reported to act as an inhibitor of prolyl endopeptidase (IC_{50}=1.47 μM) (33) transcription factor AP-1 (IC_{50} 5.45 nM-54.5 μM) (34) and xanthine oxidase



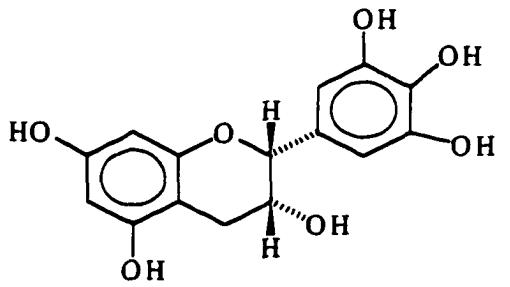
(+)-Catechin



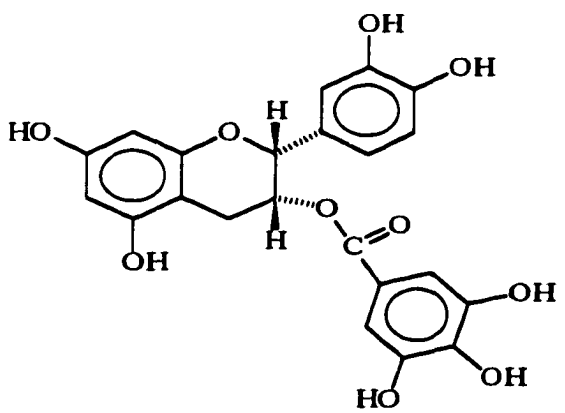
(-)-Epicatechin



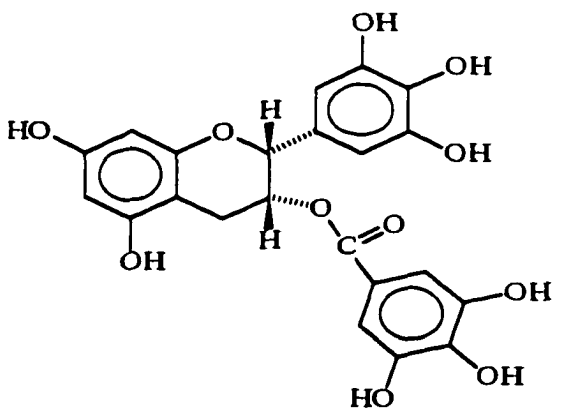
(+)-Gallocatechin



(-)-Epigallocatechin



(-)-Epicatechin gallate



(-)-Epigallocatechin gallate

Figure 2. Structures of major polyphenols in green tea (23).

Table 1. Principal fresh leaf catechins (23).

Catechins	% Dry Weight
(+)-Catechin	1-2
(-)-Epicatechin	1-3
(-)-Epicatechin gallate	3-6
(+)-Gallocatechin	1-3
(-)-Epigallocatechin	3-6
(-)-Epigallocatechin gallate	7-13

(K_i = 0.76 μM, competitive) (35). EGCG has been shown to inhibit the induction (36) and expression of an inducible nitric oxide synthase protein (37) and is antihemolytic (38) in systems using *Staphylococcus aureus* α-toxin and *Vibrio cholerae* hemolysin. EGCG also has been shown to be a causative agent for asthma in workers who handle large amounts of dry green tea (39).

EGCG Effects on Coagulation

It has been previously suggested that EGCG has two effects on blood coagulation. EGCG is a suggested urokinase inhibitor, as judged from its antiangiogenic activity (40), and it has been shown to inhibit platelet aggregation (41). Kan et al. (42) reported that EGCG prevented death due to pulmonary thrombosis and prolonged the tail bleeding time in mice in vivo and inhibited human patelet aggregation in vitro. But EGCG did not change the coagulation parameters such as prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT). They suggested that the antithrombotic activities of EGCG may be due to the antiplatelet activities, but not to anticoagulation activity. EGCG has also been shown to bind to human fibrinogen (43).

A review of EGCG's biological effects is available (44).

Uptake of Orally-Consumed EGCG into Blood Plasma

Any consideration of EGCG biological effects must include its ability to be transported to the bloodstream. In rats, oral administration of 500 mg EGCG per kg body weight results in a 12.3 μM level in blood plasma and higher levels in intestinal mucosa

(45). In another study, the rat plasma EGCG concentration increased to 2.3 μM, 30 min after a single oral dose of 56 mg EGCG (46).

In human volunteers who consumed up to 4.5 g of decaffeinated green tea solids dissolved in 500 mL water, the maximum plasma concentration of EGCG was 0.71 μM (47). In another study, ingestion of 1.2 g of decaffeinated green tea in warm water resulted in a maximal plasma EGCG concentration of 0.58 μM (48).

These studies suggest that attainable EGCG levels in human plasma after oral administration are below 1 μM, while those in rats, higher EGCG administration levels can yield 2-12 μM plasma EGCG levels.

These plasma EGCG concentrations are roughly comparable to the IC_{50} values given above for EGCG mediated inhibition of prolyl endopeptidase (1.47 μM) (49) transcription factor AP-1 (5.45 nM-54.5 μM,) (50) and xanthine oxidase (0.76 μM).

The objective of present study is to isolate, purify, and characterize thrombin inhibitors from green tea.

MATERIALS AND METHODS

Isolation of Thrombin-Inhibiting Material from Tea Leaves

In Figure 3, assays of the thrombin-inhibitory activity from green and black tea were carried out. 0.05 g green tea (Ever-Green, a product of China) or black tea dried leaves (Lipton) were extracted with 100 mL boiling water for 30 minutes. 50 μL of this extract were used for the thrombin inhibitory assay (for conditions, see below.) The absorbance values in Figure 3 are normalized to remove the background absorbance caused by the teas being assayed.

Dry green tea leaves were extracted with methanol for 30 minutes. The filtered extract was subjected to preparative thin-layer chromatography on 20 x 20 cm silica gel plates in formic acid:ethyl acetate:chloroform (3:8:10) (51). Five UV-absorbing bands were collected and eluted with methanol. The methanol was dried, the residue taken up in water, and subjected to the thrombin inhibition assay. The band with thrombin-inhibitory activity was further purified on a 3.9x150 mm Novapack C_{18} RP HPLC column in a Waters Alliance chromatograph with a diode-array detection system. A 30 minutes linear elution gradient (0 to 50% acetonitrile in water containing 0.01% TFA) was used at 0.5 mL/min.

Mass Spectral Analysis

Mass spectra were taken in a Finnigan LCQ quadrupole ion trap instrument equipped with electrospray ionization (ESI). Samples at the concentration of 50 μg/ml

were dissolved in 1% acetic acid in methanol/water (1:1). They were directly injected by the syringe pump at the flow rate of 3 μl/minute.

Assay of Thrombin

Bovine thrombin was purchased from Sigma. Thrombin activity was assayed by using $5x10^{-5}$ M H-D-hexahydrotyrosyl-Ala-Arg-*p*-nitroaniline in 0.05 M phosphate buffer at pH 7.0. Substrate hydrolysis was observed at 405 nm in a microplate reader using initial rate assays for 5 minutes.

In order to exclude a possibility that EGCG reacts with the chromophoric thrombin substrate, HPLC was employed to determine if the substrate changes during the incubation of EGCG and the substrate. 0.5 mM EGCG was mixed with 0.1 mM thrombin substrate in 0.05 M phosphate buffer (pH 7.0) at room temperature for 30 minutes. The mixture was then injected to HPLC with the same conditions mentioned above except that a 20 minutes linear elution gradient (0 to 50% acetonitrile in water containing 0.01% TFA) was used at 0.5 mL/min.

To determine if thrombin inhibitory activity of EGCG results from oxidation of thrombin by EGCG, a reducing reagent, mercaptoethanol (0.05 M), was added to a mixture of thrombin inhibitory assays in the presence of 60 μM EGCG.

To investigate a possibility that EGCG is an irreversible inhibitor which can covalently modify thrombin, thrombin (50 μM) was incubated with 250 μM of EGCG in phosphate buffer at room temperature. After 60 minutes, the mixture (0.5 μl) was added to 250 μl of a phosphate buffer solution containing $1x10^{-5}$ μM thrombin substrate. Kinetic data were recorded using a microplate reader.

Proton Magnetic Resonance

Proton NMR was done in a Bruker 300 MHz digital NMR spectrometer at Fordham University. Spectra were taken in CD_3OD at a concentration of 1mg/mL.

Clotting Assay – the APPT Test

This test was performed using Sigma Diagnostics APTT Reagent. Coagulation Control (Level I) from Sigma was used as a control. 0.1 mL of control plasma was mixed with various concentrations of EGCG for 5 minutes at 37°C. Pre-warmed APTT was added to the above mixture and incubated at 37°C for exactly 3 minutes. The recorded APPT time is the time (in seconds) needed for clot formation after addition of 0.1 mL of 0.02M $CaCl_2$ solution.

RESULTS AND DISCUSSION

The goal of this work was to determine if green tea contains a thrombin inhibitor and, if one was found, to chemically identify the responsible compound. Figure 3 shows the results of a thrombin assay for green and commercial black tea. The more active green tea was analyzed by preparative thin layer chromatography (TLC) (See materials and methods).

Characterization of an Active Component of Green Tea:

A methanol extract from green tea was directly applied to preparative TLC plates developed in formic acid:ethyl acetate:chloroform (3:8:10) (51). Five bands, which showed strong absorbance at the wavelength of 254 nM, were collected. Thrombin inhibitory assays showed that only the R_f 0.3 band gave a positive result. Preparative HPLC of this band displayed one compound with a 257.7 nm absorbance maximum eluting at 11.3 minutes (

Figure 4). The mass spectrum showed a molecular ion of 458.8 (Figure 5). The value calculated for EGCG is 458.37. The proton NMR spectrum (Figure 6) and the mass spectrum of the isolated compound were identical to that of commercial EGCG. The assignment of protons of EGCG is listed in Table 2, which is consistent with the reference (47). The TLC and HPLC characteristics of the isolated compound were also identical to those of authentic EGCG purchased from the Aldrich Chemical Company.

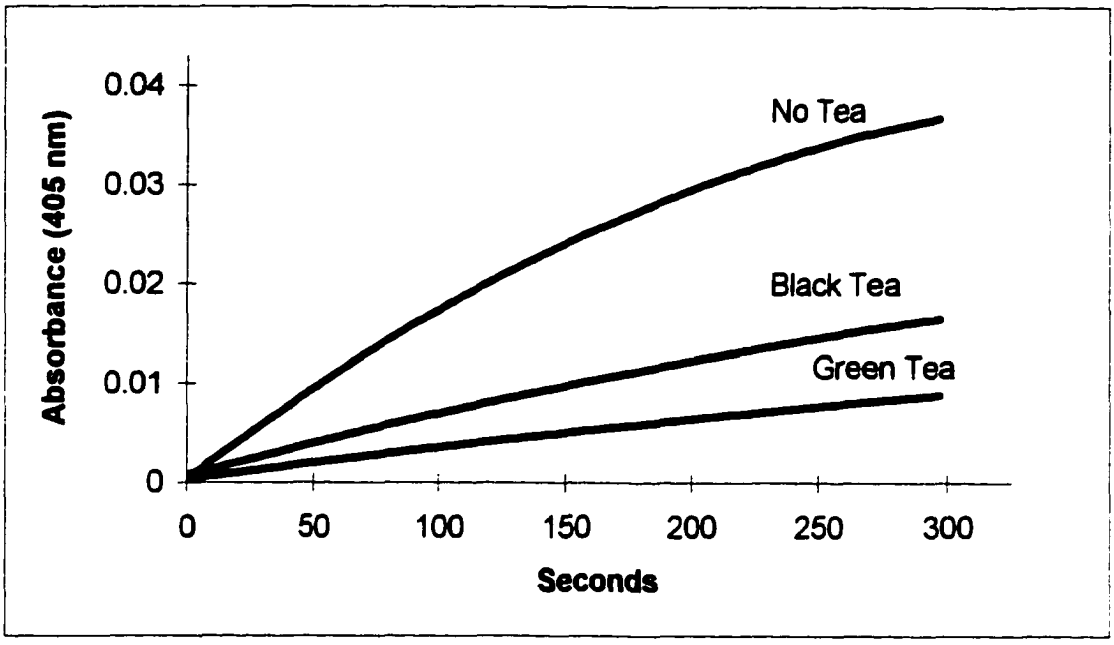


Figure 3. Time course of bovine thrombin substrate hydrolysis in the presence and absence of black and green tea.

Reaction conditions are given in the text.

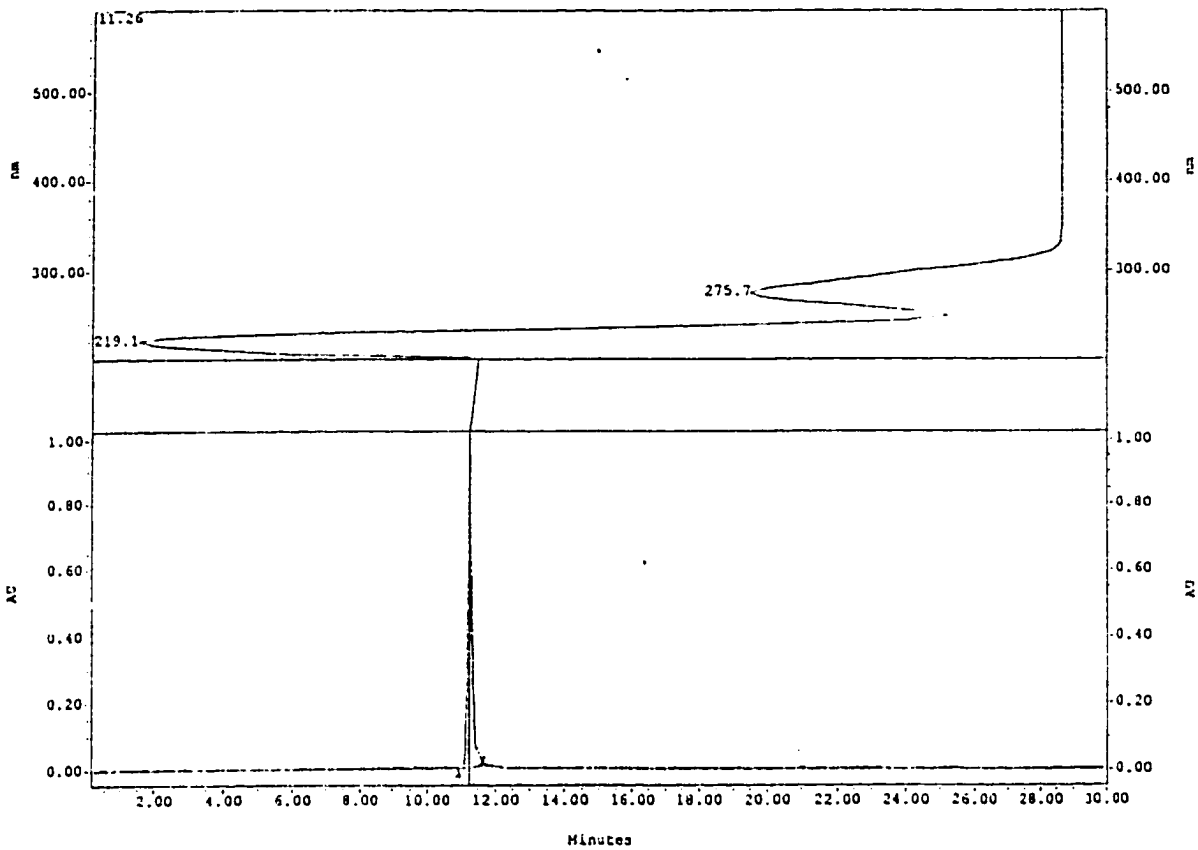


Figure 4. HPLC chromatography and UV spectrum of the R_f 0.3 band from preparative TLC.

See Materials and Methods for the experimental procedure.

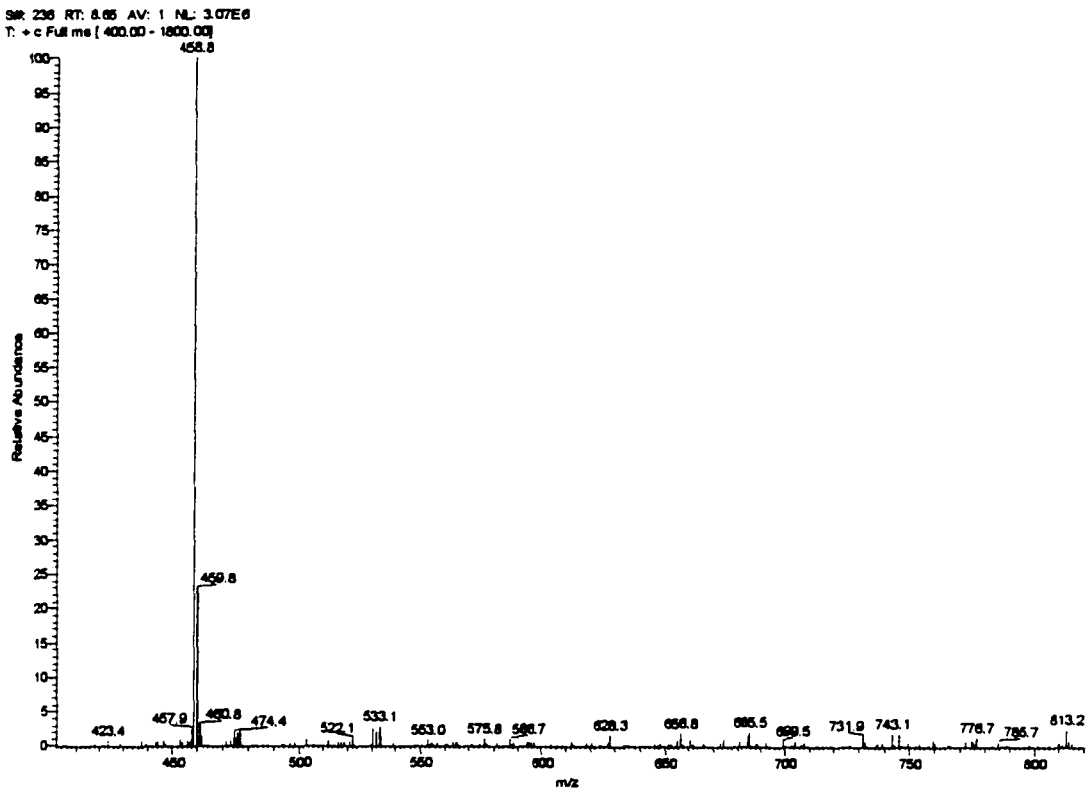


Figure 5. ESI-MS spectrum of the active component from green tea.

See Materials and Methods for the experimental procedure.

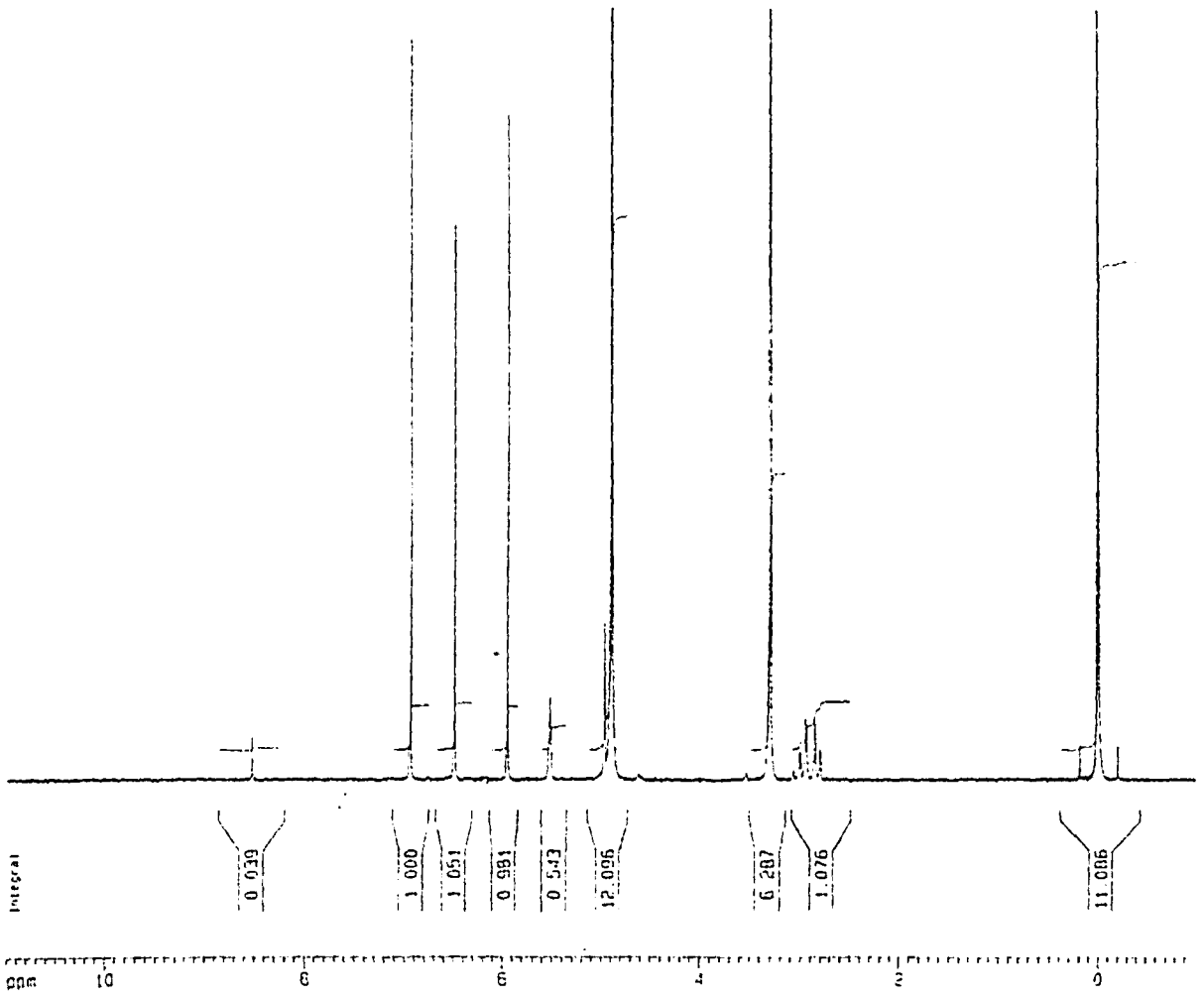
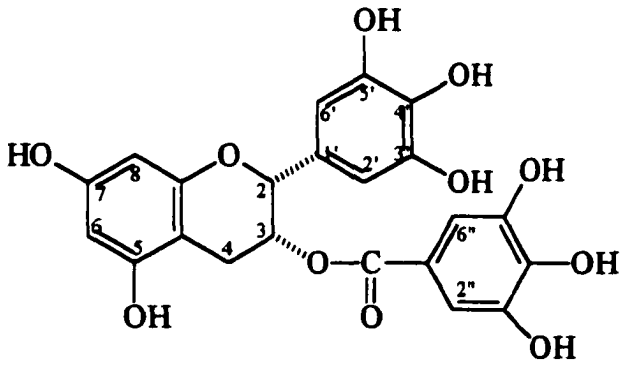


Figure 6. ^{1}H NMR spectrum of the active component from green tea.

See Materials and Methods for the experimental procedure.

Table 2. ^{1}H NMR spectral assignment of EGCG.

Protons	ppm
H-2	5.08
H-3	5.56
2H-4	2.8 -3.2
H-6	6.08
H-8	6.08
H-2'	6.64
H-6'	6.64
H-2"	7.04
H-6"	7.04



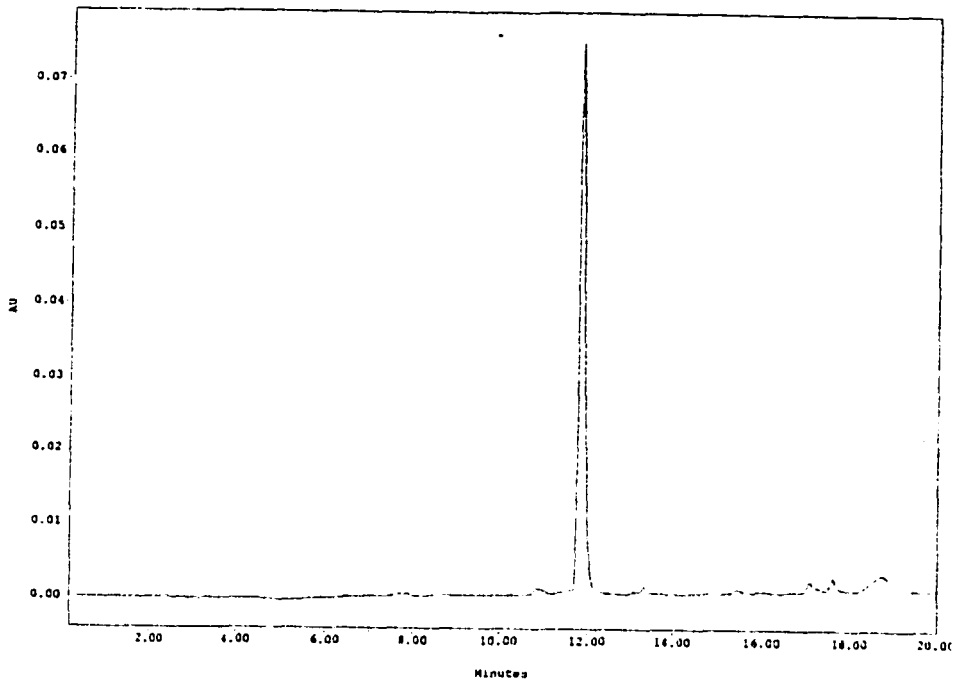
Thrombin Inhibition:

Under the conditions of the thrombin inhibition assay, there was no reaction between EGCG and thrombin substrate after incubation of 30 minutes. This mixture had a 5:1 EGCG to substrate ratio and was analyzed by HPLC chromatography (Figure 7). The peak height of the substrate did not change in the absence or presence of EGCG. This experiment clearly indicates that the inhibition of thrombin activity is solely caused by EGCG in the thrombin inhibition assays.

The results of the inhibition assays are seen in Figure 8, which is shown in an Eadie-Hofstee plot. For convenience, these assays were done using commercial EGCG. Figure 9 shows that EGCG affects the V_{max} but not the K_m of thrombin – substrate interactions. This binding pattern is consistent with either EGCG binding away from the active site (a noncompetitive inhibitor) or binding combined with slow irreversible inhibition. One possible mechanism for slow irreversible inhibition is acylation by the EGCG trihydroxybenzoyl group. Further study is needed to fully elucidate the nature of this inhibition. The dissociation constant (k_i) calculated from the relationship between V_{max} and v/S (Figure 9) is 80 μM. Previously observed EGCG binding to fibrinogen is not a cause of the thrombin observed here since fibrinogen is not part of this thrombin assay. EGCG was shown not to inhibit coagulation Factor Xa or the related digestive enzyme, trypsin, suggesting EGCG does have specificity to thrombin.

To test if EGCG is an irreversible inhibitor, excess EGCG (250μM) was preincubated with thrombin (50 μM) for one hour at room temperature. The mixture was diluted by a factor 500 and was used in thrombin activity assays. The preincubation of

A:



B:

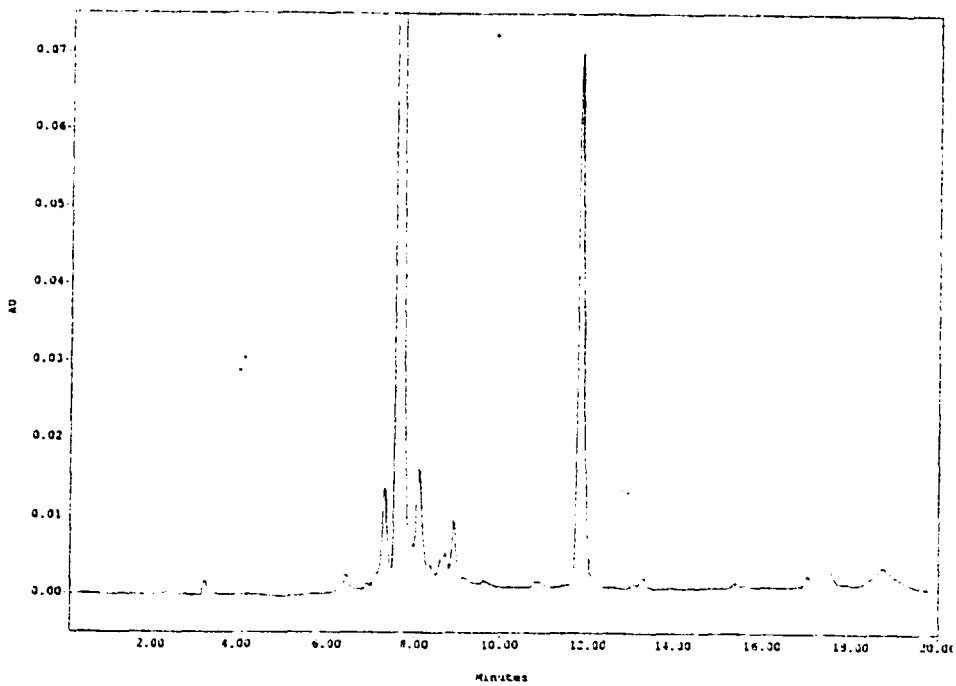


Figure 7 HPLC chromatography of thrombin substrate (A) and the mixture of the substrate and EGCG (1:5) after 30 minutes incubation (B).

Reaction conditions are given in the text.

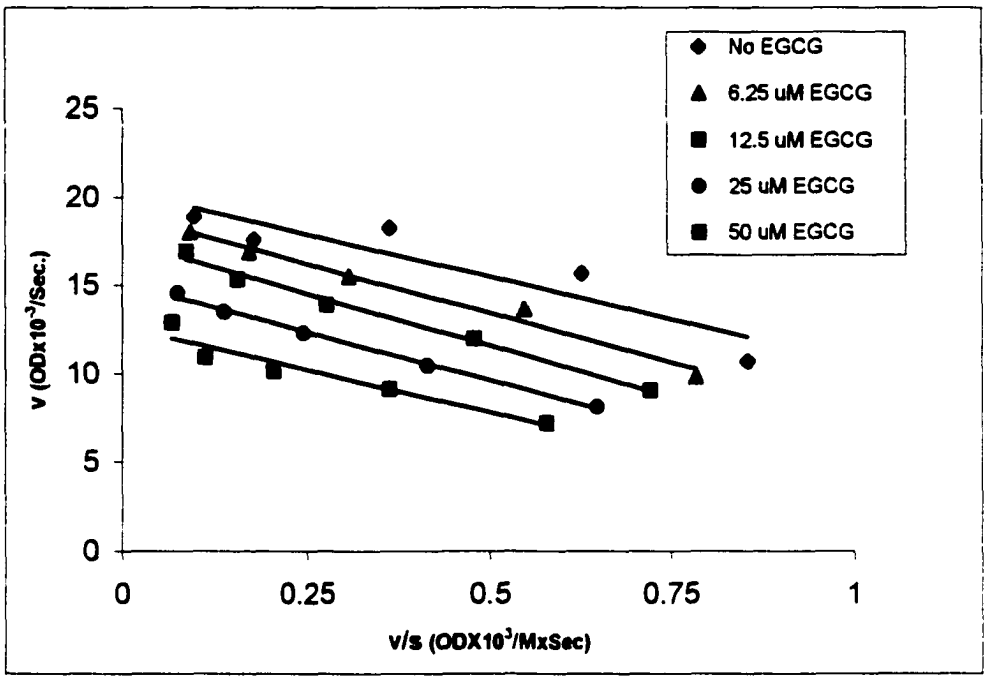


Figure 8. Inhibition of bovine thrombin by EGCG.

Reaction conditions are given in the text.

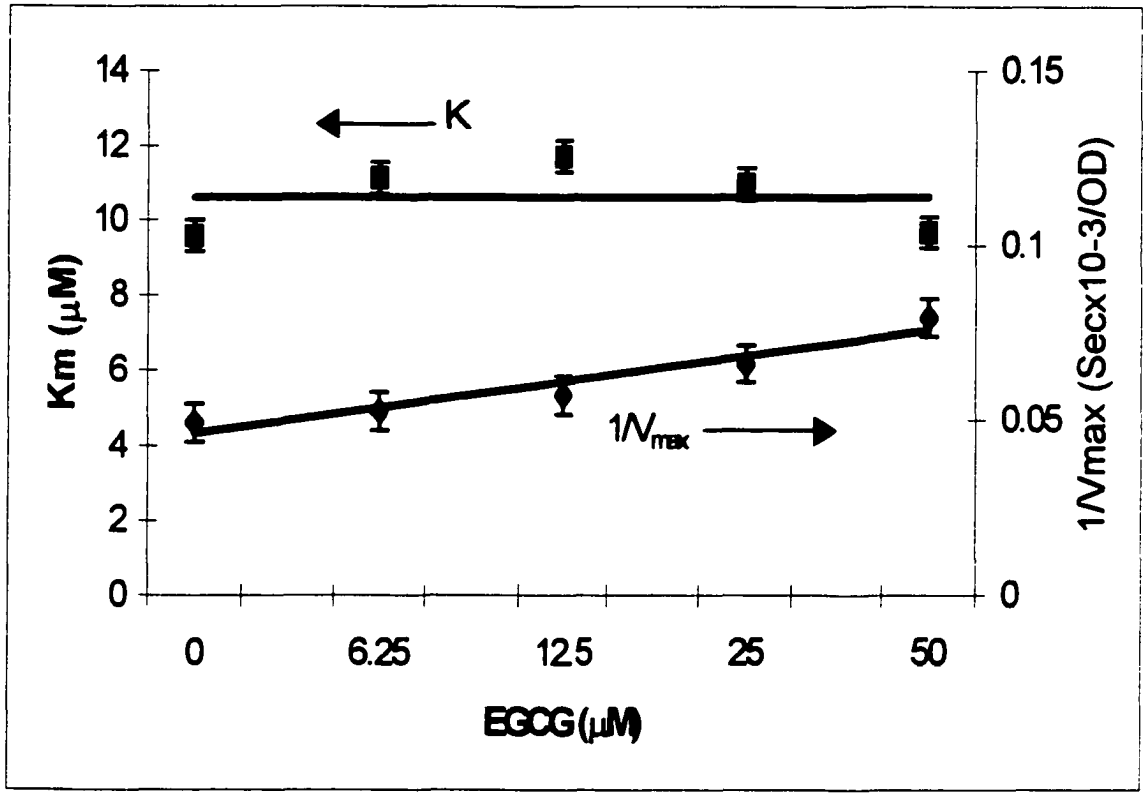


Figure 9. Dixon Plot of EGCG – mediated inhibition of bovine thrombin.

Data are derived from Figure 8.

thrombin with EGCG did not change the thrombin activity as compared with the control (Figure 10). This experiment clearly indicates there is no covalently modification of thrombin by EGCG.

EGCG is an antioxidant agent (32). Its thrombin inhibitory activity may be caused by oxidation of thrombin. This possible mechanism can be tested by addition of a reducing reagent, mercaptoethanol, in thrombin inhibitory assays. The presence of 50 mM mercaptoethanol has no effect on inhibition of thrombin by EGCG (60 μM) (Figure 11). There are no reduction-oxidation reactions involved in thrombin and EGCG.

(-)-Epigallocatechin (see structure in Figure 2) was assayed towards thrombin and has an IC_{50} above 500 μM. It is not a potent thrombin inhibitor. (-)-Epigallocatechin is the hydrolysis product of EGCG and does not have the galloyl ester group.

Inhibition of Blood Clotting

The APPT test was used as a model for blood coagulation *in vivo*. This test is sensitive to deficiencies or abnormalities of plasma proteins specific to the intrinsic pathway (see Scheme 1). The presence of inhibitors of blood coagulation also causes prolonged APTT values. The effect of EGCG on APTT is shown in Table 3. The clotting time is prolonged in proportion to the level of EGCG. At 600 μM EGCG, the clotting time is double that of the control. While this result is consistent with that obtained from direct *in vitro* thrombin inhibition, it suggests that other factors in the assay sequester EGCG.

In direct contradiction to the results from Kan et al. (42), it was found that EGCG prolongs the APTT time. This assay reports a deficiency of any clotting factors except for

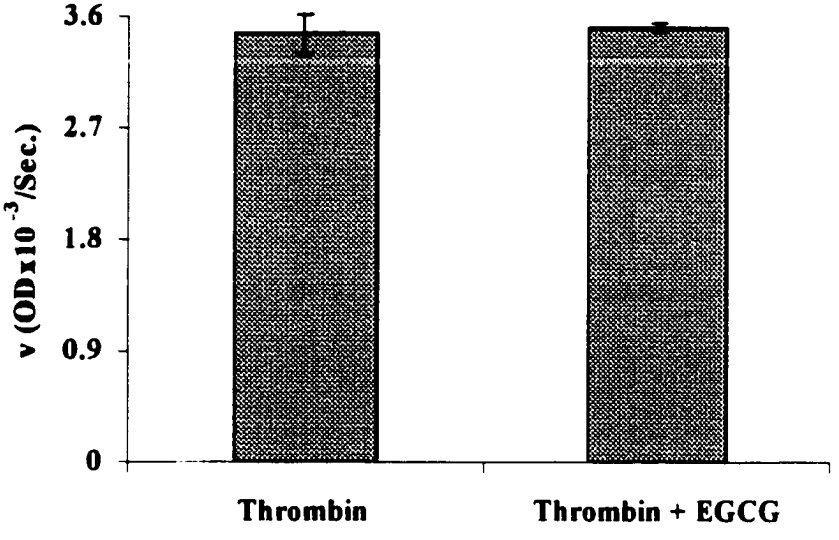


Figure 10 Effect of preincubation of EGCG (250 μM) with thrombin (50 μM) on thrombin activity.

Reaction conditions are given in the text. All values represent the means of triplicates (error bars indicate ± SD).

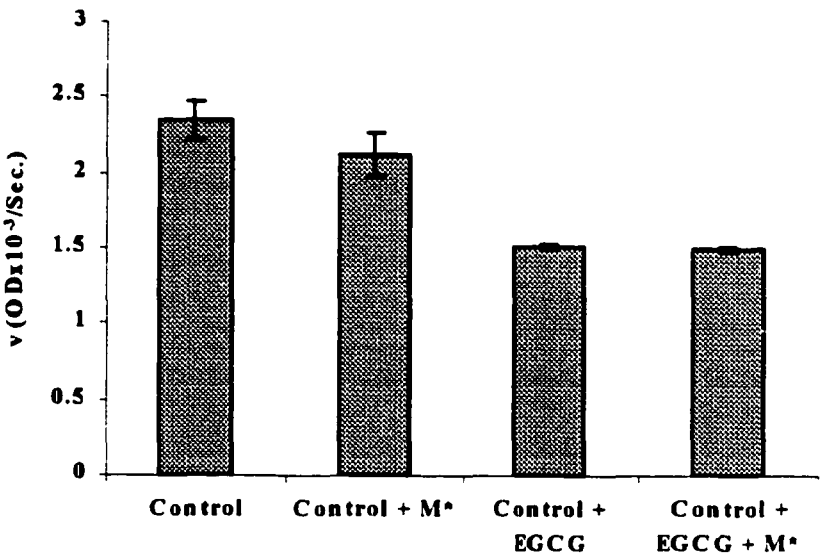


Figure 11 Effect of 50 mM mercaptoethanol (M*) on EGCG inhibitory activity of thrombin.

Reaction conditions are given in the text. All values represent the mean of duplicates (error bars indicate ranges of measurements).

Table 3. Effect of EGCG on the ATPP assay.

See Material and Methods for the experiment procedure. Values are means of triplicates.

EGCG (μM)	APTT (Seconds)
0	31.7
90	36.3
180	38.3
300	45.0
600	62.0

factor VII or presence of any inhibitor of one of those factors. Combined with the report that EGCG binds to human fibrinogen (43), these results show that EGCG is a thrombin inhibitor and causes prolonged APTT time. This makes it possible that EGCG is an anticoagulant. Epidemiological studies have reported a reduced risk of coronary heart disease in subjects with a high intake of tea components (53). The protective effect of these components has been attributed to antioxidative activity. These results suggest that the anticoagulant activity of tea components should also be considered in interpreting such studies.

CONCLUSIONS

This study demonstrates that ECGC is able to inhibit thrombin and is able to inhibit blood coagulation. It is not an irreversible inhibitor of thrombin and this inhibition is not caused by thrombin oxidation by EGCG. The concentration needed for the inhibition of purified thrombin is over 10-fold higher (K_i = 80 μM) than those concentrations that have been observed in blood plasma after oral ingestion of green tea. The concentration needed for inhibition of blood coagulation in the APPT test is 600 μM, 50-fold higher than that seen *in vivo*. Despite the fact that ECGC is consumed by millions of individuals daily, the variety of biological activities previously observed for ECGC and the high concentrations needed to affect blood coagulation make EGCG a poor candidate for clinical testing as an anticoagulant. However, the data presented here may be useful to alert those studying ECGC as an anticancer agent that ECGC may have a side effect on coagulation at high dosage levels.

REFERENCES

1. Jordan, R.E., G.M. Oosta, W.T. Gardner and R.D. Rosenberg (1980) *J. Biol. Chem.*, **255**: 10081.

2. Suttie, J.W. (1987) *Adv Exp Med Biol* **214**: 3.

3. Fevig, J. and R.R Wexler (1999) *Annual Reports in Medicinal Chemistry* **34**: 81.

4. Davie, E.W. (1995) *Thromb. Haemost.* **74**: 2.

5. Claeson, G., L. Aurell, G. Karlsson and P. Friberger, (1977) *New Methods for the Analysis of Coagulation using Chromogenic Substrates*, I. Witt (ed.), Walter de Gruyter, p37.

6. Collen, D. (1980) *Thromb. Haemost.* **43**, 77

7. Fenton, II J.W. (1986) *Ann. N.Y. Acad. Sci.* **485**: 5.

8. Fenton, II J.W., F.A. Ofosu, D.G. Moon, J.M. Maraganore, (1991) *Coagulation and Fibrinolysis* **2**: 69.

9. Berliner, L.T. (1992) *Thrombin Structure and Function*, Plenum Press, New York,

10. Schechter, I. and A. Berger (1967). *Biochem. Biophys. Res. Commun.* **27**: 157.

11. Petersen, T.E., H.R. Roberts, L. Sottrup-Jensen, and S. Magnusson (1976) *Protides. Biol. Fluids Proc. Colloq.* **23**: 145.

12. Rydel, T.J., K.G. Ravichandran, A. Tulinsky, W. Bode, R. Huber, and et al. (1990) *Science* **249**: 277.

13. Rydel, T.J., A. Tulinsky, W. Bode, and R. Huber (1991) *J. Mol. Bio.* **221**, 583.

14. Dimaio, J., B. Gibbs, D. Munn, J. Lefebvre, F. Ni,, and Y. Konishi (1990) *J. Biol. Chem.* **265**: 21698.

15. Weir, M.P., S.S. Bethell, A. Cleasby, C.J. Campbell, R.J. Dennis, and et al. (1998) *Biochemistry*, **37**: 6645.

16. Philipp, M. and M.L. Bender (1971) *Proc. Nat. Acad. Sci. USA* **68**: 478.

17. Bone, R., D. Frank, C.A. Kettner, and D.A. Agard (1989) *Biochem.* **28**: 7600.

18. Kettner, C., L. Mersinger, and R.J. Knabb (1990) *J. Biol. Chem.* **265**: 18289.

19. Weber, P.C., S. Lee, F.A. Lewandowski, M.C. Schadt, C. Chang, and et al. (1995) *Biochem.* **34**: 3751.

20. Claeson, G., M. Philipp, E. Agner, M.F. Scully, R. Metternich, and et al (1993) *Biochem. J.* **290**: 309.

21. Brady, S. (1995) *Bioorg. Med. Chem.* **3**: 1063.

22. Iwanowicz, E.J., J. Lin, D.G.M. Roberts, I.M. Michel, and S.M. Seiler (1992) *Bio. Med. Chem. Lett.* **2**: 1607.

23. Lunder, T. (1989) *Farm Tijdschr. Belg.* **66**: 34.

24. Fujiki, H., S. Yoshizawa, T. Horiuchi, M. Suganuma, J. Yatsunami, and et al (1992) *Prev. Med.* **21**: 503.

25. Taniguchi, S., H. Fujiki, H. Kobayashi, H. Go, K. Miyado, H. Sadano, and et al. (1992) *Cancer Lett.* **65**: 51.

26. Yoshioka, H., G. Akai, K. Yoshinaga, K. Hasegawa, and H. Yoshioka. (1996) *Biosci. Biotechnol. Biochem.* **60**: 117.

27. Meckes, M., F. Calzada, A. Tapia-Contreras, and R. Cedillo-Rivera (1999) *Phytother. Res.* **13**: 102.

28. Okubo, S., T. Sasaki, Y. Hara, F. Mori, and T. Shimamura (1998) *Kansenshogaku Zasshi* **72**: 211.

29. Ikigai, H., T. Nakae, Y. Hara, and T. Shimamura (1993) *Biochim. Biophys. Acta.* **1147**: 132.

30. Hernaez, J.F., M. Xu, and R.H. Dashwood (1998) *Mutat. Res.* **402**: 299.

31. Zenda, N., S. Okubo, Z.Q. Hu, Y. Hara, and T. Shimamura, (1997) *Int. J. Immunopharmacol.* **19**: 399.

32. Nakagawa, K., S. Okuda, and T. Miyazawa (1997) *Biosci. Biotechnol. Biochem.* **61**: 1981.

33. Fan, W., Y. Tezuka, K. Komatsu, T. Namba, and S. Kadota (1999) *Biol. Pharm. Bull.* **22**: 157-61.

34. Barthelman, M., W.B. Bair, 3rd., K.K. Stickland, W. Chen, B.N. Timmermann, and et al. (1998) *Carcinogenesis* **19**: 2201.

35. Aucamp, J., A. Gaspar, Y. Hara, and Z. Apostolides (1997) *Anticancer Res.* **17**: 4381.

36. Lin, Y.L. and J.K. Lin. (1997) *Mol. Pharmacol.* **52**, 465-72

37. Chan, M.M., D. Fong, C.T. Ho, and H.I. Huang (1997) *Biochem. Pharmacol.* **54**: 1281.

38. Ikigai, H., M. Toda, S. Okubo, Y. Hara, and T. Shimamura (1990) *Jpn. J. Bacteriol.* **45**: 913.

39. Shirai, T., A. Sato, and Y. Hara (1994) *Chest* **106**: 1801.

40. Swiercz, R., E. Skrzypczak-Jankun, M.M. Merrell, S.H. Selman, and J. Jankun (1999) *Oncol. Rep.* **6**: 523.

41. Sagesaka-Mitane, Y., M. Miwa, and S. Okada (1990) *Chem. Pharm. Bull.* **38**: 790.

42. Kang, W.S., I.H. Lim, D.Y. Yuk, K.H. Chung,J.B. Park, and et al. (1999) *Thrombosis Research* **96**: 229.

43. Sazuka, M., T. Itoi,, Y. Suzuki, S. Odani, T. Koide, and M. Isemura (1996) *Biosci. Biotechnol. Biochem.* **60**: 1317.

44. Chung, K-T., T.Y. Wong, C-I., Wei, Y-W. Huang, and Y. Lin (1998) *Critical Reviews in Food, Science and Nutrition* **38**: 421-464.

45. Nakagawa, K. and T. Miyazawa (1997) *J. Nutr. Sci. Vitaminol.* **43**: 679

46. Nakagawa, K. and T. Miyazawa (1997) *Anal. Biochem.* **248**: 41.

47. Yang, C.S., L. Chen, M.J. Lee, D. Balentine, M.C. Kuo, and Schantz, S.P. (1998) *Cancer Epidemiol. Biomarkers, Prev.* 7: 351.

48. Lee, M.J., Z.Y. Wang, H. Li, L. Chen, Y. Sun, and et al. (1995) *Cancer Epidemiol. Biomarkers, Prev.* **4**: 393.

49. Fan, W., Y. Tezuka, K. Komatsu, T. Namba, and S. Kadota (1999) *Biol. Pharm. Bull.* **22**: 157.

50. Barthelman, M., W.B. Bair, 3rd., K.K. Stickland, W. Chen, B.N. Timmermann, and et al. (1998) *Carcinogenesis*, **19**: 2201.

51. Coxon, D.T., A. Holmes, W.D. Ollis, V.C. Vora, M.S. Grant and et, al. (1972) *Tetrahedron*, **28**: 2819.

52. Lee, M.W., S. Morimoto, G.I. Nonaka, and I. Nishioka (1992) *Phytochemistry*, **31**: 2117.

53. Geleijnse, J.M., L.J. Launer, A. Hofman, H.A.P. Pols, and J.C.W. Witteman (1999) *Arch Intern Med.***159**, 2170.