COMPARATIVE STUDIES OF THE PURIFIED CYSTEINE PROTEASES OF *TRYPANOSOMA BRUCEI* AND *TRYPANOSOMA CONGOLENSE*

BY

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A DISSERTATION SUBMITTED FOR THE DEGREE OF MASTERS OF SCIENCE IN BIOCHEMISTRY TO THE SAMORA MACHEL SCHOOL OF VETERINARY MEDICINE

UNIVERSITY OF ZAMBIA

ZAMBIA

1996

DECLARATION

I DECLARE THAT THE CONTENTS OF THIS THESIS ARE THE WORK OF THE AUTHOR. I FURTHER DECLARE THAT THE THESIS HAS NOT BEEN PREVIOUSLY SUBMITTED TO ANY UNIVERSITY FOR THE AWARD OF A DEGREE OR ANY OTHER QUALIFICATION.

V. KONDE

ACKNOWLEDGEMENTS

I would like to thank all the members of staff of the Department of Biomedical Sciences for the help they gave me and for having accommodated me comfortably for the whole period of my study. I wish to thank Prof. D. Hogg, Mr. J. Daka, Mr. B. Sakala and Mr. I. Nyirenda for giving me access to all the facilities of the Department of Biomedical Sciences. I would like to thank also Dr. A. Nambota and Dr. H. Chitambo for their kindness and help in times of need and for criticising most of my work. I would like to extend special thanks to all members of the School of Veterinary Medicine who were so helpful and for having considered me as one of their own.

I wish to thank Dr. R. Eley, Dr. N. Murphy, Dr. A. Peregrine and Dr. E. Authie for having given me the chance to work with them at the International Livestock Research Institute and for all their scientific advice. I would like to extend special thanks to Dr. Z. R. Nkhungulu (late) for having accepted to supervise me and from whom I learnt most of the scientific knowledge reflected in this thesis. Special thanks also goes to Prof. C.E.A. Lovelace for having accepted to take me on during the period when my supervisor was ill and after she departed and for having been there for me whenever I needed help, advice and criticism.

I would like to thank Dr. S. Phiri and Prof. E. Kiremire of the Department of Chemistry for having given me the chance to do this work in the School of Veterinary Medicine. I wish to thank Prof. I. Takatori for his help and advice on the cell culture experiments.

DEDICATION

This thesis is dedicated to my wife Queen and my son Victor junior who allowed me to leave full-time employment to come and take up full time studies and for having been very supportive.

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ABSTRACT

The cysteine protease enzymes from *T. congolense* and *T. brucei* were purified from pure blood stream forms of the trypanosomes grown in adult albino rats. The trypanosomes were harvested when the number of trypanosomes had reached 10^8/ml of infected blood or higher. The rats were supplied with 10% glucose in drinking water when the parasiteamia was 10^8/ml which enabled the rats to survive for at least two extra days. In case of *T. brucei* infections, the level of stumpy forms in blood was monitored and when the number of stumpy forms reached 50% or more the trypanosomes were harvested. This resulted in an increase in cysteine protease recovery by at least two fold.

The enzymes were successfully purified using egg white cystatin immobilised on sepharose. There were no significant differences in the yields of the two cysteine proteases and both enzymes had similar activity. The activity of the two enzymes was assayed with carbobenzoxy-phenylalaninyl-argininyl 7-amido-4-methylcoumarin by monitoring the fluorescence at 365 nm. The reactions were performed at pH 6 at room temperature (28 - 30°C). *T. congolense* cysteine protease was purified using a monoclonal antibody, prepared against the *T. congolense* cysteine protease, immobilised on sepharose. The enzyme was of similar molecular weight as that purified by the cystatin-sepharose column.

The enzyme was incubated with a variety of host macromolecules, mainly connective tissue proteins and the digestion products were followed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In other cases the proteins were co-polymerized in SDS-PAGE and then digestion monitored in zymograms. The enzymes expressed proteolytic activity against collagen type I, collagen type IV, glycoproteins, fibronectin, laminin and to a small extent against elastin. Fibronectin was the best substrate of all the connective tissue proteins while elastin was least reactive. All the above connective tissue proteins were not denatured prior to digestion as reported in similar experiments.

The two trypanosome cysteine proteases failed to round off and detach cells in culture at our working enzyme concentration (160 μg/ml). The enzymes had greater effect on the cell membrane than on the intercellular matrix, an effect that is similar to papain. The enzymes have high proteolytic activity against cell surface proteins and this may account for the failure to round off cells, as the enzymes are likely to preferentially attack cell surface proteins rather than proteoglycans in the extracellular matrix.

The enzymes were inhibited by trans-epoxysuccinyl-l-leucylamido (4-guanidino) butane (E-64), egg white cystatin, and Nα-ρ-tosyl-1-l-lysine chloromethyl ketone (TLCK) while phenylmethylsulphonyl-fluoride

(PMSF) and aprotinin failed to inhibit the enzymes to any noticeable extent.

The enzymes digested bovine serum albumin (BSA), fibrinogen and casein and their proteolytic products were resolved on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were resolved at similar regions and the patterns of bands produced by the two trypanosome cysteine proteases were identical. This strongly suggests that the trypanosome cysteine proteases under study have preference for the same peptide bonds. The two trypanosome enzymes have similar proteolytic properties and are cysteine proteases in nature as shown by their inhibition by cysteine protease inhibitors.

1. INTRODUCTION

1.1 GENERAL

1.1.1 OCCURRENCE

African trypanosomosis occurs across more than a third of the African continent and is arguably the most important livestock disease in Africa (ILRAD, 1991). It is caused by a protozoan parasite, the trypanosome, and in Africa most trypanosomes of importance to livestock are transmitted by the tsetse fly, the vector of the disease. The trypanosomes infect a variety of domestic and wild animals as well as human beings. The wide occurrence of the disease in people and livestock is a great constraint to agricultural and economic development and consequent improvement of human welfare.

The tsetse fly, which occurs only in Africa, inhabits mainly potentially agriculturally productive areas. Some of its favourable breeding places include river banks, valleys and the open savannah and forests. It was estimated in 1974 that the threat of trypanosomosis persists throughout ten million square kilometres of tropical Africa, including seven million square kilometres that would be suitable for grazing of livestock, should the disease be controlled (FAO, 1974). The situation has not changed and it is estimated that, two decades later, thirty percent of Africa's cattle, a total population of about one hundred and forty seven

million, together with comparable numbers of small ruminants are at risk of acquiring the disease. Annual losses in meat production alone are estimated at about five billion United States dollars (ILRAD, 1991). This figure can even double if the costs due to loss in animal draught power, abortions, low milk yields and the cost of running trypanosomosis control programs were to be included. This means that some money, manpower and time which is supposed to be devoted to improving the agricultural sector is diverted to running trypanosomosis programmes. In Zambia for example, tsetse control receives almost as much personnel and funding as all the other programmes of the Department of Veterinary and Tsetse Control Services combined. Cattle farming will develop considerably if the disease is controlled especially in the small scale farming sector. Fig.1 shows the distribution of tsetse fly and cattle on the African continent.

In Zambia, over 60% of the two and a half million cattle in the traditional farming sector, which constitutes about 80% of the total national herd, together with a small fraction of the commercially kept cattle are at risk of being infected with trypanosomes (Mumba and Chizyuka, 1987). Cattle farming in Zambia is not expanding mainly due to the prevalence of tsetse flies in potential agriculturally productive areas. Fig. 2. shows the distribution of tsetse flies and cattle in Zambia. There is little overlap in the distribution of tsetse fly and cattle populations. This is due to the fact

Fig.1

The map of Africa showing the distribution of tsetse fly and the area of cattle farming on the continent. It is to be noted how tsetse fly belts share boundaries with cattle (map after ILRAD, 1991).

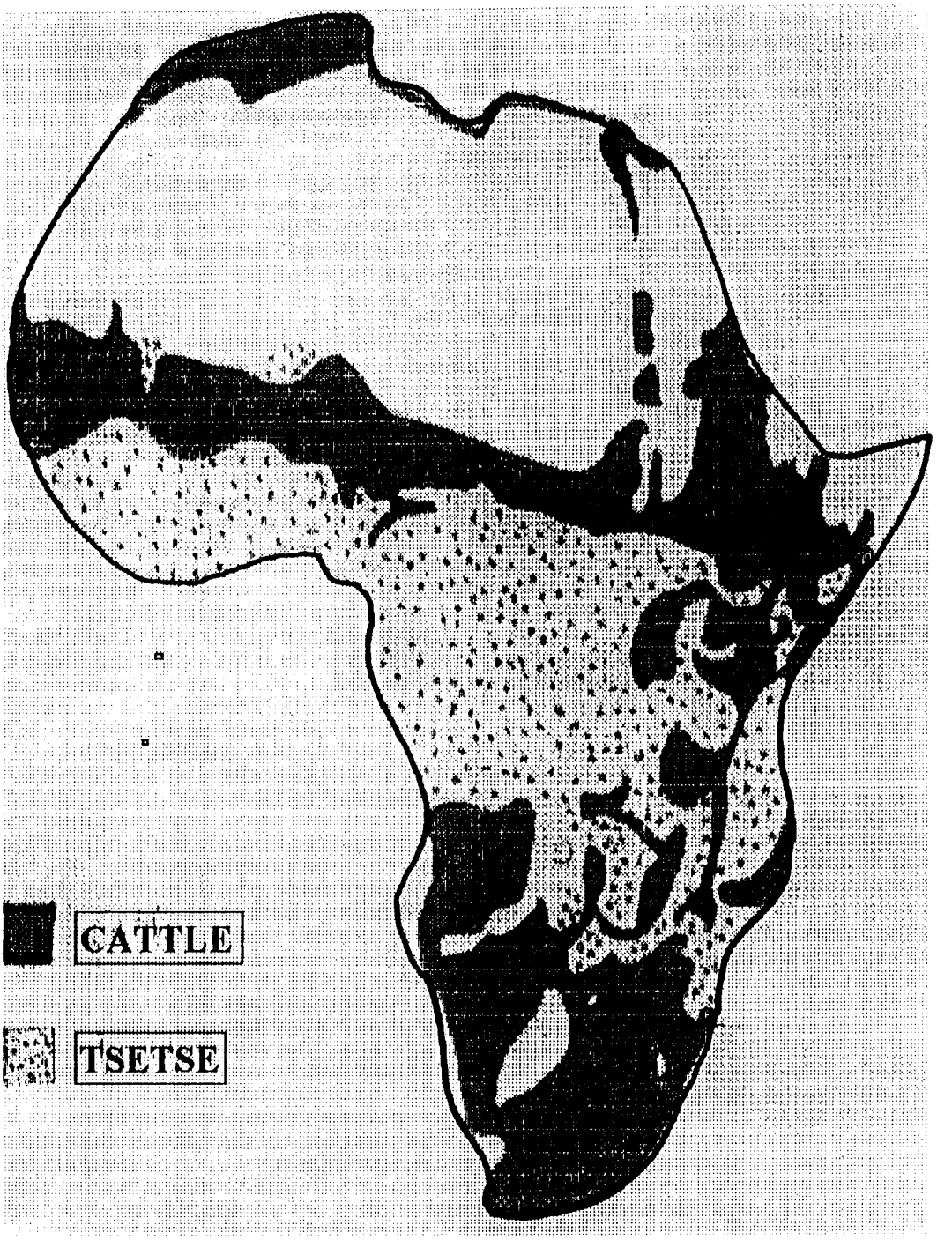
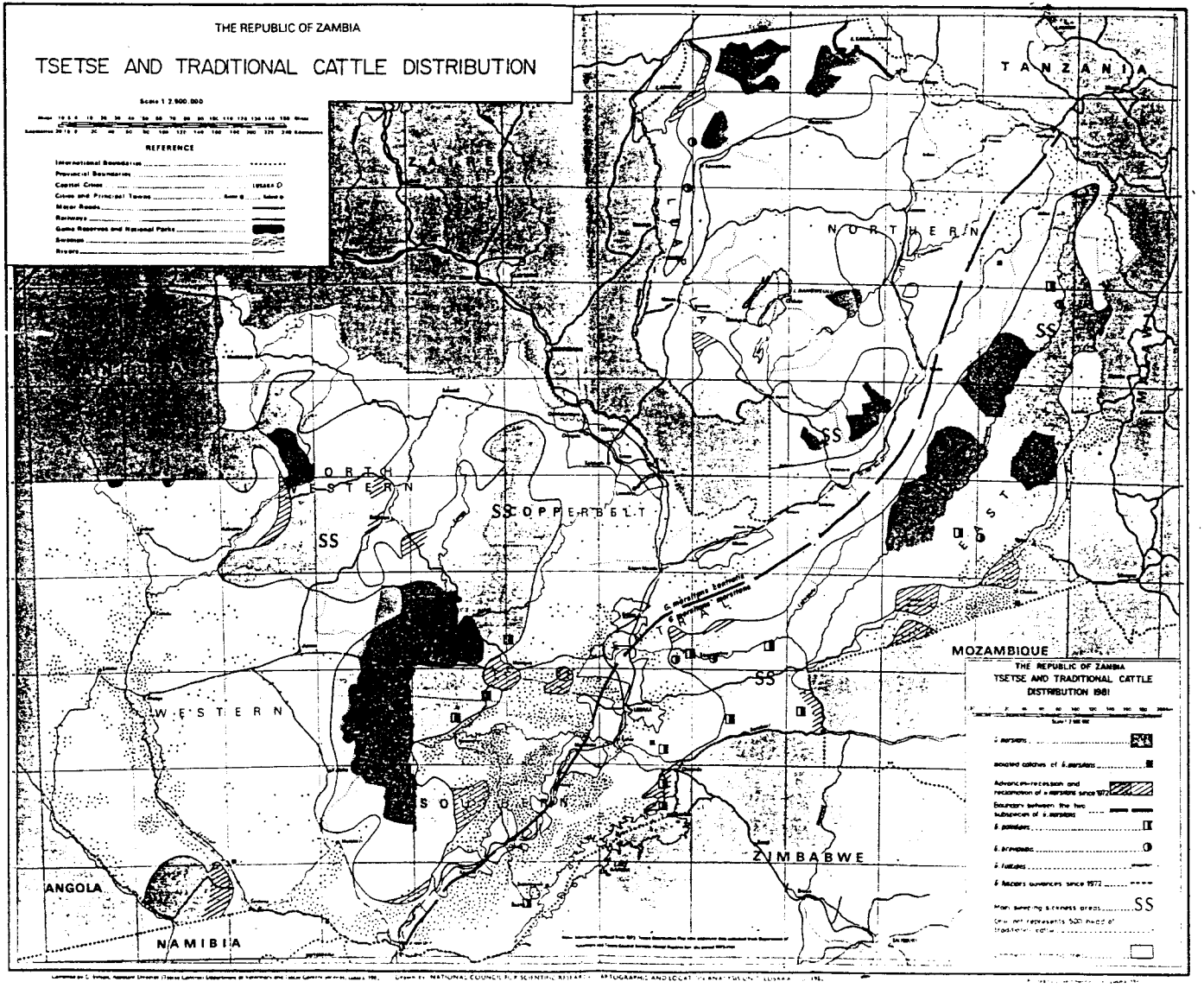

CATTLE
TSETSE

Fig. 2

The map of Zambia showing the cattle population and the tsetse fly distribution. Each dot (.) represents 500 head of traditional cattle and the shaded areas represent tsetse fly belts while SS represent main sleeping sickness areas. Map with the courtesy of the Department of Veterinary and Tsetse Control Services, Lusaka, Zambia.


THE REPUBLIC OF ZAMBIA
TSETSE AND TRADITIONAL CATTLE DISTRIBUTION
Scale 1:2,500,000
REFERENCE
International Boundaries
Provincial Boundaries
Capital Cities
Towns and Principal Towns
Major Roads
Railways
Game Reserves and National Parks
Swamps
Rivers
ZAIRE
TANZANIA
NORTHERN
NORTH WESTERN
COPPERBELT
EASTERN
LUAPULA
LUSAKA
WESTERN
SOUTHERN
MOZAMBIQUE
ZIMBABWE
ANGOLA
NAMIBIA
SS
G. morsitans centralis
G. pallidipes
G. brevipalpis
THE REPUBLIC OF ZAMBIA
TSETSE AND TRADITIONAL CATTLE DISTRIBUTION 1981
G. morsitans
associated catchers of G. morsitans
Advance/Re-invasion and recolonisation of G. morsitans since 1972
Boundary between the two subspecies of G. morsitans
G. pallidipes
G. brevipalpis
G. fuscipes
G. fuscipes outriders since 1972
Main sleeping sickness areas
Grid unit represents 500 square miles of traditional cattle
Traditional cattle

that the main traditional method of rearing cattle by allowing the animals to graze freely in the savannah woodlands and in valleys or on river banks results in infection of the animals as these are some of the preferred dwelling and breeding places of the tsetse fly. In some areas of Zambia such as Kasempa District in the North-Western Province, there is almost no cattle farming because of trypanosomosis (personal experience). It is therefore true to say that cattle farming in the country is being seriously affected by African trypanosomosis.

1.1.2 CONTROL METHODS

The need to control trypanosomosis has attracted attention from international world bodies including Food and Agricultural Organisation, United Nations Development Program and the World Health Organisation and has lead to the formation of the Regional Tsetse and Trypanosomiasis Control Program. The methods used in the control of this disease can be categorised into three groups: firstly, those targeted at eradicating or controlling the population of the vector (tsetse fly), secondly, those targeted at the trypanosomes (chemotherapy), and thirdly, those targeted at increasing the host's resistance to the trypanosome.

The first two methods have been widely practised over the past five decades. The major method of controlling the vector was spraying tsetse fly belts with insecticides and cutting of the forests where the tsetse fly

preferred to stay. In a few cases the slaughter or cropping of game animals, the parasite reservoirs of the trypanosomes, was encouraged. Wild animals were considered trypanosome reservoirs as they carried the disease without showing serious consequences. In other cases, erection of fences to minimise or limit the game animal movement was also supported. All these methods employed were not fully reliable.

Insecticide spraying, especially aerospraying, has almost been abandoned in most African countries because it is very expensive for their economies. This constraint has therefore made spraying of large tsetse fly belts very difficult. These methods were once successfully used in Zambia (Personal communication with Mr. Mumba, Assistant Director, Tsetse Control Services), but have since almost been abandoned because uncontrolled widespread spraying is condemned as a source of indiscriminate pollution.

Recently, another method of controlling tsetse flies was developed, which involves the use of targets. A target (screen) is a blue/black piece of cloth mounted on a steel rods frame and anchored into the soil, and the cloth is impregnated with insecticide(s) such as deltamethrin. When a fly lands on a target, the insecticide kills the fly on contact. Targets have proven to be efficient in reducing the population of flies with minimal pollution of the environment. This method may also have its own set-backs and limitations such as the risk of being stolen in the forest by local

people due to the valuable cloth they are made of and the need of resetting the targets now and then which makes it more labour intensive.

Traps are specially designed to allow flies to enter but once inside the tsetse fly gets trapped (can not get out). Traps, like targets are normally impregnated with insecticides. Traps if set properly, can help reduce fly populations but often, traps are used to assess the population of flies in a given area by comparing the numbers of flies being trapped over a period of time. If progressively less flies are caught in a control area, in properly set traps, it is an indicator that the population of flies is likely to be reducing. Targets and traps are now being used widely, including here in Zambia, in collaboration with the local people thereby reducing the theft incidence as they are part of the programme (Jordan, 1986).

Treatment of animals infected with trypanosomes using trypanocides will remain the main method of controlling the disease for a long time to come. The emergence of drug resistant trypanosomes is however, becoming a major source of concern and one wonders for how long the commonly used trypanocidal drugs such as Berenil (ρ-amidino-phenyldiazoamino-ρ-benzamidine) and samorin (isometamidium) will be effective for use in the field as there seems to be no hope of any new drugs coming on the market in the near future (Chitambo and Arakawa, 1991). Since the 1950's almost no new drugs have been developed. This has been attributed to the high cost of developing and marketing of such drugs to third world markets

with small budgets for drugs of veterinary use. This does not make business sense for large pharmaceutical companies.

Development of drug resistant trypanosomes has partly been attributed to the lack of knowledge by the majority of the users, both the commercial and peasant farmers, resulting in indiscriminate use of drugs. The lack of quick, efficient and constant screening of animals under various treatments received over prolonged periods of time is one of the main factors in the development of drug resistant trypanosomes.

Most farmers do not involve veterinary staff to help them with pre-treatment and post treatment screening of sick animals. They resort to treating the animals with trypanocidal drugs routinely and/or whenever they suspect trypanosomosis. Unfortunately, both farmers and veterinary staff seem to be content that all animals are cured following a single dose treatment with trypanocidal drugs. There is virtually no follow up of treated cases except maybe, in the research designated areas (Chitambo and Arakawa, 1991). This has made it difficult to establish the levels of drug resistance, relapse and new cases in the field. Chitambo and Arakawa (1991) suggested a simple but quick method to estimate the possibility of a total cure or a partial cure (relapse) which may be helpful in field studies.

1.2 OBJECTIVES OF THE RESEACH

While the above methods of controlling the tsetse fly and trypanosomes will remain useful for a long time to come, increasing the host's resistance to the parasite is still the most promising method of controlling trypanosomosis in future. This method is "environmentally friendly" and has many long term benefits. To date, there have been very few attempts to try and use this method of control as most of this work is still at research level. In West Africa certain breeds have the ability to control trypanosome levels and/or undergo self cure such as the N'dama breed, which are thus termed trypanotolerant cattle. The mechanism by which the trypanotolerant breeds control the levels of parasiteamia and self-cure is not well understood. Therefore, careful study of the metabolism and development of the trypanosome in the host may provide some insight into how the trypanotolerant animals manage to control this disease. It may also reveal new areas that can be used for molecular and/or drug attack. Hence my interest in pursuing research in this area of trypanosome biochemistry.

It is hoped that research in this field will encourage new developments towards monitoring and controlling trypanosomosis. For instance, it is important to study the role of trypanosome molecules that may be virulent factors and contribute to the pathogenesis of the disease. If

the role of some of these factors were understood it may be possible to find ways of neutralising their effect or to develop them into vaccines against animal trypanosomosis. In this study I intended to investigate the possible role that the trypanosome cysteine protease might play in African trypanosomosis with greater emphasis on tissue destruction and tissue invasion. The following were the objectives of the study:

(1) Purification of the trypanosome cysteine protease from both T. *congolense* and T. *brucei.*

(2) Determination of the ability of the trypanosome cysteine proteases to degrade host macromolecules such as bovine elastin, collagen, laminin, glycoproteins, gelatin and fibrinogen.

(3) Determination of the ability of the trypanosome cysteine poteases to digest extracellular matrix in cell culture (i.e. the ability to exhibit cell rounding off and cell detachment as shown by trypsin).

(4) Comparison of the two trypanosome cysteine proteases in their ability to degrade proteins and their inhibition by a variety of protease inhibitors.

The words trypanopain Tc and trypanopain Tb will be used to mean *T. congolense* cysteine protease and *T. brucei* cysteine protease respectively as these were the first terms that were suggested to be used due to the enzymes' properties that greatly resemble those of papain (Lonsdale-Eccles and Mpimbaza, 1986) and trypanopains will be used to refer to the two above trypanosome cysteine proteases only unless stated in the text.

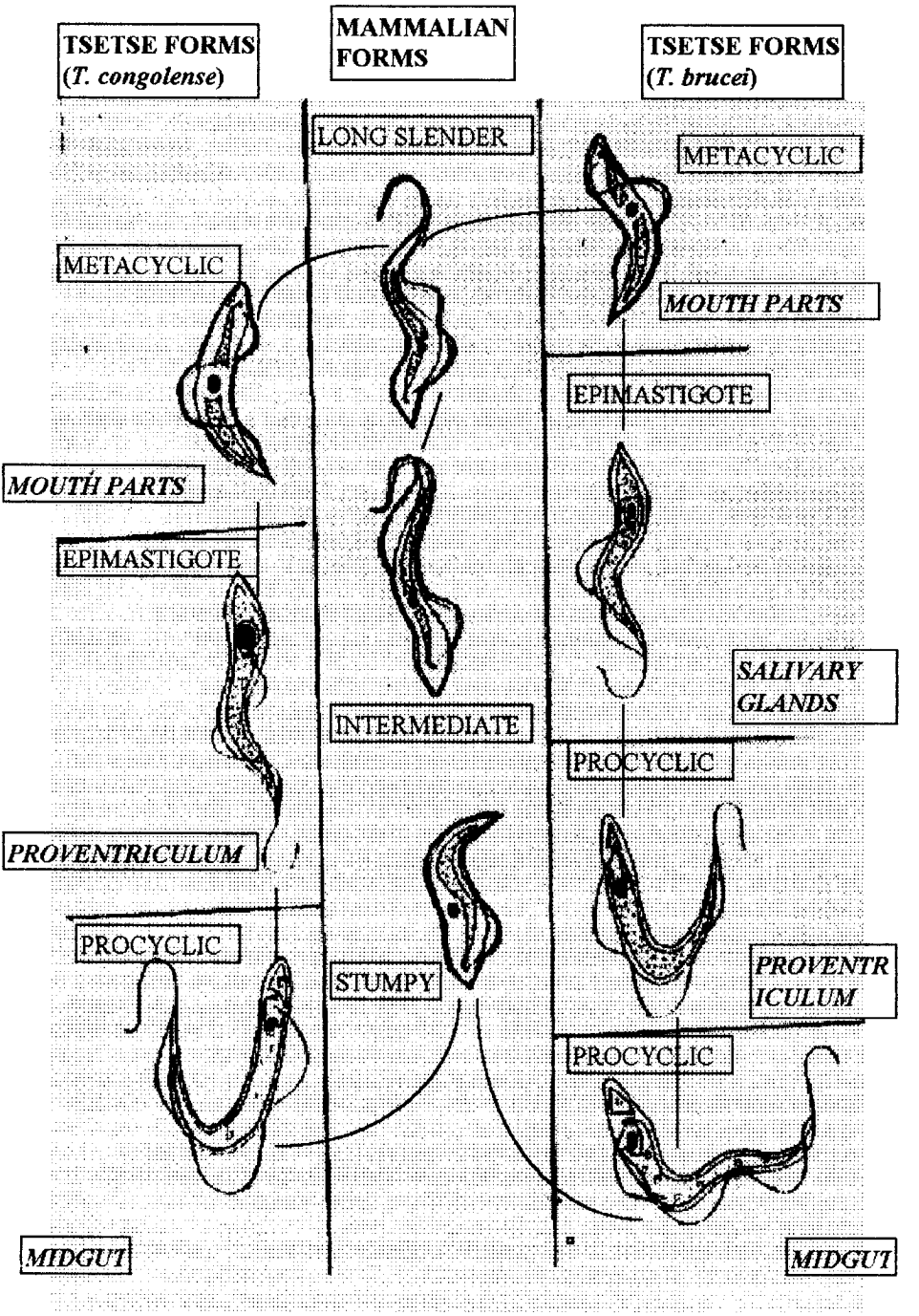
2 0 LITERATURE REVIEW

2.1 THE LIFE CYCLE OF AFRICAN TRYPANOSOMES: A GENERALIZED LIFE CYCLE BASED ON *T. BRUCEI.*

The life cycle of African trypanosomes takes place in two different hosts; the tsetse fly referred to as the vector and the mammal called the host. Fig. 3 is an illustration showing the life cycle of *T. brucei* and *T. congolense*. In nature, infection of the mammal begins when an infected tsetse fly feeds on a susceptible host. The feeding mechanism adopted by the tsetse fly when taking in its blood meal, is such that it is accompanied by injection of saliva from the hypopharynx into the intervening tissue and lumen of the capillary, presumably to avoid formation of blood clots. This saliva inoculation from infected flies results in infection of the mammal (Gordon *et al,* 1956). From the site of infection (chancre) trypanosomes transform from metacyclic (tsetse fly infective form) to the blood stream forms. In the host the trypanosomes multiply and are disseminated throughout the host to colonise and further multiply in selective tissue micro-environments such the blood and other organs. Only the metacyclic forms of the tsetse fly trypanosome are capable of infecting the mammal. There are three blood stream forms in the life cycle of the polymorphic *T. b brucei* (T.b.b): the long slender form, the intermediate form and the

Fig. 3

The life cycle of *Trypanosoma congolense* and *Trypanosoma brucei.* Note the presence of the variable surface glycoprotein (as a thick outer line on the trypanosome) on all blood stream forms and the metacyclic form and the absence of the same coat on all the parasite forms except in the metacyclics when it arises.(adapted from ILRAD, 1989 and 1991).


TSETSE FORMS
(T. congolense)
MAMMALIAN
FORMS
TSETSE FORMS
(T. brucei)
LONG SLENDER
METACYCLIC
METACYCLIC
MOUTH PARTS
EPIMASTIGOTE
MOUTH PARTS
EPIMASTIGOTE
SALIVARY
GLANDS
INTERMEDIATE
PROCYCLIC
PROVENTRICULUM
PROCYCLIC
STUMPY
PROVENTR
ICULUM
PROCYCLIC
MIDGUT
MIDGUT

stumpy form. Once the T.b.b metacyclics are deposited in the mammal, transform into the long slender form which is an actively dividing form. They multiply rapidly by binary fission and this is the way all African trypanosomes are known to multiply. This form has a long, slender and curving body with an elongated nucleus and the kinetoplast is small and normally subterminal. The flagellum is attached to the undulating membrane and is extended as a free flagellum from the exterior end for about 6 μm. The long slender forms measure about 25 μm in mean length and 3 μm in mean width.

As parasitaemia progresses, the long slender forms transform into intermediate forms. This form is best described as the interface stage between the long slender forms and the stumpy forms measuring about 20-25 μm in length and 3.3 μm in width. The kinetoplast is small, the nucleus is elongated and the flagellum is free for about 3 μm with a moderately developed undulating membrane. The intermediate forms also divide by binary fission. The intermediate forms transform into the short stumpy forms. Stumpy forms have a well developed undulating membrane, with no free or a very short free flagellum and measure about 17-22 μm in mean length and 3.5 μm in mean width. The body appears rigid and broad with an oval nucleus and the kinetoplast is normally situated close to the

posterior end. Fig. 4 shows light microscope pictures of trypanosomes in a mixed infection of *T. brucei* and *T. congolence*.

It is reported that the short stumpy forms are the only form known to infect the tsetse fly when it takes in a trypanosome infected blood meal. Once in the open posterior end of the peritrophic membrane, they then migrate in the anterior direction of the peritrophic membrane, between the outer surface of the peritrophic membrane and gut wall. The procyclic forms develop in the mid gut. This form has a kinetoplast that is normally posterior and it is part of a fully developed mitochondria and the nucleus is oval shaped and has a free flagellum at the extreme anterior end without a variable surface coat. The organisms undergo division and migrate into the proventriculus and continue their migration up to the oesophagus, the hypopharynx and later enter the salivary gland. In the salivary gland they assume the epimastigote form and undergo further division. This form has a kinetoplast that is placed almost in the middle and the nucleus is normally posterior and has a free flagellum at the extreme anterior end. The epimastigotes later transform into metacyclics, a non-dividing form which resembles the blood stream form and is found in the mouth parts. This form has a variable surface coat. The metacyclics are therefore ready to infect new hosts or re-infect host at the next tsetse fly bite and are the only forms known to infect mammals (ILRAD,1989: Stephen, 1986).

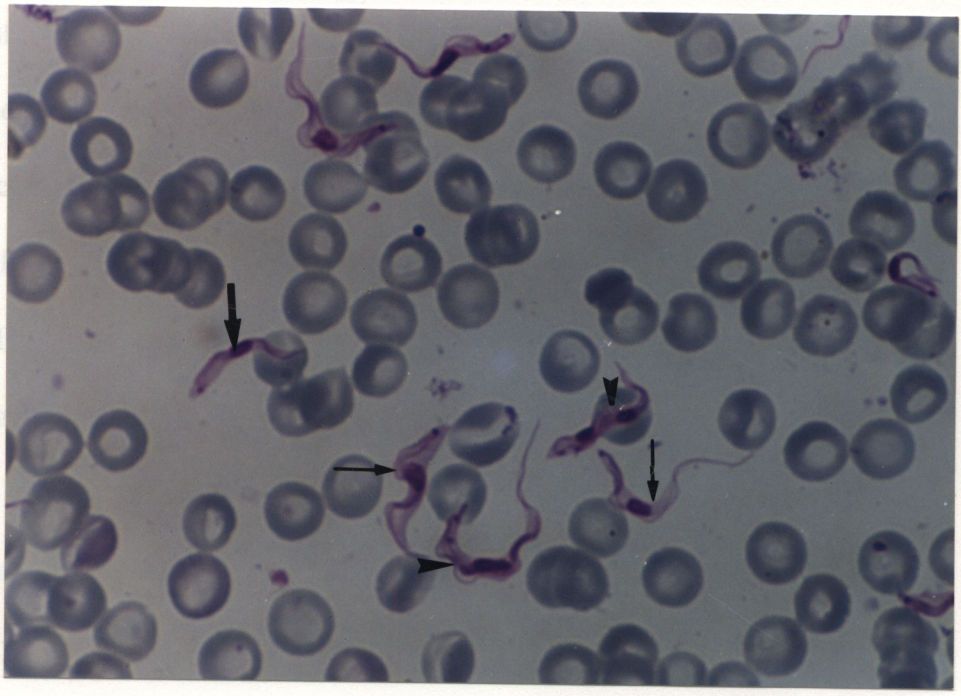
Fig. 4

Light microscope picture of trypanosome

The pictures of *T. brucei brucei* and *T. congolense* in a mixed infection as seen under a light microscope 15 x 100 magnification. Cultivated and prepared from rats. Stained with giemsa stain.

↓ indicate a *T. brucei* long slender form and →indicates a *T. brucei* stumpy form. An arrow head pointing forward indicates a dividing form of *T. brucei*.

↓ indicates a *T. congolense* and an arrow head pointing down indicates a *T. congolense* dividing form.



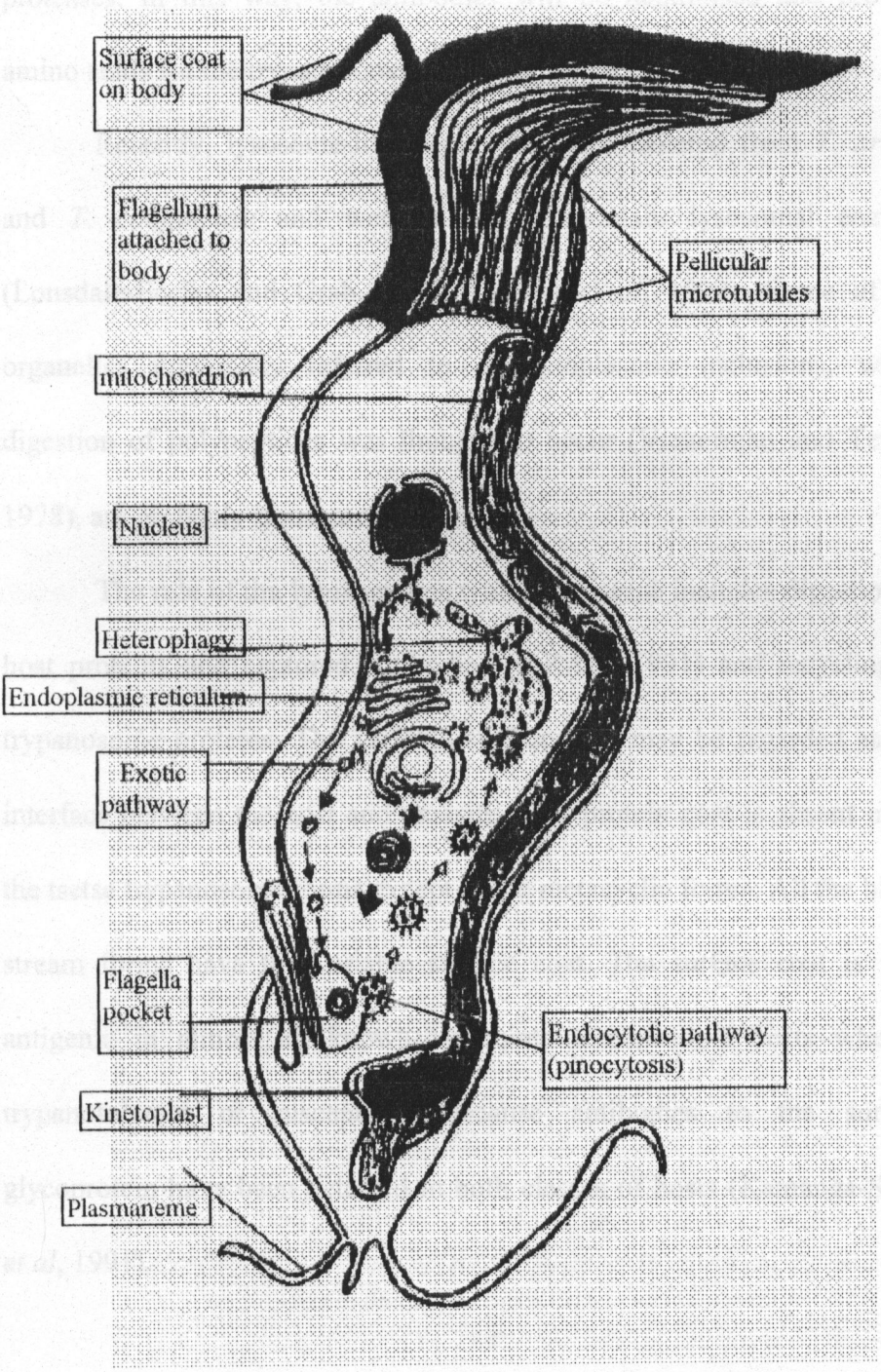
2.2 TRYPANOSOME STRUCTURES AND THEIR ROLE IN PATHOGENESIS.

A generalised structure of a salivarian trypanosome is given in Fig.5. The plasma membrane maintains the shape of the trypanosome (Steven, xx). Lying immediately below it are the cortical microtubules which maintain the elongated shape of the organism. They form the cytoskeletal tissue and are likely to function in the locomotion (movement) of the organism. The flagellum arises from the basal body between two cortical microtubules to later appear free at the anterior extremity. The free flagellum is not observed in *T. congolense* and stumpy form of *T. brucei.* The flagellum and the microtubules are thought to be vital in the ability of trypanosomes to change their shape and swim around very fast (Brooks, 1978). The flagellum has also been implicated in the ability of the trypanosomes to attach to host surfaces such as the vector's gut (Molyneux, 1977) and small diameter blood vessel (Banks, 1978).

The flagella pocket is an important organ of the trypanosome. It is the only demonstrated entry site for nutrients and captured antibodies and also appears to be the main excretory organ (Langreth and Balber, 1975; Russo *et al*, 1993). Therefore it may play a role in the disease by helping trypanosomes capture antibodies and other host proteins. The autophagic

Fig. 5

The structures of a salivarian trypanosome as revealed by the electron microscope (after Vickerman and Tetley, 1978).


Surface coat on body
Flagellum attached to body
Pellicular microtubules
mitochondrion
Nucleus
Heterophagy
Endoplasmic reticulum
Exotic pathway
Flagella pocket
Endocytotic pathway (pinocytosis)
Kinetoplast
Plasmaneme

vacuoles formed after pinocytosis (engulfing of the host macromolecules by parasite membrane) takes place at this site, and these later fuse with the primary lysosomes which contain lysosomal digestive enzymes such as proteases. In this way, the antibodies will be neutralised and provide amino acids for the trypanosome. (Russo *et al*, 1993; Stephen, 1986).

Recently, lysosome-like organelles were isolated from *T. brucei* and *T. congolense* and were shown to contain lysosomal enzyme (Lonsdale-Eccles and Grab, 1987; Mbawa *et al*, 1992). Some of the organelles previously referred to as cytoplasmic inclusions, where digestion of polypeptides was thought to occur (Vickerman and Tetley, 1978), are probably lysosomes.

The role of the lysosomal enzymes in disease includes digestion of host proteins and captured antibodies (ILRAD, 1991) and recycling of trypanosome proteins. The variable surface coat may be regarded as the interface between the host and parasite. This protein coat is absent in all the tsetse trypanosome forms except in the metacyclic forms. All the blood stream forms have the variable surface coat. The surface coat is very antigenic in almost all known vertebrate trypanosome hosts whether trypanotolerant or susceptible, because antibodies to the surface glycoprotein have been detected in both classes of hosts (Kamanga-Sollo *et al*, 1991).

remains unclear up to now what the actual mechanism is by which trypanotolerant animals control parasitaemia.

The mitochondrion is poorly developed in all the blood stream forms (ILRAD, 1991). The mitochondrion is the power house of any cell and is the main site where the tricarboxylic acid cycle takes place in the production of adenosine triphosphate. It is thought that the abundance of nutrients in the mammal especially glucose means that the trypanosome can easily satisfy its energy requirements by glycolysis alone (Stephen, 1986). However, this requires a much higher consumption of glucose and during peak parasiteamia or in chronic stages when the number of trypanosomes is high, this may result in low glucose levels in the blood. This can lead to reduced supply of glucose to specialised tissue that utilise glucose as the main energy source such as red blood cells and the brain. The mitochondrion is fully developed in all the tsetse fly forms with an active tricarboxylic acid cycle as complete oxidation of the small amount of sugar available in the environment is essential for the energy requirement of the trypanosomes.

The levels of cysteine protease activity change throughout the life cycle of the trypanosomes. The enzymes are developmentally regulated (Pamer *et al*, 1989). The highest cysteine protease activity is found in the stumpy forms and it is almost non-existent in the procyclics and epimastigotes. In general the blood stream forms have higher cysteine

protease activity than the tsetse fly forms (Mbawa *et al,* 1991). The lysosomal cysteine protease activity is higher in the trypanosome forms with the variable surface coat including the metacyclic forms.

The role of the lysosomal cysteine proteases in infection is not well understood. These enzymes have been found capable of eliciting an immune response in the host. The ability by the host to mount a detectable antibody response against the cysteine protease results in some immunity. This suggests the enzymes involvement in pathogenesis of the disease (Authie *et al*, 1993a; Pamer *et al*, 1989 and 1990). Pamer *et al,* (1989) showed that the monomorphic strain of *T. brucei* IL Tat 1.4 produced a high level of cysteine protease activity when exposed to diflouromethylornithine (DFMO). DFMO is a potent inhibitor of ornithine decarboxylase and this results in depletion of polyamine. How these changes in the levels of polyamines trigger transformation to stumpy forms or trigger a high cysteine protease activity is not well understood.

The morphological changes have also become an area of active research recently. It is suggested that the ability to limit the parasitaemia levels in trypanotolerant animals is partly due to the fact that the trypanosomes transform into the non-dividing stumpy forms faster in trypanotolerant animals than in susceptible animals (ILRAD, 1994).

2.4 THE BIOCHEMISTRY OF VARIANT ANTIGEN TYPES (VAT)

The variable surface glycoprotein (VSG) has been proposed to be a single polypeptide. Cytochemical techniques have indicated that the sugar moieties are attached to a single polypeptide chain (Cross and Johnson, 1976). The *T. congolense* VSG has been reported to be a 56 kDa molecule (Rovis *et al*, 1978) while the *T. brucei* VSG has been found to be 65 kDa (Cross, 1977). Amino acid sequence analysis of various N-terminal VSG revealed high variability while the sugar analysis revealed presence of constant carbohydrate chains (Baltz *et al*, 1977). Although the mechanism of replacement is not well documented it is likely to be due to phenotypic rather than genotypic differences. The carbohydrate chains are attached to the polypeptide and are located close to the plasma membrane and thus may be vital in attachment to host cells (Renwrantz and Schottelius, 1977).

Recently, it has been shown that the inheritance of variant surface antigens in *T. brucei* involves genetic exchange in co-transmission in the tsetse fly. Such exchange provides the mechanism for genetic variation and generation of new serodemes may occur (Turner *et al*, 1991). This seems to support the findings that trypanosomes with new metacyclic variable antigen types (M-VATs) arise from one clone of trypanosomes in the tsetse fly. This means that the host first antibody response is against all the M-VAT expressed by the particular serodeme rather than just one M-

VAT (Luckins *et al*, 1990). The genetic exchange in metacyclic forms seems to continue even in the chancre region (the site where the tsetse fly deposits trypanosomes during its feeding) as is seen by the generation of new M-VATs at this point (Luckins *et al*, 1990). However, factors that lead to generation of new VATs are not well understood.

2.5. CELLULAR INJURY

2.5.1 ANAEMIA

An obvious and striking feature of African trypanosomosis is anaemia. Although the onset of anaemia and measurement of anaemia is well understood, its cause is not clearly understood. Many likely causes of anaemia have been suggested and many trypanosome factors have been implicated. Hemodilution has been suggested as a likely cause of anaemia. The overall blood volume increases without a corresponding increase in the number of red blood cells (Mamo and Holmes, 1975). The involvement of immunological mechanism in anaemia has been the focus for a lot of research. Immunosuppressed animals have been shown to have high parasite numbers but with a delayed onset of anaemia and normally experience less anaemia than controls. In normal infection the onset of anaemia follows the decline of the first wave of parasiteamia which corresponds to the first peak of antibodies against trypanosomes (Losos and Ikede, 1972). This strongly suggests the involvement of the immune

system in destruction of red blood cells. Immunoglobulins have been eluted from erythrocytes of infected animals and such adsorption of antibodies may even result in self-destruction (Kobayashi *et al*, 1976). The mechanisms that lead to the adsorption of antibodies to red blood cells are not clear.

The activation of the complement cascade in African trypanosomosis is well documented (Jarvinen and Dalmasso, 1976). However, the trypanosome factors involved seem to be many and not all are known. Both the classical pathway and the alternative pathway are activated in African trypanosomosis (Greenwood and Whittle, 1976). Neilsen *et al* (1978) established that mechanisms other than just immune complexes are involved in complement depression. A trypanosome protease and a trypanosome glycolipid were implicated in complement component depression during infection. Recent findings seem to support this hypothesis. Sharpio and Murray (1982) showed that trypanosome molecules of varying molecular weight elicited antibody response which could be capable of activating complement. Isotype switching (IgM to IgG) has been found to be significant in animals that maintain sufficient complement component levels (Authie *et al*, 1992). A 33kDa immunodominant protein (the cysteine protease from *T. congolense*) is recognised differently by susceptible and trypanotolerant animals and the

isotype switching is very significant in trypanotolerant animals but not significant in susceptible animals (Authie *et al*, 1992).

2.5.2. TISSUE LESIONS

When the tsetse fly deposits the metacyclic forms of trypanosomes in a mammalian host, the trypanosomes develop, multiply and migrate from the chancre, point of infection, to colonise different tissues of the animal. The point of infection (chancre region) is characterised by focal disorganisation and tissue degeneration of the dermal collagen followed by an acute inflammation (Gray and Luckins, 1980). Most studies of tissue lesions have been reported in infections with *T. brucei*. Maybe it is due to the fact that most investigators regard *T. congolense* as being strictly a plasma parasite (heamatic) and *T. brucei* as a tissue parasite (humoral) (Tizard *et al*, 1978). Many animals infected with *T. brucei* develop tissue lesions in most of the organs such as the heart, kidney, central nervous system, skeletal tissue and the endocrine system (mainly the pituitary and the thymus) (Murray *et al*, 1974 ; Poltera *et al*, 1977). Given the important roles that some of these organs play such as the pituitary in hormonal balance and the thymus in immune response, their damage and/or malfunction may undoubtedly compromise the animal's overall response to infection. Ocular lesions have also been reported in sheep infected with *T. brucei* and trypanosomes were found in the vitreous and aqueous

humours of dogs infected with the trypanosomes (Losos and Ikede, 1972). About 20% of the animals developed total blindness if not treated.

Animals infected with *T. congolense* have not been found to show such pronounced tissue lesions. However, large numbers of *T. congolense* have been found in small capillaries of the brain and skeletal tissue suggesting tissue tropism or parasite adherence (Banks, 1978). Fieness (1950) reported *T. congolense* in pituitary and adrenal gland as well as in lymph nodes as a tissue parasite, evenly distributed in the tissue and not in 'nests' and 'islets' like *T. brucei*. Gray and Lukins (1980) found *T. congolense* in draining of the lymphatic system and lymph nodes even before the trypanosomes were seen in the peripheral blood. This may suggest that the amount of noticeable damage in infection with *T. brucei* will be easy to demonstrate as the trypanosomes tend to form nests or colonies while it will be difficult to show in *T. congolense* as the trypanosomes are evenly distributed.

The difference in distribution seems to be the main cause of the differences in tissue lesion development in infections with *T. brucei* and *T. congolense*. It is also important to note that the development around the chancre region is similar in infection with *T. brucei* or *T. congolense*. What is even more important is the fact that cellular damage, which is not always easy to show, leads to malfunctioning or damage of organs. It is

speculated that these lesions are caused by chemical agents, immune complexes or trypanosome proteases (Davis *et al*, 1974; ILRAD, 1990),

2.6. NORMAL PHYSIOLOGICAL ROLE OF PROTEASES.

Proteases are enzymes involved in the processing and degradation of proteins. Proteases are found in plants, animals and all other living organisms and viruses. Some hormones, antibodies, all other enzymes, the complement components and many other important biological molecules are proteins or are partly proteins. Proteases are required in processing important biological molecules such as those synthesised as precursors or proenzymes (North, 1982) and also in the degradation of protein molecules which are not required by the cell or those which are taken in as nutrients (Kirschke and Barret, 1985).

This degradation activity of proteases if left uncontrolled could result in self destruction. Nature has put in place mechanisms to control proteolytic activity when not required. For example, the soya bean protease inhibitor protects the soya bean proteins (with an average protein content of 38%) from degradation by its own proteases. However, during germination the soya bean protease inhibitor is degraded by a serine protease contained in the cotyledon of the same seed (Sheih, 1988). In this way the proteins are stored until required for nutrition during germination. Another example is the alpha-2- macroglobulins (α_2M) with a molecular

mass of 720 kDa which serve as peptide traps in sera as they are capable of trapping both foreign or self bioactive proteins or indeed proteases (Borth, 1992). The mechanism is switched on when a protease in serum cleaves the exposed 25 amino acid *bait* region of the α_2M thus activating the α_2M. This is followed by instantaneous conformational changes mediating the "trapping" of the protease which results in physical exclusion of large, but not small, substrates from the active site of the enzyme.

Aprotinin and anti-trypsin are tissue inhibitors that inactivate self or foreign proteases that can, otherwise, digest self proteins. Other digestive proteases are synthesised as pro-enzyme (inactive) forms such as chymotrypsin which is synthesised as chymotrypsinogen. The inactive forms of the enzyme require post translation modification to become mature enzymes (Stryer, 1988). These mechanisms sometimes fail and the effect of proteases can result in self destruction.

2.7. PATHOGENIC ROLE OF PROTEASES.

The role of parasite proteases in disease pathogenicity is receiving a lot of research attention. Their narrow specificity makes them good targets for drug or molecular attack. Many parasite proteases have been identified as virulent factors of the disease in parasitic infections. For instance, elastase has been found to be a virulent factor in infection with *Pseudomonas aeruginosa*. When non virulent mutants that do not secret

elastase are infected together with elastase the mutants become virulent (Jones *et al*, 1993; Tamura *et al*, 1992). Proteases produced by *Bacteriodes nodosus* are involved in foot rot (Yunhao, 1991). Only pathogenic strains of *Porphyromonas gingivalis* produce the cysteine protease argingpain. Collagenase activity expressed in the lungs of transgenic mice is the main enzyme responsible for emphysematous lung damage (Armiento *et al*, 1992). The proteases produced by mycoplasmas are speculated to be involved in the development of lesions in immunocompromised patients (Simecka *et al*, 1993).

Activated neutrophils release proteases that are involved in accelerated destruction of red blood cells by promoting IgG binding to erythrocytes (Weiss *et al* ,1992). The proteases released by activated neutrophils have been shown to change the antigenicity of the red blood cells. Self antibodies bind to the modified membrane as they now bear a different antigen. This has been suggested as one of the main causes of the accelerated destruction of red blood cells in inflammatory diseases.

Argingpain from *Porphyromonas gingivalis* is capable of degrading collagen types one and four and immunoglobulin and has the ability to disrupt the functions of polymorphonuclear leukocytes (Kadowaki *et al*, 1994). The protease produced by *Aeromonas salmonicida* is associated with the haemorrhagic lesions seen in furuncles (Soafd, 1991) while the protease released extracellulary by *Legionella*

pneumophila is involved in the pathogenesis of Legionnaires disease due to its cytotoxic, tissue-destructive and phagocyte-inhibitory properties (Rechnitzer *et al*, 1992). This shows the capacity of proteases to destroy tissue and interfere with cellular functions.

Proteases are also capable of interfering with the functions of the immune system. The protease produced by *Legionella pneumophila* is capable of inactivating interleukin-2 and cleaving CD4 on human T cells thus interfering with the immune system (Mintz *et al*, 1993). The *streptococcal* pyrogenic exotoxin, which is a cysteine protease, cleaves inactive human interleukin-1 beta to produce the biologically active interleukin-1 beta, thus directly working on the cytokine pathway (Kapur *et al*, 1993). Thus proteases can help parasites establish themselves by interfering with the host immune system (Verwaerd *et al*, 1988), by degrading host proteins (Rosenthal *et al,* 1988), by facilitating invasion of host tissue and by preventing blood coagulation (Bordier, 1987; Daudi *et al*, 1991).

2.8. AFRICAN TRYPANOSOME PROTEASES

The most economically important African trypanosomes are the *Trypanosoma congolense, Trypanosoma vivax* and *Trypanosoma brucei.* These trypanosomes, like any other living organisms have the built-in mechanisms for their survival and reproduction which include the

synthesis of necessary enzymes such as proteases. Trypanosomes have been shown to release proteases in vitro (Nwagwu *et al*, 1988) and have both lysosomal and non-lysosomal proteases (Lonsdale-Eccles and Grab, 1987). A number of trypanosome proteases have since been isolated. An 80 kDa serine protease was isolated from *T. brucei* (Kornblatt *et al,* 1992) and cysteine proteases have been isolated from *T. brucei* (Pamer *et al*, 1990) and *T. congolense* (Rautenberg *et al*, 1982). All the trypanosome species listed above have been shown to have cysteine proteases which were later characterised (Mbawa *et al* 1992, Pamer *et al*, 1991). The cysteine protease from *T. congolense* has been shown to digest the VSG of trypanosomes, antibodies and other proteins such as fibrinogen and albumin (Mbawa *et al*, 1992). Other African trypanosomes have been shown to have similar cysteine protease activity and this is developmentally regulated (Lonsdale-Eccles and Grab, 1987; Mbawa *et al*, 1991; Pamer *et al*, 1991).

2.9 CLASSIFICATION AND SPECIFITY OF PROTEASES

2.9.1 CLASSIFICATION

Proteases are classified into two groups based on the part of the peptide they hydrolyse. Those which act on the terminal peptide bonds are called exopeptidases. Exopeptidases that act on the amino terminal are called aminopeptidases while those that act on the carboxyl terminal are

known as carboxypeptidases. Those proteases that hydrolyse the inside peptide bonds in a polypeptide chain are called endopeptidase. This class of proteases is divided into four categories based on the catalytic amino acid residues or element that are found at the active site. The metallo-proteases have a catalytic metal at the active site which is normally a zinc ion. The aspartic proteases (also called acid proteases as optimal activity is achieved at acidic pH) have two aspartic residues at the active site of which one must be ionised and the other un-ionised. The serine proteases have an unusually reactive serine residue (which forms an intermediate ester bond with the substrate) which is next to a histidine and aspartate residues. The cysteine proteases, also called thiol proteases, have an unusually reactive cysteine residue, which forms an intermediate thiol ester bond with the substrate, at the active site and is next to the imidazole group of histidine (Stryer, 1988).

2.9.2 GENERAL MECHANISM OF CYSTEINE PROTEASES REACTION

The reactive cysteine side chain of enzyme -CH_2-SH first reacts with the carbonyl carbon (-CO-) of the specific substrate to form the enzyme-substrate complex. The adjacent histidine serves as the proton acceptor. The amino terminal of the peptide bond is released. The enzyme-substrate complex then reacts with water to give the active enzyme and the

other substrate fragment. The enzyme is ready to undergo another cycle. See fig. 6 for a detailed chemical mechanism.

2.9.3 THE SPECIFICITY OF THE *T. CONGOLENSE* CYSTEINE PROTEASE (TRYPANOPAIN Tc)

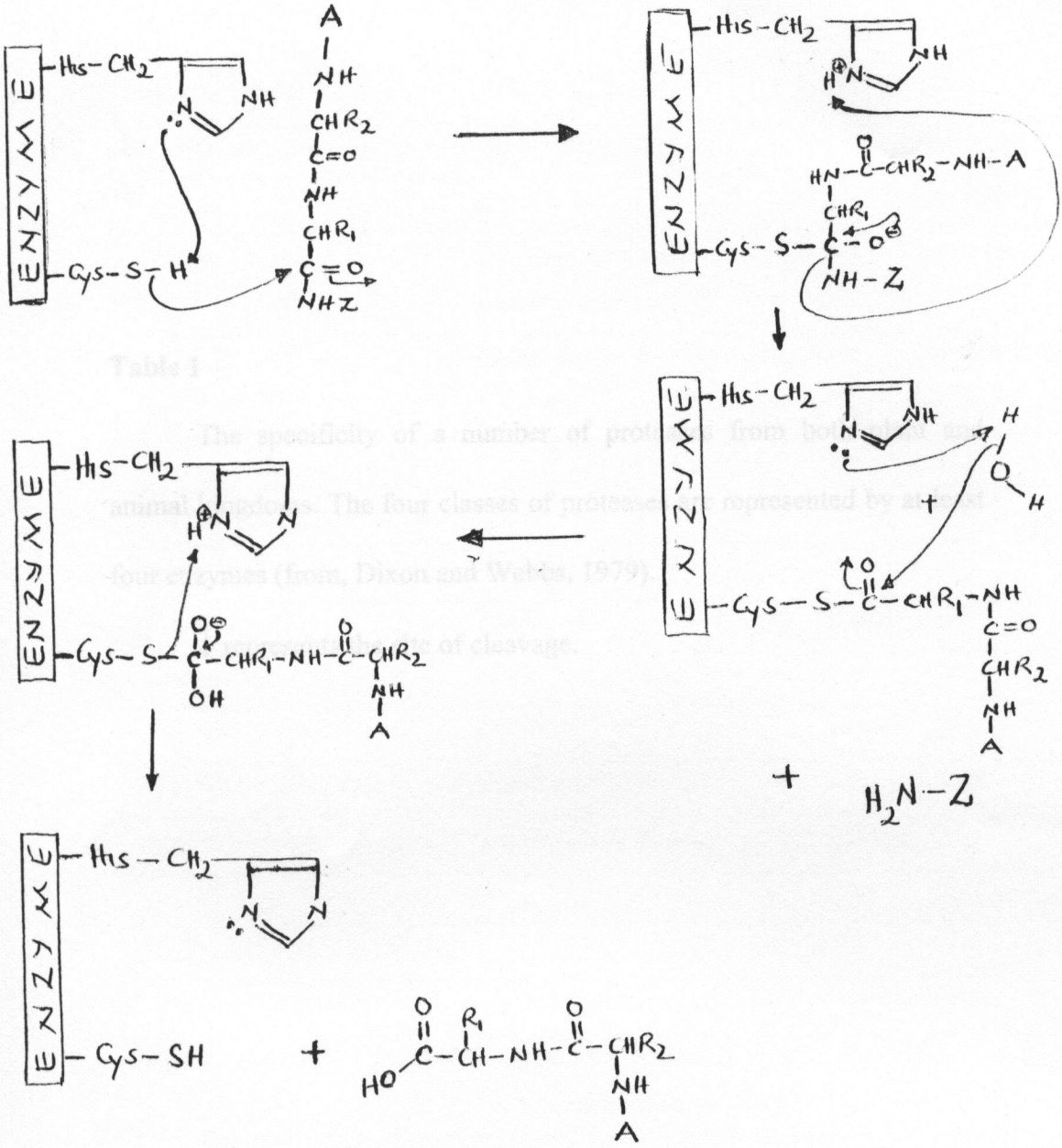
The best substrates of the of trypanopain Tc have been identified as those with either phenylalanine or leucine in P2 and P3 and either arginine or lysine in P1. This suggests that trypanopain Tc prefers bulky hydrophobic amino acids in P3 and P2 and amino acids with basic hydrophilic residue in position P1. For example, Cbz-Phe-Arg-NHMec (Cbz = carbobenzoxy) is hydrolysed very fast while Ac-Phe-Arg-NHMec (Ac = acetyl) is not hydrolysed at all thus suggesting that a bulky amino acid is required in P3 (Mbawa *et al*, 1992), where P1 and P2 are the first and second amino acids on the substrate polypeptide from the active site on the side towards the amino terminal. See figure 6 which shows the proposed amino acids at the site of cleavage. The substrates of trypanopain Tc have been found to be good substrates for the *T. brucei* cysteine protease suggesting how close these enzymes are related (Pamer *et al*, 1990). However, the anti-trypanopain Tc monoclonal antibody did not react at all with the *T. brucei* cysteine protease suggesting that the two proteases may be immunologically different (Authie *et al*, 1992).

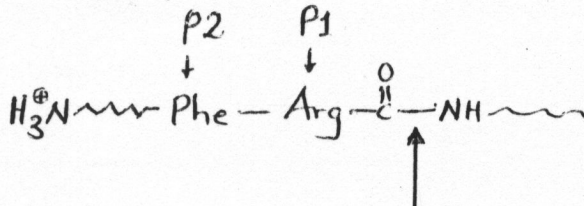
Fig. 6

MECHANISM OF CYSTEINE PROTEASES

The general mechanism of cysteine proteases showing the flow of electrons and the release of substrate and product(s) by the enzyme from the active site (prepared after explanation in 'Biochemistry', Stryer, 1988).

With respect to the *T. congolense* cysteine protease, R1= arginine given in the text as P1, R2= phenylalanine given in the text as P2. A= the rest of the substrate towards the amino teminal and Z the rest of the substrate towards the carboxyl terminal from the cleavage site of the substrate by the enzyme.


ENZYME
His–CH2
Cys–S–H
NHZ
+
H2N–Z


ENZYME
Cys–SH
HO

P2
P1
Phe
Arg
NH
COO⊖
Cleavage point
(bond)

Table 1

The specificity of a number of proteases from both plant and animal kingdoms. The four classes of proteases are represented by at least four enzymes (from, Dixon and Webbs, 1979).

↓ represents the site of cleavage.

SERINE POTEASES	SPECIFICITY	SOURCE
Chymotrypsin	Tyr-Trp-Phe-Leu-↓	Pancreas
Trypsin	Arg-↓,Lys-↓	Pancreas
Elastase	Carboxyl terminal of uncharged aromatic amino acids	*Pseudomona*
Cucumisin	Carboxyl side of acidic amino acids	*Cucumis melo* sarcocarp

ASPARTIC PROTEASES	SPECIFICITY	SOURCE
Pepsin	Phe-Leu-↓	Gastric juice
Chymosin	Phe-Leu-↓	Gastric juice
Cathepsin D	similar to pepsin but narrower	Liver/spleen
Lotus acid protease	Unknown	*Lotus* seeds
Aspergillus saitoi protease	Broad specificity	*Aspergillus saitoi*
Nepethes protease	Asp-↓ ,Lys-↓ ,Ala-↓	Thyroid

METALLO PROTEASES	SPECIFICITY	SOURCE
Crotalus atrox protease	Leu-↓,Phe-↓,Val-↓,Ile-↓	*crotalux atrox*,
Penicillum roqueforti protease	Bonds adjacent to hydrophobic amino acids	*Penicillum roqueforti*
Sea-urchin-hatching protease	Glu-↓, Asp-↓	Echinoiddea,

THIOL PROTEASES	SPECIFICITY	SOURCE
Cathepsin L	Arg-↓,Lys-↓, Phe-X-↓	Tissue enzyme
Papain	Arg-Lys-↓, Phe-X-↓	*Papaya* latex
Bromelain	Lys-Ala-Try-Gly-↓	pineaple
Staphylococcal protease	Broad specificity	*Staphylococcus* sp
Trypanopain Tc	Leu-Phe-Arg-↓	T. *congolense*

2.10. CONNECTIVE TISSUE PROTEINS

Connective tissue proteins play a critical role in cell structure, cell orientation and cell interaction. Without connective tissue proteins most tissue will just be a collection of individual cells. It can therefore be imagined that connective tissue proteins are likely to be as diverse as different roles this class of proteins play in different specialised tissues. About 75% of the total connective tissue proteins in animals is collagen, a glue like protein that is very crucial in keeping the cells intact. Elastin, a protein with elastic properties, has the ability to stretch and return back to its original form. Other members of this group include fibronectin, which plays an important role in extracellullar interaction, laminin which also plays an important role in intracellular interaction, and proteoglycans which form the bulk of the intracellular matrix (Stryer, 1988).

2.10.1 COLLAGEN

Collagen is an insoluble fibrous molecule with high tensile strength. It is the major element of skin, bone, tendon, cartilage, blood vessels and teeth. It holds cells together in discrete units. Collagen has an amino acid composition distinct from many other proteins. It is rich in glycine (30% or more) and in proline (10%) and is rich in hydroxyproline and hydroxylysine. Collagen has five (5) main groups. Collagen type I which is the main collagen form found in skin, tendon, bone and cornea.

Collagen type II is mainly found in cartilage, inveterbrate discs and vitreous body. Collagen type III is found in foetal skin, cardiovascular system, reticular fibres. Collagen type IV is found in the basement membrane and collagen type V is found in placenta and skin. Enzymes that degrade collagen are called collagenases. They are broadly grouped in two classes of collagenases depending on their specificity. The tissue collagenases are metalloproteases and are highly specific and cleave collagen at -Gly-$^{\downarrow}$-Ile- whereas the bacterial proteases such as *Clostridium histolyticum* proteases specifically cleave at the amino-peptide bond of glycine (X-$^{\downarrow}$-Gly-Pro-Y).

2.10.2 ELASTIN

Elastin is found in tissues in conjunction with collagen. Its ability to stretch many times its own length and return back to its original size and shape when the tensile is released gives it a special place in tissues like the aorta, blood vessels and neck of grazing animals, all of which require to stretch and return back to their original shape. Like collagen, elastin has a distinct amino acid composition with almost every third amino acid being glycine and it is rich in proline. Unlike collagen, it has less hydroxyproline and no hydroxylysine. It is digested by a class of proteases called elastases.

2.10.3 FIBRONECTIN

Fibronectin is a cell surface protein that is crucial in cell communication. It is involved in cell interactions with the extracellular matrix by binding to the external face of the plasma membrane irreversibly. Fibronectin consists of 250 kDa polypeptide chains that are linked through a disulphide bond near the carboxyl termini. It contains an array of domains that specifically bind molecules outside the cell. For example in blood clotting, fibronectin binds to Factor XIII transamidase, which catalyses the final step in blood clotting, and this makes it possible for fibroblasts and other cells that repair the injury sites to bind to the clot by interacting with fibronectin. It is also crucial in cell migration during the development of the embryo. Findings that malignant cells are deficient in fibronectin maybe explains their anarchic and invasive properties which maybe the result of breakdown in cell communication (Stryer, 1988). Recently, it was demonstrated that a tumour cell line U937 A which was depositing fibronectin in the intracellular matrix, was invasive and metastatic *in vivo* while it produced large, loosely packed colonies *in vitro*. A mutant cell line U937 A/R which was resistant to killing by tumour necrosis factor did not deposit fibronectin in the extracellular matrix and thus was likely to have recycled its fibronectin and was found not to be invasive or metastatic *in vivo* and produced tightly packed small colonies

in vitro (Matthews and Neale, 1990). All the above shows the central role fibronectin plays in cell communication.

2.10.4 LAMININ

Laminin is another adhesive glycoprotein of the extracellular matrix. It enables epithelial cells to attach to underlying connective tissue. It is a triple helical protein with an average molecular weight of about 1000 kDa. Like fibronectin, laminin has specific binding domains (Stryer, 1988). Laminin from different tissues has been found to have similar structure and immunochemical properties (Paulsson *et al,* 1991). Laminin has always been thought to be an extracellular protein until recently when laminin has been located in endocrine cells in the rat pituitary (Kikuta and Namiki, 1992).

2.10.5 RELATIONSHIP TO PATHOGENESIS

It is obvious from the description above that connective tissue proteins play an important role in cell communication, cell migration or multiplication and cell attachment and repair of injury sites. Destruction of such proteins can have far reaching consequences as the above processes may be damaged.

Recently, a lot of researchers have demonstrated that micro organisms that bind to cells do so by interacting with the connective

proteins especially fibronectin and to some extent laminin and collagen. Streptoccoci and staphyloccoci bind to the N-terminal domain of fibronectin and *E.coli* binds to the internal heparin binding domain of fibronectin through the proteins that these organisms express (Visai *et al,* 1991). A surface tetragonal paracrystalline array composed of a single protein (50.8 kDa) is a virulent factor in *Aeromonas salmonicida.* The protein has high affinity for fibronectin and laminin (Doig *et al*, 1992). In this way bacteria can bind to host cells through the proteins they express on the surface by interacting with the connective tissue proteins. Even specialised receptors, such as the human C1q receptor (C1q-R), which is a 65-70 kDa highly acidic glycoprotein that is expressed on a wide variety of cell surfaces and binds preferentially C1q (a complement component protein), can also bind laminin, fibronectin and C1q-inhibitor (a proteoglycan) (Ghebrehiwet *et al*, 1992). It is clear that if an organism expresses fibronectin on its surface or expresses a protein that binds fibronectin or laminin it can easily interact with a cell that will bind such proteins through its own connective tissue proteins. Through such a process infection is more likely to take place than in a case where such interaction was absent.

2.11 ENZYME PURIFICATION PROTOCOLS

2.11.1 CHOICE OF METHOD

When choosing methods of purification of an enzyme it is necessary to ensure that the activity of the enzyme is not lost during purification. It is important to know the best source of the enzyme as most enzymes do not exist in large quantities in nature. Its also important to know a fast and reliable method of assessing the presence of the enzyme so that one knows which fractions contain the enzyme. It is equally important to know a method of analysing the extent of purity of the molecule achieved after every stage. This helps avoid unnecessary steps when the enzyme is purified while in other cases helps one to take extra steps if the enzyme is still impure. The level of purity aimed at depends on the nature of the use of the enzyme after purification. Finally, one has to know how to measure the enzyme concentration and the activity of the enzyme.

2.11.2 SOURCE OF THE ENZYME

The source of the enzyme was trypanosomes. Trypanosomes can be grown either in culture or in rats or mice. Cell culture adapted trypanosomes require large amounts of media and special conditions (sterile hoods, flasks and incubators) that were not only expensive but were not available in our laboratory. The blood stream forms maintained

in culture remain in the long slender form for most of their life and the overall trypanosome yields are normally low (10^8 trypanosome per 8 T25 flasks) (personal experience). The long slender forms are not the best source of the cysteine proteases.

Mice give very little blood, about 2 ml per mouse and this means that a large number of mice (about 50 mice) would be needed to give about 100 ml of infected blood normally required for purification to give about 4 x 10^{10} trypanosomes. Adult rats on the other hand give more blood, about 10-15 ml per rat, and only about 10 rats are needed to get more than 100 ml of infected blood. It is also possible to have very good yields of parasites, about 10^{10} trypanosomes per 3 rats (personal experience), and achieve good yields of stumpy forms, at least 50% by supplementing the drinking water with glucose (personal discussion with Dr. N Murphy, ILRI, Nairobi). The stumpy forms have the highest cysteine protease activity, more than the other two blood stream forms (Mbawa *et al*, 1992; Pamer *et al*, 1991). Rats are easy to buy or breed in Zambia. The project therefore used rats for the above reasons

2.11.3 ENZYME ASSAY METHOD

Though there are many ways of determining the presence of any enzyme, most of the methods utilise the ability of enzymes to catalyse specific reactions. The reaction should be fast, reproducible and easy to

monitor by either colour change, change in absorption or by fluorescence of the product(s). The method should be very sensitive requiring only very little amount of the pure enzyme. In the study there were three methods that had been used for similar work which met all the above conditions. The chromogenic and fluorogenic substrates Cbz-Phe-Arg-ρNA, Cbz-Phe-Arg-NHMec and Cbz-Arg-Arg-NHMec (Cbz = Benzyloxycarbonyl; ρNA = paranitroanilide and NHMec = 7- amino-4-methyl coumarin) have all been used previously to assay the enzyme activity and presence during purification of the cysteine proteases from *T. congolense* and *T. brucei* (Mbawa *et al*, 1992; Pamer *et al*, 1991). The reaction depends on the release of either 7-amino-4-methylcoumarin (fluorogenic) or nitroanilide (absorbs at 400 nm). The project used Cbz-Phe-Arg-NHMec as it required less amount of enzyme and required a shorter reaction time than Cbz-Phe-Arg-ρNA.

2.11.4 CONCENTRATION AND PURITY ANALYSIS

The method of choice for analysing the purity of the enzyme was sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). It is mainly used to estimate the molecular mass of reduced proteins or even compare different samples and see the number of bands that each sample produces and the mobility of the protein bands. However, if a sample gives only one band then it is likely that the sample is composed of

only one molecule. This is the assumption one makes when using it to determine purity of a given molecule.

The concentration of the sample is very crucial in determining whether the sample will stain with coomassie blue or silver stain or whether it can not stain at all. The modified micro-lowry method of determining small concentration (in μg/ml) of proteins was adopted as described by Sigma procedure number P 5656. The method is reproducible and reliable.

2.11.5. ENZYME PURIFICATION PROCEDURES

Purification of most enzymes require many various steps that normally last for many hours. Pamer *et al*, 1991 described a method that was used to purify the cysteine protease from *T. brucei*. The method required centrifugation at 100,000 x g, salting out, affinity chromatography on two columns, elution from each column, dilution with water four fold and then application on a third column followed by elution and then lyophilisation. This procedure seems to require many steps and loss of some enzyme at each step can not be overlooked. Inactivation is also likely as the method seems to be long though time taken for each step was not reported. The procedure started with 1-1.5 $x10^{10}$ trypanosomes, however the pure enzyme did not stain with any method used to stain proteins resolved on acrylamide gel, demonstrating that the yield was very low.

Kos *et al* (1986) described a method for purification of cysteine proteases (cathepsin B from human lung) using chicken egg white cystatin (cysteine protease inhibitor) as a ligand for affinity chromatography. This method was later successfully used by Mbawa *et al* (1991) to purify the *T. congolense* cysteine protease. It is a two step purification and is thus a fast method. Mbawa (Thesis, 1992), described another method which used thiopropyl-sepharose and sephadex 25 columns. This three step purification procedure was reported to give similar yields to that of the cystatin column.

Some of the *T. congolense* cysteine protease was purified on monoclonal antibody column as described by Authie *et al* (1992). This was the quickest, simplest and, maybe, the cheapest method of purifying the enzyme. The yields were however low, about 20% lower than those given by the cystatin-sepharose method. The antibody column has been reported not to bind the homologous *T. brucei* enzyme which was another drawback of the method.

The project thus used the cystatin-sepharose affinity column for most of the purification procedures. It was relatively faster and gave better yields than other methods. The purity of the enzyme from the column was good and the enzyme was active. Its main drawback was the high cost of cystatin (British Pound 103.5=00 per mg), making it the most expensive method.

3.0 MATERIALS AND METHODS

3.1 MATERIALS

The commercial macromolecules collagen type I (from calf skin) and type IV (from membrane basement of englebreth-holm-swarm mouse sarcoma), elastin (from bovine neck), fibronectin (from bovine plasma), laminin (from membrane basement of englebreth-holm-swarm mouse sarcoma), glyco-proteins (bovine cohn fraction IV), gelatin (from bovine skin) and fibrinogen (from bovine plasma) were all purchased from Sigma Chemical Co. (St. Louis, USA). The protease inhibitors trans-epoxysuccinyl-ι-leucylamido (4-guanidino) butane (E-64), cystatin (from egg white), aprotinin, Nα-ρ-tosyl-l-ι lysine chloromethylketone (TLCK), phenylmethylsulfonyl-fluoride (PMSF), leupeptin and the fluorogenic substrate carbobenzoxy-phenylalanyl-arginyl 7-amido-4-methylcoumarin (CBZPhe-Arg-NH-Mec) were supplied by Sigma Chemical Co (St.Louis, USA).

Earle's balanced salt solution (EBSS), trypsin, l-glutamate, tryptose phosphate broth, fetal bovine serum (Fbs), antibiotics and minimum essential media (MEM) were purchased from GIBCO laboratories (New York, USA). Acrylamide, N,N'-methylene bisacrylamide (bisacrylamide), 2-mercapto-ethanol (2-ME) and sodium dodecyl sulphate (SDS) were purchased from BDH Chemicals Ltd (Poole, England). Percoll, CNBr-activated sepharose 4B, Sephacryl S-200 HR, 4-

(2-hydroxyethyl)-1-piperazine sulphonic acid (HEPES), hypoxanthine, triton X-100, dithiothreitol (DTT), dimethyl-sulphoxide (DMSO), tris (hydroxymethyl) aminomethane (Tris), ethylenediaminetetraacetic acid disodium salt (EDTA), glycine (chromatography reagent), low range and high range molecular weight protein markers for sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), ι-cysteine, ammonium persulphate (electrophoresis reagent), bromophenol blue, coomassie brilliant blue 250-R, Bradford protein concentration assay kit, bovine serum albumin (BSA) protein standard, Folin and Ciocalteu's phenol reagent, glycerol, N,N,N',N', tetramethylethylenediamine (TEMED), sodium azide, diethylamine (DEA), elastin-congo red were supplied by Sigma Chemical Co. (St.Louis, USA). Diethylamino ethyl cellulose (DE52 cellulose) was a Whatman laboratories division product (Maidstone, England). Centriprep 10 and centrico 10 concentrators were from Amicon (Uppermill, U.K.).

3.2 PREPARATION OF TRYPANOSOMES AND EXPERIMENTAL ANIMALS

3.2.1. PREPARATION OF TRYPANOSOMES

3.2.1.1. ISOLATION FROM CATTLE

One stabilate of *T. congolense* was isolated from naturally infected cattle in the Eastern Province of Zambia (Petauke area) and another

T. brucei stabilate was isolated from infected cattle under natural challenge in the Central Province of Zambia (Mumbwa area).

The blood was collected as described by Murray *et al*, (1983). Generally, the blood was collected from animals that had not been treated with any trypanocidal drugs in at least the previous six months or was collected from animals which looked ill with symptoms of trypanosomosis. The blood was initially collected from the ear veins into capillary tubes. One end of the tube was sealed with cristaseal and then centrifuged in a microhaematocrit centrifuge at 12,000 rpm for 5 minutes. The tube was cut with a diamond pencil about 1 mm below the boundary between packed red blood cells and the plasma which carried the buffy coat (i.e to include 1 mm of red blood cells). The buffy coat (a concentration of white blood cells and trypanosomes if present and some red blood cells) was placed on a microscope slide and screened for trypanosomes. When the animal was found to have trypanosomes, blood was collected from the jugular vein of the animal using vacutainer tubes treated with EDTA. The blood was diluted 1:2 (v/v) blood with phosphate buffered saline PBS, pH 7.4, and 0.02 ml was inoculated into mice intraperitoneally.

3.2.1.2 CLONING OF TRYPANOSOMES

The infected mice were screened for trypanosomes by examining wet blood film preparations every day. The mice that showed trypanosomes in the blood (10^6/ml) were bled from the tail, 4 drops in 2 ml PBS, and the blood used to infect another set of mice. This second set of mice was screened for trypanosomes as earlier explained above.

The trypanosomes were then partially cloned by counting the number of purified trypanosomes from the second set of mice using the improved neubauer cell counter chamber. The trypanosome solution was then appropriately diluted to give an average of one trypanosome per 200 µl of solution. Six mice were put in an incubator at 37°C for 30 minutes. 100 µl of the diluted final solution was intravenously administered through the dilated inferior tail veins using a 1 ml syringe and 21G needle. The mice that developed the disease, out of the six mice used for each of the trypanosome stabilate, were bled and the blood was used as the source of trypanosomes. Cloning was done as an attempt to raise a single trypanosome population or at least a single infection clone e.g *T. brucei* only. Two well-established clones: *T. congolense* IL- 3000 and *T. brucei* IL-Tat 1.1 were kindly provided by the International Livestock Research Institute (ILRI), Nairobi, Kenya.

3.2.2 EXPERIMENTAL ANIMALS

Rats were used for the cultivation of trypanosomes *in vivo*. In-bred adult male albino rats aged 5 months or older were required although on a few occasions female rats were used when stocks of male rats were low. Mice on the other hand were not used for cultivation of trypanosomes due to the large number of mice needed to obtain 50 ml or more of highly infected blood required for purification of trypanosomes. The animals were fed *ad libetum* on commercial mouse comproides (2 X 25 Kg) kindly provided or purchased from National Milling Co., Lusaka and fresh tap water was supplied twice per day. The animals were kept in metallic cages in a fly free room at the small laboratory animal facility of the School of Veterinary Medicine or the small animal unit of ILRI (for animals used at ILRI).

3.2.3 CULTIVATION OF TRYPANOSOMES.

The mice that were infected with trypanosomes in section 3.2.1.2. were bled from the tail once the parasitaemia had reached at least 10^8/ml as estimated using the neubauer cell counter and the blood collected directly into a tube containing 1 ml PBS. The trypanosomes were then diluted with phosphate buffered saline (PBS) pH 7.4 to give a final concentration of 10^5/ml. 500 µl of this diluted blood was inoculated into each rat intraperitoneally using the appropriate syringes (2.5 ml) and

needles (21G). The animals were administered with 600 rads prior to infection with *T. congolense* using the National Council for Scientific Research (Chilanga station) irradiation facility or ILRI irradiation unit. *T. brucei* infection did not require prior irradiation of animals as it normally killed by the 6th day. The parasitaemia of the infected rats was checked daily using microscopic examination of a wet blood smear until the number of trypanosomes had reached at least 10^8/ml of blood (normally on the fourth day) determined as described by Murray *et al,* (1983). The rats with high parasitaemia were anaesthetised in chloroform or carbon dioxide and then bled by cardiac puncture.

In the case of *T. brucei* the drinking water for the rats was supplemented with 10% w/v glucose when the parasitaemia had attained 10^8/ml of blood as estimated using a neubauer cell counter. The animals normally survived two days more than those not supplied with glucose in drinking water and the levels of stumpy forms was monitored microscopically using giemsa stained microscope slides. Briefly, a slide smear was prepared by drawing a drop of blood from the tail and smeared along the slide using another slide edge. The blood slide was air dried and then fixed in pure methanol for about 1 minute and then stained in 5 % geimsa solution for 5 minutes, followed by a wash under running water. The rats were bled when the level of parasitaemia of stumpy forms had achieved at least 50% or conversely, when the level of the long slender

forms dropped to less than 50%. This was necessary because it is reported that the stumpy forms have higher levels of the cysteine proteases than any other mammalian forms (Pamer *et al*, 1991: Mbawa *et al*, 1992). Measurement of percentage of the stumpy forms could not be applied to *T. congolense* which does not clearly show detectable morphological changes similar to that shown by *T. brucei*. However, *T. congolense* trypanosomes were harvested from terminally ill rats which were also supplied with glucose mainly to increase the harvest of parasite by prolonging the disease and this was hoped to increase the yield of stumpy like parasites.

3.3 COLUMN CHROMATOGRAPHY

3.3.1 ION EXCHANGE CHROMATOGRAPHY : DE52 CELLULOSECOLUMN PREPARATION

100 g of pre-swollen DE52 cellulosewas dissolved in 1000 ml of phosphate saline glucose (PSG) pH 8.0 and the final pH of the solution adjusted to 8.0 using 10% H_3PO_4 as recommended by the manufacturer on ways of equilibrating the resin (Use of Whatman ion exchange celluloses in the laboratory, IL6, Whatman, England). The solution was allowed to settle for about 15 minutes and the fines were aspirated off using a water pump. This cycle was repeated twice. If this was not done, the fines were eluted and increased the size of the trypanosome pellet.

The DE52 cellulose was stored in PSG pH 8.0 containing 2% sodium azide at 4°C or was loaded onto a column of 12 x 4 cm to give a packed (settled) height of about 5 cm (locally improvised by the author using a mice water bottle with the bottom cut) or on a 6 x 3 cm column (for purifications performed at ILRI). A Whatman No. 41 filter paper was placed inside and the slurry was loaded and allowed to pack under gravity. The column was always packed at least 60 minutes before being used for trypanosome purification. 100 ml of PSG was run through the column and its pH checked.

3.3.2 PREPARATION OF EXCLUSION CHROMATOGRAPHY COLUMN: SEPHACRYL S-200 HR

200 ml pre-swollen Sephacryl S-200-HR resin was suspended in 600 ml of degassed buffer 50 mM Tris/HCl, pH 7.5 containing 0.1M NaCl, 1 mM EDTA, 0.05% Brij 35. After the gel had settled, the fines were aspirated off using a water pump. The above procedure was repeated twice. The resin was then loaded onto a column to give a packed volume of 1.5 x 3.0 cm. The flow rate was adjusted using the flow adapter purchased together with the column to give a flow rate of about 20 ml per hour. The solution used was degassed for at least one hour using a water tap in order to avoid the formation of air bubbles which readily formed in about 20 minutes if undegassed solution was used. 0.02% sodium azide

was added to the above buffer when it was used to store the resin at 4°C until required for enzyme chromatography.

3.3.3 PREPARATION OF AFFINITY CHROMATOGRAPHY COLUMN: COUPLING CYSTATIN TO CNBr-ACTIVATED SEPHAROSE

The coupling of cystatin, the ligand, to sepharose 4B gel was done as described by Pharmacia Biotech, edition AB 71-7086-00, for immobilisation of proteins on CNBr-activated sepharose. 0.5 g of freeze dried CNBr-activated sepharose 4B was swollen for 15 min in 1 mM HCl and washed on a sintered glass filter (porosity G3) with 100 ml 1 mM HCl. 4 mg cystatin ligand was suspended in 8 ml of 10 mM Tris/HCl pH 8.0, in 50% glycerol, and mixed with 2 ml coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl). The coupling buffer contained high sodium chloride concentration (0.5 M) to minimise protein-protein adsorption and formation of protein aggregates. The resin (sepharose) and protein (cystatin) solutions were mixed on an end over end mixer for 2 hours at room temperature. The remaining active sites of the resin were blocked with 0.2 M glycerol pH 8.0 for 2 hours at room temperature. The excess adsorbed proteins on the resin were washed away with coupling buffer followed by washes with 0.1 M sodium acetate pH 4.0, 0.5 M NaCl

and by the coupling buffer again. The resin was centrifuged at 1000 rpm for 3 minutes and stored at 4°C until required for purification of enzyme.

3.4 PURIFICATION OF TRYPANOSOMES

3.4.1 PREPARATION OF PHYTOHAEMAGGLUTININ (PHA)

Phytohaemagglutinin (PHA) is a lectin which specifically binds N-acetyl-D-galactosamine, a red blood cell membrane carbohydrate. It does not agglutinate lymphocytes. The levels of lectins in local beans (*Phaseolus vulgaris L*) are sufficient for use in agglutination of red blood cells. About 200 g of dried beans were ground very finely using a coffee grinder for 5 minutes. The beans' flour was dissolved in 400 ml of PBS and stirred for ten minutes to maximise the aqueous extraction of lectins. The mixture was allowed to stand for 15 minutes. The clear supernatant was decanted into a 500 ml beaker and its pH adjusted to 8.0 (the pH of ion exchange purification of trypanosomes from rats' blood). 10 ml aliquots of the solution were transferred to centrifuge tubes. The solution was centrifuged at 4°C for 10 minutes at 12000 rpm in a Beckman centrifuge. The supernatant was either used or stored at -20°C in a freezer for future use.

3.4.2. PURIFICATION OF TRYPANOSOMES ON DE52 CELLULOSE

The infected blood was prepared in two different ways. In one method the infected blood was subjected to centrifugation over Percoll density gradient generated *in situ* as described by Grab and Bwayo (1982). The infected blood was mixed with percoll solution pH 7.4 (see appendix) in a ratio of 1:1 v/v and centrifuged in a refrigerated high speed Beckman centrifuge at 16,000 rpm for 30 minutes at 4°C. The red blood cells were packed at the bottom and the trypanosome milk-like band in the middle was carefully isolated. The trypanosome solution was diluted four times with PSG, pH 8.0, and then centrifuged at 2500 rpm for 15 minutes to wash away excess percoll. The supernatant was discarded and the trypanosome pellet re-suspended in a minimal volume (2 ml) of PSG pH 8.0. The trypanosome solution was carefully layered on the DE52 cellulose column.

In the second method, the infected blood was diluted with an equal volume of PSG pH 8.0 and then incubated with PHA (1:10 (v/v) PHA : the initial blood volume) for 10 minutes at 37°C in a water bath. It was then centrifuged at 500 rpm for 5 minutes. The red blood cells settled at the bottom leaving the trypanosomes in the supernatant. The trypanosome rich supernatant was loaded onto the column.

The trypanosomes were basically purified from blood elements by ion exchange chromatography using DE52 cellulose column as described by Lanham and Godfrey (1970), for the purification of salivary trypanosomes from rats' blood elements. In each case the elution procedure was the same. The trypanosomes were eluted with PSG pH 8.0, ionic strength (I) 0.221. The trypanosomes were collected in 100 ml of PSG pH 7.4 supplemented with 100 μM hypoxanthine (Lonsdale-Eccles, 1987).

The trypanosomes were washed twice with 10 ml of PSG, pH 7.4. The trypanosome solution was concentrated by centrifugation in a Beckman centrifuge at 2500 rpm for 15 minutes at 4°C. The trypanosome pellet was dispersed in minimal phosphate buffered glucose saline pH 7.4. The number of trypanosomes was determined using a neubauer (improved) cell counter and the trypanosomes were stored in a freezer at -70 degrees. This served as the source of enzymes.

The trypanosomes to be used for protease purification on the monoclonal antibody column were frozen down with three protease inhibitors 60 μl leupeptin (40 μM), 100 μl PMSF (40 μM) and 100 μl EDTA (5 mM). This was needed to block or minimise the digestion of the antibodies by proteases. However, the trypanosomes to be used for enzyme purification on cystatin-sepharose column were not frozen with inhibitors.

3.5. PROTEIN ELECTROPHORESIS: PREPARATION OF SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The gels were prepared according to well-established procedures like those which were in use at ILRI and in our laboratory. The resolving gel was either 8.5% acrylamide, pH 8.7 for high molecular weight range (200 kDa - 29 kDa) and 11% acrylamide, pH 8.7 for low molecular weight range (70 kDa-14 kDa) and the stacking gel was 5% acrylamide, pH 6.5 (see appendix for preparation of solutions). For any other preparations of homogenous gels the composition was as indicated in the text. The solutions wereall degassed for at least 5 minutes. The resolving gel was poured between the glass plates (14.4 x 11 cm) with seals in place (ring) and 0.1 ml of water or butanol was layered above the gel very carefully without disturbing the surface. When the resolving gel had polymerised, the layer of water or butanol was removed and the surface washed with 0.5 ml water. The stacking gel was immediately layered on the separating gel and a 10 well comb was put in place. Once the stacking gel had polymerised the comb was removed and the wells were washed with the running buffer.

The apparatus was set up and the two chambers were filled with the running buffer (2.4g Tris, 11.52g glycine and 2 ml 20% SDS were mixed and made up to 400 ml with water). The sample was mixed with its

appropriate sample buffer in a ratio of 1:1 (v/v) (see appendix for details). The mixture was boiled for 3 - 5 minutes. 10 µl of the sample was loaded on the gel. The gels were run at 25 mA/gel until the stain (bromophenol blue) front reached the bottom. The power was switched off and the gel carefully removed from the plates.

3.6 STAINING OF RESOLVED PROTEINS AND PROTEIN CONCENTRATION DETERMINATION

3.6.1. COOMASSIE BLUE STAINING

The staining of resolved proteins with coomassie blue was performed as described by Sigma Chemical Co. The proteins resolved on the gels were stained by incubating the gel with 0.1% (w/v) coomassie brilliant blue R-250 in a solution of acetic acid : methanol : water (10 : 30 : 60, v/v/v) for 3 hours or more. The gel was then destained in acetic acid:methanol:water(10 : 30 : 60, v/v/v) until bands were clear and then stored in 10% acetic acid.

3.6.2 SILVER STAINING

The method for silver staining was kindly faxed to me by Dr. Edith Authie, ILRI, Nairobi, Kenya. Silver staining was performed in clean glass ware and the gel was not touched or handled with gloves to avoid finger printing and all the process of staining took place in the same container.

The gel containing resolved proteins from section 3.5 was fixed in a solution of methanol, acetic acid and water (50 : 10: 40 v/v/v) for at least 30 minutes or more. The gel was washed twice in a solution of acetic acid, ethanol and water (5 : 10 : 85 : v/v/v) for 30 minutes or more each time. The gel was then washed twice with water for two minutes. The gel was incubated with a solution composed of 3.2 mM potassium dichromate and 3.4 mM nitric acid for 5 minutes followed by two consecutive washes with water for 2 minutes each. The gel was incubated with 2% (w/v) $AgN0_3$ solution for 25 minutes. A developing solution was made up of 3% (w/v) Na_2CO_3 and 0.019% (v/v) formaldehyde. The gel was quickly washed twice with water for one minute each time and then washed with small aliquots of the developing solution twice. It was then immersed in developing solution completely and placed on a shaker until clear bands appeared. The developing process was terminated by washing the gel with about 20 ml of 5% (v/v) acetic acid for 3 minutes. The gel was stored in 5% (v/v) acetic acid .

3.6.3 PROTEIN CONCENTRATION DETERMINATION

The protein concentration of the enzyme was assayed using the Bradford protein assay kit which is the micro-lowry method. It was used as recommended by the supplier (Sigma Chemical Company, procedure number P 5656). The working range was between 50 μg/ml to 400 μg/ml

of BSA (bovine serum albumin), as a standard, in which most of the samples fell. The BSA standard was reconstituted to give 0.0 μg (blank), 50 μg, 100 μg, 150 μg, 300 μg and 400 μg/ml using deionized water. The samples were also appropriately prepared and/or diluted to give an equal volume to that of the standards.

An equal volume of Lowry's reagent solution was added and mixed well. The tubes were allowed to stand at room temperature for 20 minutes. Folin and Ciocalteu's phenol working reagent was prepared by mixing 18 ml of Folin and Ciocalteu's phenol reagent with deionized water up to 80 ml in the bottle supplied together with the kit. With rapid and immediate mixing 0.5 ml Folin and Ciocalteu's phenol working reagent was added to the tubes. The tubes were allowed to stand for at least 30 minutes. The absorbance was read at 750 nm using a Hitachi U-2000 spectrophotometer and the readings were completed within 30 minutes. The absorbance values of the protein standards were plotted against their concentrations manually on a graph paper. The concentration of the samples were read or estimated from the graph using their absorbance.

3.7. ENZYME ASSAY AND DETERMINATION OF ENZYME CONCENTRATION

3.7.1 USING A FLUOROGENIC SUBSTANCE

A solution of 50 mM CbzPhe-Arg-NHMec (fluorogenic substrate) was made in 100% DMSO as described by Mbawa *et al,* (1992). The substrate was diluted to a final concentration of 50 μM in assay buffer (50 mM Tris, 50 mM citric acid pH 6.0, 2 mM EDTA, 5 mM DTT). 300 μl of this solution was delivered into micro ELISA wells according to the number of samples. 40 μl of the samples suspected to contain the cysteine protease or column fractions collected during elution of the enzyme were mixed with the buffer containing the fluorogenic substrate and then viewed using a Chromato-Vue C-70G viewing system at 365 nm. Samples containing appreciable amounts of the enzyme became illuminated (fluoresced with a blue like colour) within 3-5 minutes.

3.7.2 USING CONTACT ZYMOGRAM

This method utilises co-polymerization of protein substrates in the preparation of the resolving gel as described by Lonsdale-Eccles and Mpimbaza (1986). 300 μg/ml of the protein substrate of interest was included in the preparation of the solution for the resolving gel. The gel was cast as described in section 3.5. The sample buffer did not contain 2-mercaptoethanol as this has been reported to increase the rate of enzyme

auto-degradation and destaining of the gel. The sample buffer was composed of 0.04 M Tris/HCl pH 8.7, 4 M urea containing 2% (v/v) Triton X-100 and 0.1% SDS (w/v). The sample was also not boiled as this inactivated or denatured the enzyme. The gel was prepared and electrophoresis run as explained in section 3.5. After electrophoresis the gel was washed twice in cold water (4°C) for 10 minutes each time. The gel was then incubated for 6 hours to overnight in the incubating buffer (50 mM citric acid, 50 mM Tris containing 5 mM DTT and 2 mM EDTA) at pH 6.0 at 20°C. The gel was then stained and destained as described in section 3.6.1 for coomassie brilliant blue.

3.7.3. ENZYME PURITY ASSAY

The enzyme purity at any stage of purification was assayed by running the enzyme sample on SDS-PAGE. 30 µl of the concentrated enzyme solution was mixed with 30 µl of sample buffer and boiled for 3-5 minutes. 15 µl of the boiled sample was loaded on the gel (11% acrylamide) after it had cooled and low range molecular weight markers were loaded on an adjacent well. After electrophoresis the gel was silver stained as explained in section 3.6.2.

3.8 CYSTEINE PROTEASE PURIFICATION PROCEDURES

3.8.1 PURIFICATION OF TRYPANOPAIN TC USING THE MONOCLONAL ANTIBODY COLUMN

Using samples prepared as described in section 3.3.1, about 5 x 10^9 trypanosomes in a pellet were mixed with 10 ml of distilled water and vortexed for a short period. A wet microscope slide was made to confirm lysis of the trypanosomes. 2 ml 10 x PBS (ten times the concentration of standard PBS, see appendix) was then added and the mixture was centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was loaded on the monoclonal antibody column equilibrated at about 4°C in degassed PBS pH 8.0. The elution was followed by measuring the absorbance of proteins at 280 nm using a single path monitor connected to a printer. Degassed PBS at 4°C was used to elute the unbound protein until the graph returned to the base line.

The enzyme was first eluted by 50 mM DEA (diethylamine) pH 11.0. The enzyme was collected in 2 ml 50 mM Tris/HCl, pH 2.3, 0.15 M NaCl in a 50 ml tube until the baseline was attained. The column was washed with about 10 ml PBS pH 7.4. The remaining bound enzyme was eluted with 0.1 M glycine/HCl pH 3.0. The enzyme was collected in 50 ml tubes containing 5 ml 50 mM Tris/HCl pH 8.5 until the base line was achieved. The elution depended on the change in pH from that at which the enzyme was originally bound on the anti cysteine protease antibody.

The pH of the two enzyme solutions collected were adjusted to between pH 6 to 7. The two solutions were not mixed until purity tests were performed. The column was then washed with PBS followed by 20 ml PBS containing 2% sodium azide (an antifungal and antibacterial compound). The column was stored at 4°C until required for enzyme purification again.

The enzyme solution was then concentrated using Amicon centricon-10 by centrifuging at 2500 rpm at 4°C for 20 minutes each time until the volume had reached a minimal volume of 500 µl. This solution was diluted ten times with PBS pH 7.4. This enzyme solution was again concentrated until a volume of about 500 µl was achieved. Aliquots (about 100 µl/vial) of the enzyme solution were stored at -70°C until required or used immediately.

3.8.2 PURIFICATION OF TRYPANOPAIN TB AND TRYPANOPAIN TC USING CYSTATIN IMMOBILISED ON SEPHAROSE 4B

The purification was in principle performed according to the method described by Kos (1986) for the purification of cysteine proteases and Mbawa *et al*, (1992) for the purification of trypanopain Tc. A pellet containing about 2 x 10^{10} trypanosomes was suspended in 4 ml buffer (0.1 M sodium acetate/0.3 M NaCl, pH 5.5) and lysed by three cycles of

freeze - thaw from -70°C to 37°C. A wet smear microscope slide was prepared to confirm complete lysis of the trypanosomes. The solution was centrifuged in a Beckman centrifuge at 5000 rpm for 10 minutes at 4°C. The supernatant was added to the cystatin-sepharose gel prepared in section 3.3.3 to give a ratio of about 1:2 gel to buffer. The mixture was gently rocked on a rolling mixer for 2 hours. The mixture was then loaded in a jacketed column 1 x 30 cm and cooled with ice cold water. The column was washed with 10 ml buffer (0.1 M sodium acetate/0.5 M NaCl pH 5.5, 1.5% Brij 35). The enzyme was eluted with the above buffer containing 66% v/v butanol prepared by mixing 170 ml buffer with 330 ml butanol. 3 ml fractions were collected using a fraction collector. Fractions showing enzymatic activity, determined as described in section 3.7.1, were pooled together and concentrated using amicon centriprep 10 by centrifuging at 6000 rpm for 30 minutes in an angular rotor cooled at 4°C as advised by the supplier (Amicon, Operating instructions, publication number 1-259C).

3.8.3 ENZYME CHROMATOGRAPHY ON SEPHACRYL S-200 COLUMN

The chromatography of the enzymes on Sephacryl S-200HR was performed as described by Kos (1986) and Mbawa *et al*, (1992). The enzyme solution collected above was layered on a Sephacryl column (3 x

1.5 cm) equilibrated in degassed 0.05 M Tris/HCl pH 7.5, 0.1 M NaCl containing 1 mM EDTA and 0.05 % Brij 35 at 4°C. The enzyme was eluted with the same solution at a flow rate of 20 ml per hour. Column fractions of about 3 ml each were collected. Fractions with enzyme activity were pooled and concentrated as described above in section 3.8.2.

3.9. DETERMINATION OF THE PROTEOLYTIC ACTIVITY OF TRYPANOPAIN TC AND TRYPANOPAIN TB AGAINST HOST MACROMOLECULES

3.9.1 DIGESTION

All the host macromolecules were reconstituted in buffer composed of 0.113 M Na_2HPO_4, 0.043 M citric acid pH 5.5 containing 0.025% SDS as described by Mbawa *et al,* (1992) or the enzyme incubation buffer to give 0.8 mg/ml dissolved protein of interest. However, for macromolecules supplied in buffer e.g. laminin which was supplied in phosphate saline buffer at 1 mg/ml, it was used as supplied or supplemented with a small amount of incubation buffer (250 µl per ml of the original sample) to bring the concentration to 0.8 mg/ml.

150 µl of this protein solution was mixed with 60 µl of enzyme solution. The mixture was incubated at 20°C. 40 µl was removed after 0, 30, 60 and 120 minutes. To these samples 40 µl of sample buffer were added and immediately boiled for 5 minutes to stop the reaction.

3.9.2 ANALYSIS OF DIGESTION PRODUCTS

The samples from section 3.9.1 were loaded in separate wells on sodium dodecylsulphate polyacrylamide plain gels. Large proteins such as fibronectin were resolved on an 8.5% or lower SDS-PAGE separating gel while low molecular weight proteins were run on an 11.5% or higher SDS-PAGE resolving gel. Gradient gels could not be prepared as we did not have a gradient former. (Efforts to improvise one did not yield good results as the gel showed spreading of bands as the gel was not consistent). The gels were stained as earlier described for silver staining of resolved protein in section 3.6.2.

3.9.3 DETERMINATION OF ELASTASE TYPE ACTIVITY OF THE ENZYMES

Since elastin was insoluble, elastin-congo red was used to estimate the enzymes' elastinolytic properties. 10 mg of elastin-congo red was placed in four tubes. 200 µl of the incubation buffer (50 mM Tris, 50 mM citric acid pH 6.0 containing 6 mM DDT and 2 mM EDTA) were added to each of the tubes. 50 µl of trypanopain Tc, 50 µl of trypanopain Tb, 50 µl of papain (200 µg/ml) and 50µl of PBS as control were added to the appropriately labelled tubes. The tubes were incubated at 20°C. The tubes

were observed for 6 hours and then left overnight. The tubes were photographed on colour prints.

To verify the results from above, 10 mg of elastin were added to 1.5 ml of the incubation buffer and vigorously shaken for 5 minutes. It was allowed to stand for 5 minutes to allow only the settlement of large particles. The fine insoluble particles were added to 10 ml of the resolving gel solution and vigorously shaken five times. 10 µl of TEMED was added to the 10 ml gel and shaken twice. The gel solution was quickly added to the casting glass plates. The plates were then immediately tilted at an angle of about45-50° (between the glass plates and the bench) to avoid excess settlement of the insoluble particles until the gel had polymerized. The pure enzymes and their respective lysates were added to the wells and processed as earlier described in section 3.7.2.

In another experiment 120 µl of the elastin solution obtained as described in the previous paragraph was also incubated with 50 µl of the pure trypanosome enzyme and processed as described in sections 3.9.1 and 3.9.2 for protein digestion and analysis of digestion products respectively.

3.9.4 DETERMINATION OF COLLAGENASE TYPE ACTIVITY

Collagen type I and collagen type IV were separately co-polymerized in separate resolving gels at a concentration of 300 µg/ml. The gels were cast as earlier described in section 3.7.2. The pure enzymes

(trypanopain Tb and trypanopain Tc) and their respective lysates (*T. brucei* and *T. congolense*) were added to separate wells in each of the gels.

Since collagen was soluble it was reconstituted in the buffer and the digestion performed as described in section 3.9.1. Analysis of the digestion products was performed as earlier described in section 3.9.2. on an 8.5% acrylamide gel.

3.10 CELL CULTURE TECHNIQUE.

3.10.1 PREPARATION OF PRIMARY TISSUE CELL LINE.

Tissue cell lines were all prepared from bovine embryo shortly after its extraction from the mother which had just been slaughtered according to established methods which were currently in use at ILRI in the cell culture bay of Unit 3. As a general procedure, samples were handled with utmost care to avoid or at least to minimise contamination. The organs of interest were extracted from the bovine embryo carcass and immediately immersed in BSS (Earles balanced salt solution) containing 2% antibiotics (1:100 w/v penicillin : streptomycin). The samples were then transported to the laboratory in black plastic bags where the rest of the activities took place. The tissues were washed twice in fresh appropriate growth media supplemented with 2% antibiotics (see appendix for details) in a sterile hood.

The tissues were treated differently depending on their natural occurrence and the nature of cell to be prepared from them. The spleen and pericardium were finely chopped using a sharp blade while thymus was forced through a 1 ml syringe and then homogenised. The aorta was cut to give a length of 3 cm and then sliced open (length wise) to expose the interior which was washed to remove fats. It was then treated with 1 x trypsin-EDTA (a standard 1 x trypsin-EDTA contains 0.5 g trypsin and 2 g EDTA per litre of Hanks BSS) followed by incubation for 10 minutes at 37°C, in 5% CO_2, 95% air regulated incubator. The cells of interest were scraped off the interior of the aorta lightly using a scalpel. Blood fibroblast like cells were prepared by centrifuging whole blood at 2500 rpm for 10 minutes. The buffy coat was carefully removed.

The cells were seeded in the appropriate growth media in cell culture flasks (surface area = 25 cm^2). The flasks were incubated at 37°C, 5% CO_2, 95% air and left undisturbed for four days. On the fifth day the flasks were checked for any cell growth using a phase contrast microscope. The media was changed and the flasks were incubated at 37°C, 5% CO_2, 95% air. The media was replaced with fresh one every 3 days until the bottom of the flask was completely covered with a monolayer of cells, normally by the end of the second week. The cells were then trypsinised by incubating with 0.5 x trypsin-EDTA in BSS. The cells were further gently dispersed using a pipette. The media was washed away by centrifuging the

mixture at 1000 rpm for 3 minutes. The cells were resuspended in the appropriate growth media. About 2 x 10^4 cells were passaged into a fresh flask (surface area of 25 cm^2). The cells that did not attach after incubating for 60 minutes were washed away by draining out the media from the culture flask and replacing it with fresh media. This served as a process of screening bad cells. The media was replaced with fresh media and incubated at 37°C in 5% CO_2, 95% air. The media was replaced every two to three days depending on the density of cells.

On the third passage the cells were harvested once confluence (when the monolayer completely covering the surface of the flask) was achieved. After trypsinisation, the cells were spun at 1000 rpm for 3 minutes. The media was removed and the cells re-suspended in the media supplemented with 7.5% (v/v) DMSO (dimethyl sulfoxide). The cells were then stored in liquid nitrogen until required.

3.10.2 DETERMINATION OF THE ABILITY OF THE TRYPANOSOME CYSTEINE PROTEASES TO ROUND OFF AND DETACH EMBRYO BOVINE THYMUS CELL LINES.

The bovine embryo thymus cells were grown in cell culture bottles after being revived from liquid nitrogen where they were stored. When the cells were well established in the bottles they were passaged into micro-ELISA plates (96 wells). The bovine cell line was selected because its

growth rate was good and predictable, and thus it could be matched with enzyme purification dates. This was important as storing purified enzyme for a long time reduced its activity. The bovine embryo thymus monolayers were also easier to round off and detach as shown by 0.5 x trypsin-EDTA during passaging of the cell line and this was important as only limited amounts of enzymes (trypanopains) were available. The cells were grown and maintained as explained in section 3.10.1. Once the cells had attained confluence, normally by the third day, the growth media was drained out completely.

The enzyme solution used for cell culture experiments was separately prepared. After the enzyme had been purified as described in section 3.8.2 it was again diluted with 5 ml $PBS^{(-)}$ and concentrated to about 0.5 ml as described in section 3.8.2 in an effort to remove most of the solution used in enzyme chromatography that could have affected the observations.

Five wells contained trypanopain Tb, 100 µl/well of the final enzyme solution, 160 µg/ml and another five wells contained trypanopain Tc, 100 µl/well, 180 µg/ml of the enzyme solution. Control and inhibited control wells contained 100 µl buffer alone (5 wells), trypanopain Tc solution (180 µg/ml) and cystatin (50 mM) and E-64 (five wells), trypanopain Tb (160 µg/ml) and E-64 and cystatin (five wells), and five

wells contained the inhibitors and buffer alone. Details of the experimental design are in table 2.

The cells were incubated at 20°C, 5% CO_2, for up to 4 hours. The cells were examined every 15 minutes for any cytopathic effect, mainly cell rounding off and cell detachment, using a contrast light microscope. The cells were observed over a total period of 4 hours.

3.11. COMPARISON OF THE ACTIVITY OF TRYPANOPAIN TB AND TRYPANOPAIN TC

3.11.1 INHIBITION OF THE PROTEASES BY PROTEASE INHIBITORS

The ability of protease inhibitors cystatin (2 mg/ml), E-64 (2 mg/ml), PMSF (10 mg/ml), aprotinin (10mg/ml) and TLCK (4mg/ml) to effectively block the hydrolysis of CbzPhe-Arg-NHMec by the trypanosome proteases was performed by observing the fluorescence of the wells under a 365 nm lamp as earlier described in section 3.7.1. Equal amounts of CbzPhe-NHMec (20 μM, 150 μl) were added to 12 wells on a 24 well cell culture plate. 50 μl enzyme solution was placed in six separate tubes for trypanopain Tb and another six for trypanopain Tc. 30 μl of enzyme inhibitors were added to ten tubes and 30 μl of PBS pH 7.2 was added to the control tubes. The enzyme solutions were incubated for 10 minutes on a shaker. Each mixture was then added to the appropriately

Table 2

The experimental design of cell culture tests.

All the samples were added after draining the media from the cell culture wells. 100 μl of the samples were added to the appropriately labelled wells. TB stands for trypanopain Tb, TC stands for trypanopain Tc, TB+Inh stands for trypanopain Tb with inhibitors, TC+Inh stands for trypanopain Tc with inhibitors, inh stands for inhibitor in buffer and Bf for buffer alone.

E-64 (50 μM) and cystatin (40 μM) (combined) were used to inhibit the enzymes and the activity of the sample was tested prior to application into the wells using CbzPhe-Arg-NHMec as described in section 3.7.1.

	well 1	well 2	well 3	well 4	well 5
A(100 µl)	TC	TC	TC	TC	TC
B (100 µl)	TC+Inh	TC+Inh	TC+Inh	TC+Inh	TC+Inh
C (100 µl)	TB	TB	TB	TB	TB
D (100 µl)	TB+Inh	TB+Inh	TB+Inh	TB+Inh	TB+Inh
E (100 µl)	Inh+Bf	Inh+Bf	Inh+Bf	Inh+Bf	Inh+Bf
F (100 µl)	Bf	Bf	Bf	Bf	Bf

labelled wells. The reaction was allowed to proceed for 2 minutes and the samples were observed under a UV lamp as earlier described in section 3.7.1. The result was recorded and the culture plate photographed using an F4 Nikon camera on a colour film while the plate was under UV light.

3.11.2 PROTEIN DIGESTION PATTERN (SPECIFICITY) OF THE PROTEASES

In order to determine whether trypanopain Tb and trypanopain Tc have the same specificity (cleavage site), two bovine protein molecules bovine serum albumin, BSA, and laminin were used. The two molecules were subjected to enzyme digestion as earlier described in section 3.9.1 and analysis of the digestion products were performed as explained in section 3.9.2. The digestion of the protein molecule by the two pure enzymes was performed at the same time and run on the same gel. The gels were stained, viewed on a Sigma Chemical Co., T 2203 viewing box , and photographed using an F4 Nikon camera.

4.0 RESULTS

4.1 CYSTEINE PROTEASE PURIFICATION

4.1.1 PURIFICATION OF TRYPANOPAIN TC USING THE MONOCLONAL ANTIBODY COLUMN

A pellet containing 5 x 10^9 *T. congolense* was lysed by mixing with 10 ml of water and vortexed for a short period. The lysates were prepared as earlier described in section 3.8.1. The supernatant was loaded on the monoclonal antibody column equilibrated at about 4°C in degassed PBS pH 8.0. The column was washed with PBS pH 8.0 and the enzyme was eluted with 50 mM DEA pH 11.0 followed by elution with 0.1 M glycine/HCl pH 3.0. The pH of the two solutions was adjusted to 7.4.

The elution profile of the enzyme is given in figure 7. The first absorbance peak shows the elution of unbound proteins. The second peak is the elution of the bound enzyme using 50 mM DEA. The large absorbance during the elution of the enzyme by DEA was due to elution of immunoglobulin (Ig) fractions from the column. The silver stained SDS-PAGE analysis of the enzyme eluted with DEA was found to contain Ig fractions (not shown). The third peak is the elution of bound proteins using 0.1 M glycine. The enzyme was concentrated as earlier described in section 3.8.1. In one case, the unbound fraction was re-run on the column and the enzyme eluted as already dicussed above. The yield was about 60% that of the original run (mother sample). This indicates that the enzyme was not totally bound by the column. Though this seems to indicate overloading of the column, it is likely to be due to incomplete binding of the enzyme by the antibody column

because the column was not pre-incubated with the lysates. Pre-incubation of the column with the lysates may result in digestion of the antibodies (proteins) by proteases in trypanosome lysates.

4.1.2 PURIFICATION OF TRYPANOPAIN TC AND TRYPANOPAIN TB USING CYSTATIN

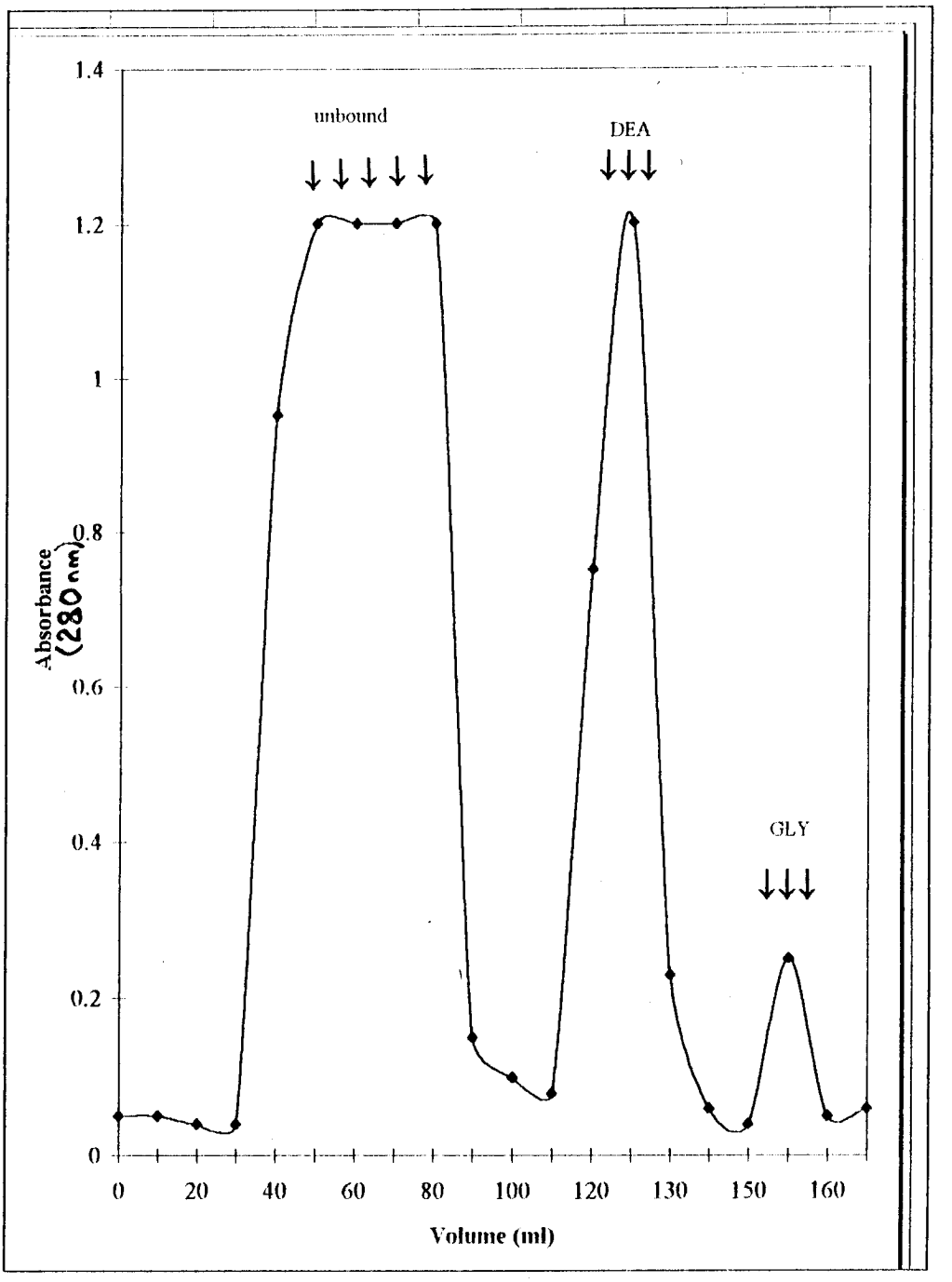
A pellet of 2 x 10^{10} trypanosomes was suspended in 4 ml starting buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 5.5) and lysed by three repeated cycles of freeze-thaw from -70°C to 37°C and processed as described in section 3.8.2. After incubation the mixture was then loaded on the cystatin column and the enzyme was eluted with the starting buffer containing 66% (v/v) butanol.

The enzymatic activity was followed using CBZ--Phe-Arg-NHMec hydrolysis as described in section 3.7.1 and protein levels were monitored by measuring the absorbance at 280 nm at 0.1 sensitivity on the Cecil CE 272 linear ultraviolet spectrophotometer. Interference in absorbance was experienced mainly due to the immiscibility of butanol and the aqueous buffer. The elution profile of the enzymes was the same and is given in figure 8. The high enzymatic activity at the beginning of the elution was likely to be due to excess unbound enzyme in the trypanosome lysates as contact zymogram analysis revealed proteolytic activity due to the trypanopains. It is unlikely that incomplete binding of the enzyme by cystatin occurred as even increase in the incubation time from 2 to 3 hours did not show any significant drop in enzymatic activity. It is likely that the amount of cystatin was not enough to

Fig. 7

PURIFICATION OF TRYPANOPAIN TC ON MONOCLONAL ANTIBODY COLUMN.

The elution of trypanopain Tc on a monoclonal column (10 x 1.5 cm) equilibrated in PBS pH 7.4. The enzyme was eluted using 50 mM DEA pH 11 and 0.1 M glycine/HCl pH 3. The elution was followed by monitoring the change in absorbance at 280 nm using a single path monitor coupled to a printer. Activity assay was performed only on the final solution as the antibody was very specific and the monitor was very sensitive to protein concentration change as shown by the large volumes of unbound proteins. The arrows show the fractions that were collected.


unbound
DEA
GLY
Absorbance (280 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
130
150
160
Volume (ml)

bind all the enzyme. From the graph, almost all the unbound enzyme was eluted within the first 5 ml while the pure enzyme was eluted in at least 25 ml. This suggests that the unbound enzyme was more concentrated (in μg/ml eluted) than the bound enzyme even though the bound enzyme was more than the unbound enzyme (in total μg eluted). This accounts for the high activity shown at the beginning of the elution process. There was very little change in absorbance during elution of the enzyme with butanol at our working level of sensitivity.

4.1.3 ENZYME CHROMATOGRAPHY ON SEPHACRYL S-200 HR COLUMN

The concentrated enzyme solution (0.5 ml) collected above was layered on a Sephacryl column (1.5 x 40 cm) equilibrated in degassed 50 mM Tris/HCl pH 7.5, containing 0.1 M NaCl, 1 mM EDTA, 0.05% Brij 35 at 4°C. The enzyme was eluted with the same solution at a flow rate of 20 ml per hour. 2.5 ml fractions were collected from the column and enzymatic activity determined using Cbz-Phe-Arg-NHMec as described in section 3.7.1. The fractions containing enzyme activity were concentrated and used or frozen at -70°C in 100 μl aliquots. When the enzyme was to be used in cell culture experiments, the fractions with enzyme activity were diluted with PBS, pH 6.5, five times and then concentrated as described in section 3.8.1. This was done to remove most of the non physiological buffer. The elution profile of the enzyme is given in figure 9. Table 3 gives a summary of the purification yields and relative activity of the enzyme at each purification step.

Fig. 8

PURIFICATION OF TRYPANOSOME CYSTEINE PROTEASE USING CYSTATIN-SEPHAROSE 4B COLUMN.

The elution profile of trypanosome cysteine proteases on cystatin-sepharose column after incubating the resin with the trypanosome lysate supernatant for 2 hours. Both enzymes were eluted with 66% butanol in the above buffer and followed a similar elution profile although trypanopain Tb was eluted in normally a smaller volume than trypanopain Tc. Absorbance was measured at 280 nm and activity using Cbz-Phe-Arg-NHMec at 365 nm. The graph gives the average elution profile of both enzymes as the graphs were the same. The arrows represent the elution of the enzyme and fractions collected.

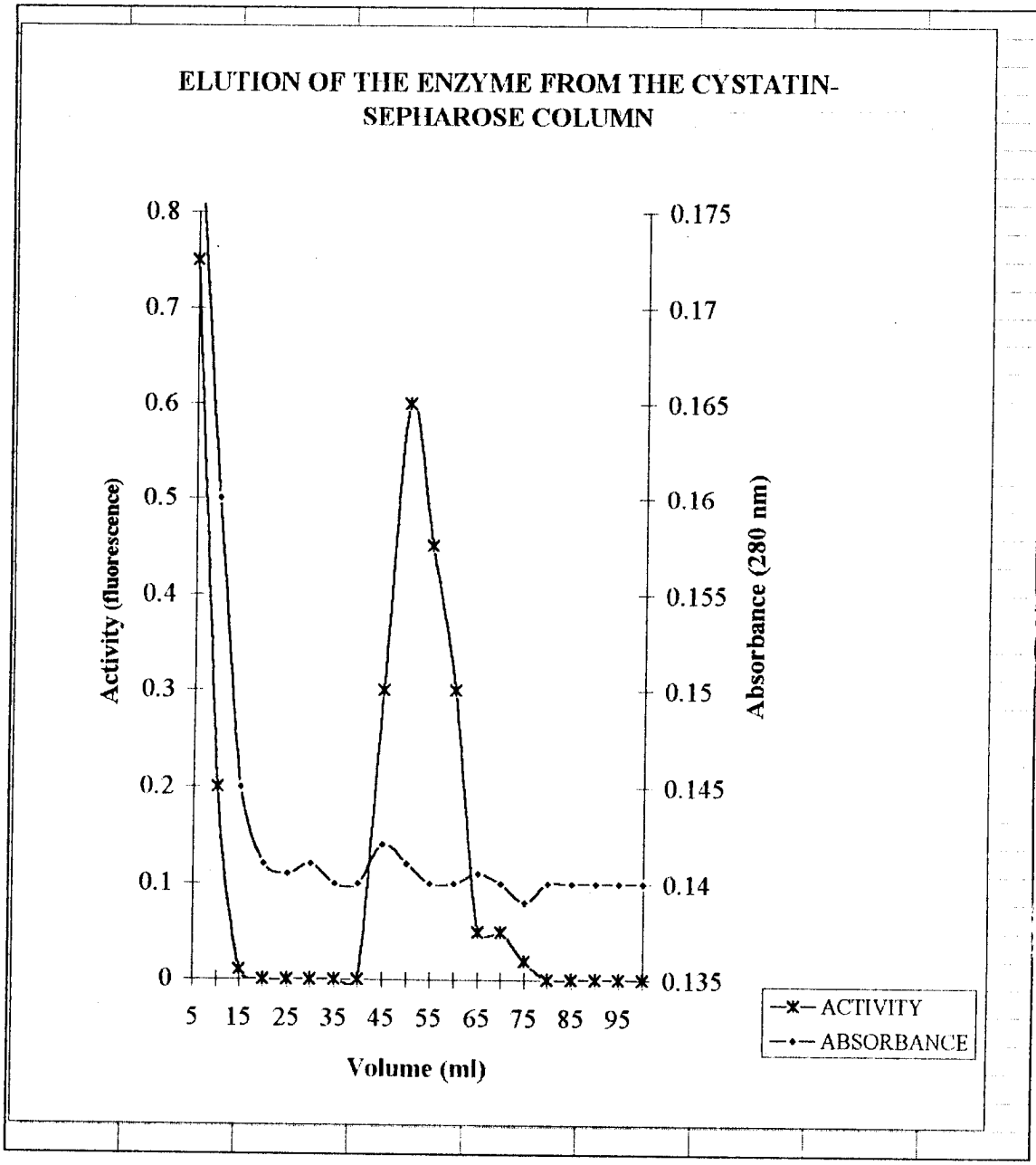
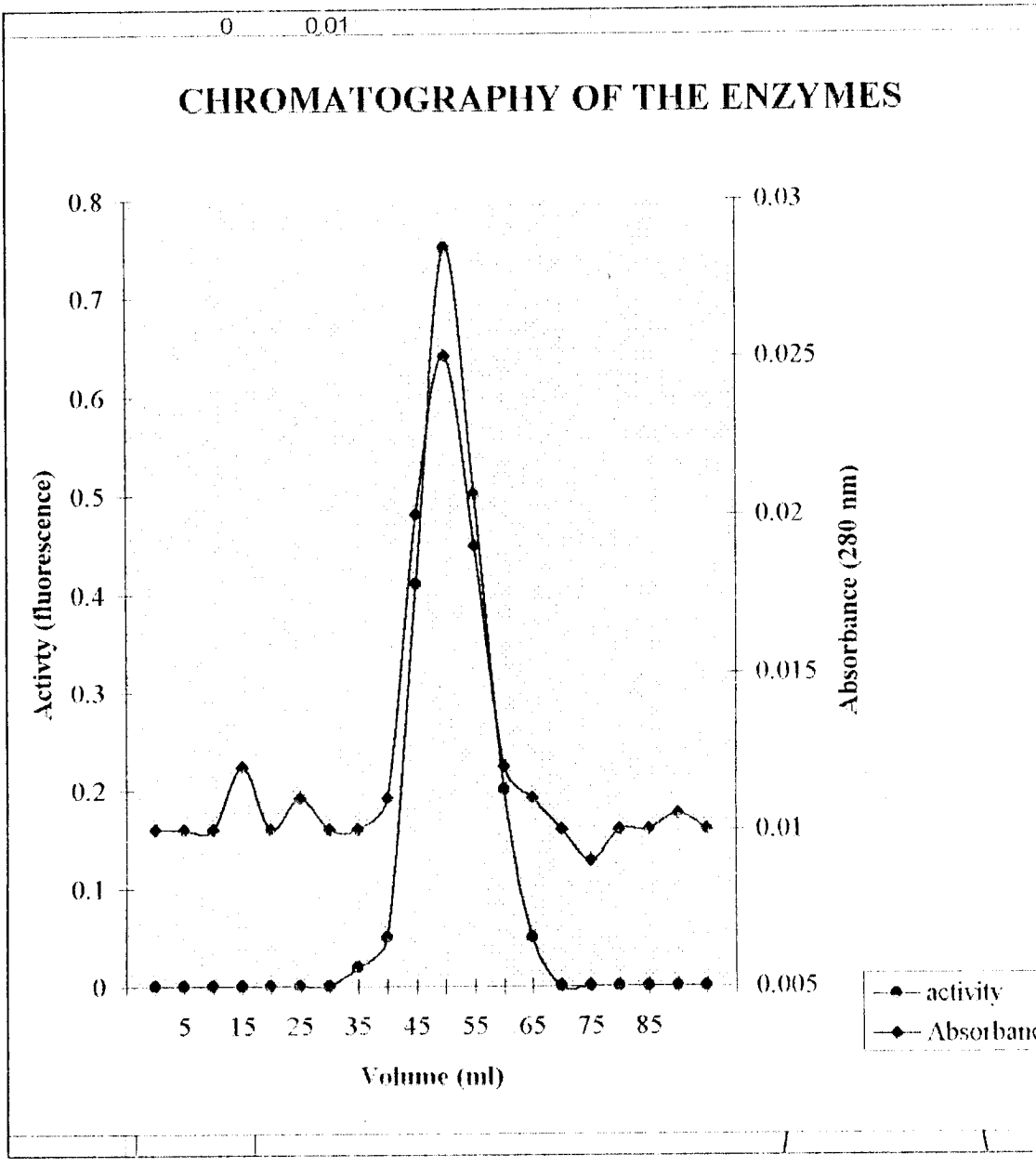

ELUTION OF THE ENZYME FROM THE CYSTATIN-SEPHAROSE COLUMN
Activity (fluorescence)
Absorbance (280 nm)
Volume (ml)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.175
0.17
0.165
0.16
0.155
0.15
0.145
0.14
0.135
5 15 25 35 45 55 65 75 85 95
ACTIVITY
ABSORBANCE

Fig. 9.

EXCLUSION CHROMATOGRAPHY OF THE CYSTEINE PROTEASES ON SEPHACRYL S 200HR.

The purified enzyme was layered on Sephacryl column (1.5 x 40 cm packed volume) equilibrated in 50 mM Tris / HCl pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.05% glycerol, and eluted using the same solution. The column was run at 20 ml per hour. Absorbance was determined at 280 nm. The activity was followed using Cbz-Phe-Arg-NHMec. The activity values were based on a score of 0.00 to 1.00 with the activity of fresh lysate being 1 (or 100%).


0 0.01
CHROMATOGRAPHY OF THE ENZYMES
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Activity (fluorescence)
0.03
0.025
0.02
0.015
0.01
0.005
Absorbance (280 nm)
5 15 25 35 45 55 65 75 85
Volume (ml)
activity
Absorban

4.2 ENZYME PURITY AND CONCENTRATION ANALYSIS

4.2 1 ENZYME PURITY

The purity of the enzyme was assayed on SDS-PAGE as earlier described in section 3.4. At every stage of the purification process, 20 µl of the enzyme solution were mixed with 20 µl sample buffer and boiled. 15 µl of the enzyme sample were loaded in two separate wells. The gels were prepared and electrophoresis performed as described in section 3.4. The gels were stained as described for silver staining of proteins in section 3.5.2.

The enzyme purity was assessed at each purification stage. Trypanopain Tc revealed one major band corresponding to 33 kDa and a weak 31 kDa band. There was no difference in molecular weights of the molecules purified by the cystatin-sepharose and by the antibody-sepharose columns. Trypanopain Tb also showed only one major band corresponding to 29 kDa with a weak low molecular weight band (27 kDa). Figure 10 shows the purity of the enzymes as determined using SDS-PAGE analysis. Figures 11 and 12 show molecular weight determined using zymogram.

4.2.2 DETERMINATION OF ENZYME CONCENTRATION.

The concentration of the enzyme was determined as described in section 3.6.3. The bovine serum albumin (BSA) protein standard was prepared to give a concentration range of 0.0 µg/ml to 400 µg/ml. The absorbance of the blank, samples and standards was measured at 750 nm using a Hitachi U-2000 spectrophotometer. The enzyme concentration was found to be about 200 µg/ml for Trypanopain Tc and about 190 µg/ml for trypanopain Tb.

Table 3

SUMMARY OF THE PURIFICATION YIELDS AT EACH STEP

A summary of the purification procedure giving the yields as total proteins of the two methods used during purification of the cysteine proteases. The expected yield is based on the findings that the cysteine protease is at least 1% of the total trypanosome proteins (Authie et al, 1992). The yield is based on the total soluble proteins (after centrifuging at 5000 rpm for 10 minutes) after each purification step and concentration determined as described in section 3.6.3.

The recovery percentage is of limited value because the instruments for quantitative enzyme activity assay were not available.

Fig. 10.

PURITY ASSAY OF THE CYSTEINE PROTEASE USING SDS-PAGE.

The purity of the enzyme was assayed by running the enzyme sample on 11% SDS-PAGE plain gel at 25 mA per gel.

Shows electrophoresis of cystatin-sepharose purified enzyme. Lane 1 shows the molecular weight markers soya trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa) and bovine albumin (66 kDa). Lane 2 contained *T. congolense* lysates, lane 3 trypanopain Tc, lane 4 *T. brucei* lysates and lane 5 trypanopain Tb.

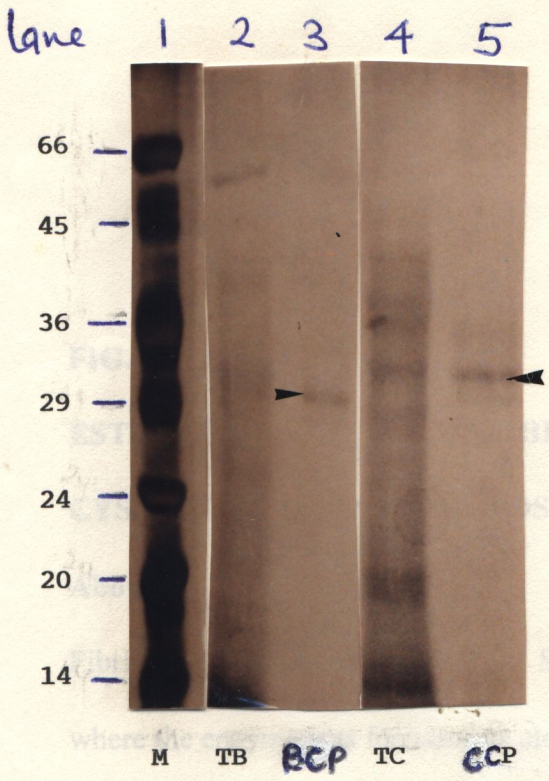

lane 1 2 3 4 5
66
45
36
29
24
20
14
M
TB
BCP
TC
CCP

FIG. 11

ESTIMATION OF THE MOBILITY OF THE TRYPANOSOME CYSTEINE PROTEASES ON SDS PAGE..

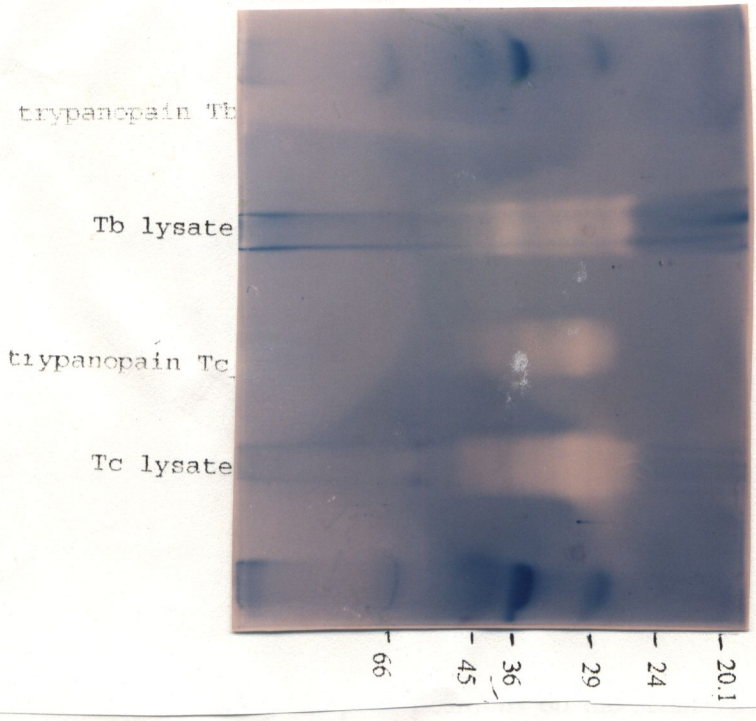
Above:

Fibrinogen was co-polymerized on SDS-PAGE. The gel shows the position where the enzyme was focussed as clear bands (not stained by coomassie blue) i.e. areas of proteolytic activity. The lanes are labelled showing the samples that were run in each lane. The outer lanes contained markers to assess whether the migration was uniform on the gel.

Below:

The gel was scanned using an HP Scanner, Hewlett packard product, coupled to a personal computer, Compaq, with the Gel Manager program (BioSystematica, Tavistock, U.K). The legend attached shows the scan of each lane showing the proteolytic bands as troughs as the protein (fibrinogen co-polymerized on the gel) around the area has been digested.

The lanes with markers were not scanned as the maximum number of lanes the gel manager could show per scan was four.


trypanopain Tb
Tb lysate
trypanopain Tc
Tc lysate
66
45
36
29
24
20.1

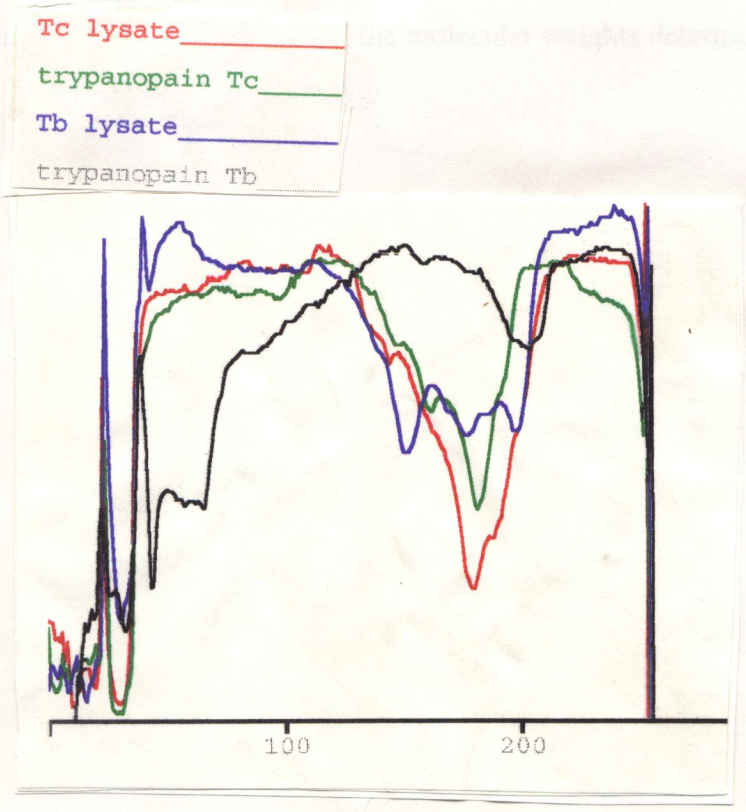
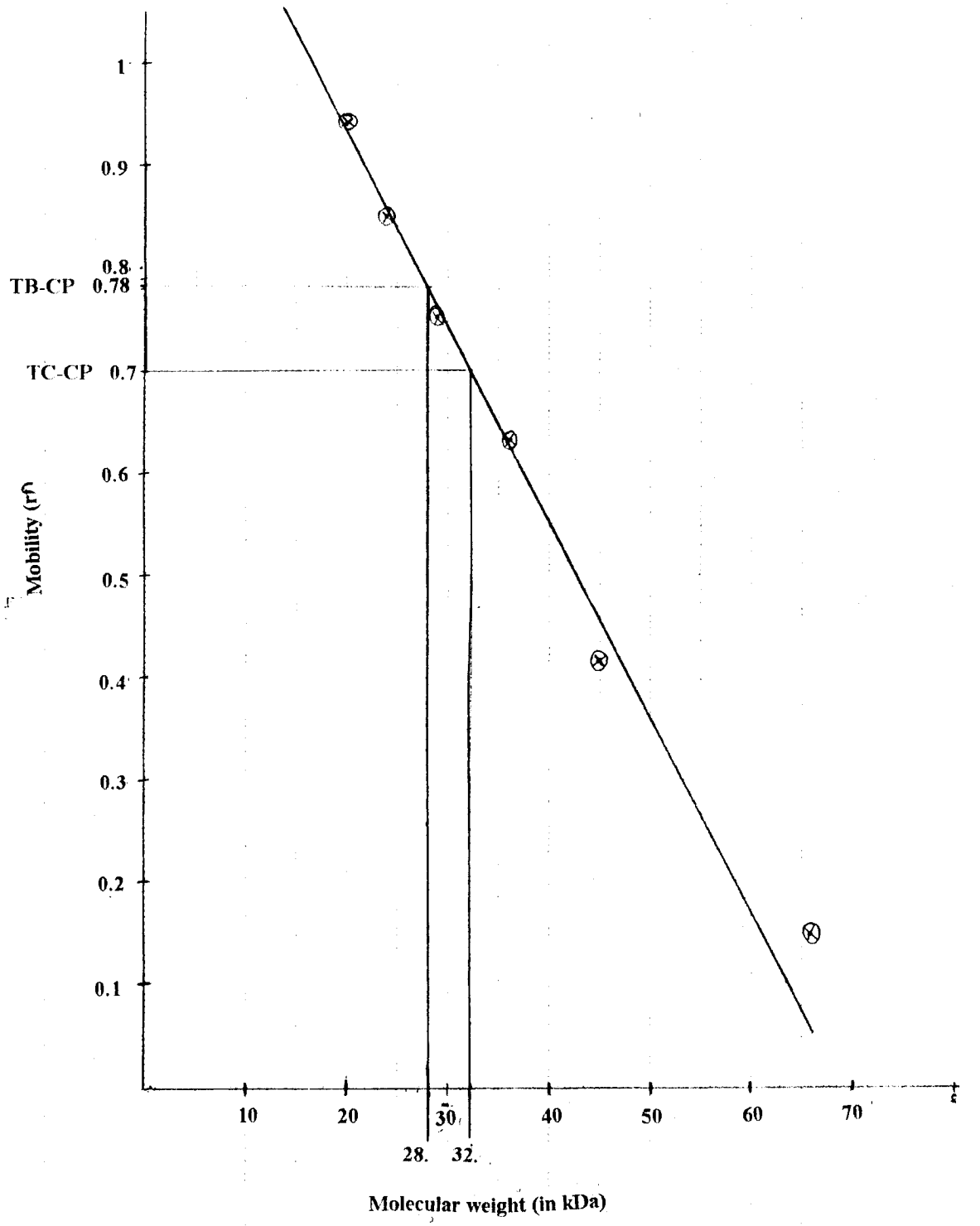

Tc lysate
trypanopain Tc
Tb lysate
trypanopain Tb
100
200

FIG. 12

THE ESTIMATION OF MOLECULAR WEIGHTS OF THE TRYPANOSOME CYSTEINE PROTEASES

From the scan digram shown in figure 11, the mobility of the enzyme was determined from the interface of stacking gel and resolving gel to the bottom of the trough. A scan of the molecular markers was also run and their mobility was determined using the peak positions on the scan. The values were then plotted as shown on the graph. and the molecular weights determined from the graph.

THE GRAPH OF MOLECULAR WEIGHT AGAINST MOBILITY OF PROTEINS (MARKERS).



4.3 DETERMINATION OF ELASTASE TYPE ACTIVITY OF TRYPANOPAIN TB AND TRYPANOPAIN TC.

4.3.1 USING ELASTIN CO-POLYMERISED SDS-PAGE ANALYSIS.

Elastin was co-polymerized in the resolving gel as explained in section 3.7.2. The two trypanosome lysates and the purified enzymes were added to the wells and electrophoresis performed at 25 mA per gel. The gel was incubated in the incubation buffer at 20°C for 18 hours. The gel was stained with coomassie blue and destained as described in section 3.6.1.

Proteolytic bands were not observed anywhere on the gel. All areas of proteolytic activity should appear as clear bands, because the proteins have been digested leaving the gel only and the gel does not stain blue with coomassie blue. However, all the gel stained blue showing that elastin co-polymerized on the gel was not digested. In one experiment the amount of enzymes were increased up to 25 µl per well from the 15 µl which was normally used but proteolytic bands were still not observed. Figure 13 shows an elastin co-polymerized gel.

4.3.2 USING ELASTIN-CONGO RED

10 mg elastin-congo red was suspended in 40 µl of the enzyme incubation buffer (50 mM Tris/citric acid pH 6.0 supplemented with 5 mM DTT and 2 mM EDTA). The tubes were incubated with 80 µl of trypanopain Tc (250 µg/ml), 80 µl of trypanopain Tb (250 µg/ml) and 75 µl of papain (300 µg/ml) at 25°C for 8 hours. Two control tubes were used. One contained only the enzyme incubation buffer while the other contained the same concentration

of enzyme (80 µl) solution as the samples and 20 µl E-64 (50 mM), a specific cysteine protease inhibitor.

The tube containing papain started changing colour from colourless, that of buffer and enzyme, towards that of congo red (brick-red) after 4 hours. The colour continued to change and after 18 hours of incubation, all the insoluble elastin congo-red had disappeared, indicating total digestion. The tubes containing trypanopain Tb and trypanopain Tc showed some colour change, about 35% of that observed with papain, after four hours of incubation. It was significantly different from that of the two positive controls (containing enzyme and inhibitor) and that of the blank. However, both enzyme tubes still contained a large amount of insoluble elastin congo-red and it was not possible to estimate how much of the substrate had been digested by the trypanosome cysteine proteases after 18 hours. Some digestion had clearly taken place as shown by the colour which was about 30% of that observed with papain and about 90% more than that of the positive controls and that of the blank.

There was a difference in colour between the two experimental tubes containing purified trypanosome cysteine proteases from *T. brucei* and *T. congolense.* Trypanopain Tc produced a slightly deeper red solution than trypanopain Tb, by about 20%. This difference was not observed during the first 6 hours of observation but was definite after 18 hours.

In order to determine the extent of digestion of elastin-congo red by trypanopain Tb and trypanopain Tc and papain, 20 µl of the incubation mixture from the above was mixed with 20 µl of sample buffer and boiled for 3 minutes. 15 µl of each were loaded in wells of the same gel (11% acrylamide) and electrophoresis performed as earlier described in section 3.5.

The gel was silver stained and the results are shown in figure 14. The gel revealed that papain had digested the insoluble elastin (elastin-congo red) to small, low molecular weight soluble proteins of about 30 kDa or smaller. Trypanopain Tc had also digested elastin to smaller, low molecular weight proteins of about 38 kDa or less and trypanopain Tb had degraded elastin to low molecular weight proteins of about 60 kDa or less. The controls did not show significant amount of digestion although the control with inhibited trypanopain Tc showed some bands but were insignificant to those observed with trypanopain Tc.

4.4 DETERMINATION OF COLLAGENASE TYPE ACTIVITY OF TRYPANOPAIN TC AND TRYPANOPAIN TB

Collagen type 1 and collagen type IV were each co-polymerized in SDS-PAGE as earlier described for contact zymogram in section 3.7.2. The trypanosome lysates and enzymes were loaded on the same gel and electrophoresis performed. When electrophoresis was complete the gels were incubated in 50 mM Tris and citric acid pH 6.0 containing 2 mM EDTA and 5 mM DTT for overnight. The gels were stained and destained as described earlier in section 3.6.1 for coomassie blue staining. The areas of proteolytic activity appeared as clear bands showing the absence of protein staining as revealed by the gel. In order to verify if the observed reaction in zymograms was due to the cysteine proteases, samples that were incubated with 50 μM E-64, a specific cysteine protease inhibitor were compared. They did not show proteolytic bands, thus it was clear that the reaction observed was due to the cysteine proteases.

Fig. 13.

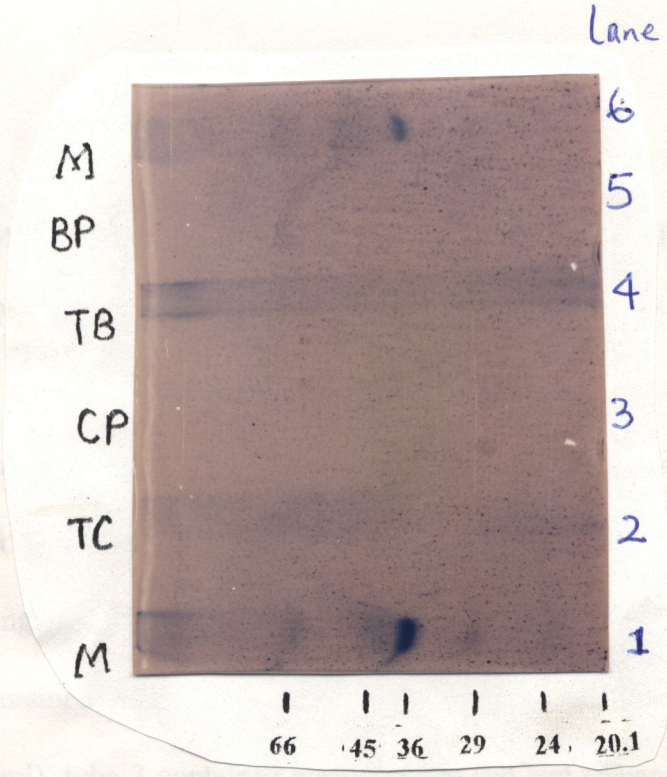
DETERMINATION OF ELASTASE TYPE ACTIVITY OF THE TRYPANOSOME CYSTEINE PROTEASES

Above:

Shows elastin co-polymerized on SDS-PAGE (contact zymogram) and incubated for 18 hours. Lane 1 and 6 low molecular markers, lane 2 *T. congolense* lysates, lane 3 trypanopain Tc, lane 4 *T. brucei* lysates and lane 5 trypanopain Tb. No digestion bands were observed.

Below:

Shows the gel scan using a scanner (Hewlett Packard, HP Scanjet 3c) coupled to a micro computer (Compaq, using the Gel Manager). The scan failed to detect areas of proteolytic activity as can be seen in the gel with fibrinogen Fig.10 (a gel run at the same time, same samples, and same equipment and solutions). Areas of proteolytic activity are supposed to appear as troughs.


Lane
M
BP
TB
CP
TC
M
6
5
4
3
2
1
66
45
36
29
24
20.1

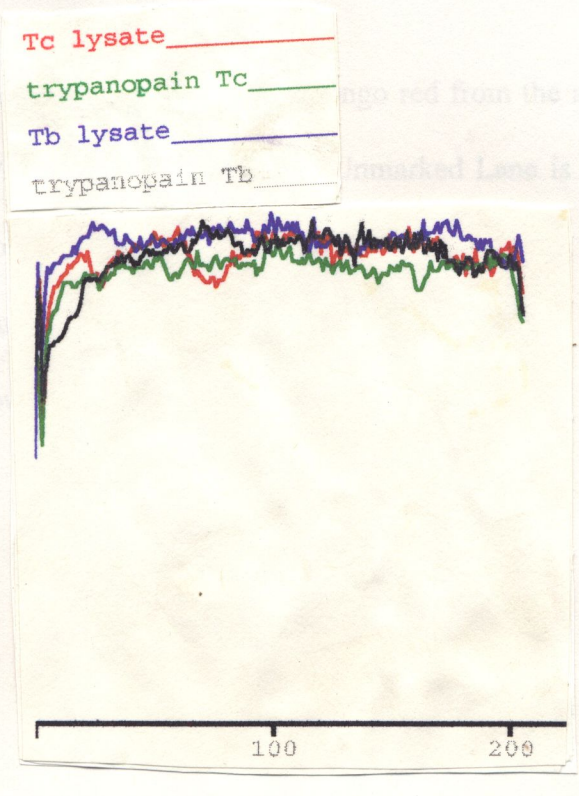

Tc lysate
trypanopain Tc
Tb lysate
trypanopain Tb
100
200

FIG. 14.

DETERMINATION OF ELASTASE TYPE ACTIVITY USING ELASTIN-CONGO RED.

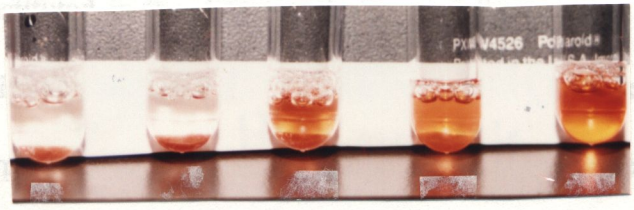
Above:

Elastin-congo red was incubated with the trypanosome cysteine proteases and papain for 18 hours and the colour change was monitored. Tube 1 contained elastin-congo red, trypanopain Tb enzyme solution and E-64 (40 µl, 2 mg/ml), tube 2 contained the elastin-congo red, trypanopain Tc solution and E-64 (40 µl, 2 mg/ml), tube 3 contained elastin-congo red and trypanopain Tb, tube 4 contained elastin-congo red and trypanopain Tc and tube 5 contained elastin-congo red and papain (Note the absence of insoluble elastin-congo red, precipitate, which is present in the other tubes).

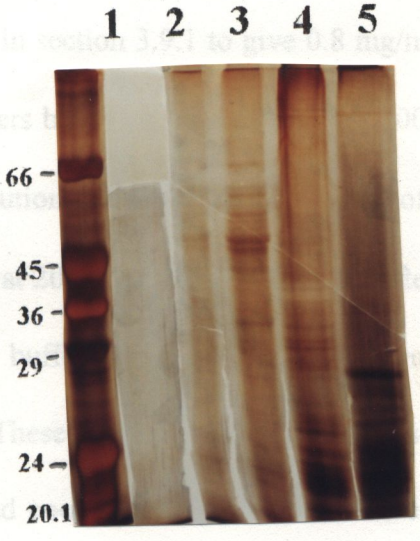
Below:

Shows the degradation products of elastin-congo red from the above tubes as revealed by SDS-PAGE (11% acrylamide). Unmarked Lane is the low range markers, lane 1 from tube 1, lane 2 from tube 2, lane 3 from tube 3, lane 4 from tube 4and lane 5 from tube 5.

The blank (Not shown) was the same as lane 1.



1 2 3 4 5



In another experiment, collagen type I and collagen type IV were reconstituted in the digestion buffer and the reaction carried out at 25°C as described in section 3.9.1 for protein digestion and then incubated with 40 µl of trypanpain Tc. Fractions collected from the mixture at fixed intervals were boiled for 5 minutes to stop the reaction and its products analysed as described in section 3.9.2 and illustrated in figure 15. Both collagen type I and collagen type IV showed progressive disappearance of the main protein bands and generation or the appearance of weak low molecular weight bands that were not present in the original reaction mixture.

4.5. PROTEOLYTIC ACTIVITY AGAINST FIBRONECTIN, GLYCOPROTEINS AND LAMININ.

All the above macromolecules were either prepared in buffer as described in section 3.9.1 to give 0.8 mg/ml dissolved protein of interest or in the suppliers buffer supplemented with 100 µl of the incubation buffer. 120 µl of the solution were mixed with 40 µl of the enzyme and the mixture was incubated at 20°C. 40 µl were removed after 0, 60, 120 and 180 minutes. 40 µl of sample buffer were added to these samples and boiled immediately for 5 minutes. These samples were loaded in separate wells immediately the last sample had cooled. The gel was run as earlier described for SDS-PAGE in section 3.4 and then silver stained as described in section 3.5.2.

The digestion of the proteins by the cysteine proteases was shown by the steady disappearance of the main bands and/or appearance of new lower

Fig. 15.

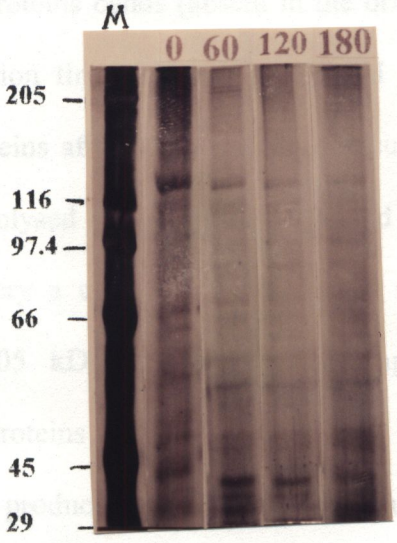
DETERMINATION OF COLLAGENASE TYPE ACTIVITY OF THE TRYPANOPAIN TC

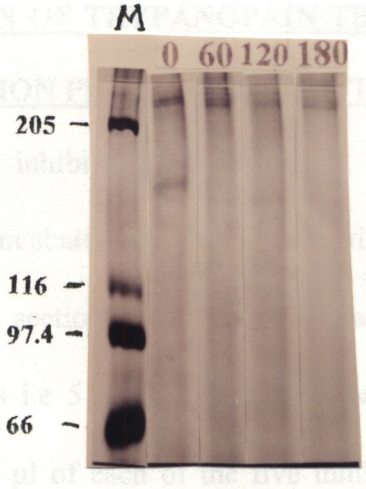
Above:

Shows the progressive degradation of collagen type I with increase in incubation time using trypanopain Tc. Lane 1 is high range markers (on an 8% acrylamide gel) 205 kDa myosin, β-galactosidase 116 kDa, phosphorylase 97 kDa, bovine albumin 66 kDa, egg albumin 45 kDa and carbonic anhydrase 29 kDa, lane 2 degradation after 0 minutes lane 3 digestion after 60 minutes, lane 4 after 120 minutes and lane 5 after 180 minutes.

Below:

Shows the degradation of collagen type IV on an 8% acrylamide gel. Lane 1 is high range markers lane 2 after 0 minutes, lane 3 after 60 minutes, lane 4 after 120 minutes and lane 5 after 180 minutes incubation.


M
0 60 120 180
205
116
97.4
66
45
29


M
0 60 120 180
205
116
97.4
66

molecular weight proteins bands (absent in the original sample mixture) with increase in incubation time. Figure 16, 17 and 18 shows the SDS-PAGE analysis of the proteins after a given time of incubation. All the above three proteins were hydrolysed by trypanopain Tc and trypanopain Tb. However, fibronectin gave very a clear digestion pattern with only one major band focussed below 205 kDa, which almost disappeared after 2 hours of incubation. Glycoproteins produced one major band which was focussed around 45 kDa and produced clear digestion products which appeared as new bands. Laminin produced a number of bands but new digestion products were observed. All these proteins were cell culture molecules and their electrophoresis properties were not specified by the suppliers. They were suitable proteins to use to investigate the effect of the trypanopains as they are connective tissue proteins used in cell attachment.

4.6 COMPARISON OF TRYPANOPAIN TC AND TRYPANOPAIN TB

4.6.1. INHIBITION OF TRYPANOPAIN TB AND TRYPANOPAIN TC BY SOME COMMON PROTEASE INHIBITORS

Five protease inhibitors, E-64, cystatin, PMSF, TLCK and aprotinin, were prepared in incubation buffer diluted with PBS, pH 6.0 1:1 (v/v) as explained earlier in section 3.11.1. 50 µl of each of the pure enzymes were added to five tubes i.e 5 tubes with trypanopain Tc and another five with trypanopain Tb. 40 µl of each of the five inhibitors were added to separate tubes containing the enzyme. The 10 different mixtures were allowed to stand for 10 minutes. 45 µl of each of these mixture were added to 200 µl of Cbz-Phe-Arg-NHMec (20 µM) and then allowed to react for 1 minute to determine

Fig. 16

THE PROGRESSIVE DEGRADATION OF FIBRONECTIN BY TRYPANOPAIN TC

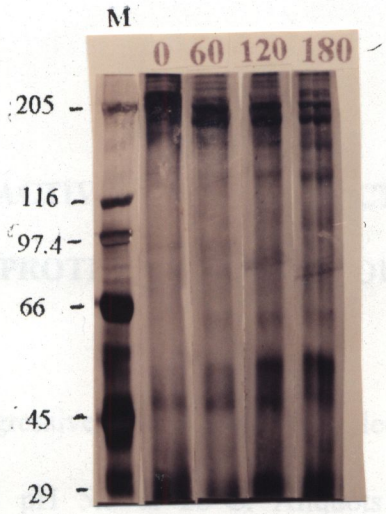
Above:

Shows the digestion of fibronectin by the pure cysteine protease. 8 μg of trypanopain Tc was used to digest 96 μg of fibronectin. Aliquots were removed at 0, 30, 60, and 120 minutes and then boiled for 5 minutes. The samples were run on SDS-PAGE 8% acrylamide plain gel immediately after the last sample had cooled. Lane 1 is high range markers, lane 2 at 0 minutes, lane 3 at 60 minutes, lane 4 at 120 minutes and lane 5 at 180 minutes.

Below:

Shows an experiment designed as the one above but run on a 9.5% gel in order to see any high molecular weight proteins that may not be revealed by the above gel. This was necessary as it was not possible to prepare gradient gel or run the samples on a gel larger than 11.3 x10 cm. The lanes have the same arrangement as above but the digestion performed using trypanopain Tb.

In each case lane 2 (at 0 minutes) shows mainly the original sample (fibronectin).


M
0 60 120 180
205
116
97.4
66
45
29

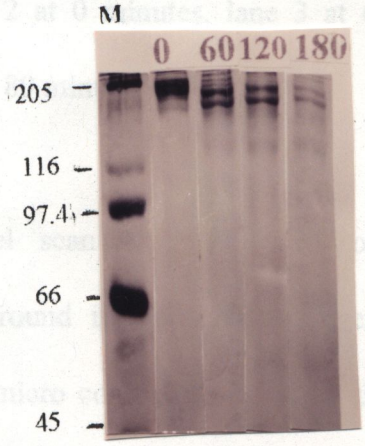

M
0 60 120 180
205
116
97.4
66
45

Fig. 17

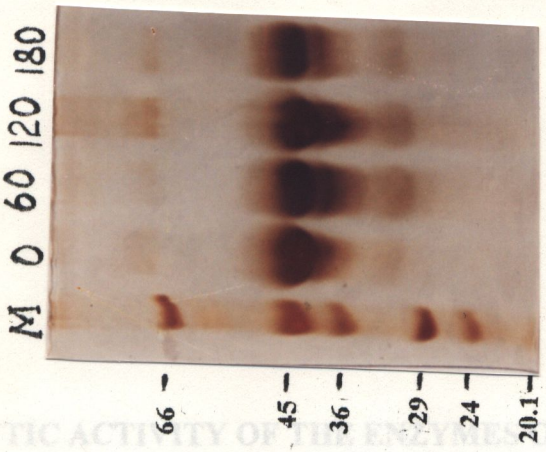
PROTEOLYTIC ACTIVITY OF THE CYSTEINE PROTEASES AGAINST GLYCOPROTEIN (OROSOMUCOID).

Above:

Shows the progressive digestion of the molecule by the trypanopain Tc after incubation at pH 5.5 at 20°C. Aliquots were removed from the incubation tube at 0 minutes, 60 minutes, 120 minutes and 180 minutes and the aliquots were immediately boiled to terminate the reaction for 5 minutes. The samples were run on 12% acrylamide gel. Lane one is the low molecular weight markers, lane 2 at 0 minutes, lane 3 at 60 minutes, lane 4 at 120 minutes and lane 5 at 180 minutes

Below:

Shows the gel scan analysis of glycoproteins (above) without subtracting the background interference. The gel was analysed using the scanner coupled to a micro computer as described on page 90. The lines in different colours show the amount of proteins in each lane after a given time according to the legend attached. The peaks show the area with more proteins on the gel.


M 0 60 120 180
66
45
36
29
24
20.1

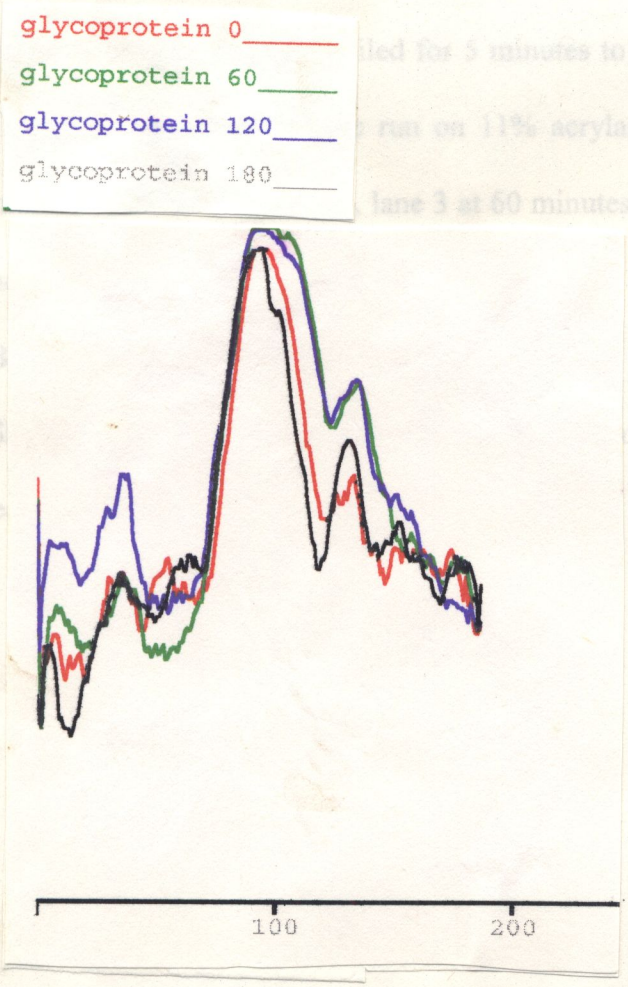

glycoprotein 0
glycoprotein 60
glycoprotein 120
glycoprotein 180
100
200

Fig. 18

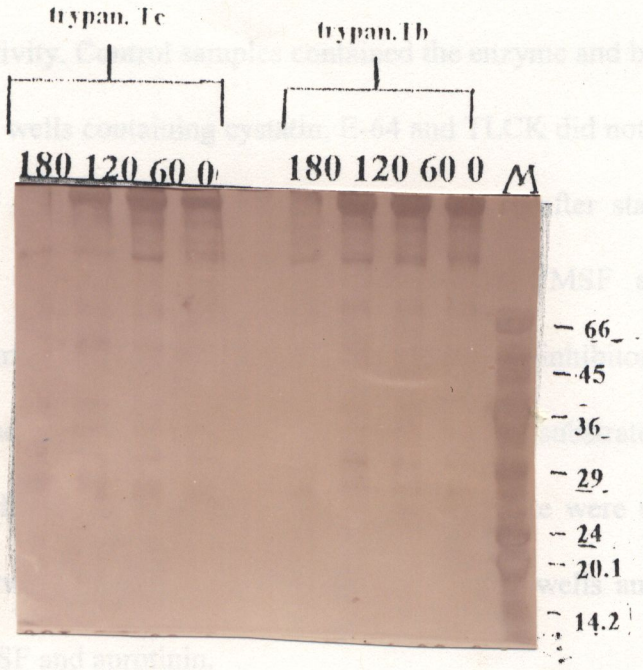
PROTEOLYTIC ACTIVITY OF THE ENZYMES ON LAMININ.

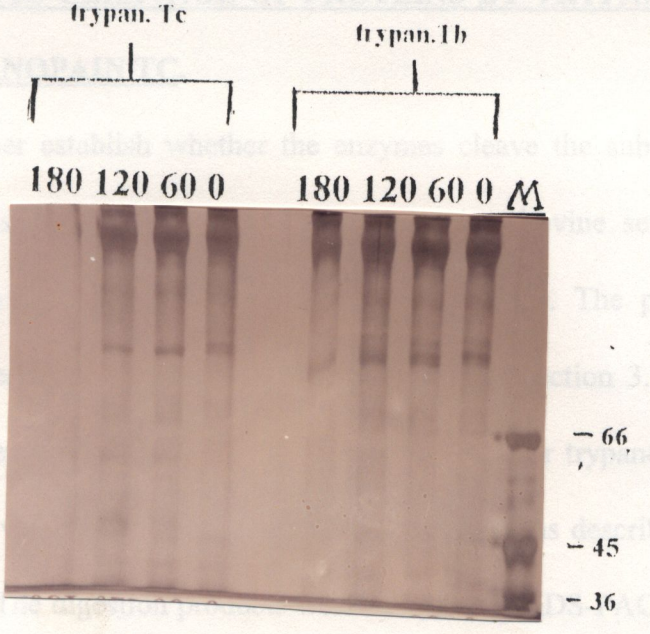
Above:

Shows the digestion of laminin by the trypanosome cysteine proteases when incubated at 20°C and pH maintained at 5.5. Aliquots were removed from the incubation tube at 0, 60, 120, and 180 minutes and then added to the sample buffer and immediately boiled for 5 minutes to terminate the reaction in the aliquots. The samples were run on 11% acrylamide gel. Lane 1 low range markers, lane 2 at 0 minutes, lane 3 at 60 minutes lane 4 at 120 minutes and lane 5 at 180 minutes.

Below:

Shows the same samples repeated on 8% acrylamide gel. The lanes are arranged as above.


trypan. Tc
trypan.Tb
180 120 60 0
180 120 60 0
M
66
45
36
29
24
20.1
14.2


trypan. Tc
trypan.Tb
180 120 60 0
180 120 60 0
M
66
45
36

the enzyme activity. Control samples contained the enzyme and buffer only.

Only the wells containing cystatin, E-64 and TLCK did not illuminate or fluoresce after 5 minutes and did not fluoresce even after standing for 30 minutes. The other wells containing buffer only, PMSF and aprotinin illuminated almost imediately (after 30 seconds). The inhibitors PMSF and aprotinin had no detectable effect on the digestion of the substrate as shown by fluorescence of the the wells under UV lamp and, there were no significant differences between the fluorescence from the control wells and from those wells with PMSF and aprotinin.

The two enzymes were affected by the inhibitors in a similar way as shown by fluorescence of similar treatments. See figure 19.

4.6.2. SPECIFIC CLEAVAGE OF PROTEINS BY TRYPANOPAIN TB AND TRYPANOPAIN TC.

To further establish whether the enzymes cleave the substrates at the same points (specific peptide bond) three proteins, bovine serum albumin (BSA), casein and fibrinogen were used as substrates. The proteins were prepared as described earlier for protein digestion in section 3.9.1. Separate tubes were prepared containing either trypanopain Tb or trypanopain Tc and fractions removed after a specific period of incubation as described earlier in section 3.9.2. The digestion products were analysed by SDS-PAGE performed as earlier described in section 3.6.

The gels revealed that the digestion patterns were the same. The protein bands which appeared after specific period of incubation with trypanopain Tb

and trypanopain Tc from the same protein were almost identical as seen by eye. The number, appearance and position of digestion products after similar time e.g 60 minutes, were the same for the same protein. See figures 20 and 21.

4.7 DETERMINATION OF THE ABILITY OF THE ENZYMES TO DETACH AND ROUND OFF CELLS

Bovine embryo cells were recovered from liquid nitrogen and seeded in cell culture bottles (25 cm^2) as earlier described in section 3.10. On the third day the cells were passaged, and seeded in micro ELISA plates (96 wells). They were grown and maintained as explained in section 3.9.1. Once the cells had attained confluence, normally by the third day, the growth media was drained out completely but the cells were not washed with $PBS^{(-)}$. The experimental solutions (100 µl each) prepared as described below were added to the wells.

The two enzymes were diluted 10 times with $PBS^{(-)}$ and concentrated again in amicon centriprep 10 as described in section 3.8.3 to increase the level of $PBS^{(-)}$, a physiological buffer. 100 µl of the enzyme solution were added to each of five wells and another 100 µl containing cystatin (1 mg/ml) and E-64 inhibitors (40 mM) were added to five control wells. The above set of experiments were performed for each enzyme. Other control wells contained the buffer and inhibitors only,and the buffer only.

Fig. 19

INHIHIBITION OF THE TRYPANOPAINS BY PROTEASE INHIBITORS

The picture of the cell culture plate shows positive and negative wells. The enzyme (50 µl) was incubated with 40 µl of the inhibitor for 10 minutes and 45 µl of the mixture was then added to the chromogenic substrate and viewed under a UV light and then the photograph was taken after 60 minutes.

The above table is a legend to the bottom photographic colour picture of a section of the cell culture plate. A legend to the table is placed just below the table.

The blue like wells seen in the photograph show the wells that were able to illuminate within 30 minutes, therefore indicating enzymatic activity.

LEGEND TO THE CELL CULTURE PLATE BELOW

	buffer only	aprotinin 10 mg/ml	TLCK 4 mg/ml	PMSF 10 mg/ml	E-64 2 mg/ml	cystatin 2 mg/ml
Trypan. Tc	+ve	+ve	-ve	+ve	-ve	-ve
Buffer	-ve	-ve	-ve	-ve	-ve	-ve
Buffer	-ve	-ve	-ve	-ve	-ve	-ve
Trypan. Tb	+ve	+ve	-ve	+ve	-ve	-ve

40 µl of the inhibitors were added to each well.

-ve = represents a well that did not illuminate after thirty minutes of incubation.
+ve.= represents a well that illuminated (all illuminated within 1 minute).

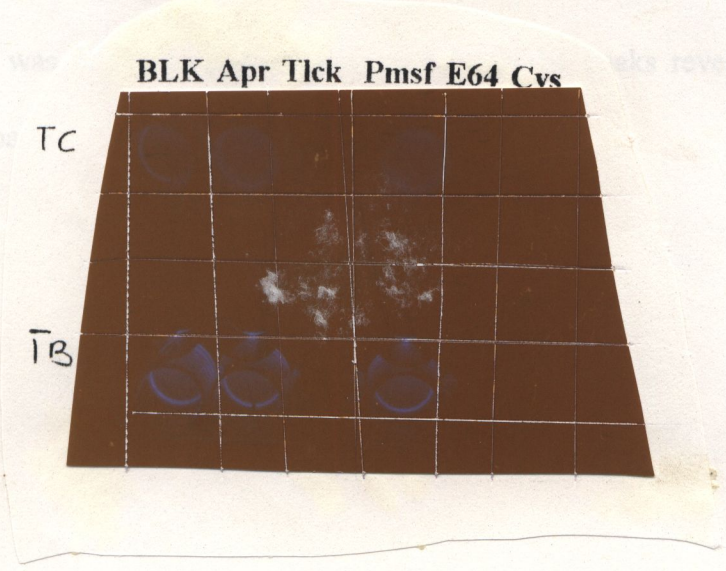


Fig. 20

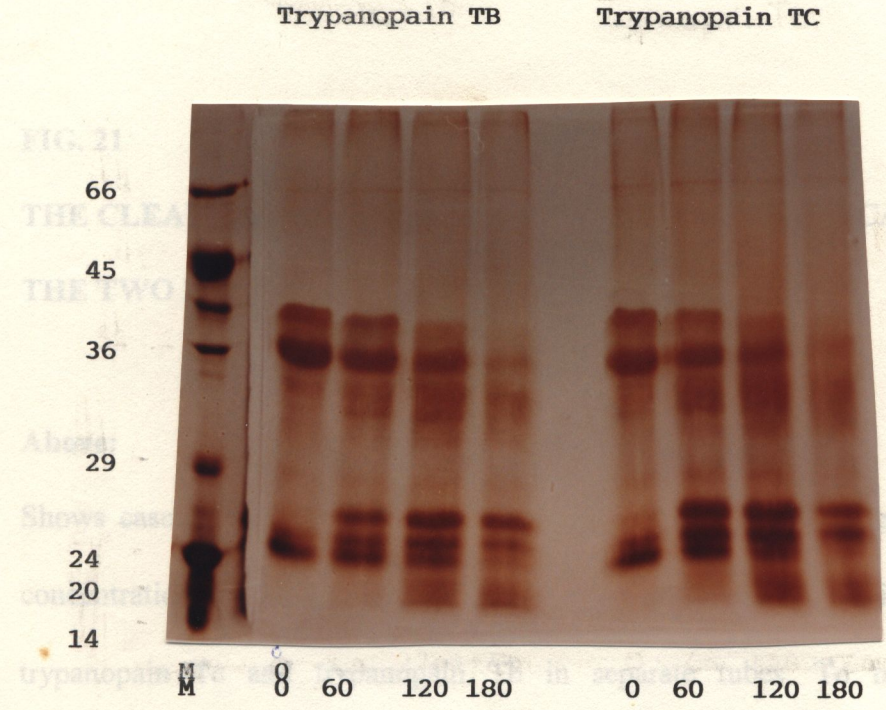
COMPARISON OF THE CLEAVAGE SITES OF THE TWO ENZYMES.

Above:

Shows bovine fibrinogen reconstituted in the enzyme incubation buffer to give a final concentration of 1.2 mg/ml. 120 µl of the protein was incubated with 50 µl of trypanopain Tc and trypanopain Tb in separate tubes. To make good observations the incubation times were increased. Aliquots were removed at 0, 60, 120, and 180 minutes and then immediately boiled. The samples were run on 11% acrylamide gel. Lane 1 low range markers, (Lanes 2-5 incubated with trypanopain Tb) lane 2 at 0 minutes, lane 3 at 60,lane 4 at 120 and lane 5 at 180 minutes , lane 6 no sample added, (Lanes 7-10 incubated with trypanopain Tc) lane 7 at 0 minutes, lane 8 at 60, lane 9 at 120 and lane 10 at 180 minutes.

Below:

Shows the above gel scanned to reveal any differences not detected by eye. The gel was scanned as previously explained. The peaks reveal areas with protein bands.


Trypanopain TB
Trypanopain TC
66
45
36
29
24
20
14
M
0
0 60 120 180
0 60 120 180

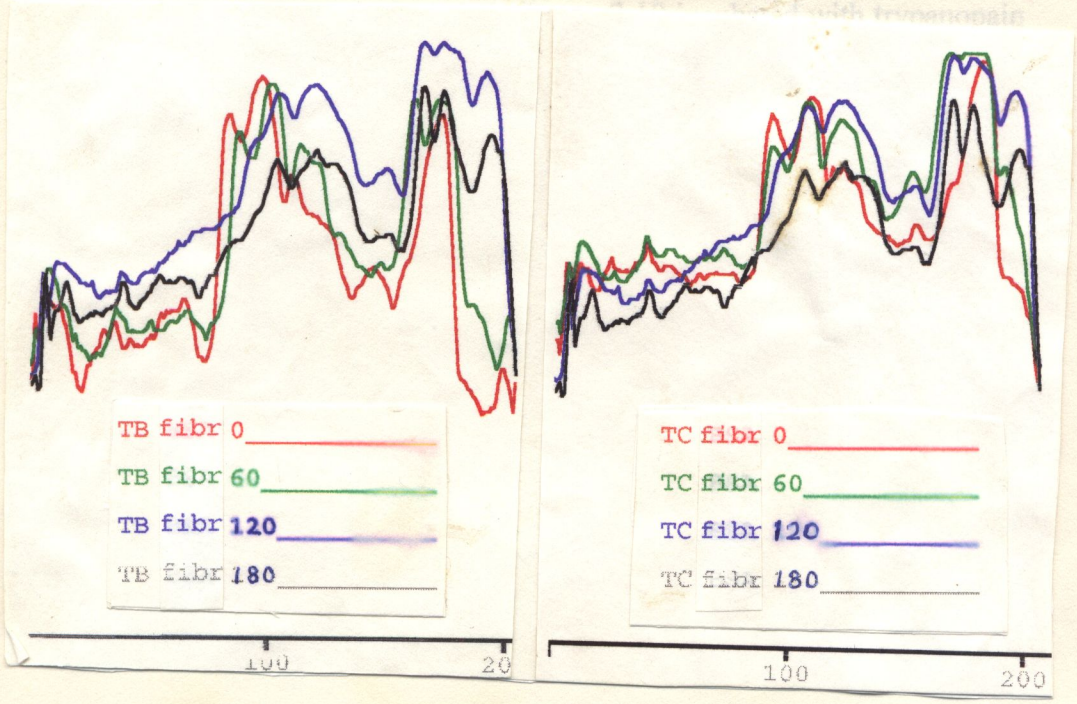

TB fibr 0
TB fibr 60
TB fibr 120
TB fibr 180
100
20
TC fibr 0
TC fibr 60
TC fibr 120
TC fibr 180
100
200

FIG. 21

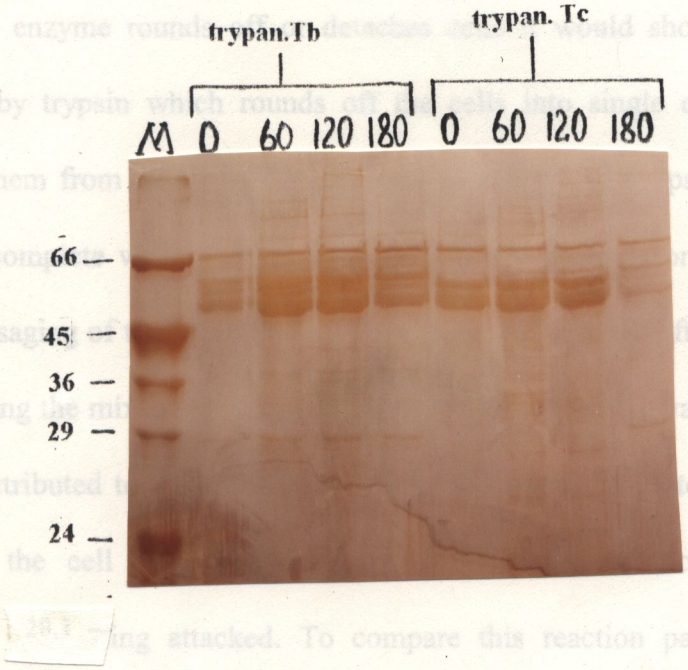
THE CLEAVAGE OF BOVINE SERUM ALBUMINN AND CASEIN BY THE TWO ENZYMES

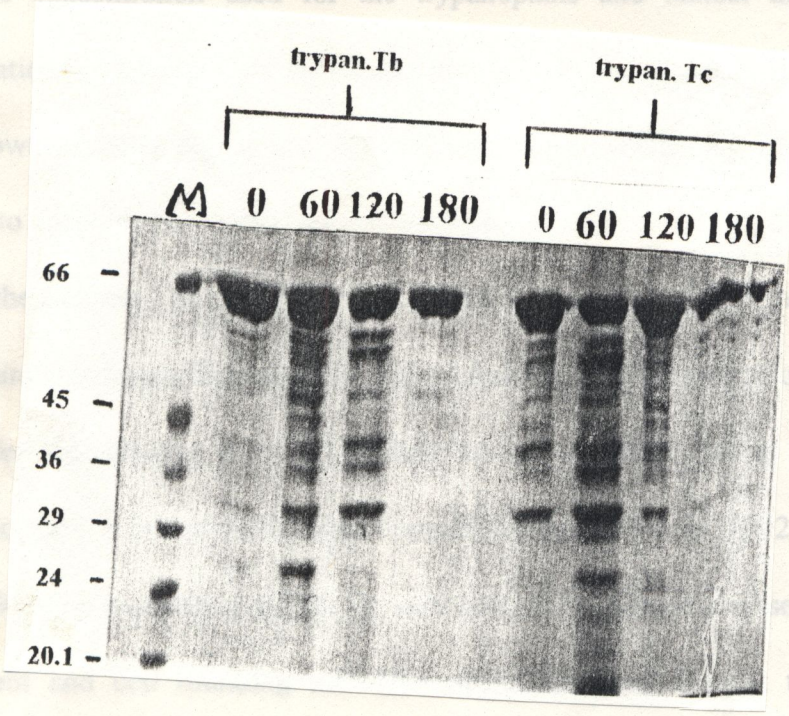
Above:

Shows casein reconstituted in the enzyme incubation buffer to give a final concentration of 1.2 mg/ml. 120 µl of the protein was incubated with 50 µl of trypanopain Tc and trypanopain Tb in separate tubes. To make good observations the incubation times were increased. Aliquots were removed at 0, 60, 120, and 180 minutes and then immediately boiled. The samples were run on 11% acrylamide gel. Lane 1 low range markers, (Lanes 2-5 incubated with trypanopain Tb) lane 2 at 0 minutes, lane 3 at 60, lane 4 at 120 and lane 5 at 180 minutes, lane 6 no sample added, (Lanes 7-10 incubated with trypanopain Tc) lane 7 at 0 minutes, lane 8 at 60, lane 9 at 120 and lane 10 at 180 minutes.

Below:

Shows a similar digestion of bovine serum albumin standard with the two enzymes. Aliquots were removed after 0, 60, 120, and 180 minutes. The samples were run on a gel exactly as above.


trypan.Tb
trypan. Tc
M 0 60 120 180 0 60 120 180
66
45
36
29
24


trypan.Tb
trypan. Tc
M 0 60 120 180 0 60 120 180
66
45
36
29
24
20.1

The cells were incubated at 20°C, up to 4 hours. The cells were examined every 20 minutes for any cytopathic effect, mainly cell rounding off and cell detachment, using a phase contrast light microscope. It was expected that if the enzyme rounds off or detaches cells it would show the effects produced by trypsin which rounds off the cells into single entities and/or detaches them from the surface of the cell culture plate. Trypsinization was normally complete within 10 minutes for this cell line (personal experience during passaging of the cells). Cell detachment was observed after 40 minutes of incubating the mixture with the cells. The cell detachment was not uniform and was attributed to the non-physiological buffer used to elute the enzyme. However, the cell outline appeared slightly cloudy as though the cell membrane was being attacked. To compare this reaction papain, another cysteine protease, was used at 200 μg/ml and 600 μg/ml. Papain, even at three times the concentration used for the trypanopains and almost the same concentrations of trypsin used for trypsinisation, failed to detach or round off cells. However, after 30 minutes the cell lines were cloudy and it was not possible to make out the individual cell outlines.

Though the enzymes (trypanopains) were about 3 times less concentrated than that required for trypsinization using trypsin and the long procedure of trying to transfer the enzyme from the elution buffer to $PBS^{(-)}$ led to loss of enzymatic activity (observed by other researchers (Mbawa *et al*, 1992; Pamer *et al*, 1991) this probably accounts for only part of the failure to observe cell detachment and cell rounding off. The results observed suggest that the

trypanopains are likely to produce the effects observed with papain, a cysteine protease and highly unlikely to produce the effects observed with trypsin, a serine protease. It was expected that since the enzyme had collagenase type activity it would dissociate cells in culture as shown by other collagenases. The results suggests that the enzyme preferantially attacked the cell outline rather than just the extracellular matrix.

DISCUSSION

The role trypanosome toxins and bioactive molecules play in infection with African trypanosomosis still remains unclear. Until the 1970s, the hypothesis that toxins released by living or dying trypanosomes were involved in the pathogenesis of trypanosomosis was out of favour because it was difficult to confirm. However several studies have reported the involvement of trypanosome factors and this idea is being looked at again (Tizard and Holmes, 1976).

Until the last decade, there was no clear evidence that Lysosomes exist in the African trypanosome. Lysosome like organelles were purified in the past two decades and were shown to contain cysteine proteases (Lonsdale-Eccles and Grab, 1987; Mbawa *et al*, 1991b). Lysosomes are the main source of proteases that degrade proteins with a long half-life. It is believed that proteins with a short half-life are degraded in the cytosol by non-lysosomal proteases.

The role cysteine proteases play during an infection with African trypanosomes is not clear. Neilsen *et al* (1978) reported that a protease accounts for a fraction, about 10%, of the total complement activating capacity of the trypanosome lysates. The cysteine protease has recently been shown to be present in the flagella pocket suggesting that it may be actively released or released through the exocytotic pathway (Mbawa *et al*,

1991b) or it may be released by dying (lysed) trypanosomes. Authie *et al,* (1992) estimated that the cysteine protease is at least one per cent of the total *Trypanosoma congolense* proteins. This clearly shows that trypanosomes contain high amounts of cysteine protease, in comparison to other enzymes, and it is likely to have an important role in the parasite.

During relapse parasiteamias, when large numbers of trypanosomes in circulation are lysed by the host's immune response, the amounts of trypanosome cysteine proteases that are released into the host may not be completely inactivated by the host's normal anti-proteases defence mechanism such as anti-trypsin or alpha-2 macroglobulins (α_2Ms) because the influx of the enzyme is likely to be sudden. The finding that animals under natural challenge produce antibodies against the enzyme indicates that the enzyme is present at significant levels, and possibly free of the host's anti-protease defence system, long enough to induce an immune response. The finding that the enzyme is immunodominant and that the immune response to the cysteine protease by trypanotolerant animals and susceptible animals is different suggests the likely involvement of the trypanosome cysteine proteases in infection (Authie *et al*, 1992). Ability to mount a detectable antibody response against the enzyme has been related to 'trypanotolerance' (Authie *et al*, 1993). Thus it is possible that the enzyme's control may be important in pathogenesis of the disease.

Pamer *et al* (1989) reported that supplementing the drinking water of rats infected with *T. brucei* with 5% DFMO increased the yield of stumpy forms and made it possible for them to get stumpy-like forms from a monomorphic strain IL Tat 1.4. In the present study the drinking water of rats infected with pleomorphic strains of *T. brucei* was supplemented with 10% glucose when the level of parasites was about 10^8/ml for two to three days and this was found to increase the yield of stumpy forms by two fold or more. Although rats infected with *T. congolense* were provided with 10% glucose as in *T. brucei* infections, the number of stumpy forms was not monitored as it was not easy to assess but the pesence of glucose prolonged the infection by at least two days. This increased the amount of enzyme in micrograms per pure 10^{10} trypanosomes by twice or more than that normally recovered from control infections (ones not supplemented with glucose).

The cysteine proteases from *T. congolense* and *T. brucei* have been purified previously in different laboratories under different conditions and using different methods and the properties of the proteases have been studied (Lonsdale-Eccles and Mpimbaza, 1986; Mbawa *et al*, 1992; Pamer *et al*, 1991). The present study has demonstrated that the cysteine proteases from *T. brucei* and *T. congolense* can easily be purified using cystatin immobilised on sepharose 4B as explained in the methods. The level of purity and yields of trypanopain Tc and trypanopain Tb were

almost the same and satisfactory as shown by the results. The elution profiles of the enzymes from the cystatin-sepharose column were similar to that of cathepsin B and L as described by Kos *et al* (1986). The graphs were slightly different from those reported by Mbawa *et al*, (1992) (they did not show the high enzymatic activity at the begining during elution of the enzyme from cystin-sepharose column) mainly because activity and protein concentration were measured (in this study) from the time the gel and the lysate supernatant mixtures were poured into the column. Any other differences may be due to the differences in equipment used in the two studies.

Some trypanopain Tc was purified on the monoclonal antibody column (mAb) but the samples obtained were never as pure as those obtained using the cystatin-sepharose column. The mAb column was about a year old and this could have affected its performance although it had been only used on few occasions, less than four times. The possibility of the column being digested by excess cysteine protease or other uninhibited trypanosome proteases was likely. The yields of the trypanopain Tc in mg enzyme/10^{10} trypanosomes were very different with the cystatin-sepharose column giving almost 3 times more enzyme than the mAb column. However, the mAb column was more efficient at binding the enzyme than the cystatin-sepharose when one takes into account the incubation time of two hours required by the cystatin sepharose column while the mAb

column did not require any incubation. No attempt was made to purify trypanopain Tb using the monoclonal antibody column as earlier reports had indicated that the mAb did not bind trypanopain Tb (Authie *et al,* . *This suggests that the pure en ymes ere immunologically di erent.*

The ability o the puri ied en ymes trypanopain Tb and trypanopain Tc to degrade elastin, collagen type , collagen type , ibronectin, glycoproteins, laminin, bovine serum albumin, casein and ibronectin as assessed. The irst our are all connective tissue proteins and ere all cell culture reagents normally used or cell attachment. This as decided upon as the aim o the study as to ind out i the en ymes are involved in tissue invasion and tissue destruction. The other protein macromolecules ere used mainly to study the ability o trypanopain Tb and trypanopain Tc to digest other host proteins and compare the cleavage sites as these molecules have relatively e main electrophoresis or migration bands.

onnective tissue proteins play a vital role in the overall per ormance o di erent organs o the body. They enable cells to attach to underlying connective tissue and are important in cell communication, interaction and migration hebrehi et et al, 1992; Mathews and Neale, 1990). They also serve as receptors of complement component and other proteins vital in blood clotting and repair of injury sites (Stryer, 1988).

The elastinolytic properties of the trypanopain Tc and trypanopain Tb were studied by zymogram analysis and the results were 'apparently negative'. It was almost impossible to tell whether there had been any reaction at all by eye as elastin was insoluble, see results, fig. 13 (above). However, the gel scanner coupled to a micro computer that was sensitive at showing proteolytic bands which appeared negative by eye, also failed to detect any digestion bands on the zymogram (see results, fig. 13 below). Lonsdale-Eccles and Mpimbaza (1986) had indicated that the trypanopains (using trypanosome lysates) degrade elastin but they also indicated that as elastin was insoluble they discontinued its use in zymogram analysis. Determination of elastinolytic properties of trypanopain Tc in agarose containing elastin also indicated that the enzyme did not have any elastinolytic properties (Mbawa *et al*, 1992).

In this study we decided to use a different method. Elastin-congo red is normally used to assess the elastase-type activity of enzymes. The reaction is fast and can easily be followed by observing the colour change. This technique demonstrated that the trypanosome cysteine proteases do possess some elastinolytic activity but this was less than that shown by papain but was significant when compared to the controls. Elastase activity in some diseases such as that expressed by *Pseudomonas aeruginosa* has been shown to be the virulence factor of the disease it causes (Jones *et al*, 1993; Tamura *et al*, 1992). However, one has doubts as to the contribution

of this level of elastase activity in trypanosomosis as it seems to be too low. Unless conditions in the host modulate this activity positively such as the host sera moiety described by Lonsdale-Eccles and Grab (1987), it is unlikely to play any significant role in disease.

The results described earlier have demonstrated that the trypanosome cysteine proteases possess collagenolytic properties. The trypanopains degraded collagen type I and collagen type IV easily. Fibronectin was also degraded far more easily than any other molecule as shown by the results. Thus fibronectin was the best substrate for the trypanosome cysteine proteases among all the connective tissue proteins studied and its rate of digestion compares very well with that of fibrinogen, described as the best substrate of trypanopain Tc (Mbawa *et al*, 1992). Similar results were obtained at 37°C which suggests that the enzyme could digest most of these molecule at the body temperature of most mammals. Glycoproteins were also easily digested by the enzymes.

The level of collagenase activity expressed by the trypanosome cysteine proteases is quite high. This high collagenase activity could play a central role in pathogenesis of African trypanosomosis such as can be seen in other diseases. Collagenase activity expressed in lung of transgenic mice is responsible for lungs damage (Armiento *et al*, 1992), that expressed in legionella disease is associated with tissue destruction (Rechnitzer *et al,* 1992) and that expressed by *Porphyromonas gingivalis*

enables the parasite invade and destroy tissue (Bedi and William, 1994). This high collagenase activity shown by trypanopain Tb and trypanopain Tc is likely also to play a similar role especially in tissue invasion and tissue destruction. The focal disorganisation, mainly collagen degeneration, seen at the chancre region in trypanosomosis (Gray and Luckins, 1980) is a possible role of the cysteine proteases. The degenerating trypanosomes seen at the chancre region (Gray and Luckins, 1980) could be the source of sufficient level of the cysteine protease that can degrade collagen.

In infections with *T. brucei* where trypanosomes are normally found in 'nests' or 'islets' in tissue, this collagenolytic activity expressed by the trypanopain Tb could account for some of the tissue lesions seen in disease. The fact that *T. congolense* is not found in colonies but almost evenly distributed within tissue (Fieness, 1950) means that even if trypanosomes release proteases the effect will be minimal. The enzyme concentration on the cells around the parasite will be very low making any enzymatic digestion hard to notice even with experienced eyes (that is the amount of enzyme in the local environment depends on the number of trypanosomes). This may be one of reasons for the differences in tissue lesions observed in the infections with *T. brucei* and *T. congolense*. Recent studies using the electron microscope have shown large numbers of *T.*

congolense parasites in the pituitary of infected animals and tissue lesions have been seen (ILRAD, 1991).

Expression of the enzyme in a host animal free of trypanosome infection may provide some insight into the enzyme's role in African trypanosomosis. Alternatively, use of monoclonal antibodies against the trypanopains during infection may show which pathogenic effects of trypanosomosis infection will be reduced.

Fibronectin and laminin are mainly cell surface proteins that play a major role in cell attachment. Fibronectin especially plays an important role in cell communication, migration and attachment. It has specialised domains that bind specific molecules involved in specialised functions such as some complement components and some blood clotting factors (Ghebrehiwet *et al*, 1992; Visai *et al,* 1991). The trypanopains' proteolytic activity against laminin and fibronectin could have an effect on cell interaction or communication and could impair some of the functions of specialised organs including those vital in fighting infection. The presence of trypanosomes in organs such as the pituitary, thymus, central nervous system, heart and other tissues including the blood may result in degradation of fibronectin and laminin. This may contribute to the poor response showed by organs like the pituitary and thymus (which both suffer tissue lesions) during trypanosomosis and this may impair the hosts' ability to fight the disease (ILRAD, 1991).

Some proteins that are virulent factors such as the 50.8 kDa protein from *Aeromonas salmonicida*, have high affinity for fibronectin and laminin (Doig *et al*, 1992). It has been demonstrated by Mbawa (1992) that trypanopain Tc has a carbohydrate moiety. The role of the carbohydrate is unknown. It may play a role in enzyme targeting. It may also help the enzyme bind to membranes through receptors of similar glycoproteins and result in digestion of cell surface proteins. Lonsdale-Eccles and Grab (1987) showed that most sera from trypanosome hosts increased the activity of the cysteine protease with calf serum having the highest activation. Instead of being inhibited, the enzyme's activity is actually enhanced by host molecules in sera and this may mean that the activity demonstrated in this *in vitro* study may be much less than that taking place *in vivo*.

In an effort to try and find out if the trypanopains have trypsin like properties of rounding off and detaching cells, cell culture experiments were performed and the results were not very clear. The major problem was how to totally remove the enzyme from the elution buffers which were not physiological buffers into $PBS^{(-)}$, a physiological buffer, without a *major or total loss of enzyme activity. Transfer could only be done by dialysis for a long time which normally resulted in significant loss of* activity. When prepared as explained in the methods section, a drop in activity occurred of up to 50% or more.

The cells in culture usually began to become detached 60 minutes after the enzyme preparation was added. Trypsin normally takes only 10 minutes to round off and detach cells in culture and therefore 60 minutes should have been more than enough to observe any cell rounding off or detachment in our experiment. The limited cell detachment observed is likely to have been caused by butanol and acetic acid which formed part of the elution buffer. However, one thing was almost certain, the trypanopains attacked the whole cell rather than just the intercellular matrix. In one control experiment using incubation with papain, another cysteine protease, this enzyme failed to detach or round off cells even after 90 minutes of incubation, though the concentration of papain was three times that of the trypanopains and equal to that of 0.5 x trypsin-EDTA normally used to dissociate cells in culture. However, papain seemed to attack the whole cell and after 60 minutes one could not easily see the outline of the cells as they all looked clouded, with some dots or dark spots. This was similar to the way trypanopains were attacking the cells in culture though this was less obvious than that shown by papain.

These rsults may indicate how closely properties of the trypanopains resemble those of papain, a cysteine protease, rather than trypsin, a serine protease. This was unexpected as trypanopain Tc has been reported to have trypsin-like activity (Mbawa *et al*, 1992; Lonsdale-Eccles and Grab, 1987) and the results have also shown that the enzymes have collagenase

type activity (collagenases are serine proteases). Collagenases are used to dissociate cells in culture. However, the results show that trypanopains hydrolyse cell surface proteins like fibronectin even better than collagen and this could mean that the trypanopains may preferably to hydrolyse or attack the cell surface proteins rather than the intercellular matrix. More experiments are required to conclusively determine the trypanopains' ability to detach or round off cells.

The results of the experiments have shown that trypanopain Tc and trypanopain Tb have similar properties. The trypanopains were inhibited by similar inhibitors. E-64, cystatin and TLCK were very effective inhibitors of both trypanopain Tb and trypanopain Tc as shown by the inability of the enzymes to hydrolyse Cbz-Phe-Arg-NHMec. Aprotinin and PMSF did not inhibit any of the trypanopains to any detectable extent, when compared to the controls. Since aprotinin is a trypsin inhibitor one would expect it to inhibit an enzyme with trypsin-like activity.

To further try and find out if the two enzymes are different in their for specific peptide bonds, three host proteins were subjected to digestion by the two trypanopains. The results showed that they had similar cleavage sites as the digestion products had similar molecular weights and the absence of detectable random digestion products. This seems to suggest that the enzymes may have preference for the same peptide bonds and may have similar specificity. This suggests that the main amino acids that take

part in the proteolytic reaction at the active site are likely to be the same or have very close properties.

Specificity studies have been done using short peptides (Lonsdale-Eccles and Grab, 1987; Mbawa *et al*, 1991; Pamer *et al*, 1991) which indicated that the enzymes prefer bulky hydrophobic amino acids and charged amino acids in positions P2 and P1 respectively. It will be interesting to further investigate and find out which bond(s) are attacked as the enzyme displayed specific cleavage instead of random cleavage. The fact that collagen is degraded can not be explained by the narrow specificity reported as one third of collagen is glycine and it contains abundant proline and hydroxyproline. This seems to suggest cleavage in collagen may be at a different site, as amino acids such as phenylalanine (and other bulky, neutral amino acids) and arginine are rare in collagen and it will be hard to find them in the proposed sequence at many points in the polypeptide chain.

Trypanopain Tc has been detected early in plasma of cattle infected with trypanosomes. It elicits an IgM response in both trypanotolerant and susceptible animals. However, only trypanotolerant animals mount a detectable IgG1 against the enzyme (Authie *et al*, 1992; 1993a). The mechanism by which the enzyme is involved in anaemia is not known but the results have shown that animals that counteracted the enzyme with antibody production do not experience severe anaemia (Authie *et al*,

1992). Anaemia may be linked to the presence of the enzyme in plasma and its ability to digest cell surface proteins such as laminin and fibronectin. This may result in alteration of the red blood cell membrane antigenicity and this could result in auto-immune destruction. It has been demonstrated that destruction of the red blood cell membrane by proteases released by activated neutrophils, presumably by destroying cell surface proteins, results in alteration of the cell antigenicity. Naturally occurring antibody (IgG) in plasma then binds to the modified red blood cell membrane. This results in self-destruction of red blood cells which results in anaemia seen in inflammatory diseases (Weiss *et al*, 1988; 1992). As trypanosomes are present in tissues such as thymus, heart, pituitary and the blood, the cysteine proteases that they may release whether actively, or through the exocytotic pathway (together with lysosomal digestion products on the way to the outside) or from lysed or dying trypanosomes, may modify the antigenicity of red blood cells. This can result in accelerated destruction of red blood cells as that shown in inflammatory diseases (Weiss *et al*, 1988; 1992).

The inability of infected animals to switch from IgM to IgG has been attributed to low levels of complement component 3 (C3) (Kobayashi *et al*, 1976). Some proteases have been shown to degrade complement C3 (Bedi and Williams, 1994) and it remains to be shown if the trypanopains too can degrade complement C3. However, activation of complement by

proteases (Neilsen *et al*, 1978) and by immune complexes formed by antibody and cysteine proteases (Authie *et al*, 1993b) may lead to low levels of complement component C3. This will lead to complement component depression. The ability to fight disease has been linked to the ability of animals to maintain significant levels of complement components.

This work has demonstrated that trypanopain Tb and trypanopain Tc can be purified using cystatin-sepharose column and the yields are quite good. The results suggest that a column with 6 mg of cystatin should be loaded with about 2 $x10^{10}$ pure trypanosomes lysate preparation. It has been demonstrated that the two enzymes may have similar specificity though they have minor differences in molecular weight (4 kDa). The study has demonstrated that the trypanopains degrade the connective tissue proteins collagen and elastin, the cell surface proteins fibronectin and laminin, and other proteins such as bovine serum albumin, casein, and fibrinogen. All the molecules used were not denatured prior to digestion with the enzymes. The molecules, elastin and casein, that were poor substrates for trypanopain Tc (Mbawa, 1992) have also been demonstrated to be poor substrates of trypanopain Tb. The likely role of the proteolytic activity of the enzyme in tissue lesion development and anaemia have been discussed.

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APPENDIX 1

PREPARATION OF BUFFERS AND SOLUTIONS USED FOR COLLECTION AND PURIFICATION OF TRYPANOSOMES.

1 *Phosphate Saline Glucose (PSG) Buffer pH 8.0.*

Na_2HPO_4	8.088g
NaH_2PO_4	0.359g
NaCl	2.500g
$C_6H_{12}O_6$	10.00g

Made up to 1 litre with water and adjusted pH to 8 using 10% phosphoric acid. The ionic strength was 0.217

2 *Phosphate Saline Glucose (PSG) Buffer pH 7.4.*

Na_2HPO_4	5.392g
NaH_2PO_4	0.239g
NaCl	1.700g
$C_6H_{12}O_6$	10.00g
$C_5H_4N_4O$	.0136g

Dissolved in water and made up to 1 litre with water and adjusted the pH to 7.4. The ionic strength was 0.145

3. ***Sodium acetate (anticoagulant)***

Trisodium citrate	29.41g
$C_6H_{12}O_6$	1.80g

Made up to 1 litre with water

4. ***Preparation of percoll solution, pH 7.4***

Sucrose	4.28g
HEPES	0.24g
Glucose	1.00g

Dissolved in 50 ml 100% percoll and adjusted pH with solid Tris then made up to 100 ml with 100% percoll.

5. ***Dulbeccos Phosphate Buffered Saline (PBS) pH 7.4***

Na_2HPO_4	1.15g
KH_2PO_4	0.20g
NaCl	8.00g
KCl	0.20g
$MgCl_2.(H_2O)$	0.10g
$CaCl_2.(H_2O)$	0.13g

Made up to 1 litre with water.

APPENDIX 2

PREPARATION OF CELL CULTURE BUFFERS AND MEDIA.

1. *Phosphate Buffered Saline Negative($PBS^{(-)}$) pH 7.4*

NaCl	8.00g
KCl	0.20g
Na_2HPO_4	1.15g
KH_2PO_4	0.20g

Made up to 1 litre with water

2. *Preparation of M199 medium*

M199 basal

10% (v/v) tryptose phosphate

Mixed and then added to the above:

15% (v/v)foetal calf serum

1% (w/v) antibiotics (penicillin : streptomycin, 1:100w/w)

1% (w/v) l-glutamate

3. *Preparation of RPMI 1640*

RPMI 1640 basal

Added 20% (v/v) foetal calf medium

1% (w/v) antibiotics (penicillin : streptomycin in 1:100 w/w)

1% (w/v) l-glutamate

4. ***Preparation of L-15 medium***

L-15 medium basal

10% (v/v) tryptose phosphate.

mixed and added:

15% (v/v) foetal bovine serum

1% (w/v) antibiotics (penicillin / streptomycin as above)

1% (w/v) l-glutamate

5. ***Earle's balanced salt solution (EBSS OR BSS) pH 7.4.***

NaCl	6.800g
KCl	0.400g
$CaCl_2$	0.200g
$MgSO_4.7H_2O$	0.100g
$NaH_2PO_4.H_2O$	0.125g
$NaHCO_3$	2.20g
$C_6H_{12}O_6$	1.00g

Dissolved in 500 ml water and then made up to 1000 ml with water

6. ***Preparation of trypsin-EDTA***

Trypsin	0.05g
EDTA	0.20g

Dissolved in 100 ml BSS.

APPENDIX 3

PREPARATION OF SODIUM DODECYL SULPHATE ACRYLAMIDE GEL SOLUTIONS.

I. *STOCK SOLUTIONS*

1. *Acrylamide/bisacrylamide 30:1*

acrylamide	30.0g
bisacrylamide	1.0g

Dissolved in 60 ml water and made up to 100 ml with water.

2. *1.5 M Tris/HCl pH 8.7*

Trisma base	18.17g

Dissolved in 70 ml water, adjusted the pH to 8.7 with 3 M HCl and made up to 100 ml with water.

3. *1.0 M Tris/HCl pH 6.8*

Trisma base	12.114g

Process as in 2. above.

4. *10% Ammonium per sulphate.*

Dissolved 0.1g ammonium per sulphate in 0.5 ml water and made up to 1 ml with water. It was prepared fresh each time required. It is stable in the refrigerator (4°C) for at least one week.

5. ***10% Sodium dodecyl sulphate (SDS)***

Dissolved 2.5g SDS in water and made up with water to 25 ml.

6.(a) ***2 x Sample buffer***

0.5 M Tris/HCl pH 6.8	10 ml
10% SDS	10 ml
Mercaptoethanol	*1.0 ml*
Glycerol	10 ml
Water	19 ml

(b) ***Urea sample buffer***

0.5 M Tris, pH 6.8	10 ml
10% SDS	10 ml
Mercaptoethanol	1.0 ml
8.8 M urea	29 ml

Stored in the refrigerator at 4°C for not more than one week.

7. ***Electrophoresis (running) buffer***

Tris	6g
Glycine	28g

Dissolved in 500 ml water then added:

10% SDS 10 ml

then made up to 1000 ml with water.

II *PREPARATION OF SDS-PAGE GELS*

1. *Preparation of 5% stacking gel*

Acrylamide/bisacrylamide	1 ml
0.5 M Tris/HCl pH 6.8	1.5 ml
Water	3.42 ml

Mixed and degassed for about 5 minutes then added:

10% SDS	60 µl
10% APS	20 µl
TEMED	10 µl

2. *Resolving gels and some common gel recipes used*

RECIPE	7.5% GEL	8.75% GEL	10% GEL	2.5% GEL
cryl/bisa (ml)	7.5	8.75	10	12.5
1.5M Tris (ml)	7.5	7.5	7.5	7.5
water (ml)	14.65	13.4	12.15	9.56

Mixed the above and degassed for about 5 minutes and then added:

0% SDS (ml)	0.6	0.6	0.6	0.6
TEMED (µl)	15	15	15	15
APS (µl)	100	100	100	100

APS is ammonium per sulphate and SDS is sodium dodecyl sulphate.

ABREVIATIONS

α_2M	alpha 2-macroglobulin
BSA	bovine serum albumin
BSS	balanced salt solution
CBZ	carbobenzoxyl
C3	complement component 3
°C	degree Celsius
°	degree
DEA	diethylamine
DE	diethyl
DFMO	difluoromethylornithine
DMSO	dimethylsulphoxide
DTT	dithiothreitol
E-64	trans-epoxysuccinyl-ι-leucyl-ι-amido (4 guanido) butane
Fbs	foetal bovine serum
Fig.	figure
HEPES	4-(2-hydroxyethyl)-l-piperazine sulphonic acid
kDa	kilodaltons
mAb	monoclonal antibody
2-ME	2-mercaptoethanol
MEM	minimum essential media

ml	millilitre
mM	millimolar
NHMec	7-amido-4-methylcoumarin
PAGE	polyacrylamide gel electrophoresis
$PBS^{(-)}$	phosphate buffered saline without Mg^{2+} and Ca^{2+}
PHA	phytoheamagglutinin
PMSF	phenylmethylsulphonylfluoride
PSG	phosphate saline glucose
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N', tetramethylethylenediamine.
TLCK	Nα-ρ tosyl-l-lysine chloromethylketone
Tris	tris (hydroxyethyl) aminomethane
μ	micro
μg	micrograms
μl	microlitre
μm	micrometer
μM	micromolar
VAT	variable antigen type
VSG	variable surface coat
v/v	volume by volume
w/v	weight by volume
/	per