Protease inhibitors and the biochemical basis of insect resistance in Vigna unguiculata

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PROTEASE INHIBITORS AND THE BIOCHEMICAL BASIS OF INSECT RESISTANCE IN *VIGNA UNGUICULATA*

by

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A thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy in the University of Durham.

September 1978

To John

To my Parents

"Abstain from the bean"

Pythagoras

ABSTRACT

The cowpea (*Vigna unguiculata*) suffers severe losses (up to 70%) when stored due to infestation by the bruchid beetle *Callosobruchus maculatus*. A variety of cowpea resistant to bruchid attack had been described (IITA); the present work confirmed its resistance. The resistance was shown not to be of a physical nature, and thus seeds of both this resistant variety and susceptible varieties were screened for all possible toxins which were likely to form the biochemical basis of the resistance. The trypsin inhibitor was the only toxin detected. Using two different methods both for extraction and determination of the inhibitory activity, the trypsin inhibitor was found to be present in about twice the concentration in seeds of the resistant variety compared to the highest level found in susceptible varieties.

The trypsin inhibitor was purified by affinity chromatography, and its toxicity towards larvae of *Callosobruchus maculatus* was demonstrated directly by feeding trials. Addition of the trypsin inhibitor, at the physiological concentration of the resistant variety, to the basic diet was found to be lethal, whereas addition at the physiological concentration found in susceptible varieties had no apparent effect. Further, addition of the inhibitor to the level of the resistant variety resulted in a susceptible variety

becoming resistant. Inactivation of the trypsin inhibitor removed its antimetabolic activity. The toxicity was also demonstrated *in vitro* by inhibition of larval proteolysis of synthetic and physiological substrates.

A partial characterisation of the cowpea trypsin inhibitor was carried out. It was shown to have a molecular weight of approximately 17,000. Complex formation with trypsin could be directly demonstrated. The inhibitor as isolated was a mixture of several isoinhibitors, some of which were able to inhibit chymotrypsin as well as trypsin. A model for the subunit structure of the inhibitor is proposed.

ACKNOWLEDGMENTS

I would like to thank Mrs. A. Kilminster and Mr. P. Dobie of the Tropical Stored Products Centre for carrying out the cowpea susceptibility trials and the insect feeding trials; also Mr. B. Joicey for carrying out the amino acid analyses.

I am most grateful to my supervisor Professor D. Boulter for his critical apprisal of the present work and for use of the department's facilities. His continued support of this research has been much appreciated. Further, my thanks are due to my colleagues in the Botany department for their advice and encouragement, in particular Dr. Ron Croy who has allowed me to mention some of his unpublished work, and Dr. Marta Evans. I would also like to thank Mrs. Ethne Ellis for typing the thesis.

The work in this thesis has been supported in part by a grant from the Vegetarian Society; however, the major financial burden was carried by my parents to whom I offer my grateful thanks.

Finally I would like to express my gratitude to Dr. John Gatehouse for his especial interest in this work throughout, and also for allowing me to mention certain of his unpublished results.

ABBREVIATIONS

The following abbreviations have been used in the thesis:

BAEE	α-N-benzoyl-L-arginine ethyl ester HCl
BAPNA	α-N-benzoyl-DL-arginine-p-nitroanilide HCl
BTEE	N-benzoyl-L-tyrosine ethyl ester
SBTI	soyabean trypsin inhibitor
CPTI	cowpea trypsin inhibitor
LAP	leucine aminopeptidase
BSA	bovine serum albumin
Bis	N,N'-Methylenebisacrylamide
TEMED	NNN'N'-Tetramethylethylenediamine
SDS	sodium dodecyl sulphate
TRIS	Tris (hydroxymethyl)aminomethane
TCA	trichloroacetic acid
PBS	phosphate buffered saline
DMSO	dimethyl sulphoxide
DTT	dithiothreitol

CONTENTS

SECTION I INTRODUCTION

PART I The Cowpea and its Major Storage Pest

The "Provisional Indicative World Plan for Agricultural Development" published by the Food and Agriculture Organisation (FAO) in 1970 stressed the vital importance of increasing the output of cheaper sources of quality protein. As was stated, edible grain legumes provide an immediate potential as food crops for alleviating human malnutrition. Among these legumes, an important species is the cowpea (Vigna unguiculata (L.) Walp.)

Origin and Production of the Cowpea

The cowpea is one of the most ancient of human food sources and has probably been used as a crop since Neolithic times (Chevalier, 1944). Because of the lack of archaeological evidence the centre of origin of cowpeas is uncertain and has been variously reported as possibly Asia, Africa, Persia or even South America (Summerfield et al., 1974). However, Steele (Summerfield et al., 1974), in agreement with (Faris, 1965), concluded that the progenitor was wild V. unguiculata, probably subspecies dekintiana, of the African Savanna zone, since no other species of Vigna produces fertile progeny when crossed with cultivars. Furthermore, Steele in agreement with (Sauer, 1952) proposed a solely Ethiopian centre of origin and

suggested that cowpeas were cultivated there with sorghum and perhaps, pearl millet. These crops subsequently formed the ancient cereal farming systems of the Savanna zone of Africa. However, others consider the cowpea to be of Asiatic origin. Burkill concluded that although of uncertain origin, the cowpea was introduced into Europe early enough for the Greeks and Romans to grow it under the names of Phaseolos, Phaseolus, or Phaselus. Despite its uncertain origin, for at present it is impossible to ascertain whether the migration started in Africa, Asia, or both, it is known that the cowpea was not introduced into the New World until the late seventeenth century and probably reached the Southern States of the USA in the early eighteenth century (Wright, 1907). It is known there as the black-eyed pea.

Cowpeas are grown extensively throughout the lowland tropics of Africa in a broad belt along the southern fringe of the Sahara, and in eastern Africa from Ethiopia to South Africa. They are mainly confined to the hot semi-arid to sub-humid areas with significant production in Nigeria (which alone produces about 61 per cent of the world crop), Niger, Upper Volta, Uganda and Senegal (Rachie and Roberts, 1974). They are also extensively grown in India, south eastern Asia, Australia, the Caribbean, lowland and coastal

areas of South and Central America and in the southern regions of the United States. Because of the habit of the plant and prolonged period of pod production of many local varieties, the cowpea is more suited to subsistence, rather than commercial, farming.

In Nigeria and the African Savanna zones the cowpea is the most important grain legume, the matured dried seed being the primary form in which the crop is consumed, although both the young pods and leaves are eaten (Steele, 1972). The vegetation also makes good quality hay which, together with surplus culled and broken seeds, is used as fodder. In the more advanced tropical agriculture of the USA, Australia, and South Africa, cowpeas have become increasingly used in the canning industry, and are also used for forage and as a cover crop.

Nutritional Value and Acceptibility of Cowpeas

The cowpea forms a major component in many African diets, not only on the basis of its high protein content, but also for calcium (90 mg/100 g), iron (6-7 mg/100 g) nictotinic acid (2.0 mg/100 g), and thiamine (0.9 mg/100 g) (Platt, 1962). In cereal based diets the lysine content of cowpeas is very important and becomes more so as the proportion of the total protein in the diet derived from cereals increases (Oema, 1963). In root and tuber diets of

the humid tropics of Africa, cowpeas are important both as a source of calories where foods of all kinds may be scarce, and a relatively cheap protein, where the staple foods contain only about 2 per cent protein. Although the percentage protein in cowpea seeds is high, varying between 19 per cent and 26 per cent (Russell, 1946; Jenkins, 1951) like other legumes the seeds are deficient in the sulphur amino acids, methionine and cystine (Sellschop, 1962; Harvey, 1970). In a survey of the amino acid profiles of six Nigerian cultivars and the wild subspecies dekintiana, methionine varied from 0.35 per cent to 0.90 per cent of total protein in the cultivars, but it was 1.47 per cent in the wild cowpea. Cystine ranged from 0.38 per cent to 0.90 per cent in cultivars though a value of 2.0 per cent has been reported (Evans and Bandemer, 1967). However, with suitable amino acid supplements (cystine and methionine) it is possible to raise the biological value of the cowpea seed protein to 95 per cent of that of egg albumin (Boulter, private communication). The chemical composition of cowpea seeds was investigated by Johnson and Raymond (1964). The mature seeds were reported to contain 11.72 to 12 per cent water, 0.69 to 1.06 per cent oil, 3.75 per cent fibre, 56.82 to 57.5 per cent carbohydrate and 23.63 to 24.56 per cent protein. Data on the vitamin A, B, C and D content

of cowpea seeds from thirty different cultivars have been reported; in general the brown-eyed seeds have the highest amounts (Ogunmodede and Oyenuga, 1968).

Although seed colour and size are important determinants of consumer preference, there is no evidence that variation in these characters is associated with variation in their nutritive value. Rough seeds are generally preferred because the testas can be removed more easily and after a shorter period of soaking than from smooth seeds. Albino seeds with rough testas are thus preferred in Nigeria and may, indeed, be a richer source of thiamine and niacine since these vitamins can be removed by soaking (Steele, 1972). In Africa cowpeas are consumed in three basic forms of which there may be many variations. Most frequently they are made into a gruel; the second form of preparation is in the form of deep-fried cakes and thirdly in the form of steamed cakes. Carrying out feeding trials with rats, Evans and Bandemer (1967) showed that cooking improved the nutritive value of the cowpeas and attributed it to the decrease in trypsin inhibitor activity on heat treatment; this will be discussed in greater detail in a later section of the Introduction.

Date of Planting and Yield

Date of planting is largely determined upon whether the

variety of cowpea is day neutral or short day sensitive, and whether there are two wet seasons or only one per annum. In northern Nigeria, where the bulk of the cowpea is grown and where there is only one rainfall, it is sown during the wet season, generally July and August, and is harvested during the dry season, usually in November and December (Booker, 1967); in this region the dry season lasts about six months, beginning in October.

In Nigeria consumer demand for cowpea seed far exceeds supply and prices are therefore high. One of the major reasons for this is that the average seed yields are of the order of only 300-400 kg/ha per season though the potential is about 2,800 kg/ha (Rachie and Roberts, 1974). Although intercropping cowpeas with sorghum or millet decreases the yield significantly, the major influencing factor on yield is insect pests. With successful control of such pests it has been possible to increase the yields of local varieties in the south from 224 kg/ha to over 1,778 kg/ha (Muller and Sellschop, 1954); much smaller increases have been achieved by fertiliser applications. In the north, when grown as a sole crop in the presence of adequate pest control, yield has been increased from 160 kg/ha to a maximum of 2,696 kg/ha with a mean of 1,534 kg/ha (Raheja and Singh, 1975).

Not only are cowpeas susceptible to a range of diseases, the principal ones being rust (Uromyces phaseoli), canker (Xanthomonas vignicola), wilt (Fusarium oxysporum), mildew (Erysiphe polygoni) and charcoal rot (Sclerotium bataticola), but they are also subject to attack by numerous pests, and virtually every part of the plant is liable to be damaged by one insect species or another. The status of all known cowpea pests has been reviewed by Libby (1968), Nyiira (1971), and Taylor (1964). A summary of the major insect pests of cowpea and time of occurrence of the pest in relation to plant growth is shown (Figure 1.1.1); the nature of damage caused by each is also given (Table 1.1.2).

The cowpea seeds are not only subject to insect attack whilst in the field, but considerable loss, as a result of insect damage, occurs during storage. In Nigeria the two major storage pests, both belonging to the beetle family Bruchidae, are Bruchidius atrolineatus (PIC) and Callosobruchus maculatus (F). Attack begins on the farms when the pods are ripening and at this stage it is predominantly by B. atrolineatus; however this infestation dies out during the early weeks of storage and is rarely found later. Infestation by C. maculatus, on the other hand, begins just before harvest and in the first few weeks of storage is not

Fig. 1.1.1.

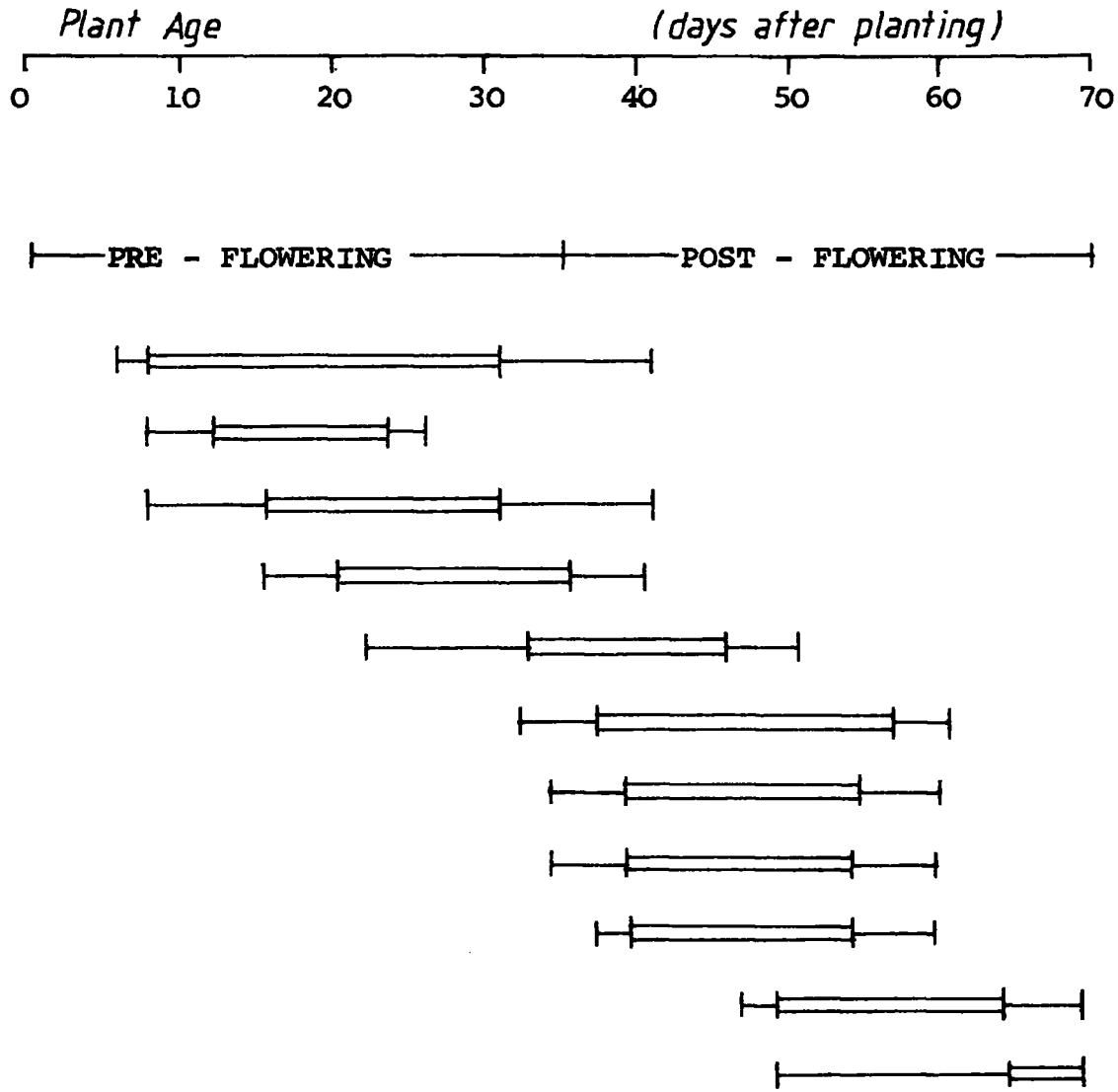


Table 1. Diagrammatic Representation of Cowpea Insect Pest Complex., Time of Occurrence and Peak Activity on Prima Cowpea. *(From IITA)*

Table 1.1.2 Cowpea insect pests and their damage to the cowpea plants

Insect Pest	Nature of Damage
1. Ootheca mutabilis	Important cowpea mosaic virus vector. Feeds on cowpea by chewing leaves.
2. Luperodes lineata	Cowpea mosaic virus vector. Feeds on cowpea by chewing tender leaves and stem.
3. Sericothrips occipitalis	Feeds on foliage by rasping and sucking plant sap. Does more damage to plant under drought stress and high temperature. Leaves become distorted, plant is stunted and loses vigour. Is cowpea mosaic virus vector and is suspected as vector for other foliage diseases.
4. Empoasca dolichi	Feeds on foliage by sucking the plant sap; leaves curl and dry. Suspected as a vector for foliage diseases.
5. Taeniothrips sjostedti	Feeds on flower buds and flowers. Causes distortion of flower buds and flowers effecting in reduced flowering and flower drop.
6. Maruca testulalis	Early generations feed on tender stems and peduncles, later feed on flowers and pods. Younger pods are completely damaged by the larva feeding on pods near peduncles. On older green pods, the larva feeds on a few developing seeds, leaving feeding holes and grass on the pods which attracts fungus on pods.
7. Anoplocnemis curvipes)	These coreids feed on green pods by sucking the sap.
8. Acanthomyia horrida)	Pods shrivel and die prematurely or few seeds develop in the pods.
9. Riptortus dentipes)	
10. Cydia ptychora	Attacks pods near maturity stage. The larva feeds inside the pod and destroys the seeds.
11. Callosobruchus maculatus	Infests pods in the field and is active as a storage pest. The larvae feed on dry cowpea seeds in storage.

(From IITA)

common, but the infestation grows rapidly in store and the species soon replaces B. atrolineatus. Booker (1967), Caswell (1961) and Prevett (1961) independently evaluated the degree of damage caused by these two species of Bruchidae. It was estimated that the overall pre-harvest seed infestation was of the order of 2 per cent (Booker, 1967), with an overall loss of 1 per cent being attributed to B. atrolineatus (Caswell, 1961). Total loss caused by C. maculatus was approximately 50 per cent, though in some cases was high as 70 per cent.

Depending upon the intended fate of the seed in storage, the amount actually consumed by the larvae of C. maculatus may only be of secondary importance when compared to the germination potential of the damaged seed. It has been shown that with three or more emergence holes, the germination of the cowpea seed was considerably reduced and that one insect can cause a 3 per cent to 5 per cent weight loss in a seed (Jotwani and Sircar, 1964; Booker, 1967). However, there is a decrease in weight loss per individual insect which is proportional to the number of larvae per seed; this is because adults emerging from seeds containing a large number of individuals tend to be smaller.

Callosobruchus maculatus (F.); the Major Storage Pest of the Cowpea

Callosobruchus maculatus was first described by Fabricius in 1775 as *Bruchus maculatus*, from material supplied by Von Rohr, with America as its habitat; however, it was undoubtedly brought across from either the West Indies or South America where the latter had been collecting. Fabricius described it again in 1792 as *B. quadrimaculatus* from material collected at St. Croix in the West Indies (Larson and Fisher, 1938). Subsequently it has been recorded from widely separated localities, and although its origin is still uncertain, it undoubtedly originated in an old world tropical, or subtropical, region alongside its primary host plant, *Vigna unguiculata*, which likewise appears to be of uncertain origin.

Callosobruchus maculatus did not gain attention as an economic pest until 1885 when cowpeas from Texas, USA, exhibited at the Cotton Exposition in Georgia were found to be heavily infested. It next attracted attention in 1893 when it virtually destroyed all the beans exhibited by Brazil and Venezuela at the World's Columbian Exposition at Chicago. At that time the Division of Entomology of the USA Department of Agriculture recorded it as common in the southern States of USA (Larson and Fisher, 1938). Although

C. maculatus causes considerable damage, and consequently loss, of mature cowpea seeds whilst in storage, it is only the larval stage which feeds. Infestation commences by the female adult laying her eggs, singly, on the outside of the pod or on exposed seeds. When laid, each egg is surrounded by a cementing fluid which, when dry, fastens it securely to the surface of the seed; this is essential in order to ensure subsequent hatching. On hatching, the larva gradually eats its way through the seed, the tunnel formed behind usually becoming blocked by egested matter. The larva moults three times and during its last instar makes a cell in which it moults a fourth time and so pupates. The pupation cell is near the surface of the seed but remains covered by a thin film known as a "window". This ensures successful emergence of the adult which does not feed, and consequently has undeveloped mouth parts; it therefore has to rely on pushing its way out of the seed by breaking the "window". As the adults can mate and lay eggs in less than one day, subsequent infestation is rapid. Maximum egg laying occurs during the first three days after emergence of the female. The duration of each stage, from the hatching of the egg to adult emergence is very much influenced both by temperature and relative humidity (Schoof, 1941; Howe and Currie, 1964; Booker, 1967).

From laboratory observations of C. maculatus Booker (1967) showed that the period of pre-adult development was negatively correlated with temperature and was also affected, though to a lesser degree, by humidity.

Howe and Currie (1964) found that the shortest mean developmental period of C. maculatus on cowpeas was twenty three days at 30°C and 70 per cent RH. There is however a certain amount of biological variation between different strains of C. maculatus. The detailed biology of the strain of C. maculatus used in the work reported in this thesis is given in a recent paper by Kilminster (In preparation).

The Breeding Programme to Find Resistant Cowpea Varieties

The International Institute of Tropical Agriculture (IITA) was established in 1967 as an autonomous non-profit corporation. It was initially conceived and subsequently developed with the mandate of improving the quantity and quality of food in the lowland humid tropics. Since grain legumes have exceptional potential for contributing universally to the availability of economical high-quality proteins and caloric energy in tropical diets, a major programme on grain legume improvement was initiated. Cowpeas were selected as a suitable crop and many varieties have already been produced. Due to the serious losses of

cowpea seed and germination potential during storage, as a result of infestation by C. maculatus, a breeding programme was set up to select for resistance against this particular pest. Out of some five thousand varieties of Vigna unguiculata one, TVu 2027, showed significant resistance towards C. maculatus (Singh, private communication). I was asked by IITA to investigate the possible biochemical basis of this resistance. Resistance is not necessarily due to biochemical factors since there may be a physical form of resistance involved. Studies have indicated that when offered a free choice, C. maculatus prefer seeds possessing a smooth testa, as opposed to rough testa seeds, for oviposition (Larson and Fisher, 1938; Booker, 1967); Nwanze et al., (1975) support these findings. However in their reported trials, the correlation obtained between seed coat texture and subsequent adult emergence was not of statistical significance; consequently preference in ovipositioning would not be relevant to infestation by C. maculatus. Further, under rural storage conditions (in which varieties are stored in separate batches) the insect does not have a free choice for oviposition. Thus this "physical resistance" does not appear to be important. It was therefore necessary to consider the various toxic constituents of legume seeds which could form a basis for biochemical resistance against this storage pest.

PART II Role of Secondary Compounds in Plant Protection

Interest in the control of insects which cause extensive damage to legume seeds in storage has arisen as a result of the importance of these seeds as a potential source of plant protein. Most legumes are grown in the developing countries, and it is precisely these countries which suffer most from protein malnutrition; partly as a result of insufficient insect control. Unfortunately the more elaborate methods of seed storage in controlled atmospheres, or by fumigation, are not an economically viable proposition to subsistence farming. Thus, in order to achieve adequate insect control it is necessary to evaluate, and hence exploit (Painter, 1941; Janzen, 1969; Whittaker and Feeny, 1971; Applebaum and Birk, 1972; Feeny, 1976; Tester, 1977), the multiple mechanisms of resistance which are present in legume seeds; providing, of course, that they do not adversely affect the nutritional value of these seeds for human consumption.

Since phytophagous (plant eating) insects do not vary widely in their nutritional requirements, that is, the basic requirements are met by all seeds (Beck and Reese, 1976), insect predation is primarily determined by the presence of secondary compounds in the host plant which affect not only the developmental and physiological

processes, but also influence behavioural patterns (Fraenkel, 1969). These compounds were called "secondary" because they play no known role in the essential metabolic processes involved in the plant's physiology, and were thus distinguished from the "primary" plant chemicals which do.

There are two schools of thought concerning the role of the secondary compounds. The first school suggest that they are either waste products of metabolism, or at best inert storage materials (Muller, 1969; Luckner, 1972). However, this suggestion does not appear plausible if their complex structure is taken into account. No cases are known where waste products are structurally much more complex than the starting materials, otherwise the metabolic costs of excretion would be prohibitive (Swain, 1977). Similarly, to be effective, storage products need to be readily synthesised and the reserves easily recoverable to primary metabolism in a directly usable form. The second school of thought, which is heavily subscribed to, propose that these secondary compounds have a protective function within the plant (Painter, 1951; Fraenkel, 1951; Whittaker and Feeny, 1971; Gill and Lewis, 1971; Feeny, 1976; Rodriguez and Levin, 1976; Mothes, 1976; Swain, 1977). However, this does not mean that every secondary compound will have a readily recognisable or even discernable role; often their

effects will be found to be marginal. The fact that seeds often contain high concentrations of such compounds, which disappear relatively quickly after germination and early seedling development, has been cited as evidence in favour of the view that they perform a protective function at the most vulnerable period of growth (Janzen, 1971a).

The number of different types of secondary compounds known to exist is large. Members of the Leguminosae, in particular the seeds, appear to be a rich source for many types (Bridwell, 1918; Janzen, 1969), although in cultivated species some of these toxic components have been lost as a result of agronomic selection for ready edibility. Despite the presence of these secondary compounds, legume seeds are still subject to insect attack. Predation of certain members of the Leguminosae by the Bruchidae has been extensively studied (Bridwell, 1925; Bridwell, 1938; Peake, 1952; Davey, 1958; Prevett, 1961; Howe and Currie, 1964; Johnson, 1966; Janzen, 1969; Janzen, 1971b); this relationship is considered as a unique case among insects (Applebaum, 1964b).

The secondary compounds which will be discussed in this Introduction are those which are likely to have a detrimental effect upon insects and could possibly be present in the seeds of Vigna unguiculata.

Protease Inhibitors

Much of the early work relating to the toxicity of legumes was carried out with species long recognised to be inedible since they contained substances which Osborne (1909) had referred to as "toxalbumins". However, it was not long before the deleterious effects of inadequately prepared edible legumes were observed in animal feeding trials. In 1917, Osborne and Mendel noticed that soyabean, unless cooked for several hours, would not support the normal growth of rats; an observation which has been extended to include many other experimental animals (Liener, 1958). The subsequent discovery (Ham and Sandstedt, 1944; Bowman, 1944) and purification (Kunitz, 1945; Kunitz, 1946) of a heat labile protein in soyabeans, which had the unique property of combining with the digestive enzyme trypsin to form an inactive complex, strengthened the concept that this trypsin inhibitor was responsible, at least in part, for the observed growth depression. Furthermore, active antitryptic fractions extracted from unheated soyabeans, when incorporated into diets, were shown to retard the growth of rats (Klose et al., 1946; Borchers et al., 1948; Liener et al., 1949), mice (Westfall et al., 1948), and chicks (Ham et al., 1945; Borchers et al., 1948). Since the protein efficiency of

partially heated soyaflours was found to increase in proportion to the destruction of the trypsin inhibitory activity, Westfall and Hauge (1948), concluded that the trypsin inhibitor was the major cause for the poor utilisation of the protein in raw soyabeans. This theory was further supported by Borchers and Ackerson (1951) who demonstrated that the addition of trypsin counteracted the growth depression obtained with rats receiving diets containing raw soyabeans. A similar effect was also observed with chicks.

From these studies there appeared to be little doubt that the poor growth-promoting quality of raw soyabeans could be attributed, to a large extent, to a trypsin inhibitor. How this inhibitor functioned was not understood. Since a difference in the digestibility between raw and cooked soyabeans did not appear to account for this observed growth depression, Melnick, on the basis of experiments involving the <u>in vitro</u> release of amino acids from soyabean protein by pancreatin, suggested that methionine was liberated more slowly by the proteolytic enzymes of the intestine than the other essential amino acids, and was thus ineffectively utilised for protein synthesis. However, later <u>in vitro</u> studies did not appear to support this hypothesis since it was shown that the

trypsin inhibitor does not specifically retard the enzymatic release of methionine, but seemed to affect all amino acids to the same degree (Riesen et al., 1947; Ingram et al., 1949; Liener and Fevold, 1949). Further, active antitryptic preparations have been shown to retard growth of rats and chicks even when added to diets containing predigested protein (Desihactar and De, 1947; Liener et al., 1949); under these conditions intestinal proteolysis would not play a part with respect to the availability of essential amino acids.

Chicks and rats, when fed raw soyabean meal (Chernick et al., 1948; Booth et al., 1960; Alumot and Nitsan, 1961), or crystalline trypsin inhibitor (Lyman and Lepkovsky, 1957) were found to develop marked hypertrophy of the pancreas. Contrary to what had been generally assumed, the amount of trypsin found in the intestines of those experimental animals was actually greater than that found in the control animals fed heated soyabean meal. From these observations it would appear that the growth depression was not a result of inhibition of proteolysis, but rather the result of an endogenous loss of essential amino acids from a hyperactive pancreas which is responding in a compensatory manner to the effects of the trypsin inhibitor (Booth et al., 1960). This is further supported by the recent findings of

Green (1972) and Niess (1972), who have presented evidence to indicate that trypsin or chymotrypsin in the intestine suppresses pancreatic enzyme secretion by feedback inhibition, and that trypsin inhibitors evoke increased enzyme secretion by counteracting the suppression produced by trypsin. Since soyabeans, and legumes in general, are deficient in the sulphur amino acids, this would explain why, on addition of cystine or methionine to unheated soyabean meal to counteract the loss of endogenous amino acids, protein utilisation was found to be improved, essentially, to the same extent as adequte heat treatment (Hayward and Hafner, 1941; Almquist *et al.*, 1942; Russel *et al.*, 1946; Evans and McGinnis, 1946; Claudimin *et al.*, 1946; McGinnis and Evans, 1947; Evans and McGinnis, 1948; Evans *et al.*, 1951).

Interest in the effects of plant protease inhibitors towards insect attack was aroused as early as 1947 when Mickel and Standish observed that larvae of certain pests were unable to develop normally on soyabean products. These observations led Lipke *et al.*, (1954) to study the toxicity of soyabean inhibitors on the complete development of *Tribolium confusum*, a common pest of stored grain. Although these results were negative with respect to trypsin inhibitors, they did however reveal the presence of a specific

inhibitor of the Tribolium larval digestive proteolysis. This inhibitor was later isolated and shown to completely inhibit larval gut proteolysis of both T. casraneum and T. confusum (Birk et al., 1963), but was found to be inactive towards trypsin and chymotrypsin (Applebaum and Konijn, 1966). However, Applebaum et al., (1964a) subsequently demonstrated that lima bean inhibitor, ovomucoid, soyabean inhibitor and Bowman-Birk inhibitor inhibited one of the midgut proteases isolated from the larvae of Tenebrio molitar which is known to contain both trypsin and chymotrypsin-like enzymes (Zwilling, 1968), whereas the Tribolium inhibitor, on the other hand, was found to be ineffective. In a recent paper Steffens et al., (1978) demonstrated that the addition of soyabean trypsin inhibitor (Kunitz), incorporated at levels of 2 to 5 per cent in the diet of young corn borer larvae Ostrinia nubilalis (Hübner), inhibited larval growth and delayed pupation. Corn inhibitors, on the other hand, which are weak inhibitors of trypsin, had no effect upon growth and metamorphosis when incorporated at levels of 2 to 3 per cent in the diet. These results led the authors to suggest that the proteolytic enzyme inhibitors of legumes may be related to the resistance of these plants to the corn borer.

Applebaum (1964) proposed that protease inhibitors in legumes evolved as a defence mechanism against insects and that digestion should be considered as a factor in host selection. Since he was unable to demonstrate the inhibition of a crude enzyme extract from the larvae of Callosobruchus chinensis L., using soyabean trypsin inhibitor, he expanded his theory by claiming that the Bruchids had developed metabolic pathways which either bypassed proteolysis or were insensitive to the presence of these inhibitors, thereby permitting larval development in the absence of competitive pressure from other insects. He used the substrates casein and azocasein to determine the proteolytic activity in a larval homogenate and found that the level of activity was very low; however, this is not surprising since the homogenate was prepared from the whole larvae and not the larval gut, and the assays are relatively insensitive. Further, the effect of crystalline soyabean trypsin inhibitor on the observed proteolysis was investigated, and no inhibition was observed. Unfortunately, since the substrates used are not specific for trypsin-like enzymes, this result cannot be definitely interpreted as a failure of the inhibitor to inhibit the larval "trypsin". No detailed results were given. Although no characterisation appears to have been carried out on the digestive proteases

of the C. maculatus larvae, it is clear from accumulated data that the digestive proteases of a number of insect genera are very similar to the mammalian trypsin and chymotrypsin (Powning et al., 1951; Zwilling, 1968; Applebaum et al., 1964a; Ito et al., 1975; Grogan and Hunt, 1977; Kunz, 1978).

Although not relevant to seeds, the possible involvement of protease inhibitors as protective agents against insects is strongly supported by the findings of Green and Ryan (1972) who demonstrated that wounding of the leaves of potato or tomato plants by adult Colorado potato beetles, or their larvae, induced a rapid accumulation of a proteinase inhibitor throughout the aerial tissues of the plants. This effect of insect damage could be stimulated by mechanical wounding of the leaves. Furthermore, Weiel and Hapner (1976) found that the distribution of inhibitors in different barley varieties correlated with the severity of grasshopper damage. Not only is there strong support for the protective role played by plant protease inhibitors against insect attack, but there is also evidence that they are active in the protection of plants from pathogenic fungi and bacteria (Mosolov et al., 1976). It seems likely that plants would have developed resistance mechanisms which are general against many predators.

Although most of the pioneering work was carried out on the trypsin inhibitors from soyabeans, proteins of similar inhibitory activity have since been isolated from a large number of other sources and appear to be ubiquitous throughout the plant kingdom (Vogel et al., 1966; Pusztai, 1967; Liener and Kakade, 1969; Laskowski and Sealock, 1971), being present in particularly high proportion among members of the Leguminosae (Borcheis et al., 1947; Abramova and Chernikov, 1965; Belew et al., 1975; Kimura et al., 1976) and Solanaceae. Their physiological function within the plant (as possible primary metabolites) is still uncertain, though in general, they are considered either as protective or regulatory proteins. This, however, will be discussed in greater detail later in the Introduction.

Lectins (phytohaemagglutinins)

The poor nutritional properties of raw legumes have not only been ascribed to the low sulphur amino acid content of the proteins (Liener, 1962; Kakade and Evans, 1965), and the presence of proteolytic enzyme inhibitors, but also to the presence of another class of proteins, the lectins. Lectins are defined as carbohydrate-binding proteins, and because of their unique property of being able to agglutinate erythrocytes, they are also known as (phyto)haemagglutinins. Like the protease inhibitors, the lectins appear to be

widespread throughout the legume family.

The first description of a phytohaemagglutinin was given by Stillmark (1889), who studied the toxicity of castor beans and press cakes from the production of castor oil. He concluded that the toxic action was due to the presence of a protein which was capable of agglutinating the red cells from human and animal blood; this he called "ricin". Landsteiner and Raubitschek (1908) later observed that extracts from many edible legume seeds would likewise agglutinate red blood cells, but that the relative haemagglutinating activities of various seeds were quite different when tested with blood cells from different animals. No toxicity was detected at that time. However, Liener subsequently isolated a haemagglutinin from soyabeans and demonstrated its ability to inhibit the growth of rats (Liener, 1953).

Among the edible legumes the seeds of Phaseolus vulgaris (kidney bean) contain about the highest lectin content, and hence most work concerning lectin toxicity has been carried out using the lectin extracted from these seeds. Jaffé et al., (1968) undertook a systematic study of the haemagglutinating activities of a large number of different varieties and cultivars of P. vulgaris, and observed that only those extracts which agglutinated trypsinised bovine erthyrocytes

were toxic when injected into rats; feeding trials confirmed this. Why this should be the case is not known; indeed the basis of lectin toxicity, when ingested, is not understood, and the fact that these proteins are readily digested and inactivated is even more perplexing. Work carried out by Evans and his colleagues strongly indicate that lectins, at least in P. vulgaris, are primarily responsible for the deleterious effects observed in rats when fed raw legume meal (Evans et al., 1973; Pusztai et al., 1975); the trypsin inhibitor did contribute to a small degree (Pusztai et al., 1975). Furthermore, in support of their findings, they demonstrated that while purified lectin preparations, when incorporated into the diet, strongly depressed the net protein utilisation of rats, a lectin-free kidney bean preparation was found to be non-toxic (Pusztai and Palmer 1977). Turner and Liener (1975) on the other hand, were unable to detect any significant difference between rats fed a crude soyabean extract from which the lectins had been removed by adsorption on to Concanavalin A, and those fed a crude soyabean extract from which the lectins had not been removed. Perhaps these conflicting results indicate that whilst some lectins may be toxic, others are not. The only lectins known to be directly toxic are of the type exemplified by ricin, from

the castor bean; in this case the protein has a carbohydrate binding subunit in association with a subunit that has no carbohydrate binding properties but is a strong inhibitor of ribosomal protein synthesis. It is thus not the "lectin" part of the molecule that is toxic.

In order to investigate the mechanism of lectin toxicity, when fed to experimental animals, Rattray et al., (1974) carried out feeding trials using both gnotobiotic and conventional rats. Previously there was evidence that germ-free chicks were significantly less affected than conventional chicks by diets containing raw soyabeans (Coates et al., 1970) and kidney beans (Hewitt et al., 1973); this tends to suggest that the gut microflora may play an intermediary role. However, Rattray found that although the toxicity of the beans was reduced in the absence of intestinal microflora it was not completely abolished. These results tend to suggest that the lectins are not the only toxic constituents influencing growth depression in raw kidney bean meal.

Although interest in lectin toxicity has similarly extended to include the possible effects on insects, this field does not appear to be so well documented compared to the possible role played by the protease inhibitors in insect resistance. Janzen et al., (1976) demonstrated that the

lectin extracted from the seeds of P. vulgaris, when incorporated at physiological levels into artificial beans, was toxic to the larvae of Callosobruchus maculatus; the artifical beans were prepared from cowpea meal as the seeds are the wild host of C. maculatus. Since these seeds have not been reported to contain lectins, whereas those of P. vulgaris which are toxic to the larvae of this beetle do, the authors proposed that the major part of the adaptive significance of lectins in legume seeds is to protect them from attack by insect predators. Unfortunately they omitted to consider the trypsin inhibitors present in the cowpea meal.

Saponins

Among other toxic compounds occurring in the seeds of many legumes in small concentrations are the saponins; the soyabean saponins being the most extensively investigated. Saponins are glycosides, which on acid hydrolysis, for example in the mammalian gut, give rise to the constituent sugar and saponogenin moieties and so are rendered harmless (Potter and Kummerow 1954). There is however a certain degree of controversy as to whether or not they are in fact toxic to endothermic (warm blooded) animals. Most saponins have a powerful haemolytic effect in vitro but large doses are required to produce haemolysis on intravenous injection

due to the protective action of cholesterol and other lipids and proteins which bind to the saponins (Liener 1969). Their effect, on ingestion, appears to be less clearly understood. Gestetner (1965) found that when mice, rats and chicks were reared on soyabean saponin supplemented diets, there were considerable amounts of the sapongenins present in the faeces, and attributed this to the hydrolysis of the saponin extract by their intestinal flora; neither the saponin nor the sapogenin are absorbed into the blood stream (Birk 1969). Other workers have also demonstrated the insensitivity of mice to the presence of soyabean saponins in their diet (Applebaum et al., 1965). Potter, on the other hand, claims that purified saponin from both alfalfa and soyabean inbibit the growth of chicks. The growth depressing effects, at least of the alfalfa saponin, has been attributed largely to a consequence of reduced food intake owing to their very bitter taste and possible irritation of the gastrointestinal tract (Cheeke 1976). Furthermore, saponins are known to inhibit smooth muscle activity which could result in reduced peristaltis.

The effect of saponins on insect development and survival appears to be less ambiguous compared to its effects in higher animals. As with other secondary compounds, the growth repellency of saponins towards certain stored-products

pests is of major interest. Ishii (1952), who was among the first to recognise the importance of the saponins with respect to insect resistance, suggested that those from P. vulgaris might be detrimental to larval development of Callosobruchus chinensis (L.) on the basis of their antagonism to essential steroids; he was unable to confirm this experimentally. However, a few years later Ishaaya et al., (1969) were able to demonstrate that addition of 1 per cent soyabean saponin extract (approximate level found in the soyabean seed) to the control diet caused a 20 per cent decrease in the average weight of the larvae of Tribolium castaneum. Bondi et al., (1962) observed similar effects, and furthermore demonstrated, in vitro, that a crude saponin extract from soyabeans also partially inhibited the proteolytic activity of the larval midgut of T. castaneum, and significantly inhibited trypsin and α-chymotrypsin. Applebaum et al., (1965) found that the developmental incompatibility of soyabeans for Callosobruchus chinensis was partly attributed to the presence of saponins. However, artifical beans composed of 25 per cent soyabean residue after saponin extraction, and 75 per cent chick-pea flour (these were used since they are devoid of saponins and are favourable for development of Callosobruchus) were also detrimental to larval development, most dying before

the second instar. These findings suggest that the saponins are not the only compounds involved in the developmental incompatibility of soyabeans for Callosobruchus. The in vitro enzymatic hydrolysis of the soyabean saponin extract by a homogenate of Callosobruchus larvae was found to be negative; this tends to suggest that if the larvae are unable to hydrolyse the saponins in vivo they will therefore be toxic (Applebaum et al., 1965). From these findings Applebaum proposes that the saponins play a positive role in the resistance of soyabean to insect attack. However, the development of Callosobruchus in soyabeans has been reported (Ishii 1952). Soyabean saponin has also been found to show high toxicity towards Sitophilus oryzae (L.) (the rice weevil) and was found to give a high degree of protection to wheat when dusted at a concentration of 300 ppm (Su et al., 1972).

Non Protein Amino Acids

The deterrent effects of certain non-protein amino acids against insects have also been studied, particularly in relation to predation of tropical legume seeds by bruchids (Janzen, 1971a; Rehr et al., 1973a; Bell et al., 1976). In many cases these are present in concentrations excess of 5 per cent of the dry weight of the seed; for example, the mature seeds from six species of the genus

Mucuna, which are conspicuously free from attack, except for the larvae of Caryedes brasiliensis and closely related species, have been shown to contain between 5 per cent and 9.5 per cent by weight free L-dopa (3,4-dihydroxyphenyl-alanine) (Rehr et al., 1973b). Feeding trials using the southern army worm, Prodenia eridania, the caterpillar of this species having an exceptional ability to detoxify many foreign compounds, have shown that the physiological concentrations of free L-dopa in the mature seeds is far in excess of that required as a feeding deterrent. Rehr et al., (1973b) showed that on addition of 5 per cent Mucuna seed powder (containing about 0.25 per cent L-dopa) to a basic diet, when fed to larvae, resulted in deformed pupae; sclerotinisation of the pupal case being incomplete. However, addition of 0.5 per cent L-dopa, which is approximately twice the concentration used in the first experiment, produced no abnormal effects; this tends to indicate that the observed effects were not entirely due to the L-dopa at the concentration used. Diets of 5 per cent L-dopa, on the other hand, were completely unacceptable. Accumulation of canavanine in the seeds of the genera Canavalia and Dioclea may be another example of such a system and it is of interest that the host specific bruchids which feed on them are also members of the genus Caryedes (Bell and Janzen, 1971).

Toxicity of non protein amino acids is due to their incorporation into proteins instead of the normal homologue resulting in non-functional protein molecules. This was discovered by the existence of a canavaine-resistant bruchid that can subsist on the seeds of Diociea megacarpa which contains up to 8 per cent of the toxin (Swain, 1977). Apparently the arginyl t-RNA synthetase of the bruchid larva is abnormal in being able to discriminate between L-arginine and L-canavanine, and so the latter is not incorporated into the protein. Because non-protein amino acids are usually present in large concentrations, a secondary function as nitrogen reserves has also been ascribed to many, such as L-dopa for example (Bell and Janzen, 1971; Rehr et al., 1973a; Rehr et al., 1973b). It is, however, important to remember that most legume seeds which contain high concentrations of non-protein amino acids are toxic, and not consumed by man.

Alkaloids

The alkaloids are a heterogeneous grouping of nitrogenous bases. Of the vast number of different alkaloids synthesised by diverse plant species, only a relatively few have been investigated for their roles in insect-plant interactions (Beck and Reese, 1976). Most of these are the major alkaloids of solanaceous plants, very

little investigation having been carried out in members of the Leguminosae. In most cases, the alkaloids have been described as either repellents, feeding inhibitors, or acute toxins, so that the insects either fail to feed or die very shortly after feeding. Janzen et al., (1977) carried out feeding trials in which they incorporated small doses of alkaloids in the diet of Callosobruchus maculatus larvae. Of eleven alkaloids tested, nine were lethal at a concentration of 0.1 per cent. However, it does not seem surprising that such potent toxins as strychnine should be very poisonous to C. maculatus larvae, which do not feed on the plant material from which they are extracted.

PART III The Cowpea Trypsin Inhibitor

The presence of a trypsin inhibitor in the cowpea (Vigna unquiculata, previously known as V. sinensis) was first reported in 1947 by Borchers et al., and later by Jaffé (1950) and others (Sohonie and Bhandarkar, 1954; Sohonie and Bhandarkar, 1955). The first work on the characterisation of the cowpea trypsin inhibitor was carried out by Ventura and Filho (1966) using a Brazilian variety of cowpea, Seridó.

The extraction procedure carried out by Ventura was as follows: the milled beans were first extracted in water and the residue removed by centrifugation (10,000 x g). Trichloroacetic acid was added to the supernatant to a final concentration of 2.5 per cent and the suspension allowed to stand (15 hours at 0-1^{o}C), after which time it was filtered. In contrast to most proteins the trypsin inhibitor stays in solution. Ammonium sulphate was then added to the filtrate to a final concentration of 31.3 per cent to precipitate the inhibitor and the solution allowed to stand overnight. The precipitate was then removed by centrifugation and redissolved in water. The trichloroacetic acid and ammonium sulphate precipitation steps were repeated. The precipitate obtained was redissolved, dialysed against water and then finally lyophilised. Further purification was carried out

by ion-exchange chromatography, using DEAE cellulose, to a final purification of 225-fold; this being calculated on the basis of chymotrypsin inhibition. Their results showed that although total inhibitory activity against chymotrypsin was increased slightly after the first precipitation with trichloroacetic acid, total antitryptic activity was reduced to approximately 25 per cent of the initial activity. On ion exchange chromatography seven elution peaks were apparent, and whereas detectable trypsin inhibitory activity was present in peaks V-VII, chymotrypsin inhibitory activity was only associated with peak VII. It was this peak which was further characterised and was subsequently shown to be an inhibitor of both trypsin and chymotrypsin. The fact that 75 per cent of the trypsin inhibitor was lost during the initial extraction, and also that the inhibitor subsequently characterised appears to be the "double-headed isoinhibitor" (q.v.), explains why Ventura claimed that the inhibitor present in the cowpea was essentially a chymotrypsin inhibitor.

Ultracentrifugation measurements showed the inhibitor to have a molecular weight of approximately 17,100; this value was in agreement with the value of 16,923 which was deduced from the amino acid composition. By determining the inhibition of one enzyme in the presence of high concentrations

of the other enzyme, using the synthetic substrates BAEE and BTEE for trypsin and chymotrypsin respectively, Ventura showed that both enzymes could be inhibited simultaneously and that the inhibition sites for the two enzymes were different. From the findings that 1 mg of inhibitor inhibited 1.56 mg of trypsin and on the assumption of a 1:1 molar stoichiometry, a molar combining weight of 15,300 g was calculated for the inhibitor with trypsin, given that the molecular weight of trypsin is 24,000. The combining weight is in reasonable agreement with the molecular weight, supporting the assumption of stoichiometry. Using the calculated molecular weight of 15,300 and a molecular weight of 24,000 for chymotrypsin, the finding that 1 mg of the inhibitor inhibited 3.03 mg of α-chymotrypsin gave a 1:2 molar stoichiometry for the inhibitor: enzyme complex. However, in a subsequent paper, using gel filtration, a molar stoichiometry of 1:1 for inhibitor: α-chymotrypsin was determined (Ventura et al., 1971). Their initial findings of a molar stoichiometry of 1:1 for inhibitor to trypsin, on the other hand, were confirmed. Furthermore they revised the values obtained for the molecular weight of the inhibitor. Gel filtration, ultracentrifugation, osmometry and amino acid analyses gave molecular weights of 13,000, 12,300 $\pm$ 900, 10,700 $\pm$ 700 and 10,000 daltons

respectively. The amino acid composition was also found to be slightly different compared to their previous findings though the inhibitor still appeared to be devoid of methionine.

During the course of the work described in this thesis Gennis and Cantor published work carried out on the isolation and characterisation of two protease inhibitors from black-eyed peas; however they state that these two inhibitors are distinct from the inhibitor isolated by Ventura. In that Gennis and Cantor do not describe their material further than as "black-eyed peas" it has to be presumed that these are in fact Vigna unguiculata; however, differences between the properties of the proteins they isolated and the proteins isolated by Ventura, and those isolated during the course of the present work may be due to varietal or even species differences rather than discrepancies in experimental results. The initial extraction procedure carried out by Gennis and Cantor (1976a) was based on that used by Ventura; however the dialysis step was replaced by gel filtration (using Bio-Gel P-2) since substantial loss of inhibitor was found to occur on dialysis. Subsequent purification was carried out either by ion-exchange chromatography (using DEAE-cellulose 52) or by affinity chromatography (using trypsin-Sepharose or

chymotrypsin-Sepharose). When the latter was used trichloroacetic acid was added to a final concentration of 10 per cent, and the ammonium sulphate precipitation step omitted. The purified inhibitors were desalted using gel filtration and finally lyophilised. The authors found that one of the inhibitors (BEPCI) inhibited both trypsin and chymotrypsin simultaneously, whilst the other (BEPTI) inhibited two molecules of trypsin simultaneously. The ability to inhibit two molecules of protease per molecule of inhibitor makes these inhibitors "double-headed". The inhibitors had similar subunit molecular weights of about 8,000 on SDS-polyacrylamide gel electrophoresis, and both had the same NH_2-terminal residue, serine. Each inhibitor gave a single band on isoelectric focussing, BEPCI at a pI of 5.1, and BEPTI at a pI of 6.2. Gennis and Cantor (1976c) investigated the subunit interactions of BEPCI and BEPTI using gel filtration and found that at either pH 8.0 or pH 3.0 there was a complex set of multiple equilibria between the monomer, dimer and tetramer, with the dimer normally the predominant species, giving an apparent molecular weight of 16-17,000, as found initially by Ventura. Extensive studies were also carried out on complex formation between the inhibitors and their respective enzymes by titration experiments and gel filtration

chromatography (Gennis and Cantor, 1976d). Due to the monomer-dimer-tetramer equilibrium of the inhibitors, many different complexes are possible between each inhibitor and the two proteases they inhibit; the experimental data were skilfully interpreted to demonstrate the presence of most of these complexes. Since each inhibitor was presumed to have two binding sites per subunit (otherwise the BEPCI dimer, which inhibits one molecule of trypsin and one of chymotrypsin simultaneously, would have to be composed of two dissimilar subunits, which was not found to be the case) half-site reactivity was invoked to explain the failure to find any evidence of two molecules of protease binding per subunit - that is, in the dimer, the binding of a protease molecule to one of the subunit binding sites renders the corresponding binding site on the other subunit, inoperative or inaccessible. Amino acid analyses were also carried out on the two protease inhibitors (Gennis and Cantor 1976b); these were significantly different to the amino acid analyses obtained for the protease inhibitor extracted by Ventura, except for the absence of methionine in BEPCI, but were similar to each other.

It has been suggested that the legume double-headed inhibitor arose by the duplication, and subsequent mutation of an ancestral single-headed structure, so acquiring their

unusual stability (Odani and Ikenaka, 1978a; Odani and Ikenaka, 1978b). This proposal was based on the fact that the soyabean Bowman-Birk inhibitor, a double-headed inhibitor of trypsin and α-chymotrypsin, when treated with cyanogen bromide followed by pepsin, yielded two active inhibitory fragments. Structural investigation showed that one of the fragments was derived from the trypsin inhibitory domain and the other from the chymotrypsin inhibitory domain of the inhibitor. In contrast to the unusual stability of the active native inhibitor, these separated domains were less stable and could be inactivated with excess proteases. This theory seems applicable also to the two cowpea inhibitors, although its validity has not been tested.

Subcellular Localisation and Possible Role of the Inhibitor in Plant Metabolism

Subcellular localisation studies of the protease inhibitors have been carried out in an attempt to determine whether or not they perform a role in seed metabolism. Despite studies on the seeds of several legumes (Hobday et al., 1973; Baumgartner and Chrispeels, 1976; Pusztai et al., 1977; Chrispeels and Baumgartner, 1978) no work appears to have been carried out on the subcellular localisation of the trypsin inhibitors in the seeds of

Vigna unguiculata. As Pusztai points out, the difficulty in unequivocably locating the trypsin inhibitors in seeds is the fact that they do not form a homogenous population (Pusztai et al., 1977). In a recent paper Pusztai et al., (1977) found that subcellular fractionation of Phaseolus vulgaris cotyledons indicated that while the protein body fraction could not have contained all the trypsin inhibitors, an appreciable part of this activity was definitely associated with it. Hobday et al., (1973) found similar results for Pisum sativum. Fifty per cent of the inhibitory activity associated with the unwashed protein body fractions could be removed by washing with extractant and then rebound. Furthermore, during a number of washes the protein bodies lost inhibitor faster than they lost total soluble protein. The authors concluded that the trypsin inhibitors could not be located within the protein bodies but were probably bound to the outside. This fact, they considered, appeared to exclude the trypsin inhibitors from regulating proteolysis during germination by inhibition of those proteases actively engaged in proteolysis within these organelles. Royer et al., (1974), on the other hand, found that a trypsin inhibitor isolated from the seeds of V. unguiculata partially inhibited endogenous caseolytic activity but not endogenous BAPNA-ase activity. From these results they suggested that the trypsin inhibitor in seeds

plays a role in the regulation of seed proteolysis although no further evidence was presented to support this hypothesis, nor was the inhibitor in any way characterised. Similarly, Gennis and Cantor (1976a), on the basis of the isolation of an apparent protease-BEPCI complex from black-eyed pea suggest that protease inhibitors play a role in seed metabolism. Furthermore, since the purified endogenous protease is very unstable in the absence of the inhibitor, the authors suggest a second possible physiological function of seed protease inhibitors in the protection of the unstable seed protease. They speculate that when dormancy is broken, the seed protease is released from the inhibitor-protease complex as a result of the destruction of inhibitor by protein disulphide reductase. They did not, however, carry out any subcellular fractionation studies of the protease inhibitor.

Baumgartner and Chrispeels (1976) isolated a proteolytic inhibitor which specifically interacts with the major endopeptidase synthesised during the germination of Phaseolus aureus seeds. Fractionation of the cellular organelles on sucrose gradients showed that the inhibitory activity was not associated with the protein bodies but rather with the cystol; the endopeptidase activity on the other hand, was predominantly within the protein bodies.

These results suggested that the endopeptidase inhibitor(s) does not regulate the increase in endopeptidase activity which accompanies germination or the metabolism of storage protein. From their results they postulated that the inhibitor(s) may function in protecting the cytoplasm from accidental rupturing of the protease-containing protein bodies. In a recent paper Chrispeels and Baumgartner (1978) isolated and characterised a trypsin inhibitor from the seeds of Vigna radiata (L.) Wilczek (formerly Phaseolus aureus Roxb). Contrary to the findings of Pusztai et al., (1977) and Hobday et al., (1973), fractionation of crude extracts on isopycnic sucrose gradients and cytochemistry with fluorescent antibodies indicated that this inhibitor was also associated with the cytoplasm and not with the protein bodies where reserve protein hydrolysis occurs. They found that this inhibitor did not inhibit the activity of vicilin peptidohydrolase, the enzyme which digests vicilin, and additionally, the removal of the inhibitor by trypsin affinity chromatography did not unmask or activate any previously inactive proteolytic enzymes. These results led the authors to conclude that the catabolism of the trypsin inhibitor during germination and seedling growth was not causally related to the onset of reserve protein breakdown. Since the findings of Hobday et al., (1973) and Chrispeels and

Baumgartner (1978) indicate that the trypsin inhibitor does not play a role in plant metabolism the hypothesis that they play a defensive role within the seed against predation is strengthened. Additionally, Ofelt (1955) after studying the presence of protease inhibitors in soyabeans concluded that in these seeds, at least, they do not function as inhibitors of endogenous proteases; this conclusion was later affirmed by Birk (1965).

In order to determine the biochemical basis of resistance in Vigna unquiculata TVu 2027 towards Callosobruchus maculatus I proposed to screen and compare the different varieties for toxins. On the basis of these results it would then be possible to isolate and purify the toxin and to characterise it chemically as far as possible. The purified substance would then be used in feeding trials to demonstrate its toxicity in vivo towards C. maculatus, and in vitro studies to elucidate the biochemistry of its detrimental effect on insect metabolism.

SECTION II RESULTS

PART I Trials to Determine the Susceptibility of Different Varieties of *Vigna unguiculata* towards attack by *Callosobruchus maculatus*.

A. Cowpea variety trial using *Callosobruchus maculatus*

Vigna unguiculata variety TVu 2027, and this variety only, was claimed to show significant resistance towards *Callosobruchus maculatus* at IITA when mass screening trials were carried out (Singh, private communication). In order to confirm the validity of these results a cowpea variety trial was carried out at the Tropical Stored Products Centre, Slough, using seven different varieties. For each variety from two to four replicates, each containing twenty seeds, were set up; replicate number being dependent upon the availability of material.

A summary of the results is given in Table 2.1.1. Although only 2 replicates were carried out for TVu 2027, due to shortage of material, it is obviously significantly resistant towards *C. maculatus* as only one adult emerged from at least 40 eggs. The other varieties, on the other hand, supported between 85 per cent to 100 per cent adult emergence. No morphological differences were apparent between the emerged adults from the different varieties.

Table 2.1.1.

COWPEA VARIETY TRIAL USING CALLOSOBRUCHUS MACULATUS

TABLE REPRESENTS NO. ADULTS EMERGED

variety	replicate 1	replicate 2	replicate 3
TVU 57	15	18	20
TVU 1502 ID	19	22	16
TVU 76	20	18	17
TVU 37	18	18	17
TVU 1190E	18	18	17
TVU 4557	18	18	19
TVU 2027	1	0	

Each replicate contained 20 seeds with one egg per seed (as far as possible)

Table 2.1.2

Cowpea Variety Trial using *Callosobruchus maculatus*

Variety	Total No. Eggs Laid	Total No. Adults Emerged	% Adult Emergence ± 1 standard deviation
TVu 37	116	110	86.6 ± 4.3
TVu 57	105	96	91.7 ± 6.7
TVu 76	116	104	90.0 ± 2.5
TVu 1190E	168	150	89.0 ± 6.7
TVu 1502-1D	151	124	92.0 ± 6.0
TVu 2027	105	0	0.0 ± 0.0
TVu 3629	70	62	90.6 ± 9.5
TVu 4557	85	81	95.1 ± 6.2
* Farrin Wake	83	74	89.0 ± 4.9
** Subsp. *dekintiana*	68	53	75.0 ± 7.0

foot note : For each variety 5 replicates of 5 seeds were used.
* Seed was collected from a farm in Northern Nigeria. It is not an IITA cultivar.
** *Vigna unguiculata* subsp. dekintiana is a wild host of *C. maculatus*

A subsequent cowpea variety trial was set up using 9 varieties of cowpea (TVu 37, TVu 57, TVu 76, TVu 1190E, TVu 1502-1D, TVu 2027, TVu 3629, TVu 4557 and Farrin Wake) and in addition one of the wild hosts of C. maculatus, namely Vigna unguiculata subspecies dekintiana. The results are shown in Table 2.1.2. These results demonstrate very clearly the resistance of TVu 2027 towards C. maculatus as no adults emerged, compared to emergence values of 86.6 $\pm$ 4.3 per cent to 92.0 $\pm$ 6.0 per cent for the other cowpea varieties. Although the wild subspecies dekintiana was also significantly susceptible towards C. maculatus, it is interesting to note that it only supported a 75.0 $\pm$ 7.0 per cent adult emergence. Furthermore these results demonstrate that there is no ovipositional avoidance of TVu 2027 compared to the other varieties.

Thus these two cowpea variety trials confirm the initial findings that TVu 2027 showed significant resistance towards C. maculatus.

B. Determination of the Survival of Callosobruchus maculatus to Adult on two different Harvests of TVu 2027

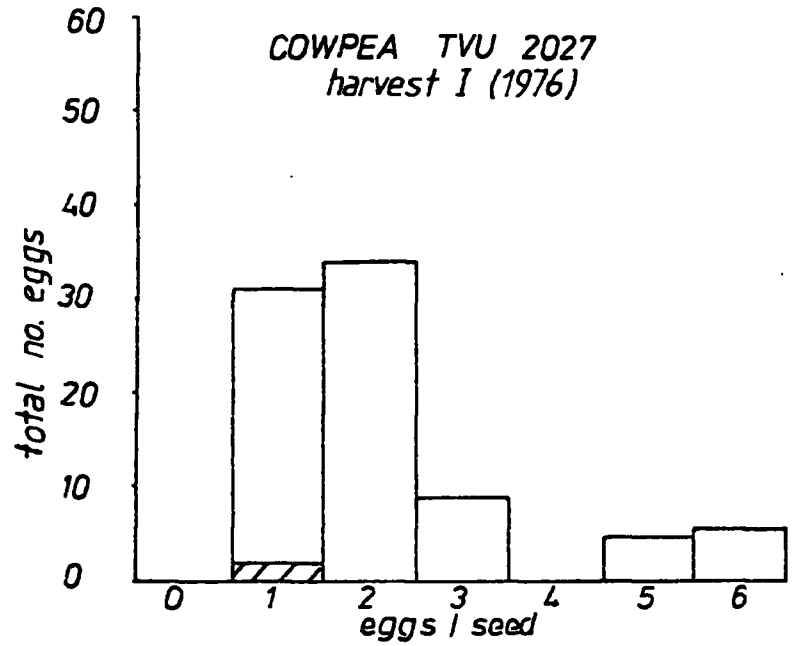
Although the cowpea variety trials clearly demonstrate the significant resistance of TVu 2027 towards C. maculatus, it was necessary not only to demonstrate the reproducibility of this result using seeds of TVu 2027 from the same harvest,

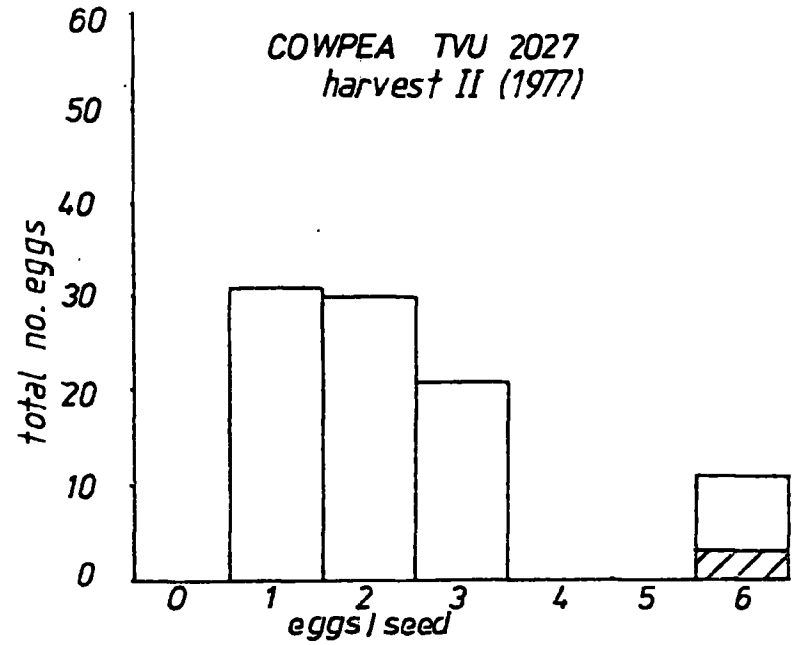
but also to demonstrate its resistance in different harvests to show that resistance is a stable genetic characteristic. This was carried out using two different harvests of TVu 2027 and comparing the results to a control variety, TVu 76.

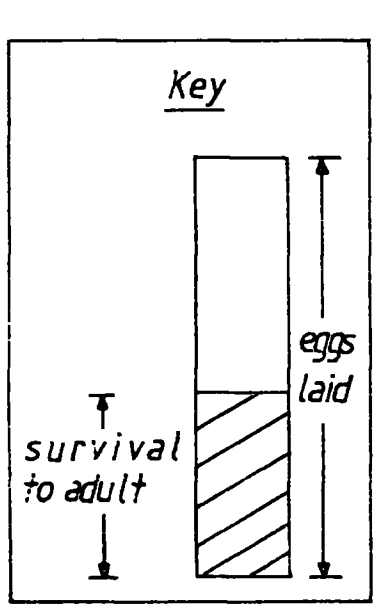
The results are summarised in histograms shown in Figure 2.1.3. Not only do these results confirm the resistance of TVu 2027 towards C. maculatus, when compared with the control, but also demonstrate that this resistance was not due to a freak harvest but is an inherent characteristic of Vigna unquiculata variety TVu 2027.

C. Oviposition (egg laying) trials of Callosobruchus maculatus on different varieties of Vigna unquiculata

Once the resistance of TVu 2027 towards C. maculatus had been established it was necessary to determine whether it was physical or biochemical in origin. Physical resistance could be expressed as the seed testa being of a nature that repelled the female beetle from egg-laying, or being too hard to allow the larva to enter the seed. Thus one indication of a physical form of resistance would be lack of oviposition by the female beetle. Consequently oviposition trials were set up incorporating both rough testa and smooth testa seeded cowpea varieties.







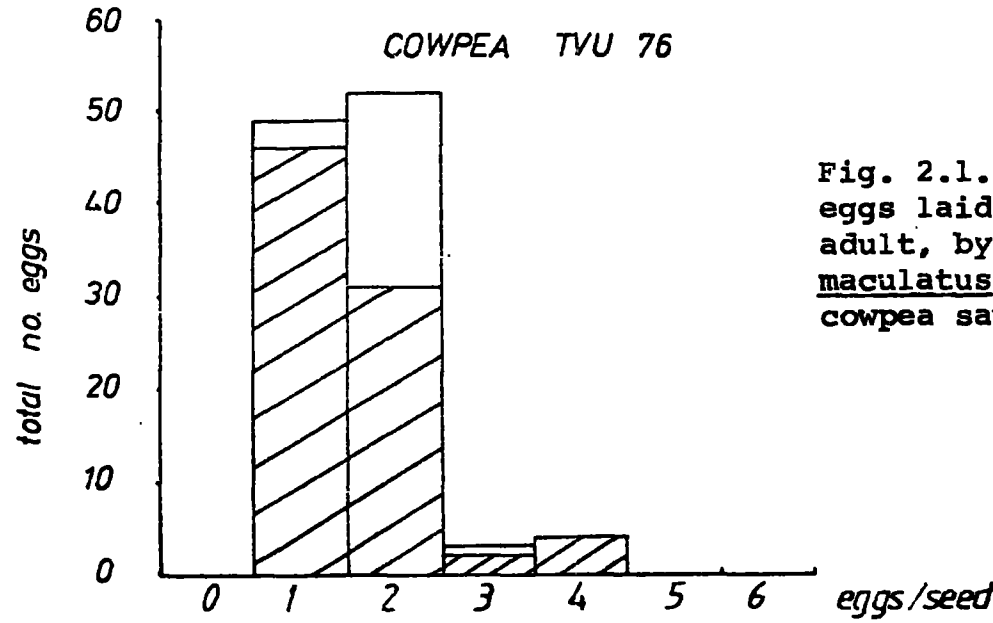


Fig. 2.1.3. Histogram showing eggs laid, and survival to adult, by Callosobruchus maculatus on three different cowpea samples.

The first set of trials were carried out on five varieties of cowpea. The results are given in Table 2.1.4. These results indicate that the oviposition is not reponsible for this particular example of resistance as there were no fewer eggs laid on the resistant variety TVu 2027 than the other varieties.

In a second set of trials nine different varieties of cowpea were investigated together with a wild host of C. maculatus, V. unguiculata subspecies dekintiana. The results are shown in Table 2.1.5. Similarly it was demonstrated that no less eggs were laid on TVu 2027 compared to many of the other varieties. The lower number of eggs laid on subspecies dekirtiana is most probably due to the seeds being significantly smaller in size than those of the cultivated species.

Thus these results eliminate the possible role of oviposition in resistance towards Callosobruchus maculatus.

D. X-ray Analysis of Infested Seeds of TVu 2027 and a Control Variety TVu 76.

To investigate the possibility of physical resistance due to prevention of larval entry into the seed, X-ray analyses were carried out on infested seeds of the resistant

Table 2.1.4

Comparison of Oviposition by *Callosobruchus maculatus* on five Varieties of *Vigna unguiculata*

Total No. of Eggs Laid Per Female in Five Days
(± 1 standard deviation)

TVu 1398	TVu 2027	TVu 1090	TVu 3629	TVu 4557
69	65	74	64	65
53	57	44	32	48
55	51	27	47	35
53	75	64	51	50
38	51	53	34	57
39	43	69	31	46
70	51	56	66	58
58	54	50	49	50
55	33		70	90
42	61		37	58
53.2 ± 10.6	54.1 ± 11.6	54.6 ± 12.5	48.1 ± 13.9	56.0 ± 13.6

1 pair of virgin adults were put in a vial with 2 seeds. These were left for 1 day and then transferred to 2 new seeds. Between 8 to 10 replicates were carried out for each variety (depending upon availability of material).

Table 2.1.5

Comparison of Oviposition by *Callosobruchus maculatus* on nine Varieties of *Vigna unguiculata* and a Wild Host, Subspecies *dekintiana*, over a period of Four Days

Variety	Replicate I	Replicate II	Replicate III	Replicate IV	Replicate V	Total No. Eggs Laid/ Variety	Eggs laid/ Five Seed + 1 St. Dev*
TVu 37	20	20	29	20	27	116	23.2 ± 4.7
TVu 57	19	27	24	17	18	105	21.0 ± 3.8
TVu 76	18	20	18	30	30	116	23.2 ± 5.6
TVu 1190E	23	31	38	37	40	169	33.8 ± 6.2
TVu 1502-1D	17	34	40	36	24	151	30.2 ± 8.4
TVu 2027	9	8	30	24	34	105	21.0 ± 10.7
TVu 3629	6	11	16	16	21	70	14.0 ± 5.1
TVu 4557	15	15	19	17	19	85	17.0 ± 1.8
Farrin Wake	11	18	21	15	18	83	16.6 ± 3.4
Subsp. dekintiana	9	10	14	16	19	68	13.6 ± 3.7

5 replicates, each containing 5 seeds, were carried out for each of the varieties

* ± 1 Standard deviation

variety, TVu 2027, and a control variety, TVu 76. They clearly demonstrate the presence of larval tunnelling in both varieties.

Photographs of these X-rays are shown in Figure 2.1.6; different harvests of TVu 2027 were also analysed. The presence of larval tunnelling in the resistant variety shows unequivocably that this resistance is not due to a physical barrier. Furthermore, when seeds of TVu 2027 were opened up, at a stage when adults were emerging from the other varieties, the larvae were still alive though at a very early instar. This finding tends to suggest the presence of an antimetabolite in the seeds, such as the trypsin inhibitor for example, rather than a potent toxin.

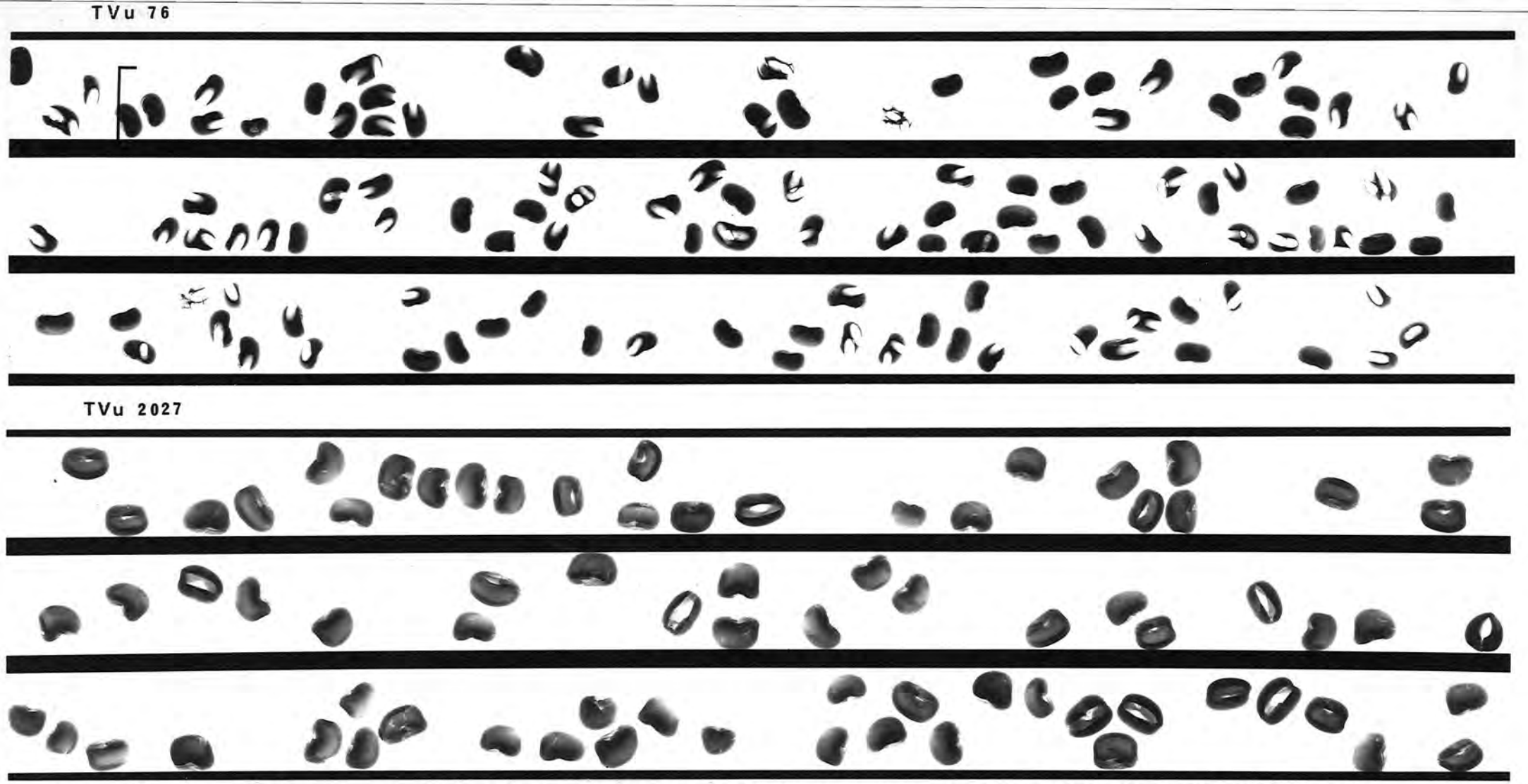


Figure 2.1.6. X-ray photographs of two varieties of cowpea seeds infested by Callosobruchus maculatus. Larval tunneling is present in both the control variety, TVu 76, and the resistant variety, TVu 2027. However, the tunnels are much narrower in the resistant variety, indicating that larval development has been halted at a much earlier stage.

PART II Tests carried out for various Toxic Compounds in Cowpea (Vigna unguiculata) seeds

Legume seeds are known to contain compounds which are toxic to insects and their larvae. Thus in order to determine the biochemical basis of resistance in the cowpea variety TVu 2027, it was necessary to screen cowpeas for these compounds, and to compare the levels of such toxic compounds as may be found in resistant and susceptible varieties.

A. Determination of the presence of Saponins by the Haemolytic Method

Several varieties of cowpeas were screened for saponins to determine whether or not they are involved in the resistance of TVu 2027 towards Callosobruchus maculatus.

Although several methods exist for detecting the presence of saponins, the most sensitive and hence most commonly used is based on the ability of saponins to haemolyse erythrocytes. It is thought that the steroid moiety of the saponin complexes with the cholesterol of the erythrocyte membrane so causing a rearrangement of the molecules; this results in the membrane rupturing and the cell haemolysing. The degree of haemolysis was determined spectrophotometrically after centrifuging off intact erythrocytes. This method is simple, sensitive and fairly

quantitative. However, not all saponins possess this haemolytic activity.

Using a 2 per cent suspension of washed rabbit erythrocytes in phosphate buffered saline (PBS), a calibration curve was constructed for digitonin and gypsophila root saponin as standards, and the concentrations necessary to give 50 per cent haemolysis determined; the values of 2.1 µg/ml and 9.8 µg/ml were obtained respectively (Figures 2.2.1. and 2.2.2). A calibration curve was also carried out for gypsophila root saponin using a 100 per cent erythrocyte suspension. Since soyabeans are known to contain saponins, both the extraction procedure and haemolytic assay were checked using hexane defatted soyabean meal. The extraction procedure of Birk et al., (1963) was followed to give a saponin extract. Using a 2 per cent erythrocyte suspension the soyabean saponin extract at 0.92 mg/ml (in PBS) caused 50 per cent haemolysis (Figure 2.2.3). Since a similar glycosidic extract of cowpea TVu 76 at a concentration of 5 mg/ml caused no haemolysis using a 2 per cent erythrocyte suspension, the assay was repeated using a 100 per cent erythrocyte suspension so as to achieve maximal saponin effect. Despite this modification

Figure 2.2.1. Calibration for the Determination of Haemolysis by Digitonin using 2 per cent Rabbit Erythrocytes

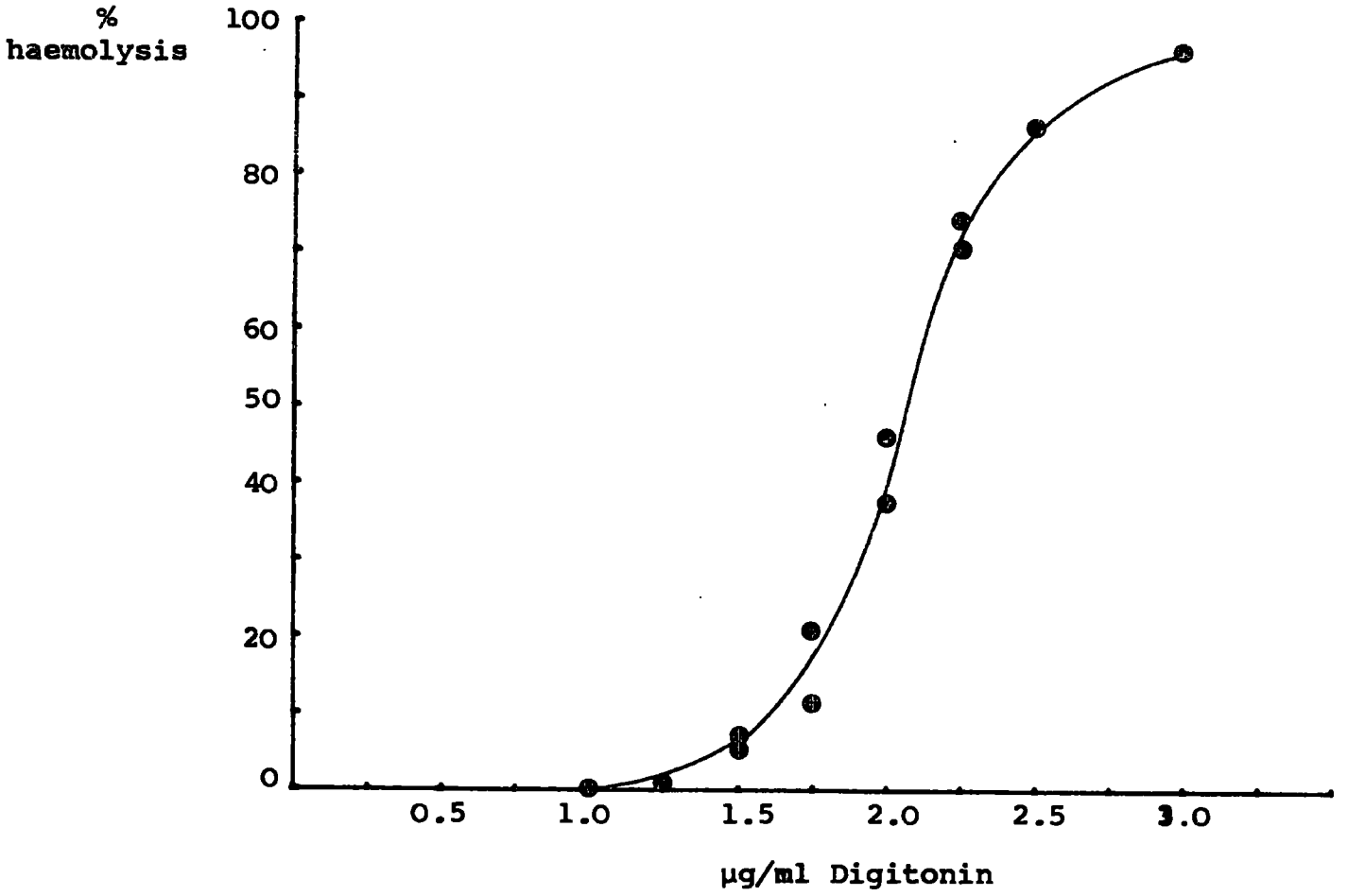
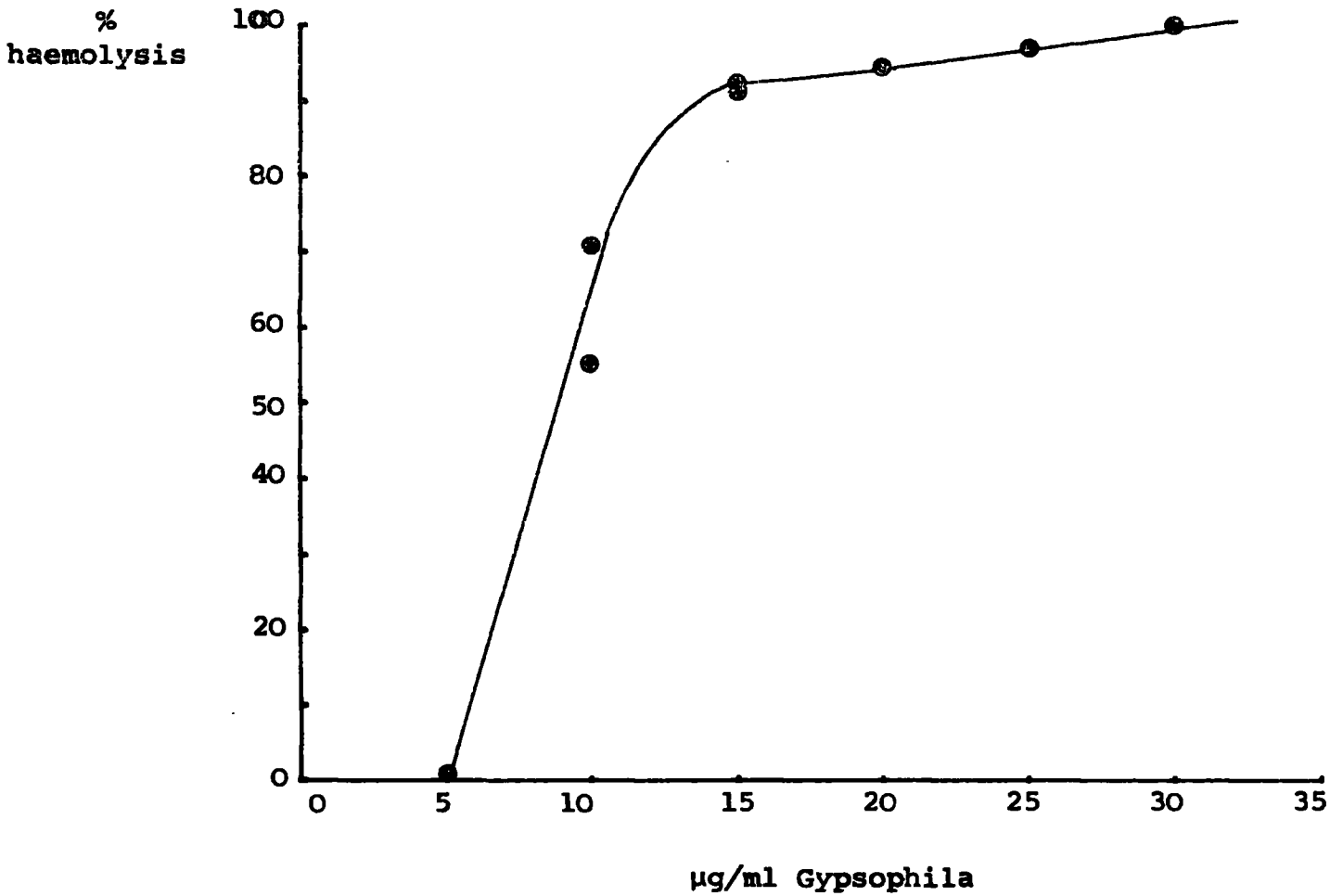


Figure 2.2.2. Calibration for the Determination of Haemolysis by Gypsophila using 2 per cent Rabbit Erythrocytes



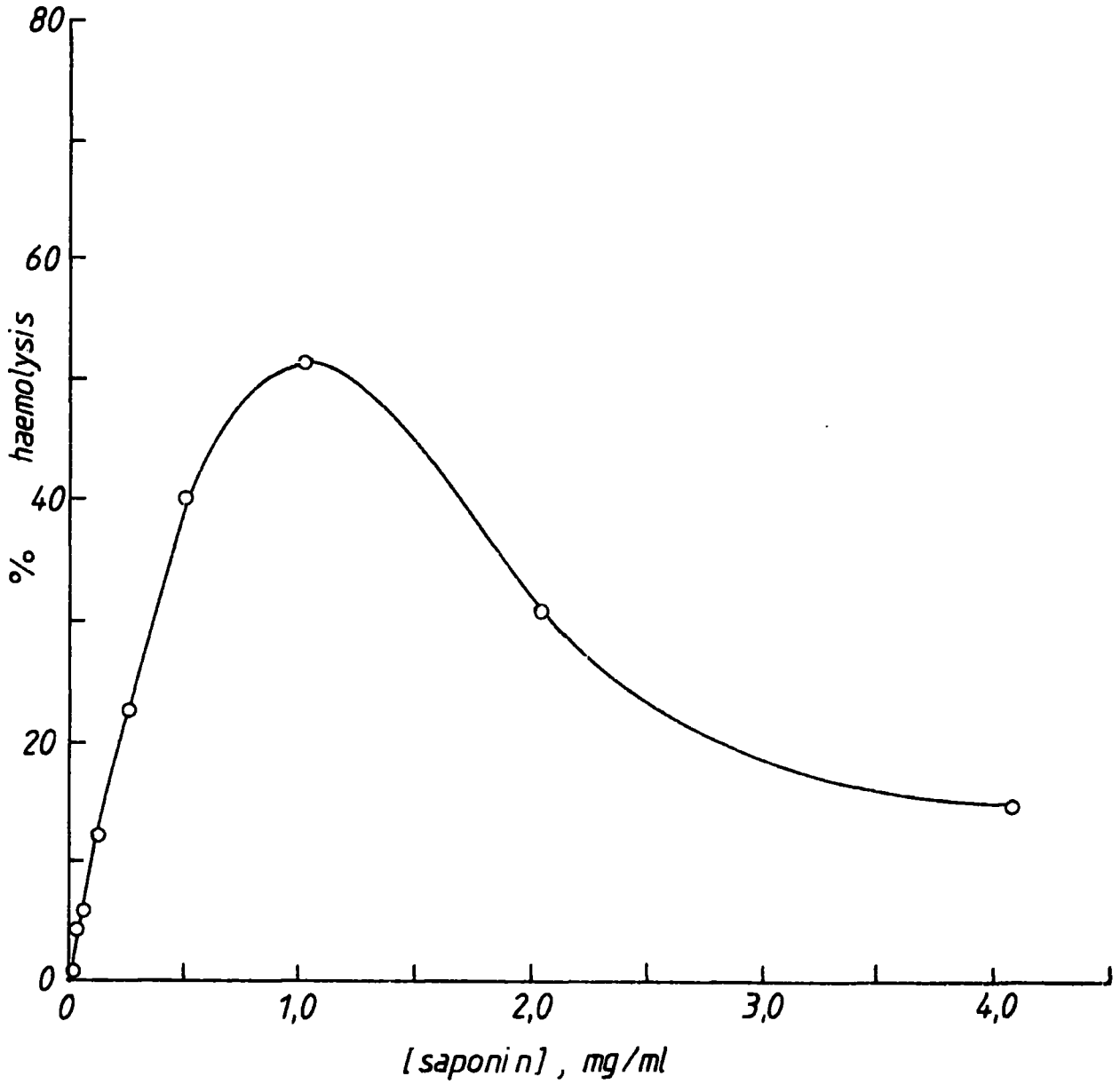


Figure 2.2.3. Haemolysis of a 2% erythrocyte suspension by varying amounts of soyabean saponin. The apparent decrease in haemolysis above 1 mg/ml saponin was due to destruction of haemoglobin by the soyabean extract. For conditions, see text.

neither an extract of cowpea TVu 76 nor an extract of cowpea TVu 2027 gave any significant haemolysis. Because the cowpea glycosidic extract is purple in colour, the relevant control had to be carried out for each incubation so as to ensure that this colouration was not masking any haemolysis. Digitonin was used as a control to achieve 100 per cent haemolysis. To confirm the absence of saponins in the cowpea seed further assay methods were attempted, these being the foaming method and the acetylation method. The former involves shaking varying concentrations of the extract or saponin in potassium phosphate buffer (67 mM) and measuring the height of foam produced after a given time. The acetylation method is based on a colour production. In the presence of saponins, a mixture of chloroform, acetic anhydride, and concentrated sulphuric acid produce either a brown or blue/green colour. Neither method produced positive results when using a cowpea extract. However in the presence of gypsophila root saponin and soyabean saponin the acetylation method was positive; in this experiment bovine serum albumin and soyabean trypsin inhibitor were used as the negative controls. Since saponins were not detected in the varieties of cowpea seed tested using these methods, it can be concluded that saponins are not present in any significant quantity.

A summary of these results obtained using the haemolytic assay for the detection of saponins is given in Table 2.2.4.

B. Agglutination Test for Lectins

Haemagglutination assays were carried out to test for the presence of lectins as a possible basis for toxicity in cowpea. Lectin solutions, usually Concanavalin A, but also Vicia faba lectin, were used as positive agglutination controls. Due to the high degree of specificity shown by some lectins, agglutination assays were not only carried out using several different types of blood, but also under different assay conditions.

Using the following types of blood, human group O (+ and - BSA), human group A (+ and - BSA), rabbit (+ and - BSA), pig, sheep and cow, no agglutination occurred in the presence of albumin extracts, at a concentration of 10 mg/ml, from 4 varieties of cowpea. A total protein extract also gave no agglutination. Concanavalin A, on the other hand, showed a strong agglutination reaction towards all the blood types. Bovine serum albumin (BSA) was added to some of the assays, at a concentration of 1 mg/ml, since some lectins appear to require added protein for activity (Toms and Western, 1971); however, the results were still negative with respect

Table 2.2.4

Summary of the Haemolytic Activity Caused by Different Sources of Saponin and Glycosidic Extract

Source of Saponin	Blood Suspension vol (ml)	concn (%)	Saponin Solution vol (ml)	concn.	Haemolysis % obtained
Digitonin	1.4	2	0.6	2.1µg/ml	50
Gypsophila	1.4	2	0.6	9.8µg/ml	50
Soyabean	1.4	2	0.6	0.92mg/ml	50
Cowpea TVu 76	1.4	2	0.6	5mg/ml	0
Digitonin	1.4	+100	0.6	21µg/ml	destroyed haemoglobin
Gypsophila	1.4	+100	0.6	21µg/ml	50
Cowpea TVu 76	1.4	+100	0.6	5mg/ml	5.75
Cowpea TVu 2027	1.4	+100	0.6	5mg/ml	5.0

+Undiluted blood was used to achieve maximal saponin effect. Incubation was carried out at 37°C for 2 hours.

to the cowpea extracts. Many lectins contain calcium and manganese ions and hence require these metals for activity, for example Concanavalin A (Lis and Sharon, 1973). Some agglutination assays were therefore carried out in the presence of these ions (1 mM), but again negative results were obtained for the cowpea varieties. Both trypsin and neuraminidase increase the sensitivity of these agglutination assays. Trypsin treatment exposes carbohydrate residues on the erythrocyte surface masked by proteins; it consequently renders the erythrocytes more susceptible to agglutination (Lis and Sharon, 1973). For example rabbit erythrocytes are 4 to 6 times as readily agglutinated by Vicia faba lectin (Gatehouse, private communication). Neuraminidase removes sialic acid from the ends of carbohydrate chains on the erythrocyte surface so rendering them more liable to agglutination; this treatment increases the sensitivity to about the same level as trypsin treatment. Peanut lectin, for example, will only agglutinate neuraminidase treated erythrocytes (Lotan et al., 1975). However, in the presence of either trypsin treated or neuraminidase treated erythrocytes no agglutination occurred with the cowpea albumin extracts. Thus under no conditions used could any agglutination be detected in the seeds of Vigna unguiculata. Furthermore, no material from V. unguiculata seed extracts will bind to either Sephadex-G100

or Sepharose 4B (Gatehouse, private communication) whereas most lectins will bind to one or other of these resins.

It has been reported that the roots of 4-day old germinated seedlings of V. unquiculata do show haemagglutinating activity towards rabbit erythrocytes (Gatehouse, private communication). Thus it seems reasonable to conclude that the seeds in fact do not contain lectins, rather than the negative results being due to wrong assay conditions. The results are summarised in Table 2.2.5.

C. Test for Alkaloids

A preliminary investigation was carried out to determine the presence of alkaloids in a 90 per cent ethanol extract from cowpea TVu 2027 meal using Dragendorff's Reagent (Dawson, 1969). Since an orange colour is produced only in the presence of tertiary substituted amines, both tertiary substituted amines (2,4,6-trimethyl pyridine and N-ethyl-morpholine) and secondary substituted amines (L-tryptophan and L-proline) were used as controls. The extract and controls were spotted onto filter paper and sprayed with the reagent. The results were negative with respect to the cowpea ethanol extract but positive for the appropriate controls.

Table 2.2.5

Summary of Agglutination Assays using Albumin Extracts from four Varieties of *Vigna unguiculata*

Erythrocytes	Agglutination by *V. unguiculata* extracts	Controls *
Human Gp O	None (TVu 76)	*V. faba* 120
Gp O + BSA	None (TVu 76)	*V. faba* 120
Gp A	None (TVu 76)	*V. faba* 480
Gp A + BSA	None (TVu 76)	*V. faba* 480
Gp B	None (TVu 76)	*V. faba* 240
Gp B + BSA	None (TVu 76)	*V. faba* 240
Rabbit	None (TVu 76, TVu 4557, TVu 57)	*V. faba* 5120; Con A 3200
Rabbit + BSA	None (TVu 76)	*V. faba* 5120; Con A 2560
Rabbit, trypsin treated	None (TVu 76)	*V. faba* 25000; Con A 25000
Rabbit, neuraminidase treated	None (TVu 76)	*V. faba* 16000; Con A 6000
Rabbit + Mn^{2+}, Ca^{2+}	None (TVu 2027)	*V. faba* 5120; Con A 2560
Pig	None (TVu 76)	*V. faba* 960
Sheep	None (TVu 76)	*V. faba* 0
Cow	None (TVu 76)	*V. faba* 0

*Assay figure represents highest dilution from 1 mg/ml solution to give agglutination

V. faba = lectin from *Vicia faba* seeds

Con A = concanavalin A

If present, alkaloids will be extracted into 90 per cent ethanol, and since most alkaloids possess tertiary substituted amines, it would appear from these preliminary investigations that they are absent in the seeds of Vigna unguiculata, at least in this particular variety.

D. Screening for Non-Protein Amino Acids

Since certain legume seeds are rich sources of non protein amino acids, many of which are toxic, the following varieties of Vigna unguiculata seeds were kindly assayed by Professor Bell's laboratory at my request for the presence of these non protein amino acids: TVu 37, TVu 57, TVu 76, TVu 1190E, TVu 1502-1D, TVu 2027, TVu 2030, TVu 3629 and TVu 4557. No positive results were obtained. Unfortunately, however, no further details were given.

E. Protease Inhibitors

Cowpea seeds were screened for the presence of various potentially toxic protease inhibitors. Since phytophagous insects do not have an acid fore-gut (Anstee, private communication) analagous to the mammalian stomach, but rather a neutral or alkaline pH of digestion, it was not thought necessary to investigate the presence of inhibitors towards acidic proteases in the seeds of different varieties of Vigna unguiculata. The inhibitors of the following endopeptidase enzymes, trypsin and chymotrypsin, and the

exopeptidase enzymes, leucine aminopeptidase and carboxy-peptidase were screened for. All four enzymes have been reported to be present in several insects.

(i) Trypsin Inhibitors

Trypsin inhibitory activity is very readily detected in cowpeas. Trypsin inhibitor content was assayed using the following substrates: casein, α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and α-N-benzoyl-L-arginine ethyl ester HCl (BAEE). In all cases inhibition of trypsin hydrolysis was detected with extracts from V. unguiculata seeds. Further details are given later.

(ii) Chymotrypsin Inhibitors

Chymotrypsin inhibitory activity was determined in cowpea seed extracts using the synthetic substrate N-benzoyl-L-tyrosine ethyl ester (BTEE). Inhibition of chymotrypsin hydrolysis was obtained with extracts from all varieties of cowpea tested although this was far less pronounced than trypsin inhibition. Further details are given later.

(iii) Leucine Aminopeptidase Inhibition

Endogenous leucine aminopeptidase (LAP) activity and an inhibitor of this enzyme were assayed for in a total protein extract of cowpea TVu 76, an albumin extract of TVu 2027 and TVu 4557 and a globulin extract of TVu 57 and TVy 2027. Inhibitory activity was determined by measuring

the rates of hydrolysis of the synthetic substrate leucineamide in the presence of cowpea extracts. Before addition of the substrate the enzyme and varying amounts of seed extract were preincubated for five minutes. Endogenous activity was measured by replacing the enzyme with a seed extract.

Using 3.1 units of the enzyme no inhibition was observed with up to 500 µl of albumin extract (2 mg/ml), or up to 100 µl of globulin extract (10 mg/ml), or up to 100 µl of total protein extract (200 mg/ml). No endogenous leucine aminopeptidase was observed in any of the protein fractions. Thus it appears that there are no inhibitors present of this exopeptidase present in seeds of Vigna unguiculata.

(iv) Carboxypeptidase A Inhibitors

Varieties of V. unguiculata were screened for both carboxypeptidase A inhibitors and activity of the respective enzyme by a method similar to LAP screening. After a five minute preincubation of enzyme and varying amounts of seed extract, the rate of hydrolysis was followed at 259 nM using the synthetic substrate hippuryl-L-phenylalanine (1mM).

Using 0.37 units of the enzyme no inhibition was observed with up to 500 µl albumin extract (2 mg/ml), or up to 150 µl of globulin extract (10 mg/ml), or up to 100 µl

of total protein extract (200 mg/ml). However, a small degree of endogenous enzyme activity was observed in a total protein extract, though it was too small to determine the rate. A summary of the results is given in Table 2.2.6.

It can be concluded that no inhibitors of the two exopeptidases screened for were present in the seeds of *Vigna unquiculata*.

Table 2.2.6

Determination of Carboxypeptidase A Inhibitory Activity in an Albumin Extract, a Globulin Extract and a Total Protein Extract from Different Varieties of Vigna unquiculata seeds

A Albumin Extract of TVu 2027 and TVu 4557 (2 mg/ml)		
No. Assay	Rate Hydrolysis	Enzyme Activity u/mg
1 2.9 ml S + 10µl E	3.3×10^{-2}	14.36
2 2.9 ml S + 500µl TVu 4557	0	0
3 2.9 ml S + 500µl TVu 2027	0	0
4 2.9 ml S + (10µl E + 100µl TVu 4557)	4.07×10^{-2}	15.80
5 2.9 ml S + (10µl E + 100µl TVu 4557)	4.00×10^{-2}	15.53
6 2.9 ml S + (10µl E + 500µl TVu 4557)	3.46×10^{-2}	13.44
7 2.9 ml S + (10µl E + 500µl TVu 2027)	3.41×10^{-2}	13.24
B Total Protein Extract TVu 76 (200 mg/ml)		
No. Assay	Rate Hydrolysis	Enzyme Activity u/mg
1 2.9 ml S + 10µl E	3.55×10^{-2}	13.78
2 2.9 ml S + 100µl total protein	+ve v.small	v. small
3 2.9 ml S + (10µl E + 50µl total protein)	3.94×10^{-2}	15.3
4 2.9 ml S + (10µl E + 75µl total "	3.24×10^{-2}	12.58
5 2.9 ml S + (10µl E + 100µl total "	3.35×10^{-2}	13.00
6 2.9 ml S + (10µl E + 125µl total "	3.34×10^{-2}	12.96
C Globulin Extract of TVu 57 and TVu 2027 (10 mg/ml)		
No. Assay	Rate Hydrolysis	Enzyme Activity u/mg
1 2.9 ml S + 10µl E	4.31×10^{-2}	16.73
2 2.9 ml S + 50µl TVu 2027	0	0
3 2.9 ml S + 50µl TVu 57	0	0
4 2.9 ml S + (10µl E + 50µl TVu 2027)	4.12×10^{-2}	16.00
5 2.9 ml S + (10µl E + 50µl TVu 57)	4.35×10^{-2}	16.88
6 2.9 ml S + (10µl E + 100µl TVu 2027)	4.53×10^{-2}	17.59
7 2.9 ml S + (10µl E + 100µl TVu 57)	4.34×10^{-2}	16.84
8 2.9 ml S + (10µl E + 150µl TVu 2027)	4.42×10^{-2}	17.15
9 2.9 ml S + (10µl E + 150µl TVu 57)	4.42×10^{-2}	17.15

foot note: A different batch of enzyme was used for each set of assays. Rate of hydrolysis $\equiv \Delta$ OD/min at 259 nm.
S = substrate
E = enzyme
( ) = denotes preincubation of E + I.

PART III Comparison of Trypsin Inhibitor Content and Chymotrypsin Inhibitor Content of Vigna unquiculata Varieties.

Since the trypsin inhibitor, and to a much lesser extent the chymotrypsin inhibitor, were the only toxic substances detected in the seeds of V. unquiculata it was necessary to screen both the resistant variety, TVu 2027, and susceptible varieties to see whether there was any correlation between the resistance and the level of trypsin inhibitor.

A. Comparison of Trypsin Inhibitor Content

The trypsin inhibitor content of the different varieties was determined using both casein and BAPNA as substrates for trypsin. Casein assays were carried out on the purified inhibitors whereas BAPNA assays were initially carried out on crude albumin extracts.

(i) Using Casein as Substrate

To estimate the inhibitor content, cowpea meal was initially extracted in acetate buffer (pH 4.4) and the protein was precipitated with ammonium sulphate to 70 per cent saturation. The precipitate was redissolved and the inhibitor purified by ion-exchange chromatography at pH 8 using DEAE-Cellulose. Essentially six peaks of protein were eluted by a linear sodium chloride gradient (0-0.5M)

collecting about 130 fractions per gradient. Trypsin inhibitory activity was assayed for in each fraction and the total inhibitory activity calculated by summing the individual activities. The OD of all the fractions was measured at 280 nm. This procedure was carried out for each of the varieties.

A summary of the results is shown in Table 2.3.1. As can be seen from these results, the resistant variety of V. unguiculata contained about twice as much inhibitory activity as the other susceptible varieties, both in terms of total trypsin inhibitory units (TIU) per 100 g of meal, and trypsin inhibitory units per mg of protein.

(ii) Using α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as substrate

The findings of the previous section were repeated and confirmed by a more direct method.

Inhibitory assays were carried out on both freshly prepared albumin extracts and the lyophilised albumins (2 mg/ml) for each variety. The albumin fraction extracted at pH 4.4 from cowpea meal contains virtually all the inhibitory activity. The degree of inhibition was measured by following the rate of inhibition of trypsin hydrolysis of BAPNA, at 410 nm in the presence of varying amounts of albumin extract; prior to addition of the substrate the

Table 2.3.1

Determination of the Specific Activity of the Trypsin Inhibitor from eight Varieties of Cowpea, Purified by Ion-Exchange Chromatography

Variety	Total Protein from 100 g seed *mg*	Total TIU (Σ of fractional activity)	Specific Activity TIU/mg Protein
TVu 57	36.5	96×10^{-2}	2.64×10^{-2}
TVu 1502-1D	46.3	142×10^{-2}	3.06×10^{-2}
TVu 76	52.8	182×10^{-2}	3.44×10^{-2}
TVu 1190E	37.1	131×10^{-2}	3.55×10^{-2}
TVu 3629	36.7	142×10^{-2}	3.88×10^{-2}
TVu 37	35.5	150×10^{-2}	4.22×10^{-2}
TVu 4557	45.4	213×10^{-2}	4.68×10^{-2}
TVu 2027	52.0	364×10^{-2}	7.00×10^{-2}

foot note : Trypsin inhibitory activity was determined using casein as substrate.
Definition of Activity: 1 trypsin unit (TU) is expressed as the amount of trypsin which under the defined conditions liberates sufficient TCA soluble hydrolysis product that the OD at 280 nm increases by 1.00 unit/minute.
Trypsin inhibitory activity (TIU) is expressed as the amount of inhibitor required to inhibit 1 unit of trypsin.

enzyme and albumin extract were preincubated for 5 minutes. Endogenous BAPNA-ase activity for each variety was determined by replacing the enzyme with the albumin extract. Trypsin inhibitor content was determined by plotting the rate of hydrolysis against the volume of albumin extract; the point of intercept along the y axis gave the amount of extract required to give 100 per cent inhibition (Figure 2.3.2). Based on a molecular weight of 23,281 for bovine trypsin (Hoffman, 1964) the amount of crude inhibitor equivalent to one μmole of enzyme was calculated (Figure 2.3.2).

A summary of the results using a lyophilized albumin extract (2 mg/ml) is given in Table 2.3.3. Since these extracts possessed endogenous BAPNA-ase activity, the amount varying slightly between the varieties, the inhibition values were thus accordingly corrected before being plotted. As can be seen from the results, the resistant variety TVu 2027 contained significantly more trypsin inhibitory activity than the other varieties, so confirming the initial findings obtained using casein. Furthermore, these findings were also confirmed using fresh albumin extracts. Although the results obtained from the casein assays and BAPNA assays were in agreement at the upper and lower limits, when placed in order of trypsin inhibitor content there were, however,

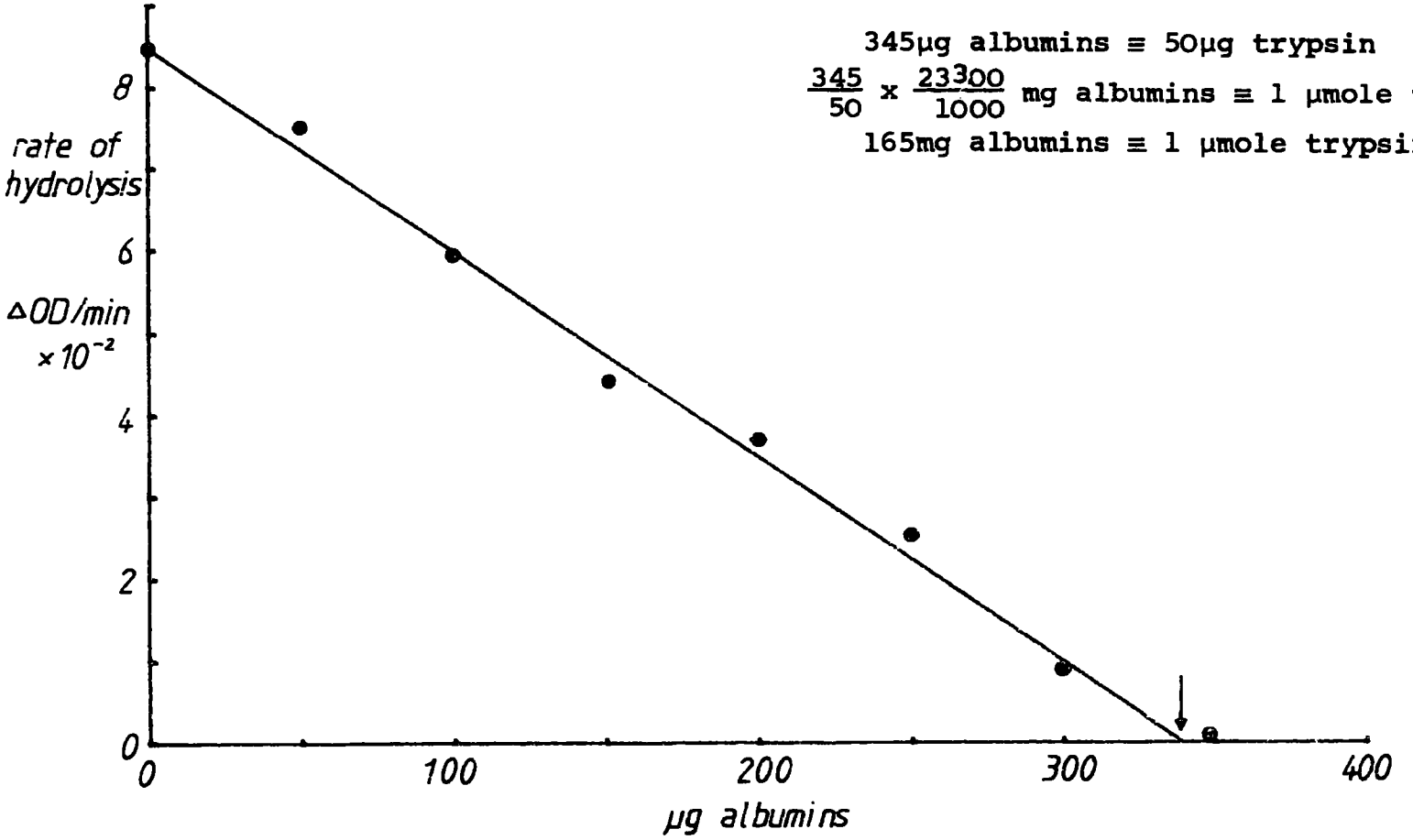


Figure 2.3.2. Inhibition of trypsin by albumin fraction from cowpea variety TVu 1190E. Assay: trypsin-catalysed hydrolysis of BAPNA, followed spectrophotometrically at 410nm.

Table 2.3.3

Comparison of the Trypsin Inhibitor Content of nine Varieties of Cowpea (Vigna unquiculata)

Variety	Trypsin Inhibitor Content
TVu 2027	41 mg extract ≡ 1 µmole trypsin
TVu 4557	85 mg extract ≡ 1 µmole trypsin
TVu 76	112 mg extract ≡ 1 µmole trypsin
TVu 3629	127 mg extract ≡ 1 µmole trypsin
TVu 37	147 mg extract ≡ 1 µmole trypsin
TVu 57	151 mg extract ≡ 1 µmole trypsin
TVu 1190E	165 mg extract ≡ 1 µmole trypsin
TVu 1502-1D	201 mg extract ≡ 1 µmole trypsin

foot note : Trypsin inhibitory activity was determined using BAPNA as substrate. Values given are corrected for endogenous BAPNA-ase activity.
The estimated error in the figures is $\leq 5\%$.

slight discrepancies of order between varieties having very similar inhibitory activity. More weight was put on the results obtained from the BAPNA assays as this method is more direct and more sensitive and varietal differences were more pronounced.

B. Comparison of Chymotrypsin Inhibitor Content

Since chymotrypsin inhibitors were also readily detected in the seeds of V. unguiculata, it was necessary to screen the seeds and so determine whether there was similarly a correlation between chymotrypsin inhibition and resistance.

Inhibitory activity was determined by following the rate of hydrolysis of BTEE in the presence of varying amounts of albumin extract (2 mg/ml); as with all inhibition assays, the enzyme and inhibitor were pre-incubated before addition of the substrate. No endogenous BTEE-ase activity was detected in any of the varieties. As with the trypsin inhibitory assays using BAPNA as substrate, the inhibitor content was then determined by plotting the rate of hydrolysis against the volume of albumin extract; the point of intercept along the y axis gave the amount of extract required to give total inhibition (Figure 2.3.4). Based on a molecular weight of 25,310 for bovine pancreatic chymotrypsin A (Eck and Dayhoff, 1966), the amount of crude

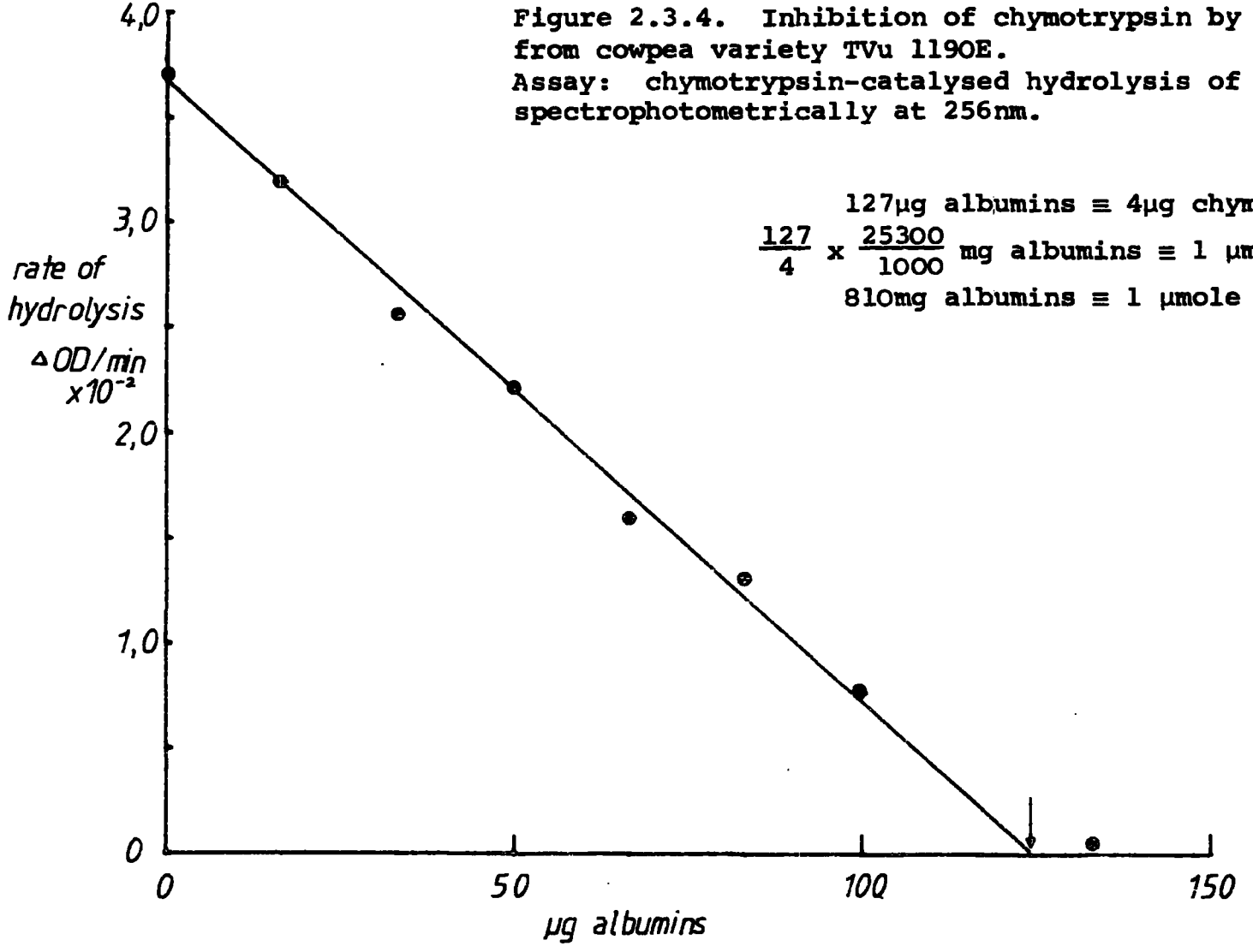


Figure 2.3.4. Inhibition of chymotrypsin by albumin fraction from cowpea variety TVu 1190E.
Assay: chymotrypsin-catalysed hydrolysis of BTEE, followed spectrophotometrically at 256nm.

127µg albumins ≡ 4µg chymotrypsin

$\frac{127}{4} \times \frac{25300}{1000}$ mg albumins ≡ 1 µmole chymotrypsin

810mg albumins ≡ 1 µmole chymotrypsin

inhibitor equivalent to one μmole of enzyme was calculated.

A summary of the results is given in Table 2.3.5. The results show virtually the same order of chymotrypsin inhibitory activity within the varieties as for trypsin inhibitory activity; however there was only about 10 to 20 per cent chymotrypsin inhibitory activity compared to trypsin inhibitory activity present in the seed. The maintenance of a rough proportionality between trypsin and chymotrypsin inhibitory activities across the varieties indicates that these two endogenous protease inhibitory activities are closely associated with one another. It was considered justifiable to work subsequently on the basis of trypsin inhibitory activity.

Table 2.3.5

Comparison of the Chymotrypsin Inhibitor Content of nine Varieties of Cowpea (Vigna unguiculata)

Variety	Chymotrypsin Inhibitor Content
TVu 2027	320 mg extract ≡ 1 µmole chymotrypsin
TVu 4557	450 mg extract ≡ 1 µmole chymotrypsin
TVu 76	650 mg extract ≡ 1 µmole chymotrypsin
TVu 3629	760 mg extract ≡ 1 µmole chymotrypsin
TVu 37	780 mg extract ≡ 1 µmole chymotrypsin
TVu 1190E	810 mg extract ≡ 1 µmole chymotrypsin
TVu 57	980 mg extract ≡ 1 µmole chymotrypsin
TVu 1502-1D	1250 mg extract ≡ 1 µmole chymotrypsin

foot note : Chymotrypsin inhibitory activity was determined using BTEE as substrate. The estimated error in the figures is $\leq$ 5%.

PART IV Purification of the Trypsin Inhibitor from Vigna unguiculata by Trypsin Affinity Chromatography. Determination of Trypsin Inhibitor Content in Eight Varieties

A. Purification of the Trypsin Inhibitor

The trypsin inhibitor from the different varieties of V. unguiculata was purified from an albumin extract of the seed by affinity chromatography on a column of trypsin linked to Sepharose. Extraction of cowpea meal at pH 4.0 left less than 2 per cent of trypsin inhibitory activity in the residue. The resulting albumin extract was loaded onto the column at pH 4.0 (the pH at which the inhibitor and enzyme forms a stable complex) and the trypsin inhibitor eluted at pH 2.0 (the pH at which the complex dissociates). The purity of the eluted trypsin inhibitor was checked by rebinding to a trypsin affinity column; all of the protein bound. The material that did not bind to the column was tested and found to be devoid of inhibitory activity. Thus the purification was virtually quantitative provided the column was not overloaded. The trypsin inhibitor was desalted by gel filtration using Sephadex G-25 and finally lyophilised.

B. Determination of Trypsin Inhibitor Content

The inhibitor content of the different varieties of V. unguiculata was determined from the equivalence of the

purified inhibitor and trypsin, the equivalence of the lyophilised albumin extract and trypsin, and the percentage albumins in the seed. The inhibitor content ranged from between 0.2 per cent to 0.4 per cent for the susceptible varieties and 0.9 per cent for the resistant variety, as shown in Table 2.4.1. Furthermore, direct determination of inhibitor content from yields of lyophilised material in the purification gave values of the same order. The percentage inhibitor present in the different varieties was required for the feeding trials (PART VII).

Table 2.4.1

Physiological Concentration of the Trypsin Inhibitor in the Seeds of Different Varieties of *Vigna unguiculata*

Variety	% Trypsin Inhibitor
TVu 2027	0.92
TVu 4557	0.44
TVu 76	0.34
TVu 3629	0.30
TVu 37	0.26
TVu 57	0.25
TVu 1190E	0.23
TVu 1502-1D	0.19

foot note : Trypsin inhibitor content was calculated on the basis of the specific activity of the purified inhibitor, and the inhibitory activity of the albumin extract.

Example of Calculation for TVu 57

200µl (100 µg/ml) inhibitor = 500 µl enzyme (100 µg/ml)
0.40 µg inhibitor = 1 µg enzyme
assuming a molecular weight of 23,281 for trypsin
0.40 x 23,281 µg inhibitor = 1 µmole enzyme
9,300 µg inhibitor = 1 µmole enzyme
Since 151,000 µg albumin extract = 1 µmole enzyme (measured)
and the albumin extract = 4.0% of the seed (measured)
the % inhibitor of the seed $\frac{9300 \times 100 \times 4.0}{15100 \times 100}$ = 0.25%

PART V Effect of the Trypsin Inhibitor from Vigna unguiculata on Endogenous Trypsin-Like Activity

There is conflicting evidence as to whether or not the trypsin inhibitors play a role in the primary metabolism and germination of the legume seed by inhibiting endogenous trypsin-like proteases. Furthermore, it is not clear as to whether the BAPNA-ase activity, often synonymously termed endogenous trypsin activity, mentioned in the literature is a true trypsin-like activity or whether the activity is due to the presence of dipeptidases which are also capable of hydrolysing BAPNA. In order to help elucidate these two problems, assays were carried out to determine trypsin-like activity in different fractions of V. unguiculata, and the effect of the purified cowpea trypsin inhibitor (CPTI) on this activity.

The albumin fraction of the cowpea variety TVu 57 was found to contain BAPNA-ase activity. However, neither the corresponding trypsin inhibitor, nor soyabean trypsin inhibitor caused any detectable inhibition. This lack of inhibition suggests that the BAPNA-ase activity found in the albumin fraction is not due to trypsin-like enzymes. A summary of the results is given in Table 2.5.1. The absence of BAEE-ase activity in albumin extracts from TVu 57, TVu 2027 and TVu 4557 confirms this assumption

Table 2.5.1

Effect of Purified Trypsin Inhibitor from Vigna unguiculata on Endogenous BAPNA-ase Activity from an Albumin Extract

Assay	Rate of Hydrolysis ΔOD/min at 410 nm (corrected for dilution)
Control: 2 ml BAPNA + 500µl Trypsin	12.60×10^{-2}
2 ml BAPNA + (100µl Pure CPTI TVu 57 + 500µl Trypsin)	9.05×10^{-2}
2 ml BAPNA + (200µl Pure CPTI TVu 57 + 500µl Trypsin)	7.02×10^{-2}
2 ml BAPNA + (200µl SBTI + 500µl Trypsin)	2.70×10^{-2}
Control for endogenous BAPNA-ase activity: 2 ml BAPNA + 500µl albumin extract	1.01×10^{-2}
2 ml BAPNA +(50µl SBTI + 500µl albumin)	1.04×10^{-2}
2 ml BAPNA +(100µl SBTI ")	1.05×10^{-2}
2 ml BAPNA +(200µl SBTI ")	1.03×10^{-2}
2 ml BAPNA +(50µl CPTI ")	1.00×10^{-2}
2 ml BAPNA +(100µl CPTI ")	1.03×10^{-2}
2 ml BAPNA +(200µl CPTI ")	1.00×10^{-2}

foot notes: [SBTI] = 500 µg/ml H_2O

[CPTI] = 500 µg/ml H_2O

[Trypsin] = 100 µg/ml 10^{-3} M HCl

[Albumin extract] = 2 mg/ml H_2O

( ) in table denotes preincubation of E + I

(the results are shown in Table 2.5.2). When a total protein extract of cowpea TVu 76 was assayed BAEE-ase activity was detected, suggesting that this activity is associated with the globulin fraction (Table 2.5.3). Since the soyabean trypsin inhibitor appeared to inhibit this activity it implies that trypsin-like enzymes are responsible for at least part of the BAEE-ase activity; lack of total inhibition is presumably due to the presence of esterase hydrolysis. This trypsin-like activity, however, was not affected by its own inhibitor (Table 2.5.3). To confirm that the trypsin-like activity was actually associated with the globulin fraction, globulin extracts were prepared from two varieties of cowpea, TVu 57 and TVu 2027. Both these globulin extracts showed BAEE-ase activity, but as was previously found the cowpea trypsin inhibitor, either from the same or a different variety, was inactive against it (Table 2.5.4). This protease activity appears to be very unstable. These findings agree with those of Gennis and Cantor who isolated a protease from black-eyed pea which was inhibited by SBTI and was also very unstable.

Whether this endogenous protease activity (BAPNA-ase activity of the albumin fraction or BAEE-ase activity of the globulin fraction) is due to a trypsin-like enzyme or not, the fact remains that the cowpea trypsin inhibitor caused

Table 2.5.2

Determination of Endogenous Trypsin-like Activity in Albumin Extracts from three Varieties of *Vigna unguiculata* using α-N-benzoyl-L-arginine ethyl ester (BAEE) as Substrate

Assay	Rate of Hydrolysis ΔOD/min at 253 nm
Control: 3 ml BAEE + 100µl Trypsin	5.18×10^{-2}
Control: 3 ml BAEE + 200µl Trypsin	9.6×10^{-2}
3 ml BAEE + 200µl TVu 57 albumin extract	0
3 ml BAEE + 500µl TVU 57 albumin extract	0
3 ml BAEE + 200µl TVu 2027 albumin extract	0
3 ml BAEE + 200µl TVu 4557 albumin extract	0

foot note: [Trypsin] = 100µg/ml 10^{-3} M HCl
[Albumin extract] = 2 mg/ml H_2O

Table 2.5.3

Effect of purified CPTI from Vigna unguiculata on Endogenous Trypsin-like Activity of a Total Protein Extract using α-N-benzoyl-L-arginine ethyl ester (BAEE) as Substrate

Assay	Rate of Hydrolysis ΔOD/min at 253 nm (corrected for dilution)
Control: 3 ml BAEE + 100µl trypsin	5.47×10^{-2}
Endogenous trypsin-like activity in total protein extract: 3 ml BAEE + 200µl cowpea protein extract	0.39×10^{-2}
3 ml BAEE + (100µl CPTI + 200µl cowpea protein extract)	0.35×10^{-2}
3 ml BAEE + (200µl CPTI + 200µl cowpea protein extract)	0.28×10^{-2}
3 ml BAEE + (50µl SBTI + 200µl cowpea protein extract)	0.24×10^{-2}
3 ml BAEE + (100µl SBTI + 200µl cowpea protein extract)	0.27×10^{-2}
3 ml BAEE + (200µl SBTI + 200µl cowpea protein extract)	0.15×10^{-2}

foot notes: [CPTI] = 500 µg/ml (TVu 57)

[cowpea protein extract] = 0.2 mg/ml
0.05M Tris/HCl pH 8.5

[SBTI] = 500 µg/ml

[Trypsin] = 100 µg/ml 10^{-3} M HCl

( ) = denotes preincubation of E + I

Table 2.5.4

To Demonstrate the Effect of CPTI on the Endogenous Trypsin-Like Activity of a Globulin Fraction from Different Varieties of Vigna unguiculata using α-N-benzoyl-L-arginine ethyl ester (BAEE) as Substrate

1. Effect of TVu 57 CPTI on Endogenous Activity of a TVu 57 Globulin Fraction
2. Effect of TVu 57 CPTI on Endogenous Activity of a TVu 2027 Globulin Fraction

Assay	Rate of Hydrolysis ΔOD/min 253 nm (corrected for dilution)
Control: 3 ml BAEE + 100µl trypsin	5.5×10^{-2}
endogenous activity: 3 ml BAEE + 400µl TVu 57 globulins	0.25×10^{-2}
endogenous activity: 3 ml BAEE + 400µl TVu 2027 globulins	0.26×10^{-2}
3 ml BAEE + (100µl TVu 57 CPTI + 400µl TVu 57 globulins)	0.25×10^{-2}
3 ml BAEE + (100µl TVu 57 CPTI + 400µl TVu 2027 globulins)	0.35×10^{-2}

foot note: [CPTI] = 500 µg/ml H_2O
[trypsin] = 100 µg/ml 10^{-3} M HCl
[TVu 57 globulin] = 10.15 mg/ml
[TVu 2027 globulin] = 10.16 mg/ml
( ) in table denotes preincubate E + I

no inhibition. Consequently, although incomplete, these findings do not support an active role for the inhibitor in seed metabolism and germination.

PART IV Determination of Protease Activity in a Larval Extract of Callosobruchus maculatus: Effect of the Trypsin Inhibitor from Vigna unquiculata on Larval Protease Hydrolysis

In order for the trypsin inhibitor from Vigna unquiculata to be an effective toxin to the larvae of Callosobruchus maculatus it must interfere with larval metabolism in some way. The most obvious mechanism would be for the inhibitor to bind the larval protease(s) so hindering proteolysis. For this theory to be correct, it is necessary first to demonstrate the presence of these proteases, and secondly to demonstrate in vitro inhibition by the cowpea trypsin inhibitor.

Larval proteolysis was demonstrated using the two synthetic substrates BAPNA and BAEE, and a physiological substrate, fluorescein labeled cowpea globulins. Furthermore, inhibition of this hydrolysis was also demonstrated.

A. Determination of Larval Protease Activity using Fluorescein Labeled Globulins, from Vigna unquiculata, linked to Cyanogen Bromide activated Sepharose 4B; Effect of the Trypsin Inhibitor from V. unquiculata

Since the larvae of C. maculatus feed on the cowpea seeds, they must be able to utilise the storage proteins (globulins) and hence must be capable of hydrolysing

these globulins. The major problem encountered using this physiological substrate is to make the assay system sufficiently sensitive to detect larval hydrolysis and subsequent inhibition. Increased sensitivity was achieved by labeling the globulins (from variety TVu 76) linked to an inert support, Sepharose, with fluorescein isothiocyanate. Since hydrolysis of this complex will result in the labeled products going into solution, it was hoped to measure the degree of hydrolysis fluorimetrically at 510 nm (emission wavelength); unfortunately, however, there was too much background interference to make the results meaningful, although the general indication was the same as the results obtained from reading the OD at 490 nm (excitation wavelength of fluorescein).

This assay system was checked using bovine trypsin (Figure 2.6.1) and the enzyme "pronase" from Streptomyces griseus.

It was possible to demonstrate both larval protease hydrolysis, and inhibition of hydrolysis by trypsin inhibitors using this technique. The results are given in Table 2.6.2. These results clearly demonstrate the presence of larval hydrolysis (assay nos. 5-9), the degree of hydrolysis being roughly proportional to the amount of extract added up to a volume of 500 µl (approximately

Figure 2.6.1. Time Course of the Hydrolysis of Fluorescein Labeled Cowpea Globulins linked to CNBr activated Sepharose by Trypsin

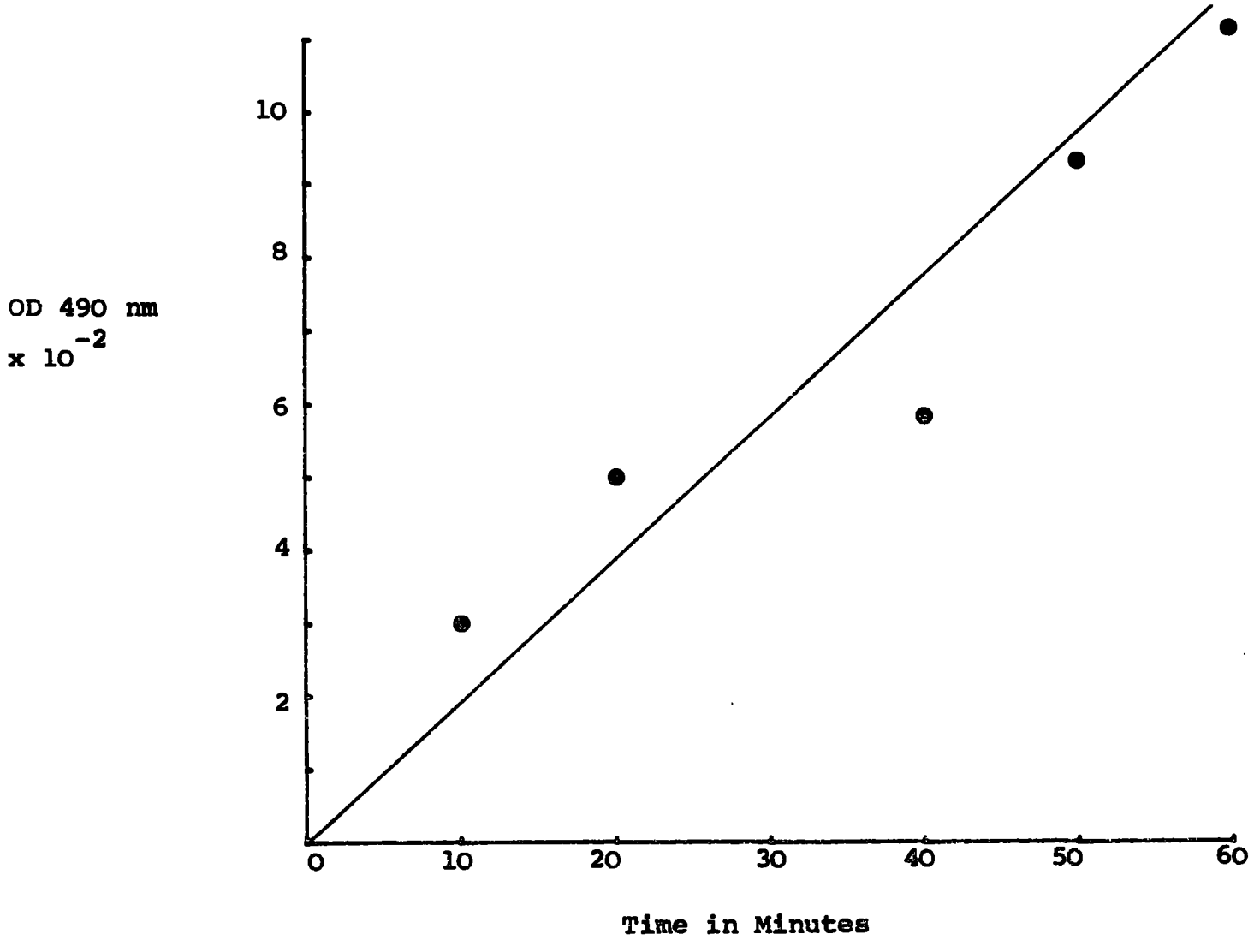


Table 2.6.2

Determination of Larval Protease Activity using Fluorescein labelled Globulins, from Vigna unguiculata, Linked to Cyanogen bromide - activated Sepharose 4B. Effect of the Trypsin Inhibitor from V. unguiculata.

Each of the following assays contained 2 ml of 0.05 M PO_4^- buffer pH 7.0 + 100µl of the fluorescein labelled globulins

No.	Assays	OD 490 nm	
	Controls		
1	+ 100µl buffer	0.04	
2	+ 200µl buffer	0.071	
3	+ 100µl pronase	0.689	
4	+ 200µl pronase	0.848	
	Determination of Larval Hydrolysis		
5	+ 100µl larval protease	0.217	0.210
6	+ 200µl larval protease	0.417	0.426
7	+ 300µl larval protease	0.540	0.508
8	+ 400µl larval protease	0.621	
9	+ 500µl larval protease	0.718	
	Effect of Inhibitors on Larval Protease Activity		
10	+ 200µl larval protease	0.417	0.426
11	+ (200µl larval protease + 200µl purified CPTI)	0.293	0.288
12	+ (200µl larval protease + 500µl purified CPTI)	0.267	0.271
13	+ *(200µl larval protease + 200µl cowpea albumins)	0.351	0.351
14	+ *(200µl larval protease + 500µl cowpea albumins)	0.307	
15	+ (200µl larval protease + 200µl SBTI)	0.334	
16	+ (200µl larval protease + 500µl SBTI)	0.282	

foot note: [Pronase] = 200 µg/ml buffer from Streptomyces griseus

[CPTI] = 500 µg/ml H_2O - from TVu 57, purified by affinity chromatography

[albumin extract] = 2 mg/ml H_2O from variety TVu 57

[SBTI] = 500 µg/ml H_2O

[larval protease] = 100 mg/ml

( ) denotes preincubation of E + I

* albumin extract had significant absorbance at 490 nm

100 mg larvae/ml). Similarly the degree of inhibition, using both the trypsin inhibitor from Vigna unguiculata purified by affinity chromatography (assay nos. 11, 12), and soyabean trypsin inhibitor (assay nos. 15, 16), was roughly proportional to the amount of inhibitor added. This therefore must represent a true stoichiometric inhibition. Furthermore, a crude albumin extract from V. unguiculata (variety TVu 57) also inhibited hydrolysis, this being related to the amount of extract added (assay nos. 13, 14); however the OD readings were quite high due to this extract having a significant absorbance at 490 nm.

In conclusion, the presence of both larval protease hydrolysis, and inhibition of this proteolysis by the cowpea trypsin inhibitor, can be demonstrated using fluorescein labeled cowpea globulins as substrate.

B. Determination of Larval Protease Activity using the synthetic Substrate α-N-benzoyl-L-arginine ethyl ester (BAEE)

The demonstration of larval protease hydrolysis and its inhibition by the cowpea trypsin inhibitor was confirmed using the synthetic substrate BAEE.

The results are given in Table 2.6.3; bovine trypsin was used as a control. That larval proteolysis is being

Table 2.6.3

Determination of Protease Activity in a Larval Extract of Callosobruchus maculatus using the Synthetic Substrate α-N-benzoyl-L-arginine ethyl ester (BAEE).

Demonstration of the Inhibition of Larval Proteolysis in the presence of the Trypsin Inhibitor from Vigna unguiculata, variety TVu 2027

No.	Assay	Rate of Hydrolysis ΔOD/min 253 nm (corrected for dilution)	
1	Control: 3 ml BAEE + 100µl trypsin	5.99×10^{-2}	5.95×10^{-2}
2	3 ml BAEE + (100µl trypsin + 50µl CPTI)	0	
3	3 ml BAEE + (100µl trypsin + 10µl CPTI)	9.23×10^{-4}	
4	3 ml BAEE + 250µl larval extract	13.11×10^{-4}	13.54×10^{-4}
5	3 ml BAEE + 125µl larval extract	7.84×10^{-4}	7.96×10^{-4}
6	3 ml BAEE + (125µl larval extract + 100µl CPTI)	5.45×10^{-4}	5.52×10^{-4}

foot note: [trypsin] = 100 µg/ml 10^{-3} M HCl
[larval extract] = 100 mg/ml chilled H_2O
[CPTI TVu 2027] = 2 mg/ml H_2O
[BAEE] = 25mM in glycine/NaOH pH 9.

measured is demonstrated by the rate of hydrolysis being proportional to the amount of larval extract added (assay nos. 4, 5). Furthermore, addition of the trypsin inhibitor from V. unquiculata (variety TVu 2027) decreased the rate of inhibition. However, total inhibition was not observed. Possibly this is due to other enzymes in the crude larval extract, for example esterases, which are capable of hydrolysing BAEE. Larval hydrolysis was also demonstrated using BAPNA as a substrate (Table 2.6.4).

Table 2.6.4

Determination of Larval Protease Activity using α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA)

Assay	Rate of Hydrolysis ΔOD/min 410 nm (corrected for dilution)
Control: 2 ml buffer + 200µl BAPNA + 500µl Trypsin	12.4×10^{-2}
2 ml buffer + 200µl BAPNA + (500µl Trypsin + 100µl CPTI)	0
2 ml buffer + 200µl BAPNA + (500µl Trypsin + 100µl SBTI)	0
2 ml buffer + 200µl BAPNA + 500µl larval protease	16.5×10^{-4}
2 ml buffer + 200µl BAPNA + 250µl larval protease	13.16×10^{-4} 13.23×10^{-4}

foot note: buffer = 0.2M Glycine/NaOH pH 9.0
[BAPNA] = 15.7 mg/ml dimethyl formamide
[SBTI] = 1 mg/ml H_2O
[CPTI] = 1 mg/ml H_2O
[larval protease] = 100 mg larvae/ml chilled H_2O

PART VII Feeding Trials using Supplemented Cowpea Meal Diets

The deletrious effect of cowpea trypsin inhibitor at the level found in TVu 2027 on the development of *C. maculatus* larvae, indicated by previous results, had to be confirmed *in vivo*. Thus larval feeding trials were used as a direct assay of the toxicity. Three different sets of feeding trials were set up, most based on the supplementation of the cowpea meal with various cowpea fractions.

The first set of feeding experiments were intended to show that supplementation of the diet with a 10 per cent cowpea albumin fraction, which contains the trypsin inhibitor, is toxic to the larvae; this was borne out in the results presented in Table 2.7.1, no adults being produced on this treatment. Unfortunately, however, very few eggs were laid on this treatment. Supplementation of the basic cowpea meal with a 10 per cent globulin fraction showed no significant difference to the control (100 per cent cowpea meal). Addition of a 10 per cent total cowpea extract (albumins and globulins) resulted in a lower emergence compared to the control, presumably due to the albumins. The addition of saponins was intended to demonstrate the vulnerability of the larvae to secondary

Table 2.7.1

Comparison of Callosobruchus maculatus Survival on six Different Treatments of Cowpea meal

Treatment	No. Beans Infested	Total No. Eggs Laid	No. Adults Emerged	% Adult Emergence
Cowpea meal + 5% saponin	22	29	0	0
Cowpea meal + 1% SBTI	9	11	6	55
Cowpea meal + 10% cowpea albumins	13	14	0	0
Cowpea meal + 10% cowpea globulin	24	33	25	76
Cowpea meal + 10% cowpea extract	6	7	4	57
100% cowpea meal CONTROL	26	30	24	80

foot note : SBTI = soyabean trypsin inhibitor
saponin: extract from gypsophila roots

compounds. The results clearly support this, since addition of 5 per cent saponins (from gypsophila root) gave 100 per cent toxicity. Addition of 1 per cent soyabean trypsin inhibitor, when compared to the control, indicated that this inhibitor is at least partially toxic to the larvae since there was a 25 per cent reduction in adult emergence. However, due to the low number of eggs laid on all treatments the results are of poor significance. A further set of trials were then set up to confirm these indications, and further to demonstrate directly the toxicity of the trypsin inhibitor, from the seeds of V. unguiculata, on larval development. The results of this second set of feeding trials is summarised in Table 2.7.2; ten replicates were carried out for each treatment. These results demonstrate that supplementation of the meal with a 10 per cent cowpea albumin fraction which had had trypsin inhibitory activity removed, by affinity chromatography, had no significant effect upon development contrasted to the effect before removal of the inhibitor. Addition of 0.5 per cent trypsin inhibitor had a marked effect upon larval development since only a few larvae were present and none had reached the pupal stage. The level of inhibitor added is greater than that found in susceptible cowpea varieties (approximately 0.2 to 0.3 per cent) but less than that found in the

Table 2.7.2.

COMPARISON OF CALLOSOBRUCHUS MACULATUS SURVIVAL ON FIVE DIFFERENT TREATMENTS OF COWPEA MEAL

addition	10% albumin minus trypsin inhibitor	5% albumin minus trypsin inhibitor	0.5% purified trypsin inhibitor	0.1% purified trypsin inhibitor	100 % autoclaved meal
eggs	163	63	139	110	73
larvae	18	6	28	17	16
pupae	104	45	0	71	41
total	122	51	28	88	57
% survival	74,9	81,0	20,1	80,0	78,1

resistant variety (≃0.9 per cent). Addition of 0.1 per cent trypsin inhibitor to the diet, as expected, had little effect.

Finally a third set of feeding trials were carried out to confirm the initial findings and also to carry out important controls. In all, thirteen different treatments were carried out, ten replicates for each treatment. (Percentage survival is based on the number of eggs laid on the artificial diet).

(i) Treatment	Larvae % survival	Pupae % survival	Total % survival
TVu 2027 meal	17.0	15.2	32.2
TVu 57 meal	4.4	64.1	68.5

These results show that the observed resistance to C. maculatus by the seeds of TVu 2027 is also maintained in the meal. This in itself suggests that this resistance is not of a physical nature.

(ii) Treatment	Larvae % survival	Pupae % survival	Total % survival
TVu 57 meal	4.4	64.1	68.5
TVu 57 meal + 10% albumins	24.8	8.0	32.8
TVu 57 meal + 10% albumin - CPTI	47.2	15.5	62.6

The greatly reduced survival on cowpea meal with 10 per cent albumins added, confirms the previous result that the cowpea albumin fraction is deleterious to growth

of C. maculatus, but as shown, the toxicity is removed by removal of the trypsin inhibitor since the survival on 10 per cent albumins minus inhibitor was nearly equal to the control.

(iii) Treatment	Larvae % survival	Pupae % survival	Total % survival
autoclaved TVu 2027 meal	34.6	0	34.6
autoclaved TVu 2027 meal + 0.25% CPTI	42.0	0	42.0
autoclaved TVu 2027 + 0.8% CPTI	0	0	0

From these results it is clear that when the purified inhibitor is added at 0.25 per cent it has no effect upon larval development. However, when added at 0.8 per cent it has a very dramatic effect since none of the larvae survived. (The basic meal was autoclaved for all 3 treatments to inactivate the inhibitor, but unfortunately it also appears to denature some necessary component of the diet, most probably vitamins).

(iv) Treatment	Larvae % survival	Pupae % survival	Total % survival
TVu 2027 meal	17.0	15.2	32.2
TVu 2027 ethanol extracted meal	27.8	3.7	31.5

90 per cent ethanol will extract compounds such as non protein amino acids, alkaloids and other small molecule

toxins, but was shown not to extract the trypsin inhibitors. The results from the above set of treatments demonstrate that there is no significant difference between percentage total survival on the resistant meal and the ethanol extracted resistant meal. Consequently it rules out the possibility of resistance being ascribed to such toxins. This conclusion is also indicated from the results obtained in the following treatments whereby adding back the ethanol extractant at over twice the concentration had little effect.

(v) Treatment	Larvae % survival	Pupae % survival	Total % survival
autoclaved TVu 2027 ethanol extracted meal	23.1	0	23.1
autoclaved TVu 2027 ethanol extracted meal + X2 ethanol extractant	15.0	0	15.0

(vi) Treatment	Larvae % survival	Pupae % survival	Total % survival
autoclaved TVu 2027 meal	34.6	0	34.6
autoclaved TVu 2027 meal + 0.8% CPTI	0	0	0
autoclaved TVu 2027 meal + autoclaved 0.8% CPTI	8.1	9.5	17.6

These results show that autoclaving the trypsin inhibitor, which was shown to partially inactivate it,

removes much of the growth depressing activity.

A final control was carried out whereby pepsin digested inhibitor was added at a level of 0.8 per cent to the basic diet. This had no effect upon larval development. The pepsin digestion destroyed approximately 90 per cent of the trypsin inhibitor's activity. Thus the inhibitor is antimetabolic through its functional properties rather than its chemical composition.

(vi)	*Treatment	Total % Survival
	TVu 1502-1D meal	82.0
	TVu 1502-1D meal + 0.8% pepsin digested CPTI	84.5
	(*TVu 1502-1D meal was used as it has the lowest trypsin inhibitory activity out of the varieties screened for in this work).	

All the conclusions drawn from the feeding trials were shown to be statistically significant to the 5 per cent level or better using one way analysis of variance and Duncan's multiple range test.

PART VIII Characterisation of the Trypsin Inhibitor from Vigna unguiculata

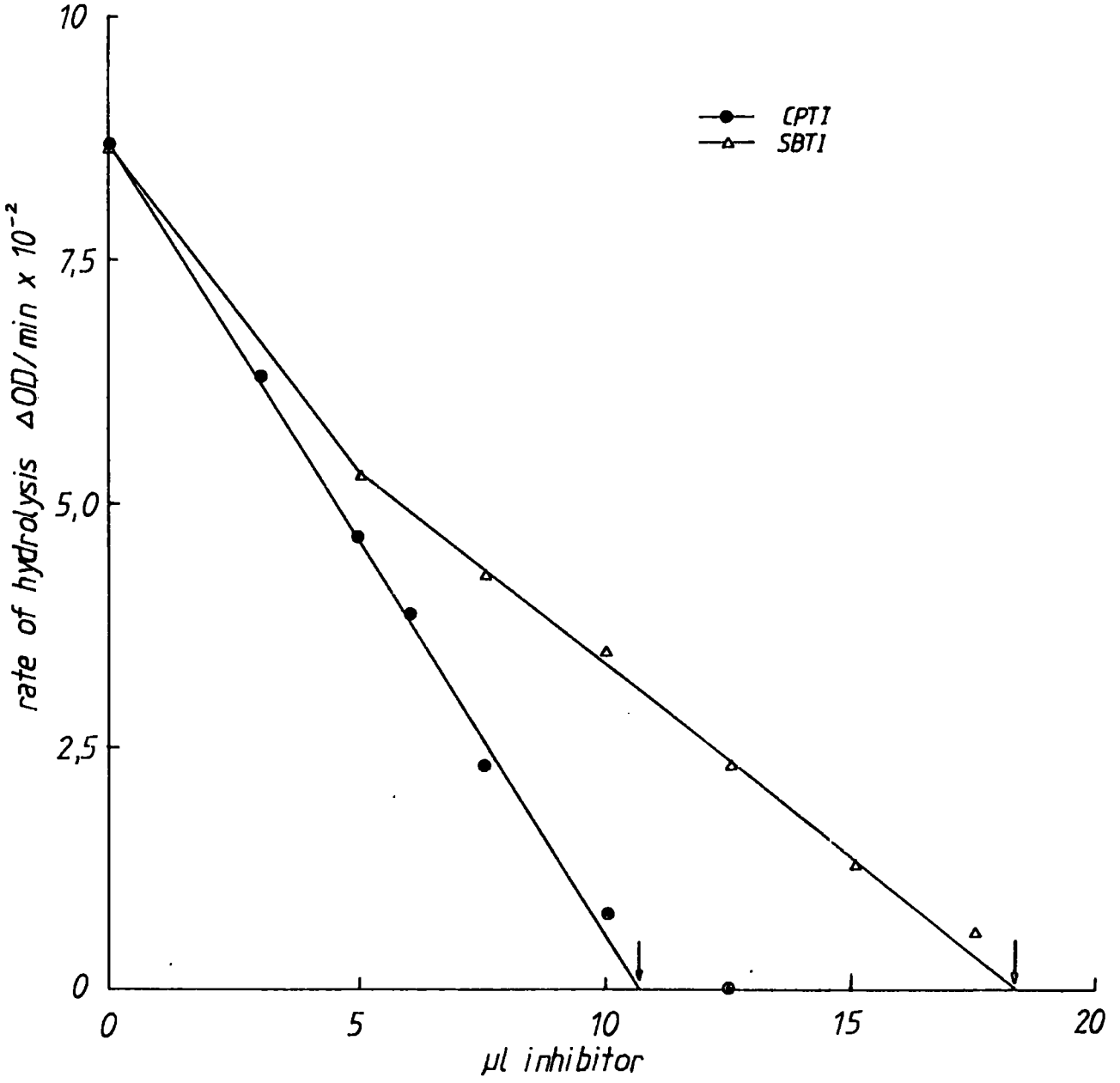
A. Determination of the Equivalence of Cowpea Trypsin Inhibitor to Trypsin

The combining weight of the cowpea trypsin inhibitor (CPTI) was determined by measuring the inhibition of the hydrolysis of BAPNA by a known amount of trypsin in the presence of varying amounts of purified inhibitor solution of known concentration. The rate of hydrolysis was plotted against the amount of inhibitor added (Figure 2.8.1). Extrapolation of the linear plot gave the amount of inhibitor required for the complete inhibition of hydrolysis, that is, the amount of inhibitor equivalent to the amount of trypsin used.

Since the combining weight of soyabean trypsin inhibitor is known, a similar experiment was carried out to check the activity of the trypsin (Figure 2.8.1).

The resulting data gave an equivalence of 0.40 mg of inhibitor to 1 mg of trypsin and hence a combining weight of 9400 for the cowpea trypsin inhibitor (calculation in Figure 2.8.1).

Figure 2.8.1. Inhibition of trypsin by cowpea trypsin inhibitor and soyabean trypsin inhibitor.
Assay: trypsin-catalysed hydrolysis of BAPNA, followed spectrophotometrically at 410 nm.



10.65μl CPTI (1.02 mg/ml) ≡ 500μl trypsin ≡ 18.3μl SBTI (1.02 mg/ml)

i.e. 0.206μg CPTI ≡ 0.352μg SBTI

but 1mg SBTI ≡ 1,5mg trypsin

thus $\frac{0.206}{0.352} \times \frac{23300}{1.5}$ μg CPTI ≡ 1 μmole trypsin

i.e. 9400μg CPTI ≡ 1 μmole trypsin

B. Determination of the Molecular Weight of the Cowpea Trypsin Inhibitor by Gel Filtration

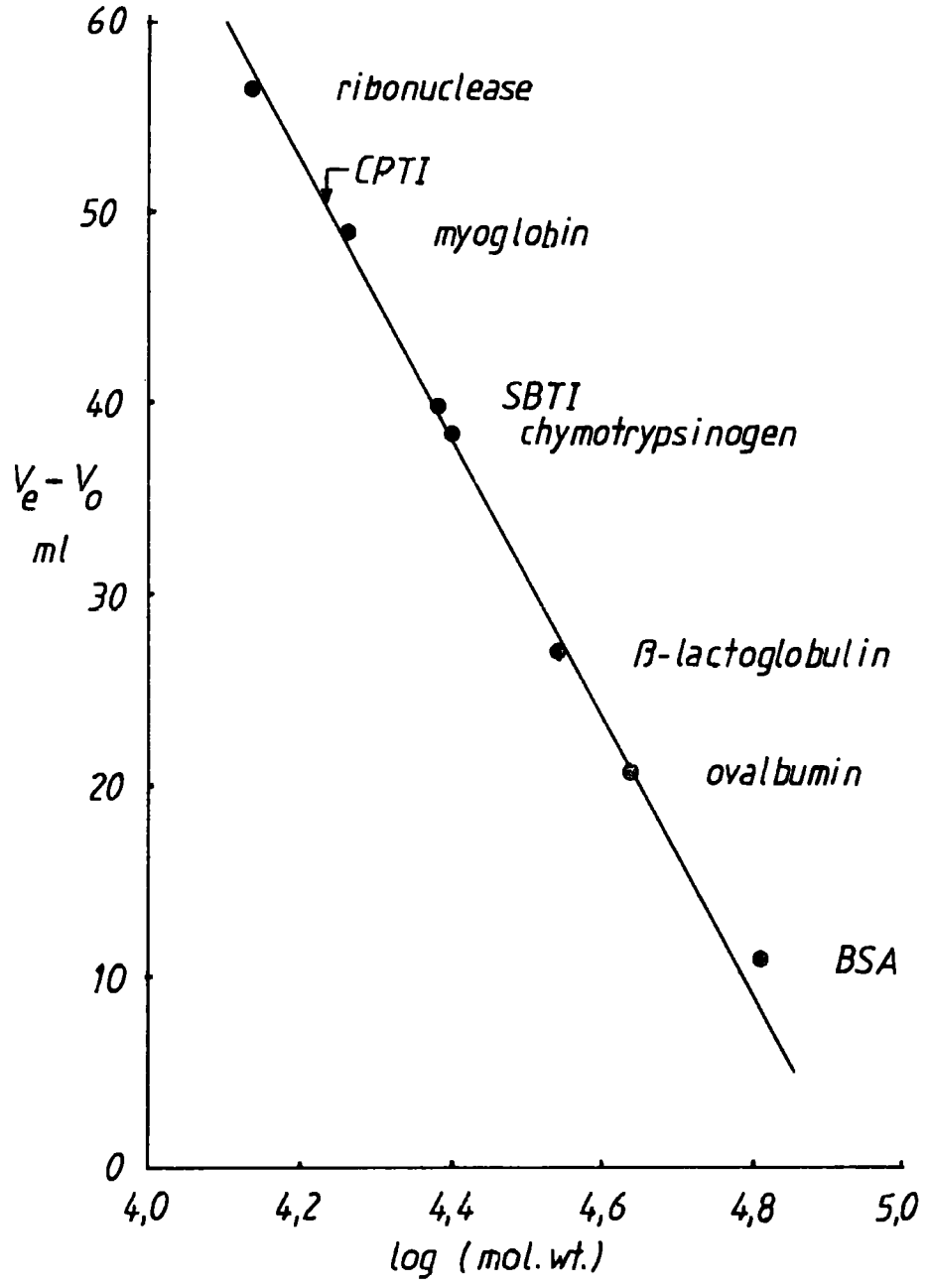
The molecular weight of the trypsin inhibitor was determined by gel filtration on a column of Sephadex G-75. The column was calibrated with blue dextran (so as to determine the void volume) and the following standard proteins: ribonuclease (13,700 mol. wt.), whale myoglobin (18,400 mol. wt.), soyabean trypsin inhibitor (24,000 mol. wt.), chmotrypsinogen (25,100 mol. wt.), β-lactoglobulin (35,000 mol. wt.), ovalbumin (43,500 mol. wt.) and bovine serum albumin (67,000 mol. wt.). A plot of the elution volume minus the void volume, against the log of the molecular weight was made; a linear calibration was obtained (Figure 2.8.2).

The cowpea trypsin inhibitor chromatographed as a single peak on gel filtration. However, this peak was slightly broader than those for the standard proteins indicating a small degree of heterogeneity in molecular weight. The mean molecular weight (3 determinations) was calculated from the calibration as 17,100 ± 800.

C. Determination of Complex Formation between Cowpea Trypsin Inhibitor and Trypsin by Gel Filtration

Gel filtration on a Sephadex G-75 column of mixtures of cowpea trypsin inhibitor and trypsin gave peaks that

Figure 2.8.2. Calibration of Sephadex G-75 column for determination of molecular weight of CPTI



could not be due to either molecule alone and thus must represent complexes of the two. Elution profiles for the various mixtures are given in Figure 2.8.3, together with the elution profiles for the pure inhibitor and trypsin. Since trypsin alone, or trypsin in excess to that complexed, was found to digest itself almost completely during passage through the column, trypsin inactivated with phenylmethyl sulphonyl fluoride was used to give an elution profile for intact trypsin molecules.

The complex species present are at indicated molecular weights of 33,500 and 51,000. The most reasonable explanation for this behaviour is that the former complex represents the addition of one molecule of trypsin to one molecule of inhibitor, the latter the addition of two molecules of trypsin to one molecule of inhibitor, since the combining weight of the inhibitor is about half its molecular weight and therefore one molecule of inhibitor should be able to inhibit two molecules of trypsin. (The molecular weights would not necessarily be expected to be directly additive). If this explanation is correct it indicates that both these complexes are stable enough that they do not dissociate significantly on the time scale of gel filtration (approximately 10 hrs).

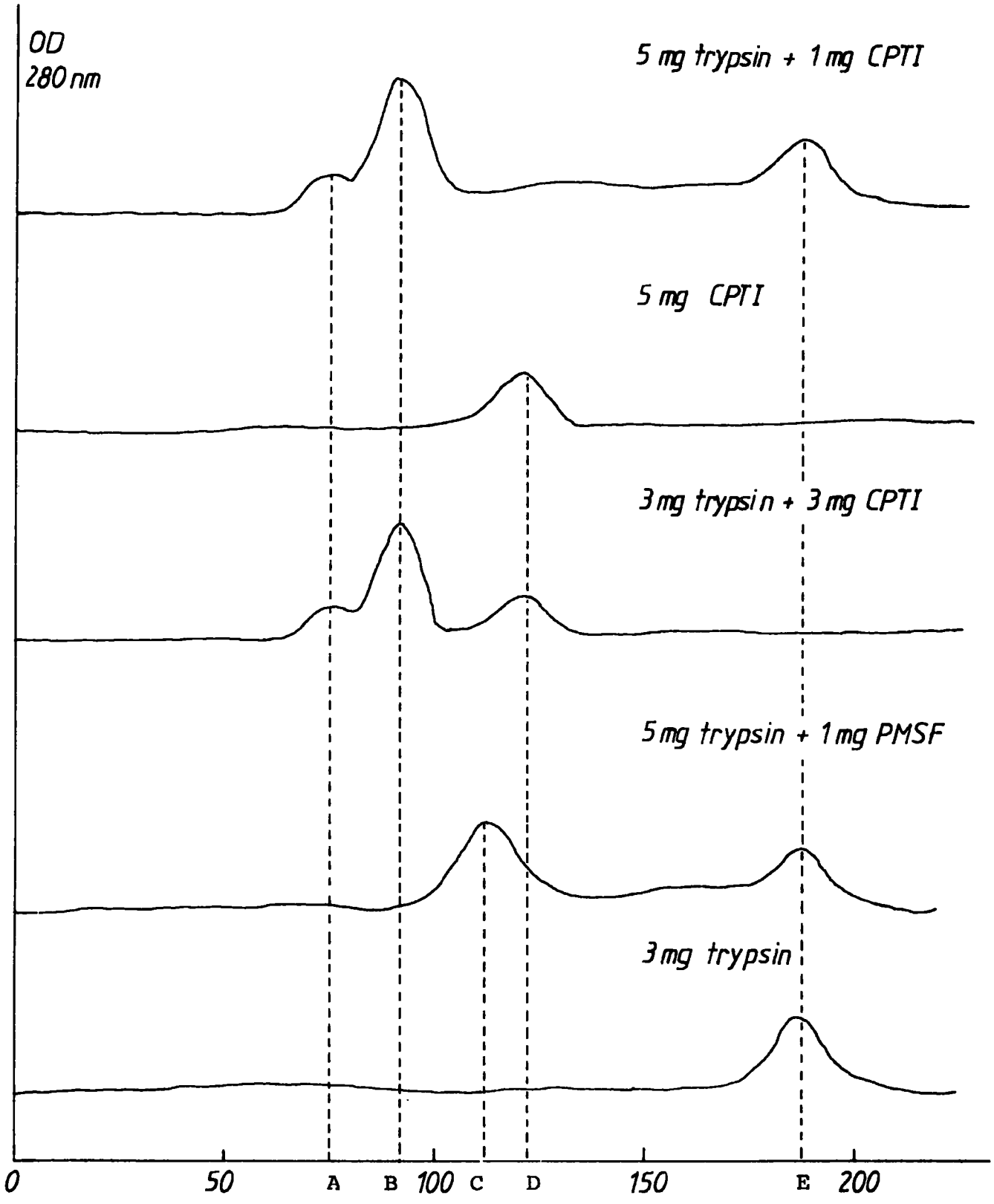


Figure 2.8.3. Chromatography of trypsin - CPTI complexes on Sephadex G-75.

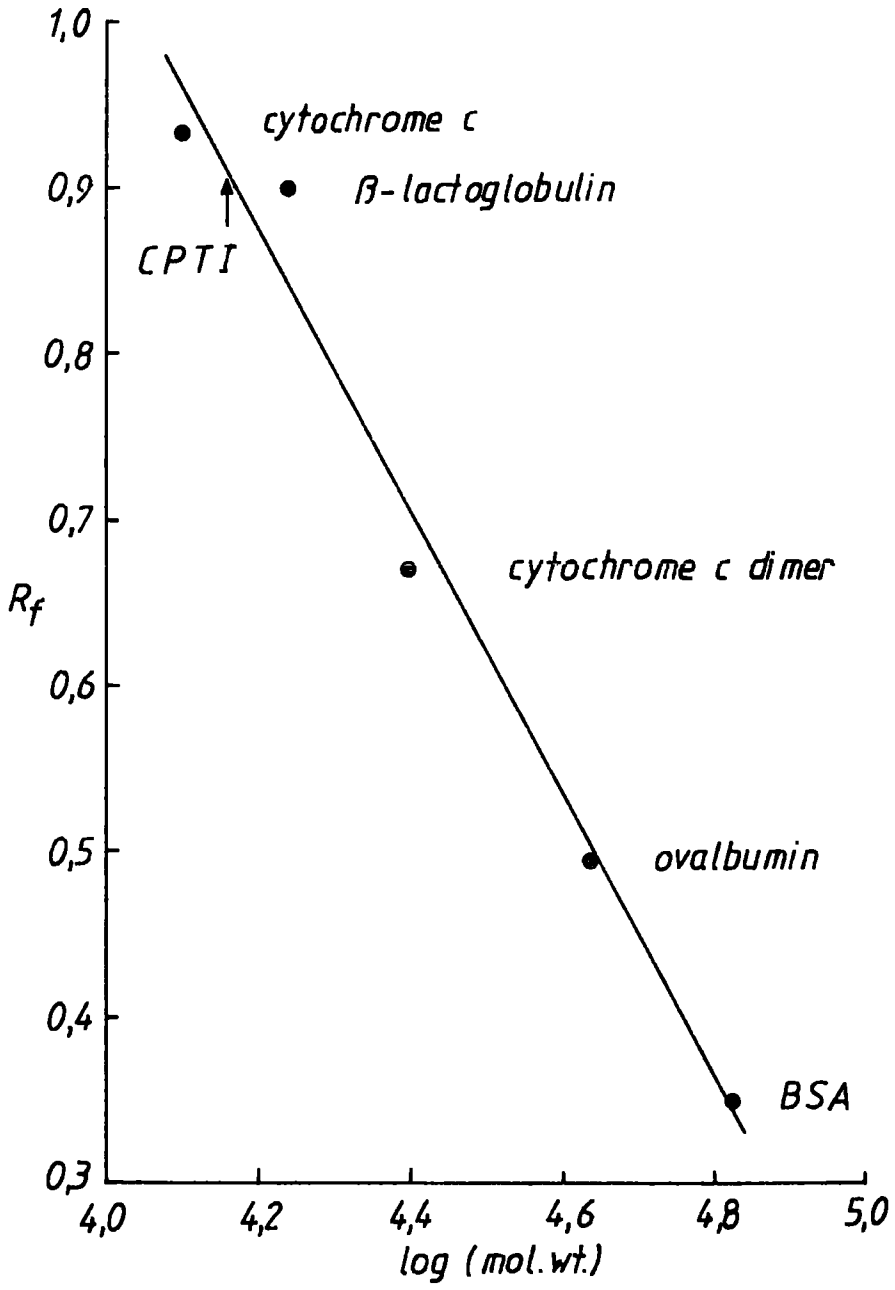
A = Trypsin - CPTI complex I
B = Trypsin - CPTI complex II
C = Trypsin
D = CPTI
E = Autolysed trypsin
PMSF = Phenylmethylsulphonyl fluoride

D. SDS-Gel Electrophoresis of the Cowpea Trypsin Inhibitor

SDS gel electrophoresis of the cowpea trypsin inhibitor was performed in a variety of acrylamide gels, using both a continuous buffer system and a discontinuous buffer system. Electrophoresis of samples not treated with 2-mercaptoethanol gave a pattern consisting of a major band, and two minor bands at higher molecular weight, as shown in Figure 2.8.5. The major band was determined to represent a molecular weight of 13,000 ± 500 (as a mean of six determinations) by electrophoresis on 10 per cent acrylamide gels in a continuous buffer system. The relative mobility to bromophenol blue was measured, and the gel system calibrated with the following standard proteins: cytochrome c monomer (12,400 mol. wt.), β-lactoglobulin (17,500 mol. wt.), cytochrome c dimer (24,800 mol. wt.), ovalbumin (43,500 mol. wt.) and bovine serum albumin (67,000 mol. wt.); these gave a linear relationship of relative mobility to log of the molecular weight (Figure 2.8.4). The minor bands had estimated molecular weights of 16,000 and 18,000.

In the presence of 2-mercaptoethanol the band at 13,000 mol. wt. is unchanged; but under these conditions the minor

Figure 2.8.4. Calibration of molecular weights by SDS-polyacrylamide gel electrophoresis. For conditions, see text.



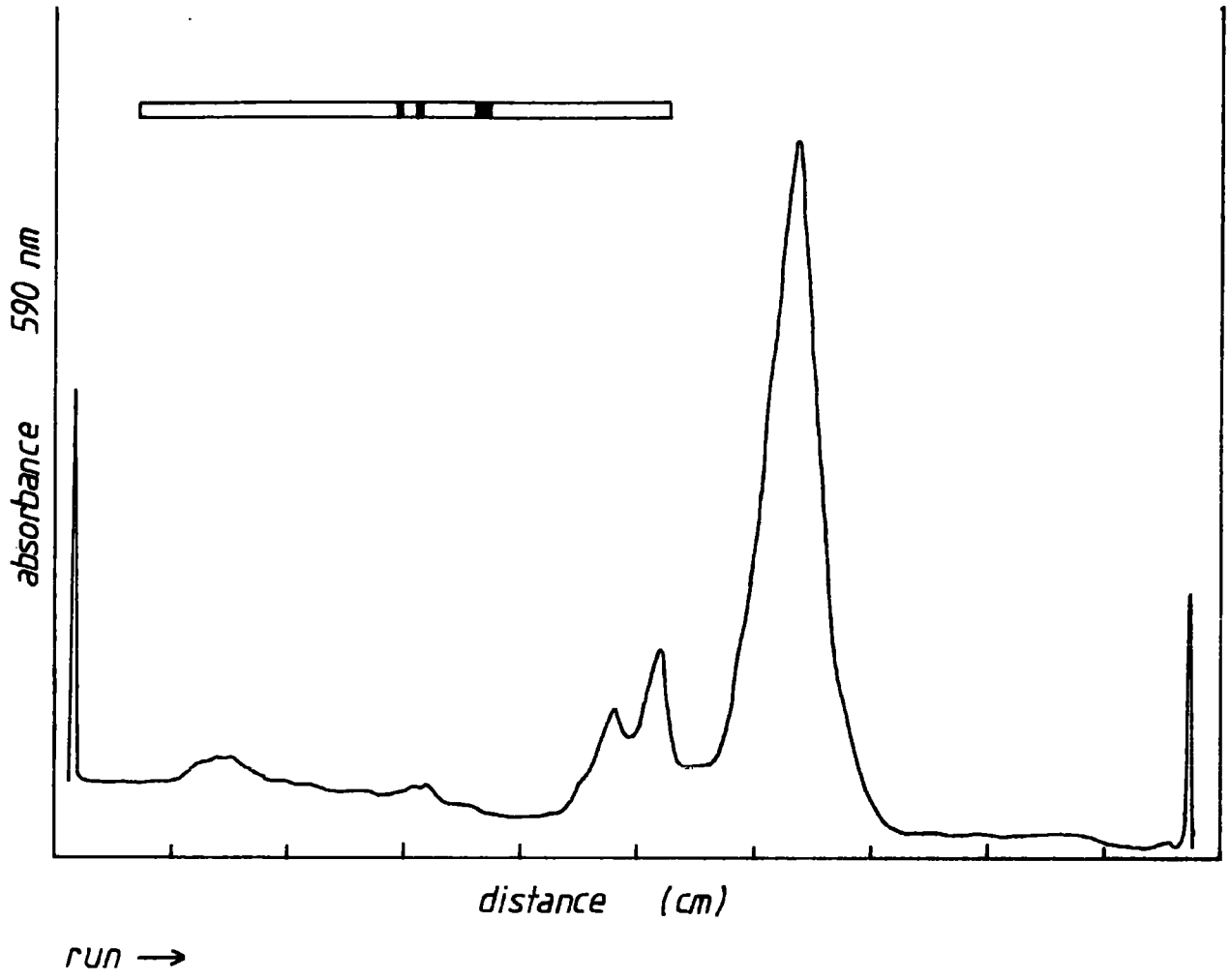


Figure 2.8.5. SDS-polyacrylamide gel electrophoresis of CPTI, with no β-mercaptoethanol present. Densitometric scan of 12% acrylamide gel after staining with Coomassie Blue.

bands at higher molecular weight are very much lessened, and indistinct bands at lower molecular weight (about 8,000) appear (Figure 2.8.6). Carboxymethylation of the protein after reduction with 2-mercaptoethanol removes the higher molecular weight bands.

The behavious of the cowpea trypsin inhibitor on SDS electrophoresis is not easily understood. A tentative explanation for the observations will be found in the Discussion section. Inhibitors purified from all the varieties tested gave essentially identical results on SDS gel electrophoresis.

E. Investigation of possible Differences between the Trypsin Inhibitors Purified from different Varieties of Vigna unguiculata

(i) Non-SDS Gel electrophoresis was performed at pH 8.9 in 7 per cent acrylamide gels on the trypsin inhibitor from several varieties of cowpea including TVu 2027. Although the resulting band patterns were complex they were all similar. This indicates that the difference in trypsin inhibitory activity between the varieties is quantitative rather than qualitative. Band patterns and densitometric scans are shown in Figure 2.8.7.

(ii) Isoelectric focussing was carried out on the trypsin inhibitor from seven varieties of V. unguiculata. This was

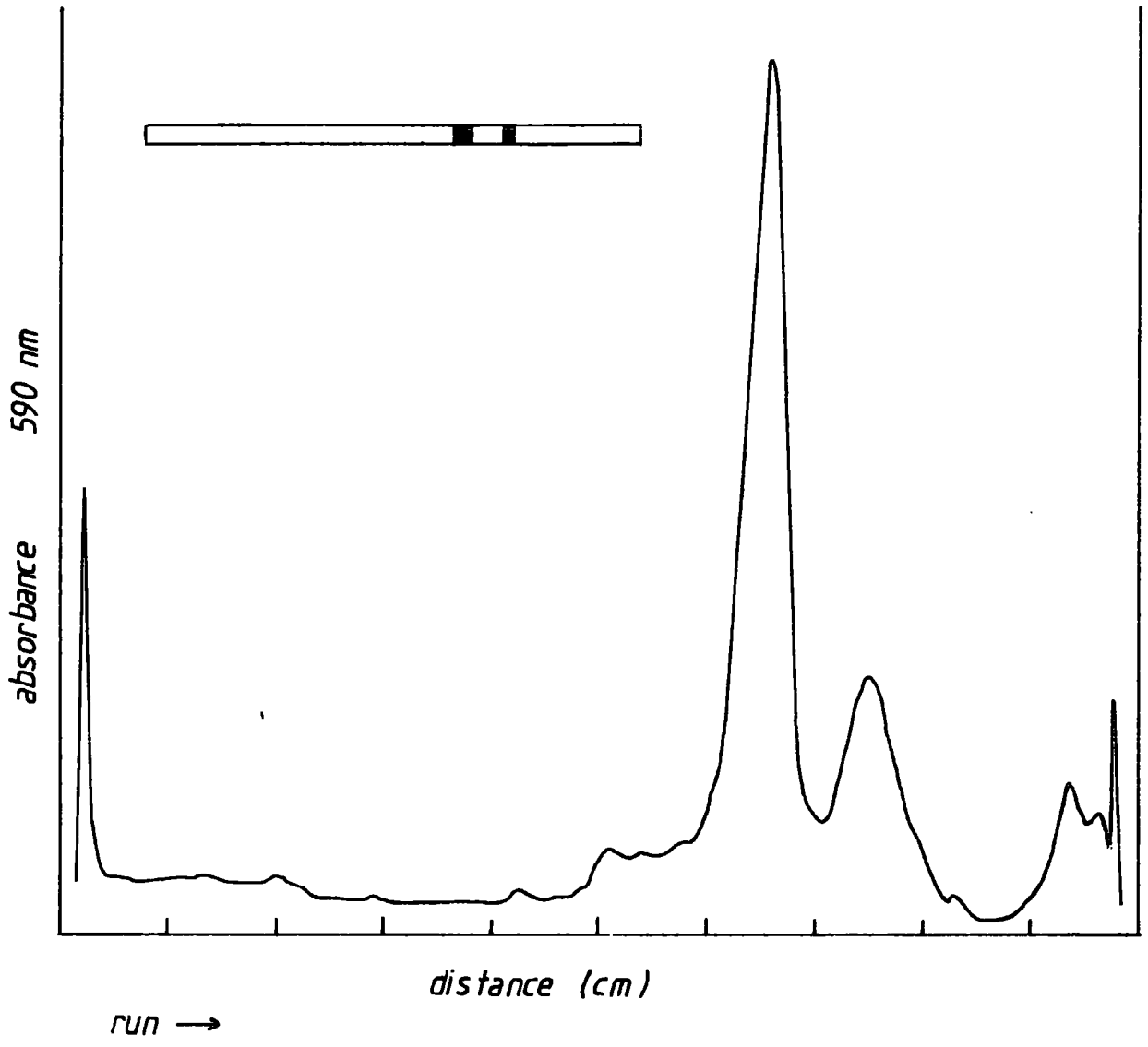


Figure 2.8.6. SDS-polyacrylamide gel electrophoresis of CPTI fully reduced with β-mercaptoethanol. Densitometric scan of 12% acrylamide gel after staining with Coomassie Blue.

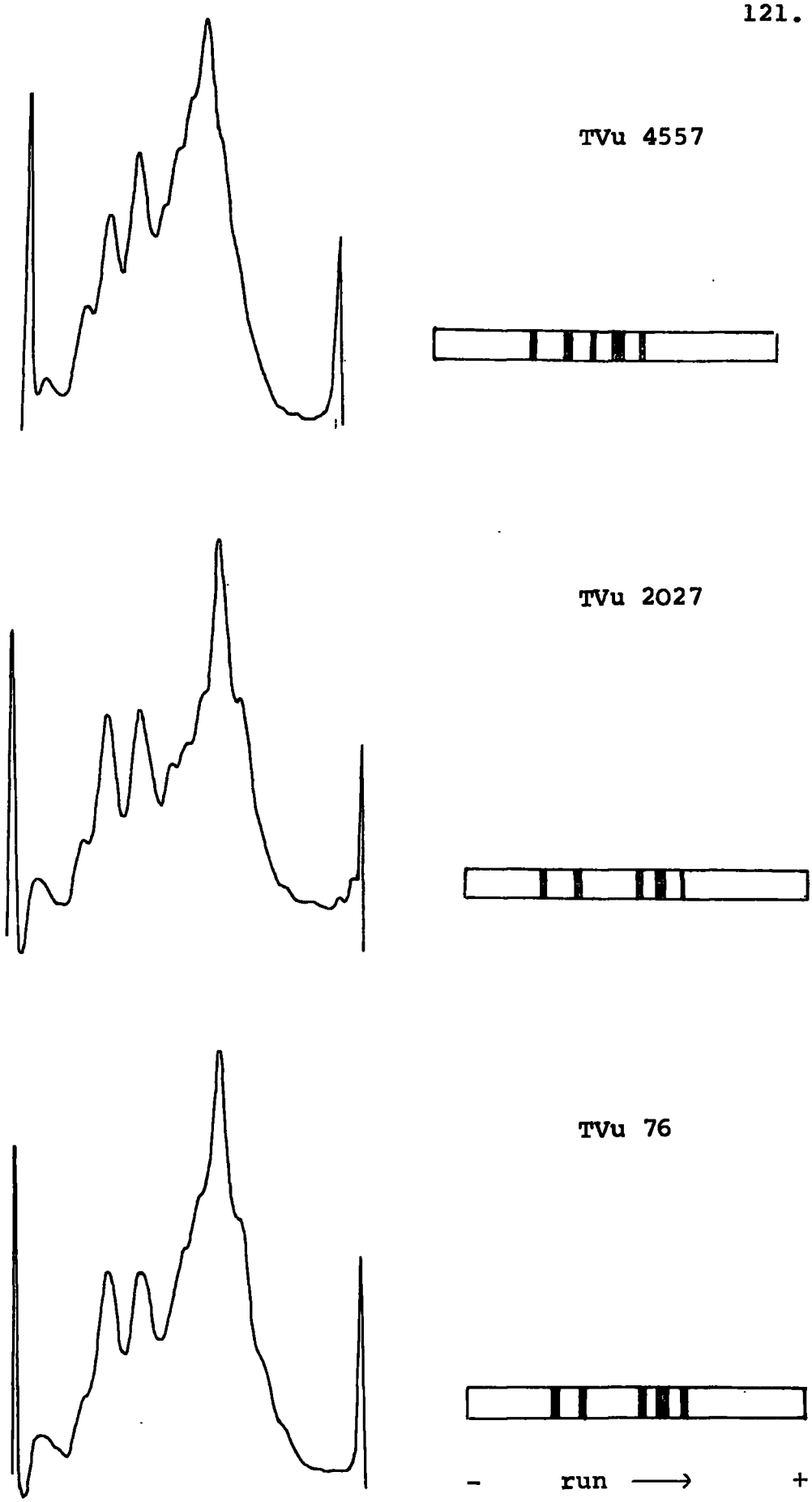


Figure 2.8.7. Polyacrylamide gel electrophoresis at pH 8.3 of the trypsin inhibitors from three varieties of cowpea. Densitometric scans of gels stained with Coomassie Blue.

performed in 5 per cent acrylamide slab gels in the pH ranges 3.5 to 10, and 3 to 7, using the following proteins as standards: ferritin (pI 4.4), albumin (pI 4.7), β-lactoglobulin (pI 5.34), conalbumin (pI 5.9), horse myoglobin (pI 7.3), whale myoglobin (pI 8.3), ribonuclease (pI 9.45), and cytochrome c (pI 10.65); haemoglobin was used as a marker. Since the inhibitors bind Coomassie Blue protein stain only weakly, and the staining is fugitive, it was necessary to copy the band pattern directly from the gel by tracing. A typical isoelectric banding pattern is shown in Figure 2.8.8.

The band patterns of the inhibitors tested were all qualitatively the same, and although a complex pattern was given, essentially all the inhibitor focussed in the pH range 4-5, as indicated by the standard proteins run on the same slab.

From these experiments, and the gel electrophoresis experiments, it was concluded that all the varieties, including TVu 2027, contain the same isoinhibitors, although their relative proportions may differ between varieties.

F. Ion-Exchange Chromatography of the Trypsin Inhibitor from Vigna unguiculata, Varieties TVu 76 and TVu 2027

The trypsin inhibitor from V. unguiculata, previously purified by trypsin affinity chromatography, was separated

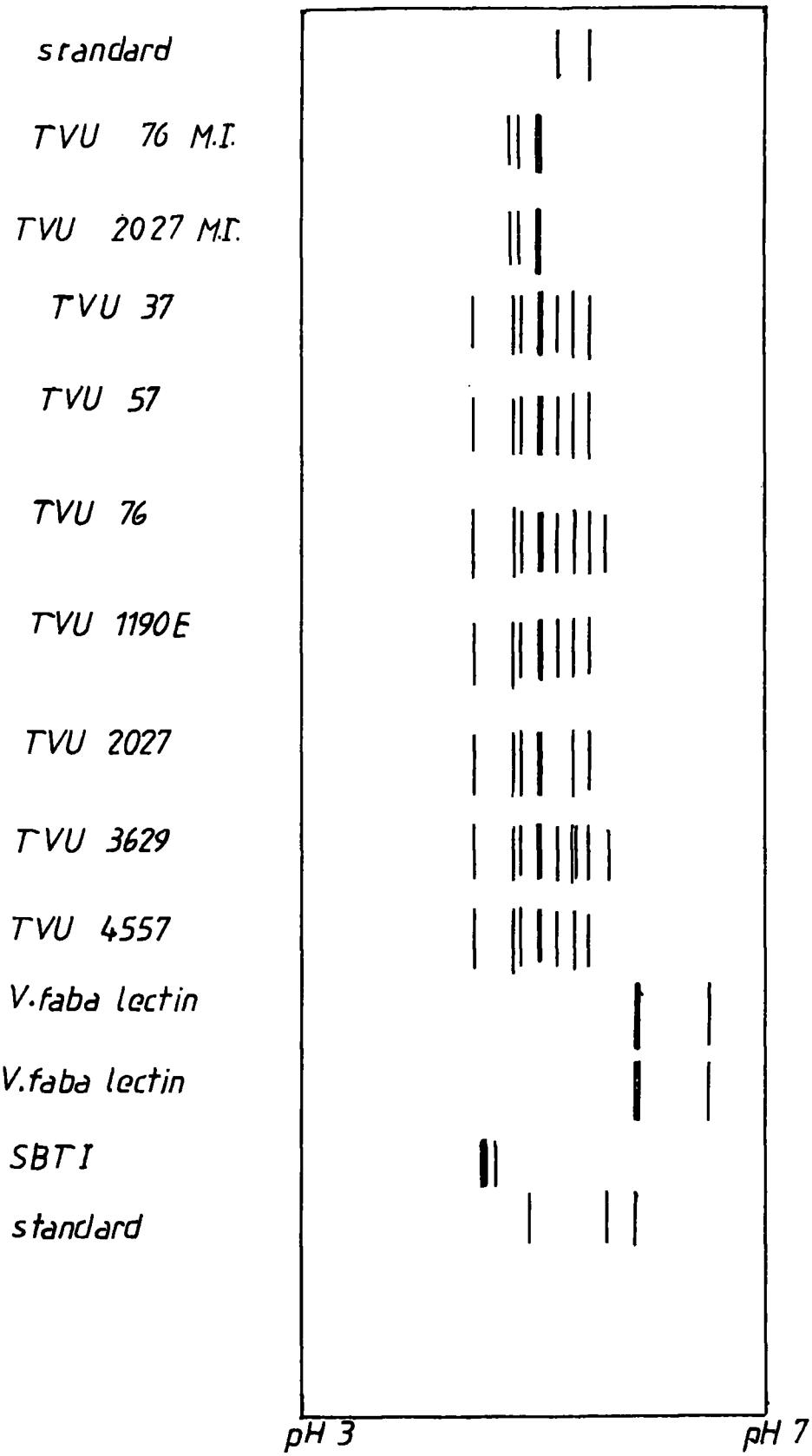


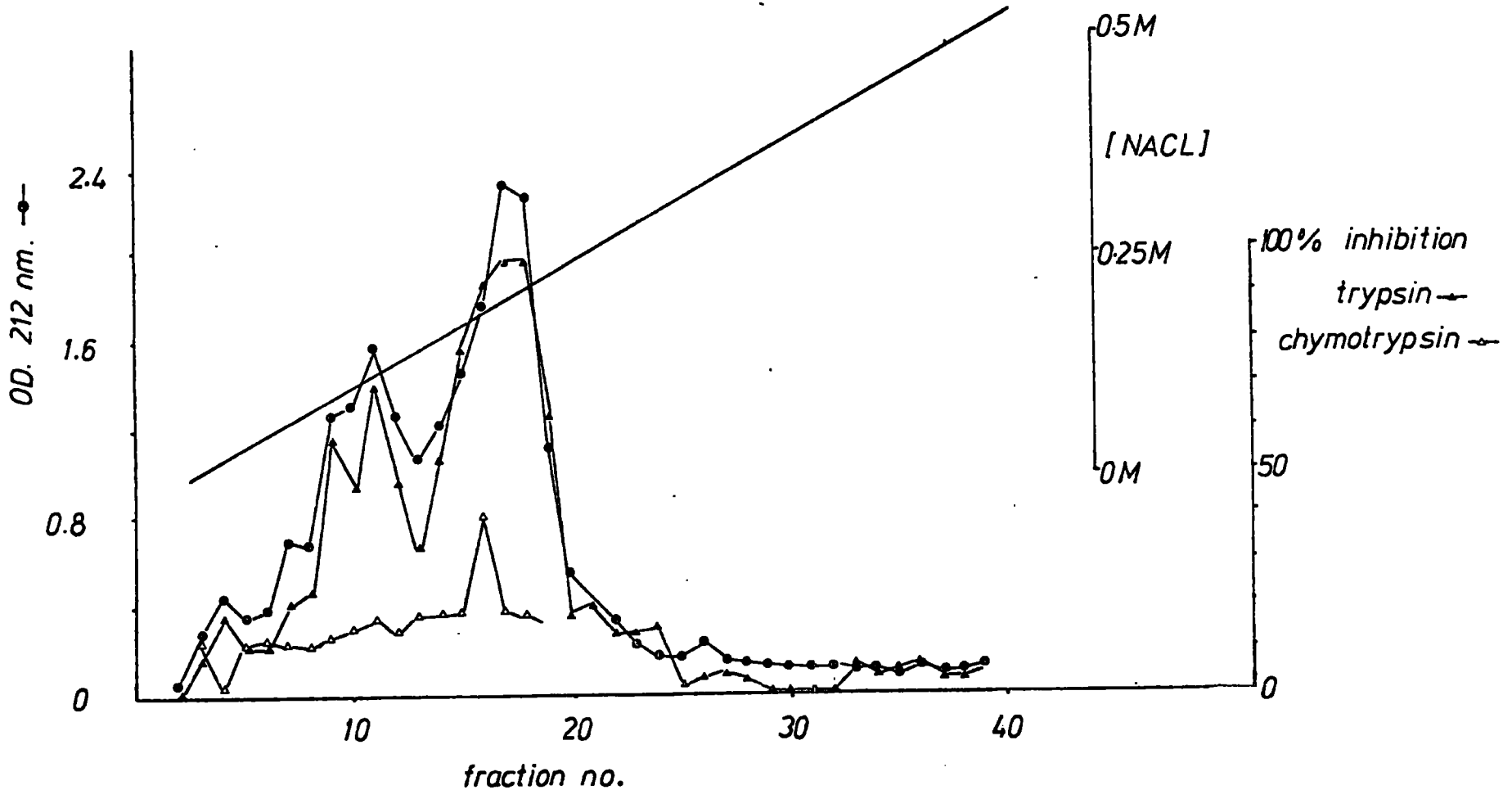
Figure 2.8.8. Isoelectric focussing of cowpea trypsin inhibitors from different varieties on a slab gel.

M.I. = Major Isoinhibitor

into the constituent isoinhibitors, indicated by isoelectric focussing and gel electrophoresis, by ion-exchange chromatography on a column of DEAE-cellulose 52 at pH 8.0. The inhibitors were applied in 20 mM buffer and eluted with a linear sodium chloride gradient (0-0.5M). The eluted fractions were assayed for both trypsin and chymotrypsin inhibitory activity using the substrates BAPNA and BTEE, respectively. This procedure was carried out for a susceptible variety of cowpea, TVu 76, and a resistant variety, TVu 2027, and the two compared.

The elution profile for the trypsin inhibitor from TVu 2027 is shown in Figure 2.8.9. Since the inhibitor has a low absorbance at 280 nm only the OD at 212 nm was measured. Inhibitor from TVu 76 gave a similar result, with no qualitative differences although slight quantitative differences in the relative heights of the peaks were evident. Each variety shows one major peak of protein with inhibitory activity against trypsin and to a lesser degree chymotrypsin, and several smaller peaks, possessing only trypsin inhibitory activity, which eluted before it. The major peak of inhibitor from the two purifications was pooled and lyophilised. Analysis by isoelectric focussing showed it to be still heterogeneous (Figure 2.8.8) but to a much lesser extent than the starting material.

Figure 2.8.9. ION EXCHANGE CHROMATOGRAPHY OF PURIFIED COWPEA TVU 2027 TRYPSIN INHIBITOR



G. Amino Acid Composition of the Major Isoinhibitor from two Varieties of Vigna unquiculata: TVu 76 and TVu 2027: Determination of the Minimum Molecular Weight

The amino acid compositions of the major isoinhibitors from two varieties of V. unquiculata, TVu 76 and the resistant variety TVu 2027, were determined using a Locarte automatic loading amino acid analyser. The two sulphur amino acids, cystine and methionine, were determined after performic acid oxidation using asparagine as a standard.

The results are shown in Table 2.8.10 and are presented as the number of amino acid residues per molecule of inhibitor; the number of residues per molecule being expressed as an integer, the lowest value being taken as one. As can be seen, the amino acid composition of the major isoinhibitor from the two varieties are virtually identical except that there appears to be less proline in TVu 2027; this may be due to experimental error as this amino acid is difficult to estimate.

Using these integral values for the number of amino acid residues per molecule, the minimum molecular weight of the cowpea trypsin and chymotrypsin inhibitor can be calculated as 9,030 and 8,640 for TVu 76 and TVu 2027, respectively, or a mean of 8,840. This value is approximately half the molecular weight determined by gel filtration.

Table 2.8.10

Amino Acid Composition of the Major Isoinhibitor of Cowpea TVu 76 and Cowpea TVu 2027

Amino acid	TVu 76 Residues per molecule	TVu 2027 Residues per molecule
Lysine	5	5
Histidine	3	3
Arginine	4	3
Cystine	13	14
Aspartic acid	11	11
Threonine	3	3
Serine	11	11
Glutamic acid	6	6
Proline	6	(1-2)
Glycine	2	2
Alanine	3	2
Valine	6	7
Methionine	1	1
Isoleucine	3	3
Leucine	2	2
Tyrosine	1	1
Phenylalanine	1	1
Minimum Molecular Weight	9,030	8,640

NB Residues per molecule are expressed as an integer.

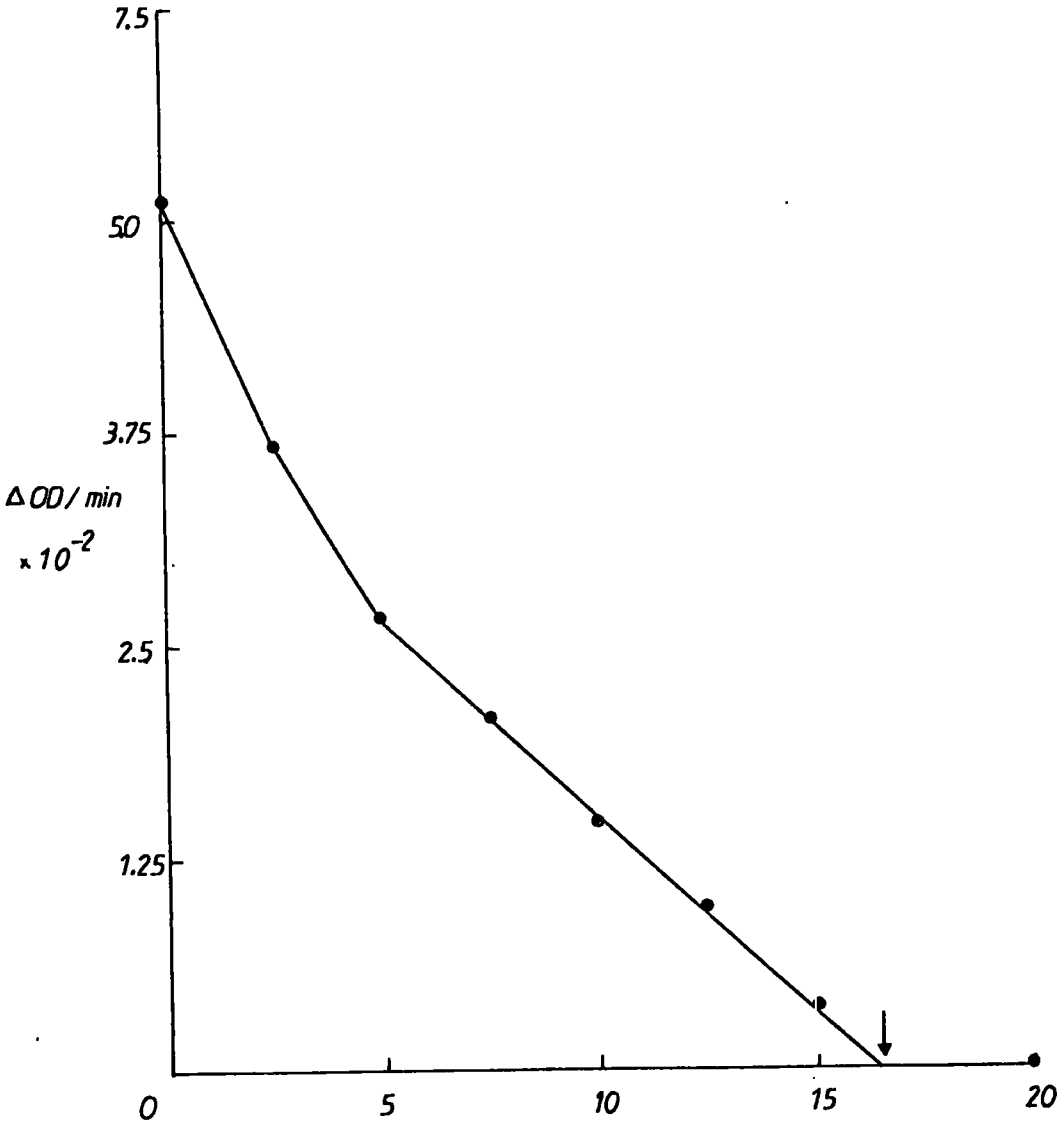
H. Determination of Chymotrypsin Inhibitory Activity of Cowpea Inhibitor (CPTI). Purification of CPTI by Removal of the Chymotrypsin Inhibitory Activity

The cowpea trypsin inhibitor purified by affinity chromatography (CPTI) was found to inhibit chymotrypsin hydrolysis of BTEE; virtually complete inhibition could be achieved (Figure 2.8.11). By using known quantities of inhibitor and enzyme, the equivalence was shown to be 4.2 mg CPTI to 1 mg chymotrypsin. Thus the chymotrypsin inhibitory activity of the cowpea inhibitor is approximately one-tenth that of the trypsin inhibitory activity; this is about the same ratio as found in the seed, indicating that all the chymotrypsin inhibitory activity can be accounted for by the trypsin inhibitors.

The chymotrypsin inhibitory activity could be removed from the trypsin inhibitor by passage through a column of chymotrypsin linked to Sepharose at pH 4.0. Material that did not bind was shown by assay to be totally devoid of inhibitory activity against chymotrypsin, but still inhibited trypsin. Unfortunately, the protein which bound to the chymotrypsin of the affinity column could not be recovered.

When the purified trypsin inhibitor, from which the chymotrypsin inhibitor had been removed, was run on SDS-gel

Figure 2.8.11. Inhibition of chymotrypsin by cowpea trypsin inhibitor.
Assay: chymotrypsin-catalysed hydrolysis of BTEE, followed spectrophotometrically at 410 nm.



16.5µl inhibitor (1.02 mg/ml) = 4µg chymotrypsin

i.e. 4.2µg inhibitor = 1µg chymotrypsin

electrophoresis, the higher molecular weight band of the two minor bands was not present (Figure 2.8.12). This band then, presumably, represents an isoinhibitor that will bind to both chymotrypsin and trypsin. Further experiments are in progress to clarify these results.

I. Gel filtration in 6M Guanidine Hydrochloride

The subunit structure of cowpea trypsin inhibitor was investigated by gel filtration in 6M guanidine hydrochloride on a column of Sephacryl S-200 superfine. The elution profile of fully reduced and carboxymethylated CPTI is shown in Figure 2.8.13. Three peaks are given. On the basis of the chromatographic behaviour of standard proteins (Legumin, vicilin, IgG, SBTI, ribonuclease, and insulin) under similar conditions the peaks could be assigned molecular weights of 8,000, 5,000 and 3,000 although there is some uncertainty (less than 20 per cent) in these values as the calibration is not very good. No peak, or trace of a peak, was seen at elution volumes corresponding to molecular weights of 16-18,000 or 13,000.

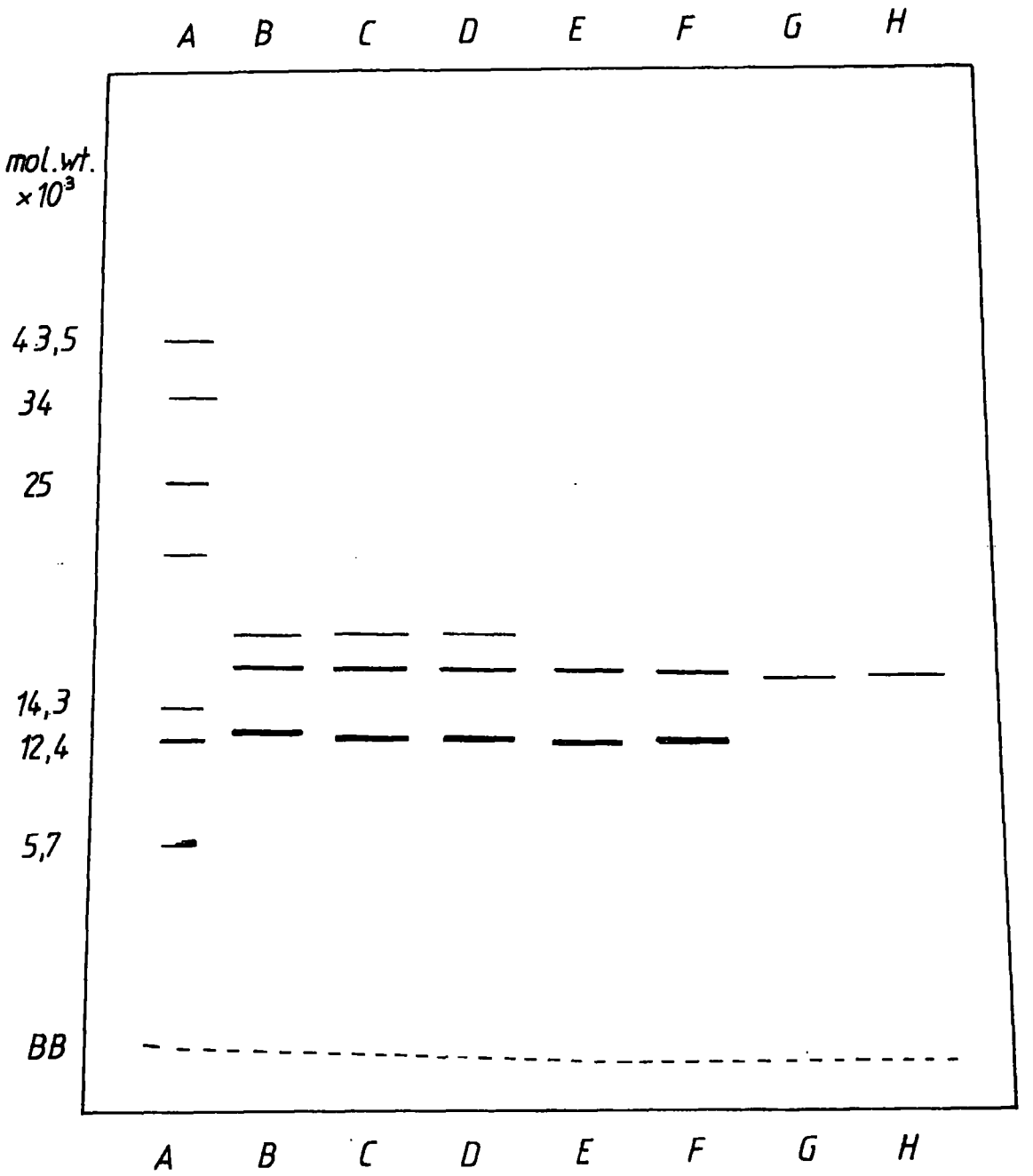


Figure 2.8.12. SDS gel electrophoresis; slab gel containing 7.5% (top) - 26% (bottom) concentration gradient of acrylamide.

A = Molecular weight markers (ovalbumin, carboxypeptidase A, lysozyme, cytochrome c, insulin).

B,C,D = Cowpea trypsin inhibitor, no β-mercaptoethanol.

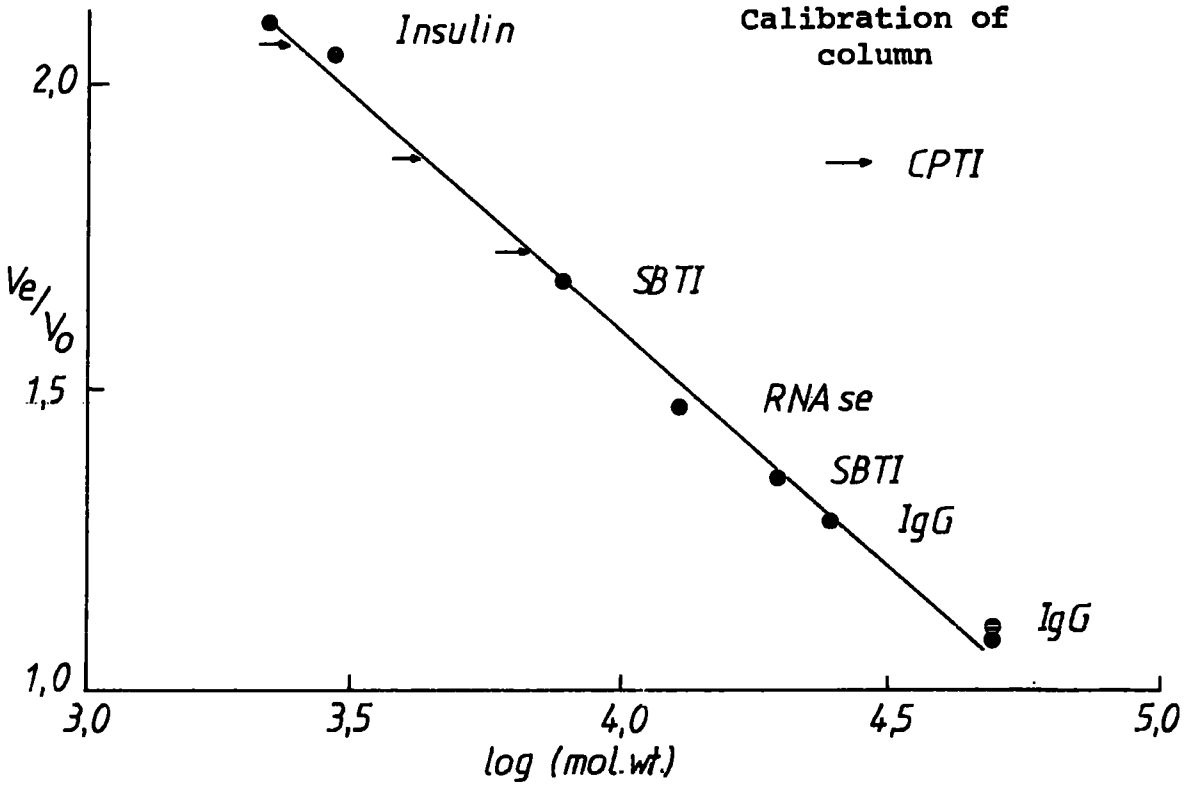
E,F = Cowpea trypsin inhibitor minus chymotrypsin inhibitor, no β-mercaptoethanol.

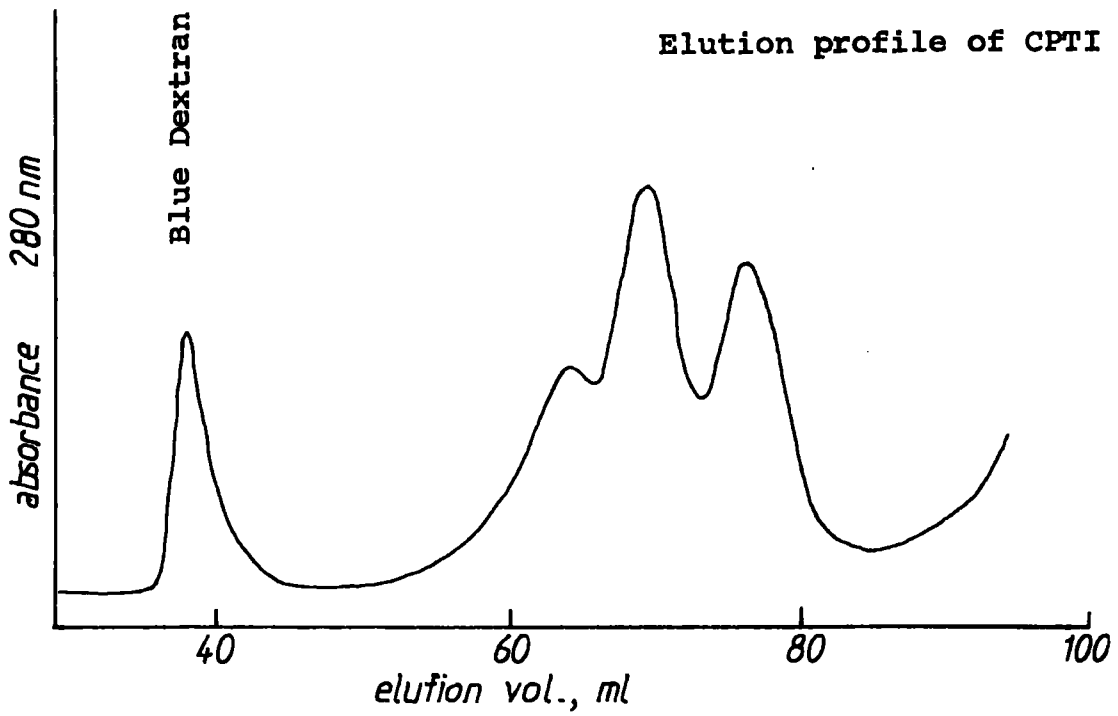
G,H = Soyabean trypsin inhibitor, Bowman-Birk type. No β-mercaptoethanol.

BB = Bromophenol blue marker dye.

(Gel prepared by Dr. R. Croy)

Figure 2.8.13. Chromatography on Sephacryl S-200 superfine of fully reduced, carboxymethylated CPTI in 6M guanidine hydrochloride.





SECTION III DISCUSSION

PART I

Resistance of TVu 2027

Results from screening trials carried out at IITA, and confirmed in this thesis, have clearly demonstrated the resistance of TVu 2027 to *Callosobruchus maculatus* infestation; no other varieties of *Vigna unguiculata* resistant to this storage pest have been found. This in intself is a considerable disadvantage to formulating a general principal of resistance since the basis of resistance found for TVu 2027 may be unique to this variety. It is conceivable that other possible resistant varieties of cowpea would have different bases of resistance. However this possibility does not invalidate or detract from the results of this thesis which are specific for this particular variety, although it would be very desirable to find another variety resistant to *C. maculatus* in order to ascertain whether or not the results from TVu 2027 could be extended. The IITA cowpea variety collection on which the preliminary screening trials were conducted comprises the existing world germplasm collection. However, since a large proportion of these IITA varieties are cultivars rather than true "wild" varieties the variability of the IITA collection may not be representative of the variability in nature. Thus there is

a possibility of further resistant varieties being found. Indeed, TVu 2027 itself is not an IITA cultivar but was found in the "wild".

It has been shown (Section II, Part I) that the resistance of TVu 2027 to C. maculatus is due neither to repulsion of oviposition by the adult female nor to a physical barrier preventing entry of larvae into the seed and subsequent tunnelling. Indeed, since the larvae are capable of tunnelling into perspex, physical resistance would seem unlikely. Involvement of an antimetabolite is clearly indicated (Section II, Part I.D).

Screening for Toxins

The toxins screened for in Vigna unguiculata were those characteristic of the Leguminosae which are known to be toxic to insects and their larvae (Section II, Part II). In screening for toxins it is only the positive results which are of real significance since negative results may be due to the inefficiency of the assay system used rather than the absence of toxins. For example, although the most sensitive assay for detecting saponins is the haemolytic assay method, not all saponins possess this haemolysing activity. This possibility was partially overcome by using, in addition to the haemolytic assay method, two other methods for detecting saponins, the Foaming method and the Acetylation method;

unfortunately, although giving positive results for most saponins, they are not sufficiently specific for this class of compound. However, the negative results obtained with all three types of assay method strongly indicates the absence of saponins in the cowpea. Similarly lectins may be difficult to detect owing to the wide range of specificities encountered by this class of proteins. It was therefore necessary to modify the assay conditions so as to cater for all the known specificities. The fact that roots from four day old seedlings gave a positive agglutination reaction with 2 per cent rabbit erythrocytes (Gatehouse, private communication) tends to support the finding of their absence in cowpea seeds, as opposed to the negative results being attributed to incorrect assay conditions. The screening method for alkaloids can claim only to be preliminary, although there appears to be no mention of their presence in V. unguiculata in the literature (Johnson and Raymond, 1964; Janzen et al., 1977). Finally the presence of a toxin of a novel type, not screened for, cannot be excluded. However the results of the feeding trials (Section II, Part VII) strongly indicate that the trypsin inhibitor accounts for the antimetabolic activity present.

The ready detection of inhibition of bovine trypsin, and to a lesser extent chymotrypsin, encouraged further investigation into the presence of inhibitors of digestive enzymes in the seeds of Vigna unguiculata. However, with reference to insect resistance obviously only inhibitors of enzymes of the types known to be present in insects were tested for. Although no characterisation of the gut enzymes of the larvae of Callosobruchus maculatus appears to have been carried out, being a phytophagous insect it will have a neutral to alkaline gut pH; such insects have been shown to possess trypsin-like enzymes, leucine amino-peptidase-like enzymes and carboxypeptidases (Grogan and Hunt, 1977; Kunz, 1978). Grogan and Hunt (1977) demonstrated the presence of trypsin, chymotrypsin and carboxypeptidase A and B-like activities in adults of two species of wasp, Vespula germanica and V. maculifrons. For both species the trypsin-like and chymotrypsin-like activities were examined with specific chemical inhibitors to determine their similarity to the corresponding bovine enzymes. Both types of enzyme possessed the amino acids serine and histidine in the active centre, and the trypsin-like enzyme possessed specificity for basic amino acids while the chymotrypsin-like enzymes possessed specificity for aromatic amino acids; in these respects the insect

proteases closely resembled the corresponding bovine proteases. Inhibition of bovine trypsin and chymotrypsin was thus considered as a good indication of the presence of inhibitors of Callosobruchus maculatus endopeptidases. The use of mammalian exopeptidases for screening purposes can be justified on similar grounds; however, the instability and inavailability of carboxypeptidase B precluded screening for inhibitors of this enzyme. Phytophagous insects do not possess an acid foregut, and thus acid proteases of the pepsin type were not considered.

The negative results for inhibition of the enzymes tested may nevertheless be due to specificity of the enzyme-inhibitor interaction rather than the lack of a particular type of inhibitor in the cowpea. The specificity of protease inhibitors is both wide and narrow. For example, soyabean trypsin inhibitor only partially inhibits human trypsin (probably by inhibiting only one component of a mixture of enzymes) despite its complete stoichiometric inhibition of bovine trypsin (Feeny et al., 1969; Laskowski and Sealock, 1971). Similarly bovine secretory trypsin inhibitor does not inhibit human trypsin, but inhibits bovine trypsin (Feeney et al., 1969). In contrast to this discrimination between enzymes from species closely related (in biochemical terms), penguin ovomucoid at the same site inhibits both bovine

α-chymotrypsin and the bacterial protease subtilisn, enzymes which are dissimilar apart from their function (Bigler and Feeney, 1971). Overall, there is no justification for doing otherwise than assuming substances not to be present until it is shown they are.

Inhibitors of other types of digestive enzymes were not screened for. Amylases, for example, are likely to be present in the larval gut of C. maculatus, and protein inhibitors of mammalian and insect α-amylases have been shown to be present in some cereals (Buonocore et al., 1977). However, although the presence of amylase inhibitors has been suggested to contribute to insect resistance, it is hard to see how these proteins would survive proteolysis in the insect gut. Further, amylase inhibitors do not cause complete inhibition of α-amylase since the inhibitor-enzyme complex is still active (Buonocore et al., 1976). Lacking any convincing demonstration of the antimetabolic nature of these proteins, it was not considered necessary to assay for them at this stage in the cowpea. The presence of amylase inhibitors has only been reported for one legume species (Phaseolus vulgaris; Marshall and Lauda, 1975).

Since inhibitors of insect endopeptidases were found to be present in the seeds of V. unguiculata, it would be interesting to look for inhibitors of bacterial or fungal-

type proteases, for example subtilisn and carboxypeptidase C. It would appear reasonable, and would be beneficial, for a plant to possess a general defensive mechanism for combating a wide range of predators. This concept is strengthened by the findings of Mosolov et al., (1976) who demonstrated the presence of inhibitors of certain pathogenic bacterial proteases and fungal proteases in plants.

Comparison of Trypsin Inhibitor Content in Different Varieties of Vigna unguiculata

Assays for trypsin inhibitor content of the varieties of cowpea screened showed a four to five fold variation between highest and lowest inhibitor content (Table 2.3.1 and Table 2.3.3). Using two totally different methods of assay the results are roughly consistent strengthening the conclusion that a real difference in inhibitor content is being measured. This variation in content might seem to be more than expected between varieties; however, the content of legumin, a major storage protein, varies by four-fold (13 per cent to 41 per cent of the total protein) in the seeds of different lines of Pisum sativum (Thomson et al., 1978), and the lectin content of the seeds of Phaseolus vulgaris varies from virtually nothing to 30 per cent of the total protein among different varieties (Pusztai, private communication).

The most significant result is that the resistant variety contains at least twice the trypsin inhibitor content as any other variety. Thus at this stage it was proposed as a working hypothesis that the resistance of TVu 2027 was due to its elevated trypsin inhibitor content. It could be argued against this proposal that the other varieties tested show variation in their inhibitor content but were nevertheless all equally susceptible to attack by C. maculatus. Consequently it must be supposed that there is a certain threshold level above which the anti-metabolic effects of the trypsin inhibitor can no longer be resisted by the larvae, but that below this level the trypsin inhibitor has no apparent effect upon larval development. Similar responses have been shown in many insects regarding the effective toxicity of many pesticides (Finney, 1971).

Purification of the Trypsin Inhibitor from Vigna unguiculata

Trypsin inhibitor was readily purified from the seeds of the cowpea varieties by affinity chromatography. The resulting product was fully active against trypsin, that is, it contained no material which did not bind to and inactivate trypsin. On the basis of the inhibitory activity of the purified inhibitor against trypsin, the proportion, by weight, of the inhibitor in the different varieties of cowpea could

be deduced (Table 2.4.1). As a consequence, biological assays of the toxicity of this trypsin inhibitor at physiological levels could be carried out.

Feeding Trials

Feeding trials were carried out using the larvae of C. maculatus to demonstrate the toxicity of the trypsin inhibitor, and to determine the critical threshold level. The major problem encountered was the inability to use an artifical growth medium. As yet no satisfactory artificial medium has been developed which is able to support the larval growth. In this work artifical media were prepared containing from 5 to 20 per cent cowpea meal in 2 per cent agar, and 20 per cent protein (casein) and 60 per cent carbohydrate (glucose) in 2 per cent agar; all media contained 15 per cent nystatin as a fungicide and were autoclaved prior to infestation. Both diets were found to be inadequate to support larval growth. The reason for the failure of the second diet being unable to support larval growth is probably the result of the absence of certain basic nutritional requirements, such as vitamin B_{12} for example. However, it is more difficult to determine why the former was inadequate; texture is not a likely drawback since the larvae are able to grow in meal as opposed to the hard cotyledon of the seed; presumably lack of

development was a result of lack of oviposition by the adult female. This growth medium would not strictly be classified as "artificial". The author has discussed the importance of developing an artifical medium with members of the TSPC, and it is not until this has been achieved that a total monitoring of the feeding trials can be conducted.

Unfortunately, as far as the author is aware, all the insect feeding trials demonstrating the toxicity of the trypsin inhibitor which have been reported in the literature either do not incorporate the inhibitor into the diet at the correct physiological concentration, or do not use the correct type of trypsin inhibitor. For example, Steffens et al., (1978) claim their work to be the first of its kind in the demonstration of the toxicity of the trypsin inhibitor to the larvae of the corn borer Ostrinia nubilalis. In these feeding trials they used both the trypsin inhibitor from corn (at a concentration of 2 to 3 per cent) and the soyabean trypsin inhibitor (at a concentration of 2 to 5 per cent). Although the latter was toxic, though perhaps irrelevant since the larvae do not feed on the soyabean, the former had no effect. The failure of the corn trypsin inhibitor to be antimetabolic may well be due to the fact that the inhibitor is not a true stoichiometric inhibitor of trypsin since maximum demonstratable inhibition was less than 50 per cent.

Despite the absence of a suitable artifical growth medium, the feeding trials carried out in this work demonstrate directly the toxicity of the cowpea trypsin inhibitor to the larvae of Callosobruchus maculatus (Section II, Part VII). In the first set of feeding trials, addition of 10 per cent albumins (equivalent to 0.85% trypsin inhibitor) to the basic meal was very toxic to the larve, none surviving to adult, whereas in a subsequent set of trials addition of 10 per cent albumins devoid of trypsin inhibitory activity had no effect upon larval development. These results, however, are only indicative of the toxicity of the resistant variety, TVu 2027, being due to an elevated trypsin inhibitor content. Further evidence for the toxicity of the trypsin inhibitor was provided from feeding trials carried out whereby the purified trypsin inhibitor was added to the basic meal at different concentrations. These results suggest that the threshold level at which the inhibitor becomes toxic to the larvae lies between 0.5 to 0.8 per cent; this conclusion is also indicated by the values obtained for the trypsin inhibitor content of the different varieties of cowpea (Table 2.4.1). However, the critical threshold level will tend to vary from one strain of C. maculatus to another, and indeed one major problem with which plant breeders are faced is that resistance in the field tends

to increase the selection pressure on the predators, eventually resulting in a strain tolerant to increased levels of a particular toxin. An example of this is seen in the gradual increased tolerance of sytophilus to many "resistant" varieties of wheat (Dobie, private communication). For this reason, resistance due to a multiplicity of factors, rather than one, is a decided advantage.

Although the evidence presented so far for the nature of the resistance of TVu 2027 may be argued as being only circumstantial, further feeding trials were carried out to prove that the resistance of this variety is due largely, if not totally, to the elevated trypsin inhibitor level. TVu 2027 meal is found to be very toxic to the larvae, none surviving to adult, whereas autoclaving (which inactivates the trypsin inhibitor) was found to drastically reduce this effect; although there was no direct evidence that another toxin was not denatured by the treatment. However, addition of the purified trypsin inhibitor at a concentration of 0.8 per cent (slightly lower than that found in TVu 2027) restored resistance, whereas addition at a concentration of 0.25 per cent (level found in some of the susceptible varieties) had no effect upon larval development. Furthermore, and possibly most important of all, addition of 0.5 per cent trypsin inhibitor to a susceptible variety

resulted in this variety becoming resistant (Table 2.7.2). The fact that inactivation of the trypsin inhibitor, either by autoclaving or by pepsin digestion, removed its anti-metabolic activity indicates that it is the functional activity of the trypsin inhibitor which is responsible for its toxicity.

Expression of the Activity of the Trypsin Inhibitor towards the Larvae and the Plant Itself

Effect on Larval Proteases

The ambiguity of previous experiments using insect proteases and protease inhibitors (Section I) indicates the difficulties involved in the demonstration of stoichiometric inhibition.

The major problem encountered in the demonstration of stoichiometric inhibition of the larval proteases by the purified trypsin inhibitor arises from the fact that no previous purification or characterisation of these enzymes have been carried out. In addition, due to the minute size of the larvae, the larval enzyme extract had to be prepared from the total larvae rather than from the larval gut. Thus, not only gut proteases but also other intercellular proteases were present. This would tend to make the results less clear cut, though it must be presumed that the proteases present were predominantly of gut origin. Although inhibition

of larval proteolysis did occur both in the presence of the cowpea trypsin inhibitor and the soyabean trypsin inhibitor, complete inhibition did not occur. Presumably this is due to hydrolysis not being solely due to trypsin-like enzymes. In the case of the synthetic substrates hydrolysis could also be the result of esterase (BAEE) or dipeptidase (BAPNA) catalysis; with the labelled globulin substrate many proteases and peptidases could cause "hydrolysis". Since the larval extract contains all the cellular enzymes as well as the gut proteases, it is perhaps not surprising that an inhibitor for a specific enzyme does not completely inhibit hydrolysis of a given substrate.

Given that inhibition of the larval proteases by the trypsin inhibitor occurs, it should be possible to purify a larval protease by affinity chromatography on a trypsin inhibitor column, and to demonstrate stoichiometric inhibition of this protease. The antimetabolic activity of the inhibitor could then be described at the molecular level.

Effect on Plant Proteases

The failure of the cowpea trypsin inhibitor to inhibit either the BAPNA-ase activity found in the albumin fraction from cowpeas, or the BAEE-ase activity found in the globulin fraction may be compared with the results of Hobday *et al.*,

(1973) or Chrispeels and Baumgartner (1978) both of whom failed to find any inhibition of endogenous proteases by the inhibitor in related species. In contrast, Royer *et al.*, (1974) and Gennis and Cantor (1976a) speculate, on the basis of incomplete results, that the inhibitor is involved in the control of endogenous proteases, and hence plays an active role in seed metabolism and germination. Part of this confusion may be due to different proteolytic enzymes being investigated. In the absence of any characterisation of the enzymic activities assayed for in the present study, no firm conclusions can be drawn from the results.

Cowpea Breeding Programme

Susceptibility trials using different harvests of TVu 2027 demonstrated that this resistance towards *Callosobruchus maculatus* was a genetic characteristic and was not due to a freak harvest (Section II, Part I). Additionally, since there was no significant variation in the levels of trypsin inhibitor in batches of seeds from different harvests for a given variety, presumably the trypsin inhibitor content for a particular variety is also genetically determined. However, it must be pointed out that all the seeds examined were grown under similar ecological conditions. It would be interesting to determine to what extent, if any, the environment influences trypsin

inhibitor content for a given variety of cowpea. Croy (private communication) showed that if the sulphur supply of Pisum sativum grown in water culture was reduced from 4 to 8 ppm to 0.06 ppm there was virtually no detectable trypsin inhibitor in the seeds. Thus it can be concluded that the environment, in this case the supply of certain elements, does have an effect upon the inhibitor content. However when grown under "field" conditions the environmental variation presumably would not be as great.

As revealed by the feeding trials, a high trypsin inhibitor content in the cowpea seeds would be very advantageous with respect to protection against C. maculatus and possibly many other predators as well. Unfortunately, although resistant to C. maculatus, TVu 2027 is susceptible to a form of viral attack and thus it is desirable to cross this variety with virus resistant and high yielding varieties. Before this can be accomplished it is essential to first determine the mechanism of inheritance of the trypsin inhibitor. A second consideration before increasing the inhibitor content of the seeds is its effect upon the subsequent human consumers. Although it has not yet been determined, the cowpea trypsin inhibitor, like the soyabean trypsin inhibitor, may have little effect upon human trypsin. However, even if it turns out to show stoichiometric

inhibition against human trypsin presumably the elevated inhibitor content will have no effect since the author has demonstrated that the inhibitor is denatured upon cooking. If perchance it is not all inactivated by cooking it will be digested and inactivated by pepsin (Section II, Part VII); pepsin digestion occurs before trypsin digestion in the human gut. Fortunately pepsin-like enzymes are not present in the gut of phytophagous insects. As the cowpea trypsin inhibitor is very resistant to hydrolysis by most digestive enzymes, even pronase (from Streptomyces griseus) which is very unspecific, it is very unlikely that it will be denatured, and so rendered harmless, by the larval gut of C. maculatus.

PART II Characterisation of the Trypsin Inhibitor from Vigna unguiculata

By analogy with other legumes the cowpea trypsin inhibitor (CPTI) had been assumed to be a protein; this was confirmed on its isolation by affinity chromatography. CPTI, although pure in terms of function, as shown by rechromatography on the trypsin affinity column and by ion-exchange chromatography (in that no material that did not inhibit trypsin was detected in the inhibitor under either treatment), was shown to be heterogenous by various criteria. The phenomenon of "isoinhibitors" being isolated from a single source is well known and well documented (see Introduction). However, the CPTI as isolated seems a mixture of fairly closely related proteins since the molecular weight determination by gel filtration gave a single peak; this is clearly incompatible with an example like soyabean which contains three classes of inhibitor: the Kunitz (mol. wt. 22,000), the Bowman-Birk (mol. wt. 16,000) and the small (mol. wt. 8,000) inhibitors. Further, on isoelectric focussing the CPTI is resolved into a large number of bands, but these bands are not widely spaced; thus the isoinhibitors must be similar in molecular weight and pI. The observed band pattern on gel electrophoresis at pH 8 can thus be assumed to be due to charge rather than size differences; in that the elution profile on ion-exchange (which depends

solely on charge) is similar to gel electrophoresis this supposition is confirmed.

It may thus be reasonably assumed that CPTI consists of a number of closely related isoinhibitors. Further, the isoinhibitors are qualitatively the same across the varieties tested; this is shown by isoelectric focussing and gel electrophoresis. The resistance of TVu 2027 might have been due to its possessing a trypsin inhibitor the other varieties do not possess; however, the results show that it merely has more of the same mixture of isoinhibitors. This is more reasonable than qualitative differences between varieties.

Amino Acid Composition

The amino acid composition of the major isoinhibitors of two varieties show them to be unusual as proteins in their very high content of cystine. This is seen in some other plant protease inhibitors in which the cystine has been shown to be involved in very extensive disulphide cross-linking of the polypeptide chain (Laskowski and Sealock, 1971). Presumably this is the case here also, as indicated by the difficulty experienced in reducing the proteins (q.v.). The tightly folded and cross-linked polypeptide chain has been suggested by Laskowski and Sealock (1971) to give these proteins their resistance to digestion by proteases in general.

The amino acid composition of the inhibitors are similar to those reported by Gennis and Cantor for inhibitors isolated from black-eyed peas.

Molecular Weight and Subunit Structure

The minimum molecular weight for CPTI as deduced from the amino acid composition of the major isoinhibitors is about 8,800. This value is obviously open to some uncertainty due to the slight heterogeneity revealed in the major isoinhibitors by isoelectric focussing. The minimum molecular weight is about half the molecular weight given by gel filtration, 17,000. In that the CPTI chromatographed as a single peak on gel filtration, it may be contrasted to the black-eyed pea inhibitor isolated by Gennis and Cantor which ran as three peaks due to a slow monomer-dimer-tetramer equilibrium, and to inhibitors such as the lima bean trypsin inhibitor which run as a very broad peak indicating a fairly rapid monomer-dimer equilibrium. Thus the 17,000 molecular weight of CPTI may represent a dimer but not a dimer in equilibrium with monomer or other oligomers, at least at pH 7-9. The slight broadening of the CPTI peak on gel filtration may be explained by the results of SDS gel electrophoresis; in the absence of reducing agents two bands may be seen in the 16,000 to 18,000 molecular weight range. These bands presumably represent whole molecules of

CPTI, giving rise to some heterogeneity in molecular weight. Thus we may reasonably conclude a mean molecular weight of 17,000 for CPTI.

Unfortunately, the subunit structure of CPTI is by no means as easy to explain. The major band indicates a "subunit" at 13,000 molecular weight on SDS-gel electrophoresis, which is not consistent with the minimum molecular weight by amino acid composition or gel filtration. The SDS-gel electrophoresis in the presence and absence of 2-mercapto-ethanol shows that the higher molecular weight bands at 16,000 to 18,000 disappear on reduction, and bands at less than 10,000 molecular weight appear; this process may be interpreted if bands round 17,000 are due to complete CPTI, which is assumed to be a dimer whose subunits are linked by disulphide bridges. In this case the minimum molecular weight corresponds to one subunit. However, even under reducing conditions the 13,000 molecular weight band is still present. All bands are very faint under reducing conditions and it is possible that material is being lost from the gel: the retention of the band at 13,000 molecular weight could be due to incomplete reduction and the selective loss of other small polypeptides. Since the gels themselves did not have reducing agent incorporated into them, the 13,000 band may be a result of recombination of fragments.

Guanidine hydrochloride chromatography of the protein thoroughly reduced and carboxymethylated, shows no 13,000 "subunit"; instead three peaks are seen at approximate molecular weight of 8,000, 5,000 and 3,000. The following additive relationships hold for the various "subunits":

$$8{,}000 \simeq 5{,}000 + 3{,}000$$
$$13{,}000 \simeq 8{,}000 + 5{,}000$$
$$17{,}000 \simeq 8{,}000 + 8{,}000 \simeq 13{,}000 + 3{,}000$$

If the model of Laskowski for soyabean trypsin inhibitor is applied to this system the results may be explained. We shall assume CPTI is a molecule of molecular weight 17,000 containing two subunits covalently bound together by disulphide bridges. It is known that reaction of soyabean inhibitor with trypsin cleaves a bond in the polypeptide chain at the active site of the inhibitor. However, the inhibitor remains fully active, and thus the break must be held together. Since CPTI is isolated by affinity chromatography on trypsin, it has been reacted with the enzyme and may be expected to be modified. This has been found to be the case in the isolation of inhibitors from other sources on trypsin affinity columns (Fritz et al., 1971). The "modified" trypsin inhibitor will behave as a complete molecule on gel filtration, but on SDS gel electrophoresis in the absence of reducing agent, if the

"break" in the polypeptide chain is not held together by a covalent (i.e. disulphide) bridge, the "end" will be lost. This is known not to be the case for soyabean trypsin inhibitor (Kunitz type) where the reactive site, and point of cleavage, is in a disulphide "loop" (Ozawa and Laskowski, 1966), but this inhibitor is not analogous to the cowpea inhibitor in other properties. The situation for the cowpea inhibitor is shown diagrammatically in Figure 3.2.1. The small fragment (3,000) will not be detected on the gel. When the inhibitor is completely reduced the disulphide bridged subunits are separated; the result is a subunit at 8,000 and two subunit fragments at 5,000 and 3,000. This is seen on guanidine hydrochloride chromatography. If reduction is not complete, as seems to be the case on SDS gels when the protein is reduced, lower molecular weight fragments are produced, but are largely lost from the gel, and the remaining unreduced 13,000 "subunit" remains the predominant species. Traces of bands at 16,000 to 18,000 are also seen, due to incomplete reduction, along with indistinct bands at molecular weight less than 10,000 due to the fragments. If this explanation is correct it would indicate that the reduction of CPTI is difficult, and only occurs under fairly severe conditions. This was observed with black-eyed pea inhibitor (Gennis and Cantor 1976a) and seems to be a general

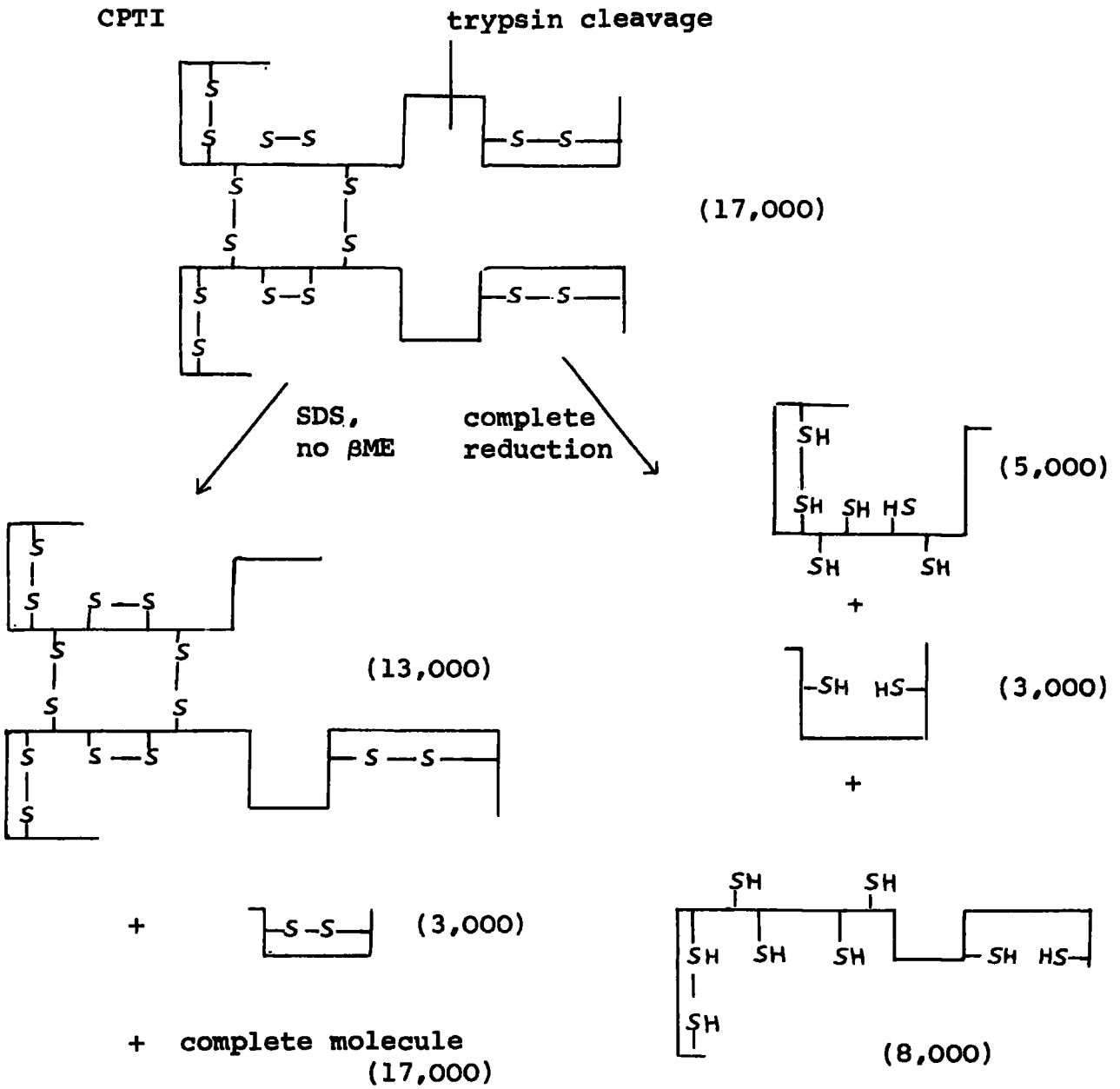


Figure 3.2.1. Model for the subunit structure of the cowpea trypsin inhibitor.
Denaturation and partial reduction will give a mixture of all the species shown.

property of these very highly cross-linked proteins.

On the basis of the present work, taken in conjunction with the known properties of protease inhibitors, it seems reasonable to conclude that CPTI is a dimer of subunits of about 8,000 molecular weight, the subunits being covalently linked by disulphide bridges. As isolated by affinity chromatography the inhibitor is modified by a break in the polypeptide chain of one subunit at the site of binding to trypsin. Since one molecule of inhibitor binds to one molecule of trypsin on the affinity column, and the complex is kinetically stable (as shown by gel filtration) there can be only one point of modification of each inhibitor molecule even though it may have more than one binding site for trypsin. Further, the results from SDS gels without reducing agent indicate that the modification is not complete since complete CPTI molecules are seen on the gel. This presumably represents the thermodynamic equilibrium

$$\underset{\text{molecule}}{17{,}000} \rightleftharpoons \underset{\text{molecule}}{13{,}000} + \underset{\text{fragment}}{3{,}000}$$

A rough estimate would be that about 10 to 20 per cent of the CPTI molecules are unmodified, but quantitation on the gels is very difficult. This modification of the CPTI may account in part for multiplicity of bands on gel electrophoresis at pH 8 since modification is known to alter electrophoretic properties (Niekamp *et al.*, 1969).

Interaction with Trypsin

The combining weight of 9,400 deduced for CPTI with trypsin indicates two binding sites for trypsin per 17,000 molecular weight molecule. The figure of 9,400 represents an upper limit for the combining weight; the true value is probably some 10 per cent lower, as will be discussed later. Since the inhibitor is dimeric, there can be presumed to be one binding site per subunit.

The existence of two complexes of the inhibitor with trypsin was demonstrated by gel filtration, making use of the extreme stability of the complexes to separate them. The interpretation of these complexes as one and two molecules of trypsin bound to one molecule of inhibitor was made on the basis of the combining weight and on the relative amounts of the complexes isolated; there is no 2:1 ratio unless trypsin is in excess. The molecular weights for the complexes, 33,000 and 51,000 are only apparent; the calibration of molecular weights by gel filtration depends on the proteins being globular. The inhibitor : trypsin complexes will not approximate well to a globular shape and thus will not run in the positions expected on the basis of added molecular weights on gel filtration. Further experiments could be done in which the complexes are isolated and their compositions determined directly; also, the 1:1

complex should still be capable of inhibiting trypsin whereas the 2:1 complex will not. This would confirm that there are two binding sites for trypsin per inhibitor molecule.

Interaction with Chymotrypsin

Purified CPTI is capable of stoichiometric inhibition of chymotrypsin as well as trypsin. However this inhibition is unlike that displayed by the Bowman-Birk inhibitor in soyabean, where one molecule of inhibitor combines with one molecule of chymotrypsin and one molecule of trypsin, since the equivalence of CPTI : chymotrypsin is very much greater than CPTI : trypsin. This could indicate that CPTI is a weak inhibitor of chymotrypsin which will compete with the substrate. However, this is made unlikely by inhibition being almost linearly related to inhibitor concentration and stoichiometric (Figure 2.8.11). Further, removal of the chymotrypsin inhibitory activity by passage through a chymotrypsin affinity column strongly suggests that one or more of the isoinhibitors in the CPTI possesses inhibitory activity towards chymotrypsin as well as trypsin but the rest do not. The removal of this "double-headed" inhibitor removes the heterogeneity in molecular weight of CPTI as indicated by SDS gel electrophoresis for complete CPTI molecules. Thus the "double-headed"

inhibitor(s) would seem to form one type of inhibitor, the trypsin inhibitor(s) another, proteins being very similar within types, and the types being similar to each other.

The "double-headed" inhibitor does not make up a large proportion of the CPTI since most CPTI does not bind to a chymotrypsin column. From the equivalences the CPTI chymotrypsin inhibitory activity is about one-tenth the trypsin inhibitory activity; this accounts for chymotrypsin inhibition in the seed and makes the presence of an inhibitor that inhibits chymotrypsin only unlikely. If one-tenth of the potential trypsin inhibitory activity of CPTI is expressed instead as chymotrypsin inhibition, the deduced combining weight with trypsin is too high by 10 per cent; the corrected value of 8,500 is closer to the minimum molecular weight, and half the measured molecular weight. By analogy with the soyabean Bowman-Birk inhibitor the most likely structure is that of a dimer with one trypsin and one chymotrypsin binding site per molecule. Further experiments are planned to isolate the "double-headed" inhibitor and to investigate its structure and interaction with the proteases.

SECTION IV MATERIALS, INSTRUMENTS AND METHODS

A. MATERIALS

I. Biological Materials

Seeds of Vigna unguiculata (L.) Walp. varieties were obtained from the International Institute of Tropical Agriculture, PMB 5320, Ibadan, Nigeria. The varieties were: TVu 37, TVu 57, TVu 76, TVu 1190E, TVu 1502-1D, TVu 2027, TVu 3629, TVu 4557 and Farrin Wake. Seeds of Glycine max were obtained locally.

The larvae of Callosobruchus maculatus were obtained from the Ministry of Overseas Development, Tropical Stored Products Centre, London Road, Slough, Berks., SL3 7HL.

II. Chemicals and Reagents

Enzymes The following enzymes were obtained from Sigma London Chemical Company Ltd., Poole, Dorset, BH17 7NH: trypsin (Type I, from bovine pancreas), leucine amino-peptidase (Type III-CP, from hog kidney), carboxypeptidase-A (Type II, from bovine pancreas), protease (Type VI, from Streptomyces griseus) and neuraminidase (Type VI, from Cl. perfringens). Alpha-chymotrypsin and pepsin were obtained from Worthington Biochemical Corp., Freehold, New Jersey.

Other Proteins The following proteins were used as standards in molecular weight determinations: bovine serum albumin (Crystallised, Sigma), cytochrome c (Type III, from horse heart, Sigma), ovalbumin (5X crystallised, Miles-Servac, Maidenhead, Berks.), β-lactoglobulin (Sigma), myoglobin (from sperm whale and from horse, Miles-Seravac), ribonuclease A (Type I-A, from bovine pancreas, Sigma), chymotrypsinogen A (Miles-Seravac) and lysozyme (Worthington Biochemical Corp.)

Soyabean trypsin inhibitor (Type I-S, Sigma) was used both as a molecular weight standard and a standard in trypsin inhibition assays.

Bovine serum albumin was also used as a standard in protein assays.

Enzyme Substrates The following enzyme substrates were obtained from Sigma Chemical Company: α-N-benzoyl-DL-arginine-p-nitroanilide HCL (BAPNA), α-N-benzoyl-L-arginine methyl ester (BAME), N-benzoyl-L-tyrosine ethyl ester (BTEE), α-N-benzoyl-L-arginine ethyl ester HCl (BAEE), L-leucine amide HCl and hippuryl-L-phenylalanine. Casein was obtained from British Drug Houses (BDH) Ltd., Poole, Dorset.

Fluorescein Fluorescein isothiocyanate isomer I (Sigma).

Saponins The two following saponins were obtained from Sigma Chemical Company: saponin from the "white roots of gypsophila" and digitonin.

Lectins Concanavalin A (grade IV, from Jack bean, Sigma).

Blood The following types of blood were used in haemolytic and agglutination assays: human group A and group O (local hospital), rabbit (Zoology dept.), and cow, pig and sheep (Houghall Agricultural College, Durham).

Column Chromatographic Materials The following materials used in the purification of the cowpea trypsin inhibitor were of the types supplied by Pharmacia Fine Chemicals, Uppsala, Sweden: Sepharose 4B, Sephadex G-25 fine and Sephadex G-25 fine. The Blue Dextran used to check the packing of

Sephadex columns was also obtained from Pharmacia. Diethylaminoethyl cellulose 52 (DE-52 Cellulose) was supplied by Whatman Biochemicals Ltd., Maidstone, Kent.

Cyanogen bromide used in the coupling of trypsin to the Sepharose 4B was obtained from Sigma Chemical Company.

Gel Electrophoresis Acrylamide and NN'-methylenebisacrylamide were "specially purified for electrophoresis" grade (BDH); Coomassie Brilliant Blue was also obtained from BDH. Ampholine carrier ampholytes of the pH ranges 2-11. 3-5, and 5-7 were supplied by Serva, Heidelberg, W. Germany, whilst ampholytes of the pH range 3.5-10 were supplied by LKB Instruments Ltd., South Croydon, Surrey.

Other Chemicals Buffer components and reagents, except as noted, were obtained from BDH, and were of analytical grade when necessary. Tris "Trizma base" was obtained from Sigma. Collidine was obtained from Hopkins and Williams Ltd., Chadwell Heath, Essex.

B. Instruments and Services

An MSE 18 centrifuge was used both in the purification of the cowpea trypsin inhibitor and in the preparation of crude larval enzyme extract.

Fractions from columns were collected by means of an LKB Ultrorac 7000 or LKB Minirac 17000; the absorbance (280 nm) of the eluant from columns was monitored by an LKB 8300 Uvichord II connected to an LKB type 6520-4 recorder. The flow of solutions through columns was pumped, where necessary, by an LKB Varioperspex 12000 peristaltic pump. Where necessary, the salt content of

column eluants was measured on a refractometer.

Protein samples were lyophilised on an Edwards Modulyo freeze drier.

All enzyme assays and optical density measurements were carried out either on a Pye Unican SP 1800 spectrophotometer, with attached Unican AR 25 recorder, a Pye Unican SP 800 spectrophotometer with attached Speedomax recorder, model XL 681, or on a Gilford 2000 spectrophotometer.

Polyacrylamide gel electrophoresis was carried out using a Shandon Southern Vokam SME 2761 power supply; destained gels were scanned by the Gilford 2000 spectrophotometer using its gel scanning attachment. Isoelectric focussing of polyacrylamide gel slabs was carried out on a Shandon U77 flat bed electrophoresis apparatus with cooled platen using an LKB 3371E power supply.

Nitrogen analyses were carried out on a Macfarlane Robson Carlo Erba, model 1512/20, with an attached Speedomax recorder, model XL 681. Amino acid analyses were carried out on a Locarte Amino Acid Analyser.

Graphs for thesis production were drawn using a Hewlett Packard Calculator Plotter, model 9862A.

C. Methods

I. Extraction and Purification of the Trypsin Inhibitor from Vigna unguiculata

Trypsin inhibitor content was analysed in eight varieties of Vigna unguiculata (L.) Walp. These were: TVu 37, TVu 57, TVu 76, TVu 1190E, TVu 1502-1D, TVu 2027, TVu 3629 and TVu 4557.

(a) Preparation of Seed Meals

Mature, dry seeds of V. unguiculata were ground for 2 minutes in a Janke and Kunkel water-cooled mill (type 10); both the testa and the cotyledons were used. The resulting fine powder would pass through a 423μm sieve.

(b) Acid Extraction, Purification by Ion-Exchange Chromatography

This was carried out according to the method of Yoshida and Yoshikawa (1975). In a typical experiment 100 g of finely ground meal were extracted at room temperature for 1 hr in sodium acetate buffer (0.02M, pH 4.4). The residue was removed by centrifugation at 15,000 g for 20 minutes. The supernatant was heated at 80°C in a water bath for 10 minutes and immediately cooled to room temperature; the precipitate formed was removed by filtration through a glass sinter. To the filtrate, solid ammonium sulphate was added to 70 per cent saturation (472 g/li), to precipitate the protein, and the suspension allowed to stand overnight at 4°C. It was then centrifuged as above and the pellet dissolved in a minimum volume and dialysed against deionised water. Finally, the dialysed solution was heated at 80°C for a further 10 minutes, and the precipitate removed by centrifugation (as above). The supernatant formed the crude inhibitor. Both the protein concentration and trypsin inhibitor content were determined at this stage.

Further purification was carried out by ion-exchange chromatography on a column of DE 52 cellulose, 2.2 cm diameter x 30 cm (114 ml volume), packed and equilibrated in Tris/HCl buffer (0.02M, pH 8.0). Elution was commenced with buffer of the same composition, then continued with a linear sodium chloride gradient (0-0.5M) in the same buffer. The

absorption of the eluate was monitored at 280 nm and fractions collected. Protein content was also estimated by the Lowry method (Lowry et al., 1951). Trypsin inhibition was determined using the casein digestion method (Kunitz, 1945).

(c) Acid Extraction; Purification by Trypsin Affinity Chromatography

Meal was extracted overnight at 4°C in 0.1M sodium acetate buffer pH 4.0, containing 0.3M NaCl, 0.01M $CaCl_2$ (buffer of the same composition was used as the washing buffer for the trypsin affinity column). The residue was removed by centrifugation at 15,000 g for 20 minutes at 4°C. The supernatant was then filtered through Whatman No. 1 filter paper and desalted by dialysing against distilled water. The desalted albumin fraction was finally lyophilised; this formed the crude inhibitor fraction. Trypsin inhibitory activity of this fraction was determined by measuring the rate of inhibition of hydrolysis of BAPNA by trypsin, correcting for endogenous BAPNA-ase activity.

Purification of the trypsin inhibitor was carried out by affinity chromatography of the non-desalted albumin extract.

A column of trypsin linked to CNBr-activated Sepharose 4B, 1 cm diameter x 28 cm (22 ml volume), was packed and equilibrated with acetate buffer (0.1M pH 4.0, containing 0.3M NaCl, 0.01M $CaCl_2$). After application of the albumin extract, the column was washed with the same buffer to remove all non-bound protein followed by an unbuffered wash (0.3M NaCl, 0.01M $CaCl_2$). The trypsin inhibitor was eluted from the column with HCl (0.01M pH 2.1, containing 0.3M NaCl, 0.01M $CaCl_2$) at a flow rate of 30 ml/hr,

collecting 5 ml fractions.

The purified trypsin inhibitor was desalted on a gel filtration column of Sephadex G-25 fine, 2.5 cm diameter x 41 cm (200 ml volume), which was packed and equilibrated with distilled water. The trypsin inhibitor from the affinity column was neutralised prior to application. Flow of buffer through the column was driven by a peristaltic pump at a rate of 38 ml/hr, collecting 10 ml fractions. The salt content of the fractions was checked by use of a refractometer; the protein fraction contained no residual salt. The desalted inhibitor was finally lyophilised.

II. Preparation of a Globulin Fraction

100 g of meal was extracted in 500 ml of sodium phosphate buffer (0.05M, pH 7.3) at 4°C overnight, and the residue was removed by centrifugation at 15,000 g for 20 minutes at 4°C. The proteins in the supernatant were then precipitated with ammonium sulphate to 100 per cent saturation at 0°C (697 g/l). This protein precipitation step was followed by further centrifugation, as above, and the protein pellet so obtained was redissolved in phosphate buffer as before; it was necessary to increase the pH slightly with 1M NaOH to increase the solubilisation of the proteins. Any protein which had not gone into solution at this stage was removed by centrifugation at 15,000 g. The supernatant was then dialysed at 4°C for a total of 24 hours against several changes of sodium acetate buffer (0.02M, pH 4.4) in order to precipitate the globulin fraction. This globulin fraction was then removed by centrifugation (15,000 g), resuspended in distilled water, and lyophilised.

III. Preparation of a Glycosidic Fraction

This was performed according to the method of Birk et al., (1963). In a typical experiment 20 g of hexane defatted meal was extracted, for a total of 6 hours, in two successive 200 ml volumes of 80 per cent ethanol at 60°C. The ethanol was removed from the combined filtrates by rotary evaporation, leaving an aqueous solution of about 40 ml volume. Accompanying materials and pigments were removed by partioning the solution with 20 ml volumes of ethyl ether until colourless. To the remaining aqueous phase 2 g of sodium chloride were added and the pH was adjusted to pH 4-5. This aqueous solution was then partioned with a 30 ml volume followed by a 15 ml volume of butanol. The combined butanol phases were then washed twice with 5 ml volumes of 5 per cent (w/v) sodium chloride solution and finally concentrated to dryness by rotary evaporation. The residue formed the glycosidic preparation.

IV. Preparation of a Crude Larval Enzyme Extract

Larvae of Callosobruchus maculatus (either freeze dried or fresh) were homogenised in chilled distilled water, using a glass pestle and mortar, to a concentration of approximately 10 per cent (w/v). The homogenate was centrifuged for 5 minutes at full speed on a bench centrifuge, so removing the debris, and the supernatant dialysed for 12 hours at 4°C against distilled water. This dialysis step was found to be necessary, since with previous larval extracts precipitation occurred during the enzyme assays. After dialysis, the precipitate was removed by further centrifugation at 7,500 g for 15 minutes at 4°C. The supernatant was filtered through a glass fibre filter; this final clear filtrate formed the crude larval enzyme

solution.

V. Enzyme and Protease Inhibitor Assays

Endogenous protease activity, and the degree of inhibition by seed extracts, was determined by monitoring the rate of hydrolysis of a substrate over a given period of time.

(a) Endopeptidase Assays

I. Trypsin Assays Trypsin inhibitory activity was determined by measuring the inhibition by seed extracts of the trypsin catalysed hydrolysis of three substrates, casein, BAPNA and BAEE. The specific activity of the purified trypsin inhibitor from different cowpea varieties, and the activity of the major isoinhibitor of two varieties, were determined using BAPNA.

(i) Trypsin Assay using Casein as Substrate (Kunitz, 1945)

Substrate: casein 10 mg/ml 0.1M phosphate buffer pH 7.6

Assay mixture:
- 1 ml trypsin (50 ug/ml 10^{-3} M HCl)
- 1 ml substrate solution
- 1 ml extract (10-fold dilution in 0.02M Tris/HCl pH 8.0) or SBTI (0-25 µg/ml H_2O) or water

3 ml total

After equilibration of the enzyme and inhibitor, or seed extract, at 35°C for 5 minutes, the assay was started by the addition of the substrate. After a period of 20 minutes the assay was terminated by the addition of 3 ml 5 per cent (w/v) trichloroacetic acid (TCA). The incubations were allowed to stand for 1 hour at room temperature and then centrifuged at full speed on a bench centrifuge (3,200 g) for 10 minutes. The OD of the supernatant was read at 280 nm. A calibration curve was constructed using

soyabean trypsin inhibitor (SBTI) as a standard (0-25 µg/ml H_2O), Figure 4.5.1.

Definition of Activity According to Kunitz [1] unit is the amount of trypsin which under the defined conditions liberates sufficient TCA soluble hydrolysis product that the OD at 280 nm increases by 1.00 unit per minute.

$$Tu^{cas}/\mu g \text{ trypsin} = \frac{OD/Min\ 280\ nm}{\mu g \text{ trypsin/ml}}$$

$$\text{Specific activity} = Tu^{cas}/mg \text{ protein}$$

Trypsin inhibitor activity is expressed in terms of trypsin units inhibited.

(ii) Trypsin Assay using α-N-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA) as substrate (Erlanger et al., 1961).

The following ingredients were placed in a 1 cm pathlength cuvette:

2.5 ml substrate (40 mg BAPNA were dissolved in 1 ml DMSO, the volume adjusted to 100 ml with 0.05M Tris pH 8.2, containing 0.02 M $CaCl_2$) Final concentration 0.92 nm
0.5 ml enzyme solution (100 µg/ml in 10^{-3} M HCl)
X ml seed extract or purified inhibitor
3.0 + X ml total

On hydrolysis of the substrate by trypsin, p-nitroaniline is produced which absorbs at 410 nm. The optical density of the solution at 410 nm is followed with time. 1 unit of enzyme activity is defined as causing an increase in OD of 0.1 per minute (corrected to a total volume of 3.0 ml) under these conditions; trypsin inhibitor activity is expressed in terms of trypsin units inhibited. When a seed extract was used as the inhibitor source, all values were corrected for endogenous BAPNA-ase activity. A calibration curve using soyabean

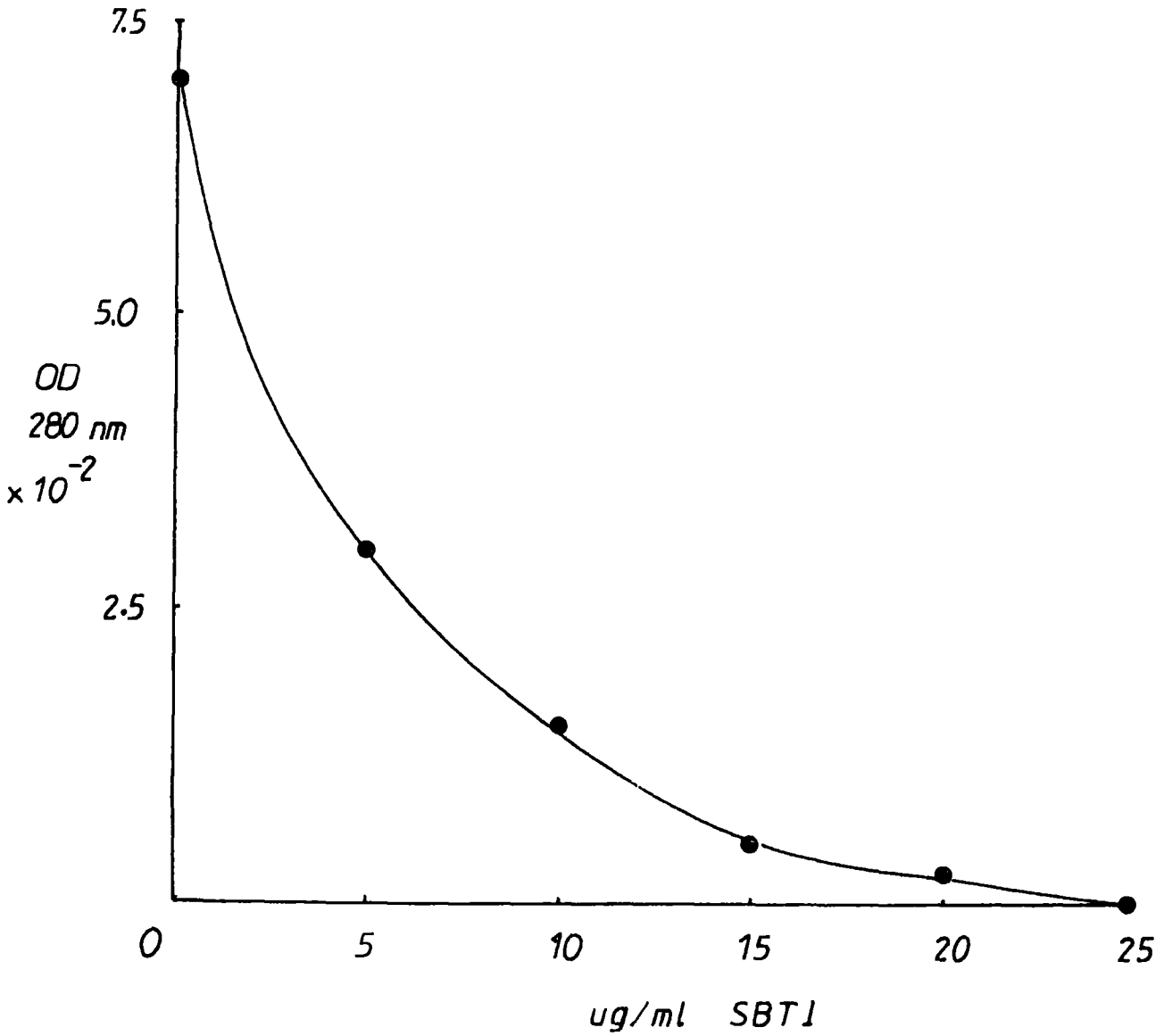
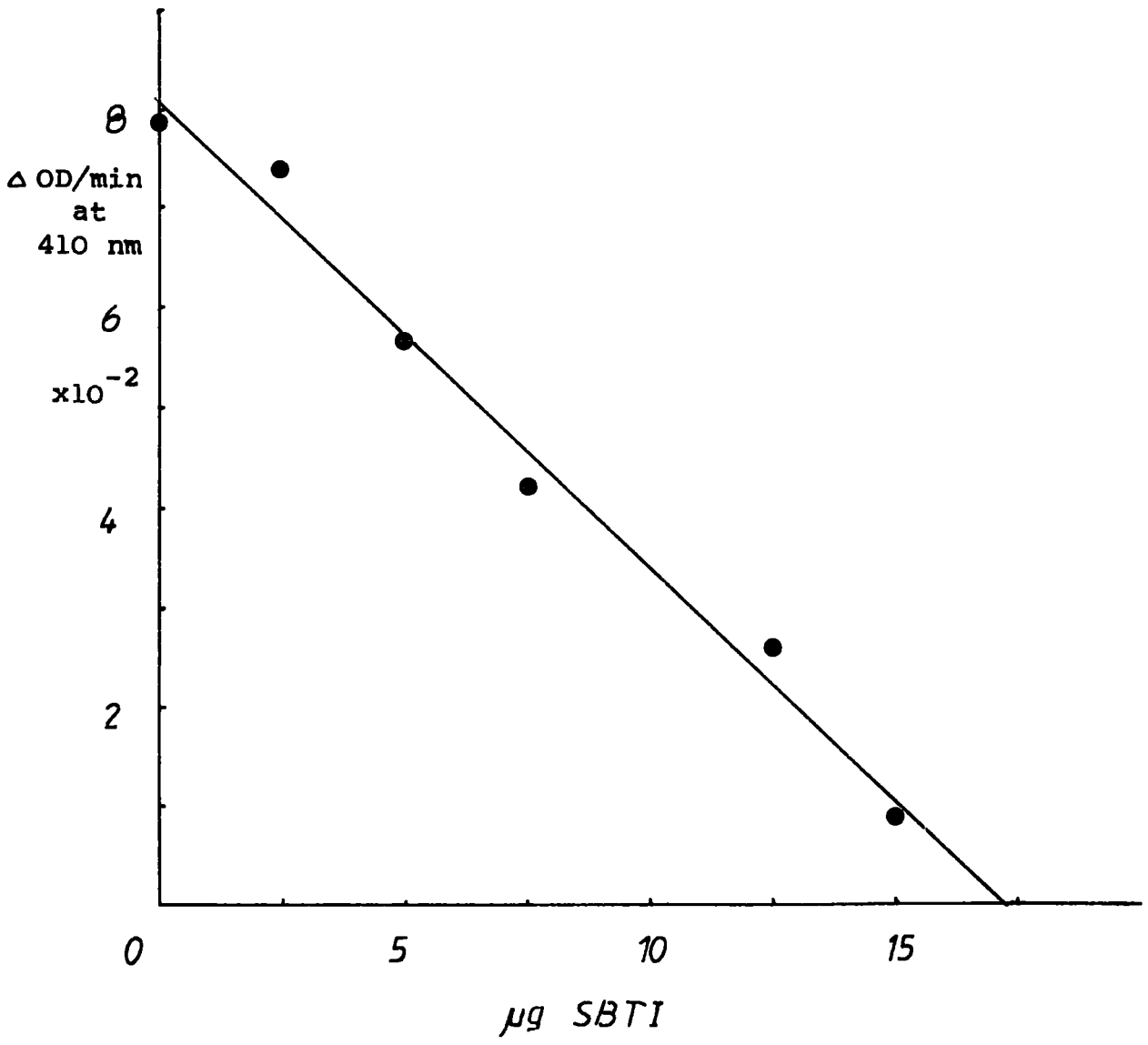


Figure 4.5.1. Calibration curve for SBTI using casein as the substrate for trypsin.

Casein; 1ml of a 10 mg/ml solution.
Trypsin; 1ml of a 25 µg/ml solution.

Figure 4.5.2. Calibration curve for SBTI using BAPNA as the substrate for trypsin.



trypsin inhibitor was constructed (Figure 4.5.2).

When a larval extract of Callosobruchus maculatus was used as the enzyme source, the above assay system was modified slightly to the following (Anstee and Charnley, 1977):

0.20 ml substrate (36 mM in dimethylformamide)
2.00 ml 0.2M glycine/NaOH buffer pH 9.0
Finally 0.25 ml enzyme solution (larval extract)
X ml purified trypsin inhibitor (500 µg/ml H_2O)

2.45 + X ml total volume

(OD/Min at 410 nm was corrected to a total volume of 2.45 ml)

(iii) Trypsin Assay using α-N-benzoyl-L-arginine ethyl ester HCl (BAEE) as substrate (Bergmann et al., 1939).

The following ingredients were placed in a 1 cm path-length cuvette:

3.0 ml substrate (25 mM BAEE in 0.1M phosphate buffer, pH 7.6)
0.1 ml enzyme solution (100 µg/ml 10^{-3}M HCl)
X ml seed extract or purified inhibitor

3.1 + X ml total volume

The optical density of the solution at 253 nm is followed with time. The rate of hydrolysis was corrected to a volume of 3.1 ml.

The following modification was carried out in the presence of larval extract as the enzyme source.

3.0 ml substrate (25 mM BAEE in 0.2M glycine/NaOH pH 9.0)
Finally 0.125 ml larval extract
X ml purified trypsin inhibitor

3.125 + X ml total volume

(OD/Min at 253 nm was corrected to a volume of 3.125 ml.)

2. α-Chymotrypsin Assays (Walsh and Wilcox, 1970).

Chymotrypsin inhibitory activity was determined by measuring the rate of inhibition of the chymotrypsin catalysed hydrolysis of the synthetic substrate N-benzoyl-L-tyrosine ethyl ester (BTEE).

The following ingredients were placed in a 1 cm path-length cuvette:

1.5 ml	substrate (0.001 M BTEE in 50% (v/v) aqueous methanol)
1.4 ml	buffer (0.05 M Tris containing 0.02M $CaCl_2$ pH 8.2)
0.1 ml	enzyme solution (40 μg/ml 10^{-3} M HCl)
X ml	seed extract or purified inhibitor
3.0 + X ml	total volume

The optical density of the solution at 256 nm is followed with time. Activity (corrected to a volume of 3.0 ml) is calculated from the slope of the linear portion of the reaction curve; this should be linear up to a reading of 0.12. 1 unit is equal to the hydrolysis of 1 micromole of substrate per minute.

(b) Exopeptidase Assays

I. Leucine Aminopeptidase (LAP) Assays (Himmelhoch, 1969)

Both endogenous leucine aminopeptidase activity and LAP inhibition, in different seed fractions, were measured using the synthetic substrate L-leucinamide HCl

The following were placed in a 1 cm pathlength cuvette:

2.50 ml	substrate (0.06M L-leucinamide in 0.5 M Tris/HCl pH 8.5)
0.20 ml	62.5 mM $MgCl_2$
0.01 ml	enzyme (2 mg/ml suspension in 0.72 saturated $(NH_4)_2SO_4$, 0.1 M Tris, 0.005 M $MgCl_2$ pH 8.0)
X ml	different fractions of seed extract
2.71 + X ml	total volume

The rate of decrease in optical density at 238 nm caused by the hydrolysis of L-leucinamide, to L-leucine and ammonia, is followed with time. Enzyme activity is expressed by the following equation:

$$u/mg = \frac{OD\ 238\ nm/Min}{0.011 \times mg\ enzyme/ml\ reaction\ mixture}$$

2. Carboxypeptidase A Assay (Folk and Schirmer, 1963).

Endogenous carboxypeptidase activity and inhibitory activity towards this enzyme, in different seed fractions, were measured using the synthetic substrate hippuryl-L-phenylalanine.

The following were placed in a 1 cm pathlength cuvette:

2.90 ml	substrate (0.001 M hippuryl-L-phenylalanine in 0.05 M Tris/HCl pH 7.5 in 1 M NaCl, 10% LiCl)
0.01 ml	enzyme (2.5 mg/ml aqueous suspension with toluene added)
X ml	different fractions of seed extract
2.91 + X ml	total volume

The rate of hydrolysis of hippuryl-L-phenylalanine is monitored by measuring the increase of L-phenylalanine at 259 nm. Enzyme activity is expressed by the following equation:

$$u/mg = \frac{OD\ 259\ nm/Min}{0.30 \times mg\ enzyme/ml\ reaction\ mixture}$$

(c) Larval Enzyme Assay using Fluorescein Labelled Cowpea Globulins as Substrate

(i) Preparation of Fluorescein labelled Cowpea Globulins

Cyanogen bromide activation of an inert matrix (Sepharose 4B) and the subsequent coupling of the globulin preparation was carried out using a similar procedure to that detailed in Section XI (March et al., 1974). The bound protein was labelled with fluorescein by adding 2 mg of

fluorescein isothiocyanate isomer I, dissolved in 2 ml of 0.1 M sodium bicarbonate pH 9.5, to 2 ml of the globulin coupled Sepharose (Bohlhool and Schmidt, 1974). (5 µl of 1 per cent merthiolate was added to prevent growth of organisms). The labelled resin bound protein was then rotated for 24 hours at 4°C, after which time it was washed with sodium bicarbonate buffer until all excess fluorescein isothiocyanate had been removed.

(ii) Larval Enzyme Assay

Assay mixture:	0.1 ml	slurry of labelled globulins (in 0.05 M Tris/0.02 M $CaCl_2$ pH 8.0)
	2.0 ml	buffer (0.05 M Tris/0.02M $CaCl_2$ pH 8.0)
Finally	0.2 ml	larval enzyme extract
	X ml	albumin extract, cowpea trypsin inhibitor, or SBTI
	2.3 + X ml	total volume

The assays were carried out at room temperature by incubating the larval enzyme extract with 100 µl of a slurry of the labelled globulins in buffer (0.05 M Tris/ 0.02 M $CaCl_2$ pH 8.0). When determining the effect of the trypsin inhibitor on the larval extract, the inhibitor and extract were preincubated before addition of the substrate. After an incubation period of given time, the assays were centrifuged at full speed on a bench centrifuge to pellet the Sepharose bound globulins, and the OD of the supernatant read at 490 nm (excitation wavelength of fluorescein). This assay system was checked using bovine trypsin (Figure 2.6.1).

IV. Test for Saponins

(a) Haemolytic Assay (Segal et al., 1974)

Haemolytic assays were carried out using both 2 per cent and 100 per cent rabbit erythrocyte suspensions; the

undiluted blood was used to achieve maximal saponin effect. When taken from the rabbit, the blood was immediately diluted 1:1 with Alserver's solution (Lis and Sharon, 1972) and stored in heparin treated tubes. Before use the erythrocytes were washed three times in phosphate buffered saline (0.006 M phosphate pH 7.4, in 0.9% NaCl) and made up to the desired concentration with phosphate buffered saline (PBS).

Incubation mixture:	1.4 ml	erthryocyte suspension (2 or 100%) in PBS
	0-0.6 ml	glycosidic extract or purified saponin in PBS
	0.6-0 ml	PBS
	2.0 ml	total incubation mixture

The assays were then incubated for 2 hours at 37^{o}C in a shaker water bath, and then spun at full speed in a bench centrifuge for 1 minute. The OD of the supernatant was read at 540 nm, against the appropriate blank, and 50 per cent haemolysis determined. Calibration curves were carried out for both digitonin, and gypsophila root saponin (Figures 2.2.1 and 2.2.2, respectively). When using the glycosidic extract from either soyabean or cowpea, digitonin was used to give 100 per cent haemolysis.

(b) Acetylation Method (Saxena, 1975)

The sample was dissolved, in excess, in 1 ml of chilled chloroform plus 1 ml of acetic anhydride. A few drops of concentrated sulphuric acid were then added. On standing, in the presence of a saponin, a red or blue/green colour is produced, and sometimes a pink ring appears. Gypsophila root saponin was used as the positive control, and bovine serum albumin and soyabean trypsin inhibitor as negative controls.

VII (a) Agglutination Test (Sharon and Lis, 1972)

A serial dilution of the protein sample (in 0.9% (w/v) sodium chloride solution, pH 7.5) was prepared, mixed with an equal volume of a 2 per cent (v/v) suspension of washed erythrocytes (in the same solution) and incubated at room temperature for at least 1 hour (total volume 1 ml). When dealing with 1 ml volume in test tubes, after incubation the erythrocytes were dispersed by gentle shaking and centrifuged at full speed for ½ minute on a bench centrifuge. The tubes were then shaken to disperse the erythrocytes and the degree of agglutination scored on the serological scale from 4* to 0 by visual detection. Lectin solutions, usually Concanavalin A but also Vicia faba lectin, were used as positive agglutination controls. Additionally assays could be carried out on 100 µl samples in microtitration plates, agglutination being judged by a failure of the erythrocytes to settle out.

Due to the high degree of specificity shown by some lectins, agglutination assays were carried out using several different types of blood and under different conditions. These were: human group O (+ and - BSA), human group A (+ and - BSA), rabbit (+ and - BSA), trypsinised rabbit, neuraminidased rabbit, rabbit (1mM calcium ions and manganese ions), pig, sheep and cow.

(b) Trypsin Treatment of Rabbit Erythrocytes (Lis and Sharon, 1973).

2 ml of rabbit blood were washed three times with phosphate buffered saline, and after the final centrifugation the supernatant was removed to leave approximately 0.5 ml of packed erythrocytes. To these, 1 ml of saline bicarbonate (0.15 M NaCl, 10 mM $NaHCO_3$) and 10 mg of trypsin were added

and incubated for 1 hour at room temperature. Subsequently the erythrocytes were again washed three times with PBS and stored at blood strength.

(c) Neuraminidase Treatment of Rabbit Erythrocytes (Lotan et al., 1975)

2 ml of rabbit erythrocytes (50 per cent) were washed with phosphate buffered saline, and after the final centrifugation the packed erythrocytes were resuspended in 1 ml of phosphate buffered saline. To this 1 unit of neuraminidase was added and incubated for 2 hours at 37°C. Subsequently the erythrocytes were again washed with PBS and diluted to 2 per cent.

VIII Test for Alkaloids (Dawson, 1969)

The presence of alkaloids in a 90 per cent ethanol extract was determined using Dragendorff's Reagent.

(Stock Solutions: I; dissolve 17 g basic bismuth nitrate and 200 g tartaric acid in 800 ml water.
II; dissolve 160 g potassium iodide in 400 ml water
Mix solution I and II and protect from light. For spraying mix 50 ml of stock with 100 g tartaric acid in 500 ml water).

The samples were spotted on to chromatography paper, and on spraying with Dragendorff's Reagent orange spots developed in the presence of compounds containing tertiary amines. Collidine and N-ethyl morpholine were used as positive controls.

IX. Characterisation of the Trypsin Inhibitor

I. Polyacrylamide Gel Electrophoresis

(a) Non-SDS Gel Electrophoresis (Gabriel, 1971)

Analytical discontinuous gel electrophoresis on 7 per cent acrylamide gels was used to analyse undissociated

proteins.

Separating gel formulation: 7% acrylamide, 0.18% BIS, pH 8.9

Stock Solutions: (a) 1 M HCl, 48 ml
Tris, 36.3 g
TEMED, 0.23 ml
(b) acrylamide, 28 g
Bis, 0.735 g
(c) ammonium persulphate, 0.14 g

working solution: 1 part (a), 1 part (b), 2 parts (c)

Stacking gel formulation: Stock solutions:

(a) Tris, 5.98 g
TEMED, 0.46 ml
1 M HCl (2.48 ml) adjust to pH 6.7
(b) acrylamide, 10 g
Bis, 2.5 g
(c) ammonium persulphate, 4.0 mg
(d) sucrose, 40 mg

working solution: 1 part (a), 1 part (b), 1 part (c), 4 parts (d), 1 part water

Samples were dissolved in running buffer (Tris/glycine pH 8.3) with sucrose added for layering prior to electrophoresis; bromophenol blue was included as a marker. Electrophoresis was carried out at 1.5 mA/gel until stacking was completed, and then at 2.5 mA/gel. After electrophoresis, the gels were fixed in 15 per cent trichloroacetic acid (TCA) and densitometrically scanned at 340 nm (acrylamide absorbs around 280 nm and below). The gels were then stained with a 1 per cent solution of Coomassie Blue (R-250) in 12.5 per cent TCA for approximately 30 minutes. Destaining was carried out in 15 per cent TCA.

(b) Sodium Dodecyl Sulphate (SDS) Gel Electrophoresis

The method of Weber and Osborn (1969) was used to determine the apparent molecular weights of proteins dissociated with SDS. 10 per cent acrylamide gels were used

routinely (the running buffer was 0.1 M sodium phosphate pH 7.2, containing 0.1 per cent SDS).

Separating gel formulation: 10% acrylamide
acrylamide, 3 g)
bis, 0.081 g) 13.5 ml H_2O
Gel buffer, 15 ml 0.2 M sodium phosphate pH 7.2 + 0.2% SDS
TEMED, 0.045 ml
ammonium persulphate, 1.5 mg in 1.5 ml H_2O

Lyophilised samples for electrophoresis were dissolved in 0.01 M phosphate buffer, containing 1 per cent 2-mercaptoethanol and 1 per cent SDS, and incubated at 37°C for 2 hours. Prior to electrophoresis bromo-phenol blue was added as a marker and sucrose added for layering. Electrophoresis was initially carried out at 5 mA/gel and they were subsequently run at 7.5 mA/gel. After electrophoresis, the total length of the gels and the distance of migration of the bromo-phenol blue marker were measured. The gels were then stained overnight with 0.25 per cent Coomassie Blue in methanol/acetic acid and destained using a 5 per cent methanol, 7 per cent acetic acid mixture in water.

(i) Estimation of Mobility and Determination of Molecular Weight (Weber and Osborn, 1969)

Using the SDS system, electrophoretic mobilities were calculated relative to the mobility of the bromo-phenol blue marker band. Assuming even swelling of the gels, the mobility was calculated as:

$$Rf = \frac{\text{distance of protein migration}}{\text{gel length after staining}} \times \frac{\text{gel length before staining}}{\text{distance of dye migration}}$$

The following proteins were used as molecular weight standards: cytochrome *c* monomer (12,400 mol. wt.), β-lactoglobulin (17,500 mol. wt.), cytochrome *c* dimer

(24,800 mol. wt.), ovalbumin (43,500 mol. wt.) and bovine serum albumin (67,000 mol. wt.). The Rf values were then plotted against the log of the molecular weight (Figure 2.8.4).

(ii) S-carboxymethylation (Konigsberg, 1972)

To denature the protein samples 2 per cent solutions in 6 M guanidine HCl containing 0.5 M Tris, pH 8.2 were flushed with nitrogen and heated at 50°C for 30 minutes. A 50-fold molar excess of dithiothreitol (DTT) was added to the samples which were flushed with nitrogen and heated at 50°C for 4 hours. The samples were then cooled and carboxymethylated with 1.5 molar excess over DTT of iodoacetamide for 40 minutes at room temperature. Samples were finally dialysed to remove excess reactants and guanidine HCl.

Samples which had been carboxymethylated were subsequently run on 12 per cent acrylamide gels, using continuous SDS electrophoresis system.

(c) Isoelectric Focussing

Isoelectric focussing was carried out on 5 per cent acrylamide slab gels.

gel formulation: 5% acrylamide
acrylamide, 1.21 g
bisacrylamide, 37.5 mg
sucrose, 3.13 g
40% ampholine carrier ampholytes pH 3.5-10, 1.25 ml
distilled water, 22.5 ml
ammonium persulphate (12 mg/ml H_2O), 1.25 ml

Samples for isoelectric focussing were prepared by dissolving the lyophilised material in distilled water at a concentration of 2 mg/ml. The protein standard, containing

the following proteins: ferritin (pI 4.4), albumin (pI 4.7), β-lactoglobulin (pI 5.34), conalbumin (pI 5.9), horse myoglobin (pI 7.3), whale myoglobin (pI 8.3), ribonuclease (pI 9.45) and cytochrome c (pI 10.65), was used at a concentration of 10 mg/ml and the haemoglobin marker at 4.5 mg/ml. Prior to loading, the gel was pre-run at approximately 260 volts for 10 minutes to establish a pH gradient. All samples, except for the haemoglobin marker were loaded centrally; the marker was loaded at both poles. The gel was run at approximately 15 watts until the two marker samples had merged together and become focussed; this took approximately 2 hours. Phosphoric acid (0.1M, pH 1.5) formed the anode, whilst ethylenediamine (1M, pH 11.5) formed the cathode.

After isoelectric focussing the gel was extensively washed in 17.5 per cent trichloroacetic acid (TCA) to remove carrier ampholytes and then stained with 0.25 per cent Coomassie Blue in 17.5 per cent TCA. Subsequent destaining was achieved with 5 per cent methanol containing 17.5 per cent TCA. (A high TCA concentration is required at all stages due to the high solubility of the trypsin inhibitor).

2. G-75 Sephadex Column: for Molecular Weight Determinations and Enzyme Inhibitor Complex Formation

A column of Sephadex G-75 fine, 1.6 cm diameter x 85 cm (170 ml volume), was packed and equilibrated with sodium phosphate buffer (25mM pH 7.2, containing 0.1M NaCl). Flow of the buffer through the column was driven by a peristaltic pump from bottom to top of the Sephadex bed. Blue dextran was used to check the packing of the column and to determine the void volume. The column was calibrated using a series of protein standards (mol. wt. range from

12,400 to 67,000); all samples were applied to the bottom of the column. The column was eluted at a flow rate of 21 ml/hour with the same buffer. For each run the Uvichord chart was calibrated by measuring the distance covered for a measured volume of solution passed through.

3. Ion-Exchange Chromatography on DEAE Cellulose 52

Ion-exchange chromatography of the trypsin inhibitor purified by affinity chromatography was carried out as detailed in part I(b) of this section.

4. Amino Acid Analysis (Evans and Boulter, 1974)

Duplicate samples of the protein in 6M HCl were hydrolysed *in vacuo* at 105°C for 22 hours; the amino acid compositions were determined on a Locarte automatic-loading amino acid analyser. The two sulphur amino acids, cystine and methionine, were determined by performic acid oxidation using asparagine as a standard (Moore, 1963).

5. Chymotrypsin Affinity Chromatography: for the Removal of Chymotrypsin Inhibitory Activity from the Trypsin Inhibitor

A column of chymotrypsin linked to CNBr activated Sepharose 4B, 1 cm diameter x 16 cm (12 ml volume) was packed and equilibrated with acetate buffer (0.1M pH 4.0, containing 0.3M NaCl, 0.01M $CaCl_2$). After application of the trypsin inhibitor, the column was washed with the same buffer to remove all unbound protein followed by an unbuffered wash (0.3M NaCl, 0.01M $CaCl_2$). The chymotrypsin inhibitor was eluted from the column with HCl (0.01M pH 2.1 containing 0.3M NaCl, 0.01M $CaCl_2$) at a flow rate of 30 ml per hour, collecting 5 ml fractions.

X. Inactivation of the Trypsin Inhibitor by Pepsin Digestion

Cowpea trypsin inhibitor was inactivated by dissolving it in 2.2% formic acid, pH 2.1, and steaming at 100°C for 15 minutes. After cooling, pepsin (0.5 mg/ml HCOOH) was added and the sample incubated at 37°C with continuous mixing for 6 hours. The sample was then boiled for a further 15 minutes to inactivate the pepsin. Trypsin inhibitory activity was determined by measuring the rate of inhibition of hydrolysis of BAPNA by trypsin. This digestion procedure resulted in approximately a 90 per cent loss in activity.

XI. Cyanogen Bromide Activation of Agarose and the Coupling of Trypsin for Affinity Chromatography

This was carried out using the method of March et al., (1974). In a typical experiment 300 ml of a slurry of washed agarose beads (Sepharose 4B), consisting of equal volumes of gel and water, were added to 300 ml of 2M sodium carbonate and mixed by stirring slowly. The rate of stirring was then increased on addition of 20 ml of an acetonitrile solution of cyanogen bromide (2 g of CNBr per ml of acetonitrile); this was added all at once. The slurry was then stirred vigourously for 2 minutes, after which it was poured on to a coarse sintered-glass funnel, washed with 0.1M sodium carbonate-bicarbonate buffer, pH 9.9, followed by distilled water and finally by 0.1M sodium bicarbonate buffer. After the last wash, the slurry was filtered under vacuum to a moist, compact cake and transferred to a plastic bottle containing 1 g of trypsin dissolved in 300 ml of sodium bicarbonate buffer (0.1M, pH 8.3 cont. 0.5M NaCl). The bottle was rotated end-over-end for 20 hours at 4°C. After coupling, the beads were washed with an alkaline buffer

(0.1M $NaHCO_3$, 0.5M NaCl), water, an acidic buffer (50 mM sodium formate, pH 4.0, 0.5M NaCl), and finally with water again. The gel was then packed into a column and equilibrated with sodium acetate buffer (0.1M, pH 4.0, containing 0.3M NaCl, 0.01M $CaCl_2$).

OD measurements at 280 nm of the coupling buffer after reaction indicated that approximately 75 per cent of the trypsin had been bound. The effectiveness of the column was tested using soyabean trypsin inhibitor; this was loaded at pH 4.0 (the pH at which the Enzyme-Inhibitor complex forms). The inhibitor was found to elute in a single peak at pH 2.1; the pH at which the Enzyme-Inhibitor complex is known to dissociate.

XII. Estimation of Protein Concentration

(a) By the Lowry Method (Lowry et al., 1951)

5 ml of freshly prepared alkaline copper solution (stock soln: 1 ml 1% copper sulphate, 1 ml 2% sodium potassium tartrate, 100 ml 2% sodium carbonate in 0.1M NaOH) were added to 1 ml aliquots of protein solution, mixed thoroughly, and left for 10 minutes. 0.5 ml of diluted Folin Reagent (standardised by titration with 0.1M NaOH using phenolpthalein as an indicator) was then added to each, mixed rapidly and left for at least 30 minutes at room temperature to develop a blue colour. The OD of the solution was then read at 500 nm. Calibrations were carried out using bovine serum albumin (BSA) as a protein standard (Figure 4.12.1). However it is important to note that each different protein will have a slightly different calibration. This method of protein estimation is only linear up to a protein concentration of approximately 200 ug/ml.

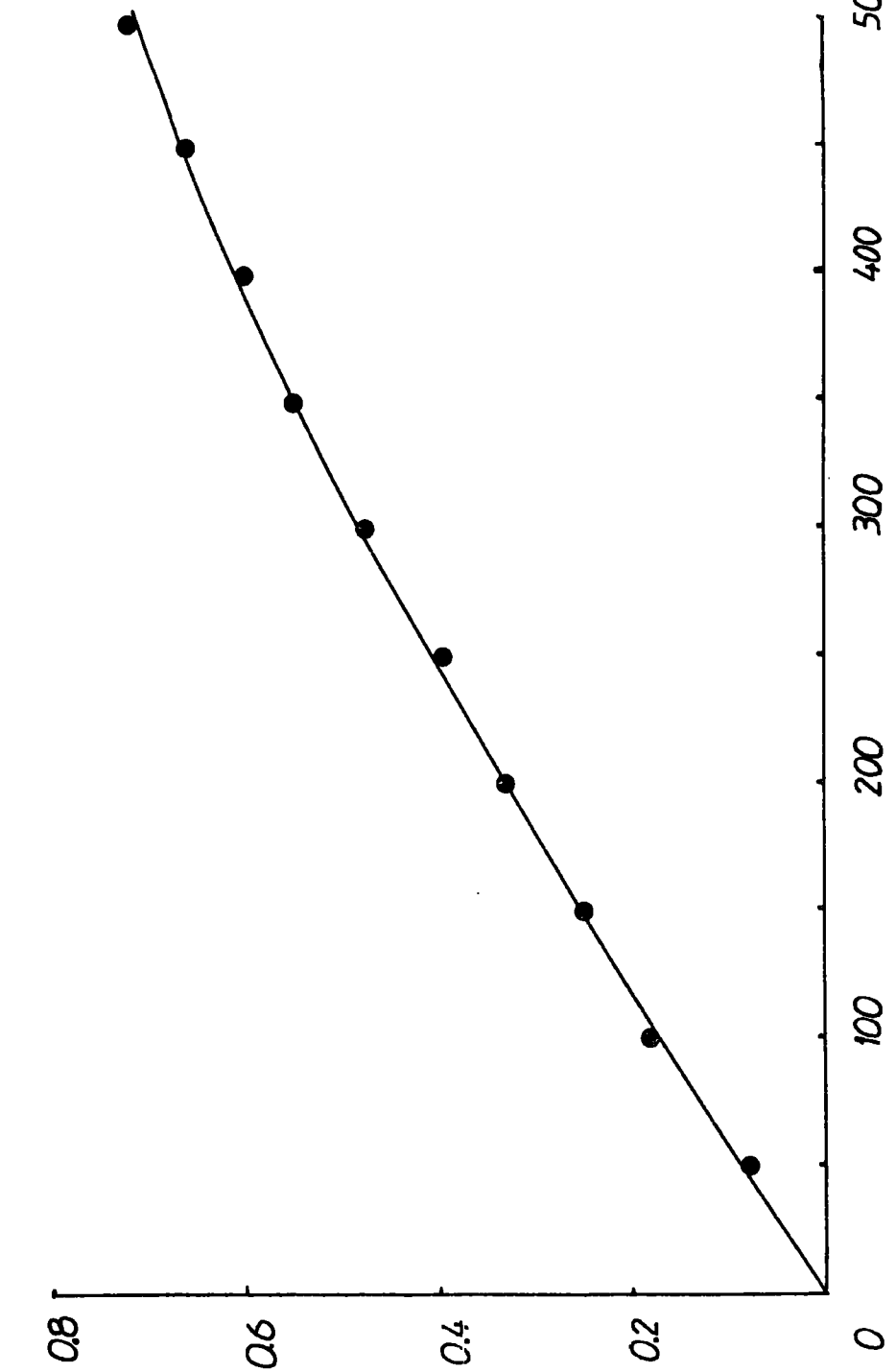


Figure 4.12.1. Protein calibration by the Lowry method using BSA as a standard.

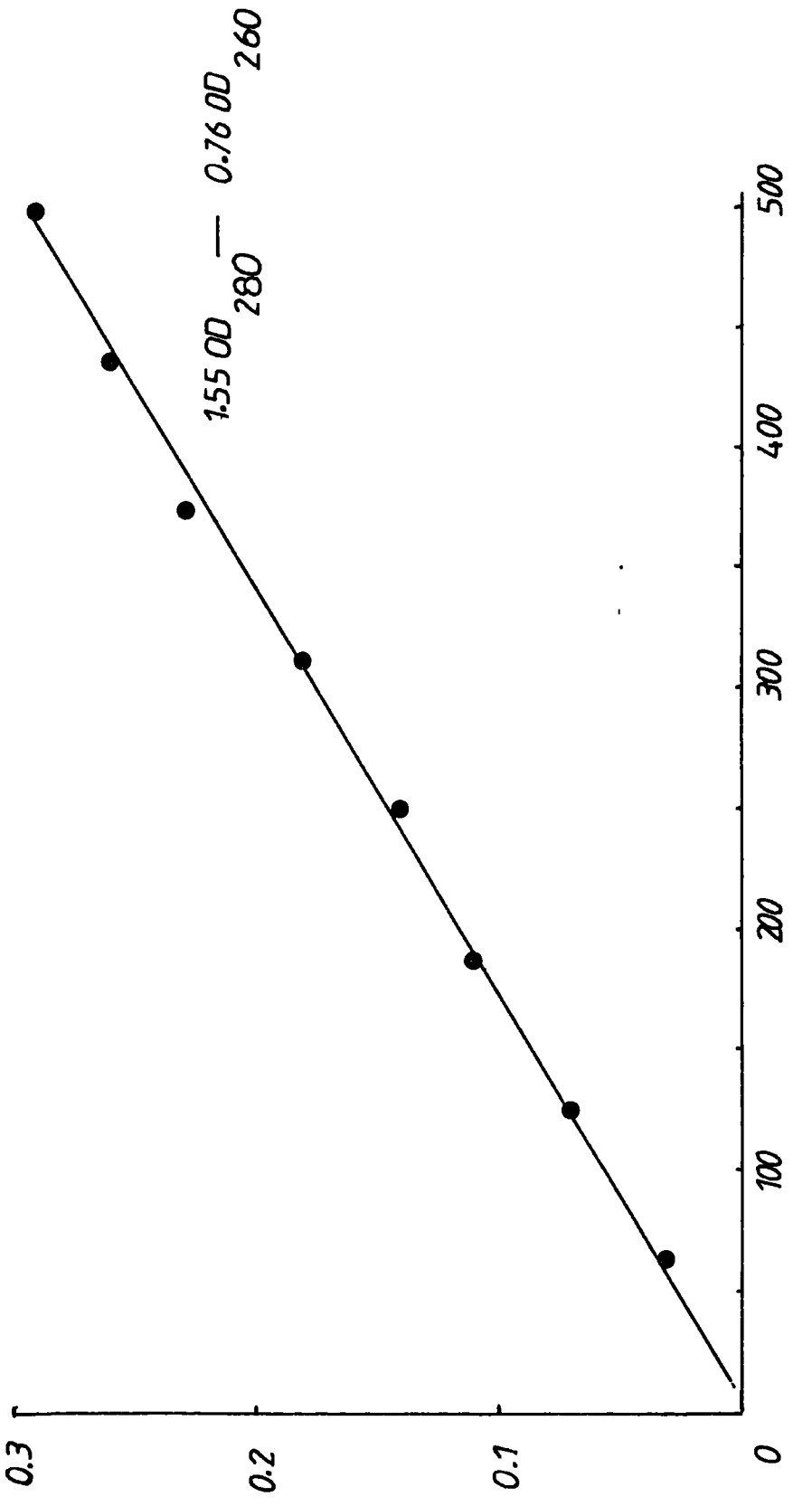


Figure 4.12.2. Protein calibration by UV absorption using BSA as a standard.

(b) Ultraviolet Absorption

Protein concentration was also determined by measuring the absorption at 280 nm and 260 nm and using the following equation (Layne, 1957):

protein concentration (mg/ml)= $1.55\ E_{280} - 0.76\ E_{260}$

A calibration was carried out using BSA as a standard protein (Figure 4.12.2).

Since the trypsin inhibitor has a very low absorbance at 280 nm measurements were taken at 212 nm.

XIII. Susceptibility Trials and Feeding Trials of Callosobruchus maculatus

Both susceptibility and feeding trials were carried out at the Tropical Stored Products Centre.

(a) Vigna unguiculata Variety Trial

Susceptibility trials were carried out on 7 different varieties of cowpea. These were: TVu 37, TVu 57, TVu 76, TVu 1190E, TVu 1502-1D, TVu 2027 and TVu 4557. Before infestation, seeds of each were placed in a vial in a controlled temperature humidity (CTH) room at 27°C and 70 per cent RH. When they had conditioned, 10 pairs of Callosobruchus maculatus, which had emerged from cowpeas within the previous 24 hours, were put into each vial and left in the CTH room to lay eggs for 24 hours. The following day each seed was examined and all eggs but one removed; this eliminates competition, so providing optimum growth conditions afforded by that particular variety. Any seeds without eggs were discarded. The seeds of each variety were then divided randomly into 4 lots of 20; however, in some cases there were only sufficient seeds with eggs to set up 2 or 3 replicas. The replicas were placed in ventilated

perspex boxes, approximately 75 cm x 40 cm x 2 cm, and returned to the CTH room until the progeny started to emerge. At this stage they were observed daily and any adults found were removed and the number noted, thus making it possible to determine the mean development period, and the total number of insects which reached the adult stage. The emerged adults were compared across the varieties for morphological differences. X-ray analyses were carried out on some of the infested cowpea varieties.

Subsequently a second, slightly modified, susceptibility trial was set up including two more varieties of cowpea, TVu 3629 and Farrin Wake, and a wild host of C. maculatus, V. unquiculata subspecies dekintiana. In this trial 25 replicas of each variety, each of 1 seed, were used. After the seeds had conditioned in the CTH room one female adult, which had emerged within the previous 48 hours, was put into each vial and left in the CTH room to lay eggs for 4 hours.

(b) Oviposition Trials of C. maculatus using Different Varieties of V. unquiculata

In the first set of oviposition trials (carried out at IITA), the following cowpea varieties were used: TVu 1090, TVu 1398, TVu 2027, TVu 2030, TVu 3629 and TVu 4557. For each variety 1 pair of virgin adults were placed in a vial containing 2 seeds and left for 24 hours. After that duration the beetles were then transferred to 2 new seeds; this was continued until 8 to 10 replicas had been obtained, replica number being dependant upon the availability of material.

In the second oviposition trial (carried out at TSPC) the following cowpea varieties were used: TVu 37, TVu 57,

TVu 76, TVu 1190E, TVu 1502-1D, TVu 2027, TVu 3629, TVu 4557, and Farrin Wake; Vigna unguiculata subspecies dekintiana was also included. For each variety, 25 replicas, of 1 seed each, were set up. Infestation of the conditioned seeds was carried out in the same way as that of the second susceptibility trial.

(c) Feeding Trials: Comparison of Callosobruchus maculatus Survival to Adult on Different Treatments of Cowpea Meal

In the first series of feeding trials artificial beans were prepared by compressing the meal, with a minimum volume of deionised water containing the fraction, into pellets of uniform size and compactness. However, these did not prove to be very satisfactory since the surface appeared to be unsuitable for egg laying. The problem was overcome by placing the treated meal into small plastic lids (approximately 15 mm diameter and 3 mm deep) and encasing each with "cling film"; this resulted in a slightly domed smooth surface of about 15 mm diameter on to which the female adult could lay eggs. Up to 10 replicas of each treatment were set up.

The lids were then placed in a C. maculatus culture in which there were large numbers of recently emerged adults, and left for 6 to 7 days. After this period they were removed from the culture and once the eggs had hatched, the number of eggs on the cling film surface immediately in contact with the meal were noted. Twenty one days later, the lids were removed from the culture, the cling film removed and the content of each were sieved individually and the number of surviving larvae and pupae (and in a very few cases, adults) were noted.

Statistical analyses were carried out for each treatment. (Where applicable, the trypsin inhibitory activity of the different fractions used was tested).

IX.6 Gel Filtration in 6M Guanidine Hydrochloride (Mann and Fish, 1972)

A column of Sephacryl S-200 superfine, 1.6 cm dia. x 52 cm (105 ml volume) was packed in water and equilibrated with 50mM tris-HCl buffer, pH 8.1, containing 6M guanidine hydrochloride and 1mM dithiothreitol. Buffer flow was pumped upwards through the column bed. Reduced, carboxymethylated protein samples (10 mg in 2 ml column buffer) were applied to the column and eluted at a flow rate of 4.1 ml/hr. The column effluent was monitored at 280 nm, and 2.7 ml fractions were collected to give the elution volume of the peaks. Elution volumes were plotted against log (molecular weight) to give a calibration (Figure 2.8.13).

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