Breeding phaseolus vulgaris (common bean) for resistance to the major pest bruchids Zabrotes subfasciatus and Acanthoscelides obtectus. Biochemical bases for seed resistance in wild lines

Minney, Benjamin Hugo

Breeding *Phaseolus vulgaris* (common bean) for resistance to the major pest bruchids *Zabrotes subfasciatus* and *Acanthoscelides obtectus*. Biochemical bases for seed resistance in wild lines

by

Benjamin Hugo Minney

Submitted for the degree of:

Doctorate of Philosophy

to:

Department of Biological Sciences

Durham University

1990

Abstract:

Phaseolus vulgaris (common bean) is an important source of protein for human and animal consumption. Economic losses post-harvest in storage are primarily due to the bruchid beetles *Acanthoscelides obtectus* (Bean Weevil) and *Zabrotes subfasciatus* (Mexican Bean Weevil). Wild-lines exhibiting resistance to these two species have been found and the mechanisms of resistance to each species are investigated. The mechanisms of resistance to the two species are found to be multiple, and different for each species.

In the case of *Z. subfasciatus*, the presence of a novel storage protein and absence of the conventional storage protein constitutes the primary mechanism. The novel protein (*"arcelin"*) is antimetabolic when included in artificial diets. *In vitro* digestibility studies indicate that it is indigestible to *Z. subfasciatus* larval gut proteases, and since *arcelin* constitutes the major protein of the seed the larvae starve. Arcelin has a similar amino-acid sequence to PHA. Also present is *WBAI*, a highly specific inhibitor of larval amylase of the two bruchid pests *Z. subfasciatus* and *Callosobruchus maculatus*, whilst having virtually no inhibitory activity on the mammalian amylase, nor on other insect, bacterial nor fungal enzymes. *WBAI* is similar in gross structure to the conventional amylase inhibitor obtainable from RKB (commercial *P. vulgaris*), and both are also immunologically similar to PHA.

Both of these mechanisms are suitable for incorporation into commercial seed, and the former has already been tested, using meal from F_2 seeds, in feeding trials using rats, confirming absence of mammalian toxicity. Resistance to *A. obtectus* damage is accompanied by reduced starch content, and high content of an acidic polysaccharide (whose structure has not been elucidated). No protein cause for resistance was found.

Inheritance of resistance to *A. obtectus* is probably recessive. Since the factor responsible for resistance is not a primary gene product and is expressed recessively, this factor is unsuitable for incorporation into breeding lines to be used for developing commercial cultivars.

Dedication

Close on four years of work is dedicated to Dr Angharad Gatehouse, always a friend and mentor.

I dedicate this volume to Ali and Emma.

Acknowledgements

I would like to thank almost everyone in the department for assistance of one kind or another at one time or another; and also to the staff of Microcomputing Ltd for help whilst writing up. Special thanks are due to Dr John Gatehouse my supervisor, Ms Julia Bryden and Ms Michelle Atterby for technical assistance, and Mr John Gilroy for extensive advice. Dr P. Dobie and Dr C. Haines, and the team at ODNRI Chatham, provided much assistance, and Professor D. Boulter allowed the use of Department facilities. The work was funded by a grant from ODA.

Declaration

The work in this thesis is all original except where acknowledged. Technical work was provided by J. Bryden and M. Atterby; bioassays on artificial diets were performed at ODNRI (Slough or Chatham), and I am indepted to Drs J and A Gatehouse for guidance.

Abbreviations

28k	Protein subunits of 28kDa relative molecular mass
35k	Protein subunits of 35kDa relative molecular mass
Arc	Arcelin (similar amino-acid sequence to *Phaseolus* lectin)
b.p.	years before present
CBAI	Cultivated Bean Amylase Inhibitor (inhibitor of animal amylase found in cultivated *Phaseolus vulgaris*)
Chi^2	A statistical test comparing frequencies observed with frequencies expected for a null hypothesis **H0**
Da	Dalton (a measure of protein molecular size)
df	degrees of freedom of a set of data
et al.	*et alias*, and other workers
F_1, F_2, F_3	generations of hybrid cross
GII	Globulin protein fraction II, *Phaseolus* globulin
H0	Null hypothesis (useful for Chi^2 tests)
ibidem	in the same place
M_r	Relative molecular mass
P	probability level for a particular null hypothesis
PHA	*Phaseolus* HemAgglutinin (*Phaseolus* lectin)
r, r^2	correlation coefficient between two variables
WBAI	Wild Bean Amylase Inhibitor (inhibitor of bruchid amylase found in wild *Phaseolus vulgaris*)

Table of Contents

List of Figures

List of Tables

List of Plates

Introduction 1
Beans and Beetles

1.1 ORIGINS OF THE COMMON BEAN *PHASEOLUS VULGARIS*

The parentage of the common cultivated bean (*P. vulgaris*) has been hard to determine, as there is no wild equivalent in the archæological record. Certain traits have been used, such as photoperiod specificity, to indicate potential origins; these factors in addition to the archæological record of the appearance of the 'modern' bean suggest two distinct sites of evolution; the first in South America (Bolivian altiplano), and the second in MesoAmerica (Mexico-Guatemala) (Kaplan 1981).

The first 'modern', or large-seeded, beans appear at least 10,000-8,000b.p. (=years before present) in South America, 7,000b.p. in Mexico, and between 7,000 and 5,500b.p. in North America. Their sudden prevalence is probably as a result of reduced pressure from weevil pests, due to human gathering practices. The advantage of large-seededness is in providing a food source for increasing seedling vigour, e.g. to reach the light (Kaplan 1981; Gentry 1969), but its disadvantages are that fewer seeds can be produced for a given investment of energy, and that the seeds are more attractive to pests. For a given cultivar seed size remains constant throughout the archæological record (Gentry 1969). These relative advantages and disadvantages may have altered the relative abundances of cultivars, rather than large-seededness being selected for by human cultivators (Gentry 1969). The pressure of pests destroying the viability of the seeds is likely to be reduced by the practise of humans gathering the pods before they can become infested, or shelling the seeds before the larvae can move from pod to seed. Collected seeds may then germinate and grow where they fall at the shelling site; however it is unlikely that cultivation of *P. vulgaris* was practised at this time (Kaplan 1981). Beans were probably first domesticated in the Andes and in MesoAmerica 7,000b.p.; modern characteristics are fully established by this time. However the record shows them becoming an important domestic crop only 1,800 to 1,200b.p..

Parentage of the cultivated lines has been ascribed to crosses between *P. vulgaris*

and *P. coccineus*, or *P. aborigineus*. However, *P. coccineus* is not found in Andean South America (Kaplan 1981), and cultivated types of *P. vulgaris* are closer in form to wild *P. vulgaris* than they are to either of these species (Gentry 1969). Today, wild types of *P. vulgaris* are found in isolated stands in a long and disjunct range, from west central Mexico through Central America and along the eastern slopes of the Andes to northwest Argentina (10° to 25°N in Sierra Madre Ocidental). Their altitude range is between 800-3000m, annual rainfall 500-1,800mm, and some of the wild types can tolerate frosts (Kaplan 1981). Cultivation of legumes is worldwide, constituting a major source of protein (see Table 1, page I.25), and *P. vulgaris* is grown in medium to high altitudes in the tropics, and in subtropical and temperate regions (Meiners and Elden 1980; Singh and Van Emden 1979).

1.2 BRUCHIDAE

Bruchids are in the superfamily Chrysomeloidea (suborder Polyphaga; series Cucujiformia (Crowson 1960)) which also includes leaf beetles (Chrysomelidae) and longhorned wood borers (Cerambycidae). Chrysomeloidea have the most diverse larval feeding habits of the insecta, as different species feed on leaves, roots, and stems, or mine leaves, or bore the stems of herbaceous plants. However the larvae of Bruchidae feed only in seeds, throughout their development, usually completing the larval stages within a single seed (Johnson 1981b). The common name 'Seed Weevils' has come about because the short snoutlike mouthparts of adult bruchids superficially resemble the mouthparts of the snout beetles (Curculionidae), and the larvae feed on seeds. 'Seed beetles' would be a more accurate description (Johnson 1981b).

At present there are 1,300 known species, within 56 genera, amongst the Bruchidae; 80% of these species are in the subfamily Bruchinae (Johnson 1981b). Some genera originated in the Old world (Africa, Europe) and some in the New world (the Americas), but now most species of economic importance can be found world-wide (Dobie *et al.* 1988; Birch *et al.* 1985).

Tracing their origins has been hampered by a lack of early fossils, but certainly an ancestor can be dated back to the early Cretaceous period (136M b.p.), probably as insects with characteristics of the Cerambycidae: the larvae would have lived in dead

woody stems, and the adult would have been short-lived and free-living, and visited the then equivalent of flowers to drink nectar, or drink sap from broken stems; ancestral adults would not have eaten foliage or solid plant tissue (Crowson 1960). The first fossils are to be found in Florissant Miocene shale beds, dating to the early Cretaceous, and possibly the Jurassic era (193M b.p.): i.e. they may have evolved as pests of Leguminosae shortly after the Leguminosae arose (Johnson 1981b). Divergence of the subfamilies can also be traced: Curculionoidea date back to Upper Jurassic (90M b.p.), and Chrysomeloidea are also likely to date from this period (Crowson 1960).

The life cycle of pest species can take two forms; uni-voltine (one cycle per host-plant growing season); or multi-voltine (many life cycles, i.e. successive generations of insect on the same season's seed). Most species that live on wild *Phaseolus* are uni-voltine since the plant seeds are widely separated in space by the time the adult emerges and seeding habit is annual (even in perennial species of *Phaseolus*), but those species that are able to be multivoltine are the species having the greatest economic impact, as they can do considerable damage to seeds in storage because the multiple generations lead to exponential population growth (Birch *et al.* 1985; Dobie *et al.* 1988).

1.2.a Life History of *Zabrotes subfasciatus* Boheman

Mexican Bean Weevil. subfamily Amblycerinae
synonyms: *Spermophagus dorsopictus* Lepesme
S. subfasciatus (Boheman)
S. musculus Boheman
S. (Zabrotes) pectoralis Sharp

Description: The oval-shaped beetles are approx 2mm broad and 3.5mm long. In the female the elytra is strongly marked with white patterning on a dark (almost black) background. The male has a uniform light-brown pubescence over a dark cuticle, with a shorter, broader, squarer elytra (Dobie *et al.* 1988). Eggs are glued onto the bean testa, and the larvae press against the wall of the egg to penetrate the testa and enter the seed. Optimum development occurs 32°C at 70% r.h., with a generation time of 24-25 days. Temperature range for sustaining development is 20° - 38°C (*ibidem*)

This species originated in the New World, and is particularly important in Central

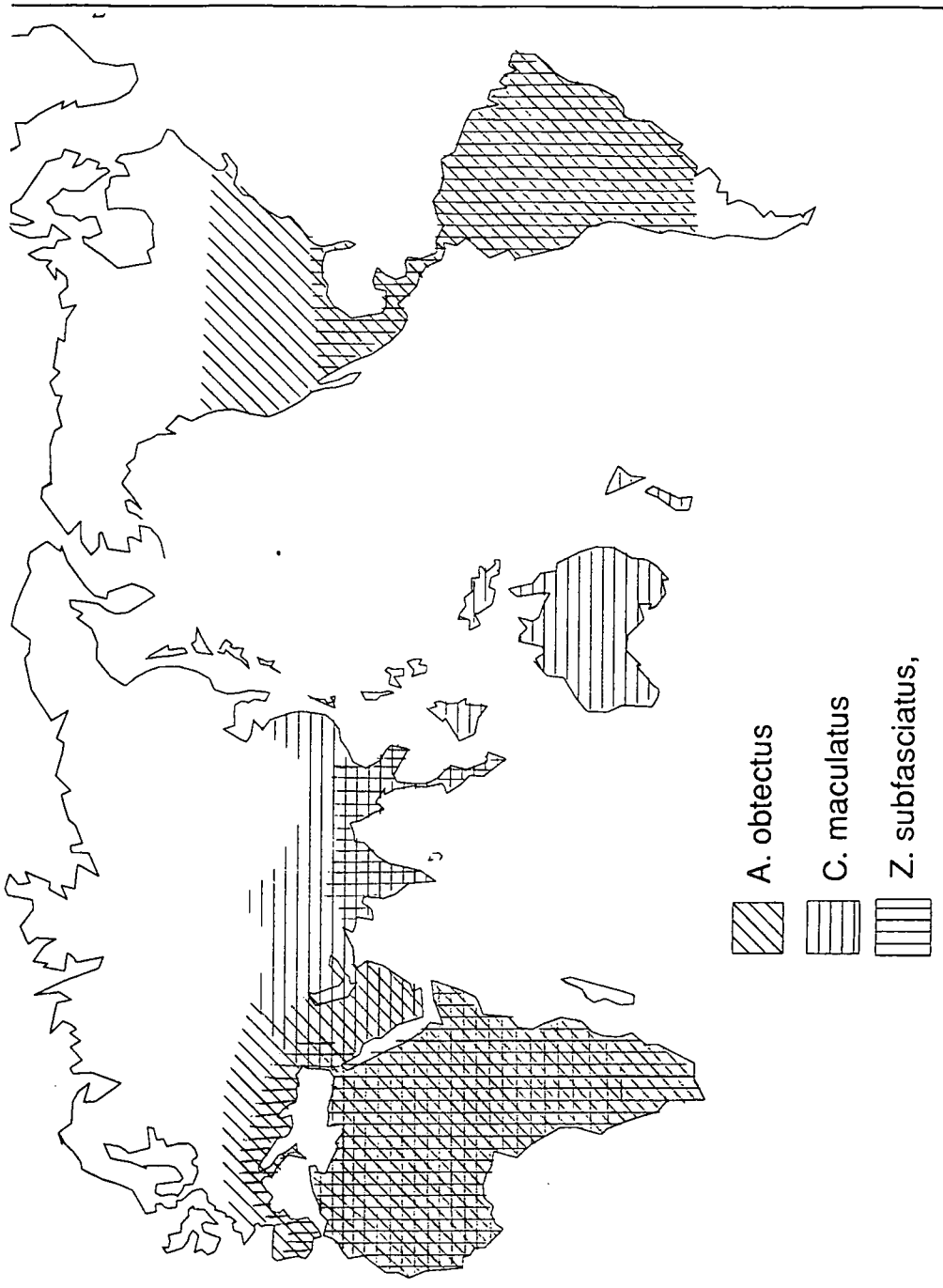


Figure 1 Distribution of the bruchid beetles *A. obtectus*, *C. maculatus* and *Z. subfasciatus*, the major pests of *P. vulgaris*

and South America. It is also found in other tropical and subtropical regions, especially Central and East Africa, Madagascar, the Mediterranean, and India (Dobie *et al.* 1988) (see Figure 1). Because of its optimal temperature range it is mainly found in the low areas in Central America, e.g. Cauca and Magdalena valleys in Colombia (Labeyrie 1981), and in the Tepoztlan region of Morelos, Mexico at altitudes lower than 1500m. Here *Z. subfasciatus* is in competition with *A. obtectus* but the high daily temperature (>35°C) sterilises *A. obtectus* males (Biemont and Bonet 1981) allowing *Z. subfasciatus* to spread widely.

Z. subfasciatus is a major pest of *P. vulgaris* and *P. lunatus*, and also attacks *Vigna unguiculata* (cowpea) (reported in Uganda and West Africa) and *V. subterranea* (groundnuts) (Hill 1983). The adults lay eggs on the crop in the field, as well as on seed in store, and populations of adults emerging from seed attacked in the field will find themselves (in the store room) well placed to continue the attack in stored beans. The multivoltine reproductive habit is facultative.

1.2.b Life History of *Acanthoscelides obtectus* (Say)

Bean Weevil subfamily Bruchinae
Synonyms *Bruchus obtectus* Say
Bruchus varicornis Mutschulsky

Description: the main body colours are greys, browns and reddish-browns, without any distinctive patterns. The antennae are dark grey except segments 1-5 and 11 which are reddish, as are the abdomen and the legs; the prothorax and elytra are yellowish-brown to brown. The male can be distinguished from the female as in the male the pygidium is vertical and only partly visible from above, whereas in the female it is oblique and fully visible (Dobie *et al.* 1988). Eggs are laid loosely, often inserted into the pod in proximity to the seeds, or lodged under cracks in the bean testa, and the larvae usually penetrate the seed using cracks or holes in the testa. This genus, consisting of 250-350 species, tolerates a wide range of temperatures from 18°C (slow breeding) to an optimum around 30°C, when the generation time may be 22.5 days (*ibidem*).

A. obtectus originated in the New World, but because its wide temperature range

encompasses temperate and sub-tropical ranges it has now spread to most warm regions with the possible exception of Australia, and to some temperate regions (Dobie *et al.* 1988) (see Figure 1). A number of features of its life cycle, e.g. no imaginal diapause, its intolerance to high temperatures, the adults showing a preference for *P. vulgaris* crops without canopy, point to a savanna type high altitude origin, probably the Colombian piedmonts altitude 1200 - 1500m (Labeyrie 1981), and it is certainly found in the Antioquia altiplano, Nariño highlands and Cordillera valleys, where there are host species throughout the year (Labeyrie 1981), and the Andean Cordillera in temperate zones between 1000m and 2000m (Huignard and Biemont 1978). *A. obtectus* did not reach Europe until 300 years after *P. vulgaris*, and its origins have been indicated as the equatorial zones in South America (Huignard and Biemont 1978).

A. obtectus is a specialist insect, attacking only the seeds of Leguminosae, particularly those of Phaseolinae: *P. vulgaris* and other *Phaseolus* are the principal and probably the original hosts of the Bean Weevil; *A. obvelatus*, *A. argillaceus* and *A. clandestinus* are also found on these. Of *Acanthoselides* species 75% pupate within a single seed: 70% of species oviposit on the pod, 29% on seed, and 2 species oviposit on both (*A. obtectus* and *A. chiricahuae*). Not many species have multiple generations (multivoltine) (Johnson 1981a). Its origins have been indicated as the equatorial zones in South America (Huignard and Biemont 1978). Of *Acanthoselides* species 50% have only one host species in *Phaseolus*, and 20% 2 hosts. The same occurs in reverse, with 60% of *Phaseolus* species only supporting one species of *Acanthoselides*, and 14% two. 90% of *Phaseolus* support development of less than 4 species of *Acanthoscelides* each (Johnson 1981a).

A number of different population types occur for this insect, depending on the environmental conditions (primarily on the availability and annual habitat of the principle host) giving rise to univoltine habit on wild Phaseolinae and plurivoltine habit on cultivated *P. vulgaris* and on the stored seeds of *P. vulgaris* (Huignard and Biemont 1978). In the fields the females lay their eggs inside the mature pods, whereas in the store the eggs are deposited near the beans. After hatching the larvae perforate the seed coat and post-embryonic development occurs inside the cotyledons.

The presence of bean seeds or bean pods may be needed to stimulate oviposition, as may copulation (Huignard and Biemont 1978). This stimulation may be followed by a latent period of oogenesis.

1.3 SPECIFICITY

Insect attack on mature seeds of Leguminosae is primarily limited to a specialised family of insects, the Bruchidae; and different species within this family show varying degrees of specialization with respect to host species (Gatehouse *et al.* 1990).

Most species of bruchid are restricted to developing on seeds of a single genus of host plant, and occasionally to a single species. Very rarely can a bruchid species develop successfully on more than one genus (Johnson 1981b); the species can be said to exhibit more or less strict oligophagy (Applebaum 1964). Because of this, indigenous bruchid species will rarely colonise introduced legumes (e.g. *Vicia faba* L. was introduced to Japan and was pest-free until the pest was also introduced; *Caryedon serratus* took over 500 years to infest the introduced groundnut (*Arachis hypogea*) in Africa (Southgate 1979)), and there is some correlation of the number of species of beetle able to develop on a particular legume's seeds and the oldness of the legume in the area (Varaigne-Labeyrie and Labeyrie 1981). This is probably due to the wide range of secondary compounds found in different legume species: many secondary compounds are likely to be toxic to some animals, and detoxification pathways are energy-consuming so few are likely to be present in any one pest species (Janzen *et al.* 1977a). The range of these secondary compounds and the predation by individual beetle species of individual legume species means that there is a limited range of host species in common to Homoptera, Coleoptera, Lepidoptera, Diptera and Hymenoptera (Jermy 1984).

Coevolution, that is, development of a species of pest in close association with the plant host so that one develops resistance and the other a means to overcome it, has demonstratedly occurred in some bruchid species. Studies of the relatedness of genes of plants with genera of bruchids suggest that in many cases plant defences are overcome and colonising species have become pests opportunistically (Jermy 1984). Credland has observed with a Turkish strain of *C. maculatus* that whilst the preferred host is cowpea (*Vigna unguiculata*) when this is not available the cowpea weevil is able to use lentil (*Lens culinaris*), though it takes a few generations before development time is nearly as it was for the original host, and no longer extended considerably (Credland 1987).

Host specificity has been quantified by estimating the percentage of all species in a particular genus of bruchid that are able to feed on seeds of any species within a particular genus of the Leguminosae. The same calculation as is applied to genera could be applied to subtribe, tribe or subfamily, and has been given a **percentage score** which indicates specificity. For the **Papilionoideae** 67-100% of *Acanthoselides*, *Bruchidius*, *Bruchus*, *Callosobruchus*, *Caryedes*, *Caryopemon*, *Conicobruchus*, *Ctenocolum*, *Kytorhinus*, *Meibomeus*, *Specularius*, *Stylantheus*, and *Zabrotes* can and do develop to adults on these seeds. Of these, *Acanthoselides* and *Bruchidius* are able to develop in seeds of **Mimosoideae**, whereas *Zabrotes* and *Caryedes* are able to develop in seeds of **Caesalpinioideae** (Johnson 1981b). 38% of accessions of *Phaseolus* supported development of the New World bruchids *A. obtectus* and *Z. subfasciatus*: 16% of accessions supported development of Old World bruchids *C. maculatus* and *C. chinensis* (Simmonds *et al.* 1989); resistance to the Old World bruchids is more prevalent in New World Papilionidae than in Old World Papilionidae (Birch *et al.* 1985). Nearly all species of *Acanthoselides* are specific to species of *Phaseolus*, and vice versa (Johnson 1981a).

Another feature of specificity is that many pests will choose to feed on only one species of host in a particular region even though others are adequate nutritionally, and may be used in other regions (Simmonds *et al.* 1989). This may be to enhance the chances of finding a mate, or for other reasons (Jermy 1984). An example of this specificity is *Caryedes brasiliensis*, which may only attack one species per habitat, in high altitude Costa Rica this is *Dioclea wilsonii*, in low altitudes in Costa Rica it is *D. reflexa*, and elsewhere the host is *D. megacarpa* (Janzen 1981). Similarly *Z. subfasciatus* may only oviposit on *P. lunatus* in Central America, even when other host species are present (Simmonds *et al.* 1989). Some species are facultatively more generalist than they are by choice, and the widest host range is that of *Stator limbatus*, which although it has only 6 host species, these are from three separate Mimosoid genera. Even so bruchid beetles exploit a very narrow range of plants; where *S. limbatus* is found exploiting six different hosts there are a total of around 2000 species of plant in the same locality (Janzen 1981).

1.4 ECONOMIC IMPORTANCE

Pulses (Leguminosae) are grown in all the major continents of the world (with the exception of Antarctica) and the seed is used as a major source of protein. Some measure of their importance can be gained from comparing production of pulses with production of other major agricultural products: cereals, roots and meat, as in Table 1. The common bean (*P. vulgaris*) is the predominant grain legume of South and Central America, and is also grown extensively in East Africa and Asia. Estimated world hectarage is 2.4 million ha; this can be compared with 8.6 million ha for chickpea (*Cicer arietinum*), 8.7 million ha for cowpea (*Vigna unguiculata*), 4 million for pigeon pea (*Cajanus cajan*), mung bean (*V. radiata*) and green gram (*V. aureus*) totalling 2.9 million, and lentil (*Lens culinaris*) 0.5 million ha (Singh and van Emden 1979). As Leguminosae species are so widespread, they have naturally attracted a wide range of pests, those which attack the growing plant as well as those which attack the seed. The most important of these pests are given in Table 2.

Species in several bruchid genera are pests of economic plants and thus have become cosmopolitan through commerce. Old World genera *Bruchus*, *Callosobruchus* and *Caryedon* are especially notorious pests as are species in New World genera *Acanthoselides* and *Zabrotes* (Johnson 1981a + b). Economically the most important species are those which are multi-voltine, able to multiply in the dry seeds during storage. The most important of these are *Callosobruchus maculatus* (attacking *Vigna unguiculata*, and some *Phaseolus* though not *P. vulgaris*), *Acanthoscelides obtectus* (*P. vulgaris*, *Voandzeia subterranea*, *Glycine max*), *Caryedon serratus* (Caesalpinaceae), and *Zabrotes subfasciatus* (*P. vulgaris* and *Vigna* species), and to a lesser extent *Bruchidus atrolineatus* (Decelle 1981). The specificity of attack by a particular species, especially within the host genera, limits the likelyhood of currently innocuous species becoming pests of economic importance on cultivated legumes (Johnson 1981a). The most recent recording of a new commercial pest species is infestation of pigeon peas (*Cajanus cajan*) by *A. zeteki* in the Caribean, with infestation reaching 40% after a few months' storage. *Specularius sulcaticollis* PIC. has also been observed attacking pigeon peas in Kenya (Southgate 1979).

Total losses due to insect damage are difficult to estimate. Many dwarf, short-

Table 1 Pulse Production Worldwide, compared with other major staples. 1000M tonnes in 1984, with +/- % change p.a. 1965-1984.

	Cereals		Roots		Meat		Pulses	
Africa	63434	+1.6	98015	+2.6	7422	+2.7	5432	+1.3
South America	77599	+3.2	39510	-0.4	11437	+2.9	3471	+0.3
Asia	774915	+3.4	233490	+2.1	32519	+4.9	23706	+0.3
Europe	309175	+2.5	112303	-1.6	40821	+3.0	4129	-1.5
Oceania	29762	+3.9	2961	+1.5	3959	+2.4	615	+11.5
North & Central America	387690	+2.7	23494	+1.1	30983	+1.8	3380	+1.6
All Developed Countries	883221	+2.2	225924	-1.2	94190	+2.5	13429	-0.4
All Developing Countries	921813	+3.4	369364	+2.0	49626	+3.9	34383	+0.6

Table 2 Major pests of Beans and Grams (*Phaseolus spp.*)

Hill 1983

Site of Infestation	Latin Name	Family	Distribution
Sap suckers, toxic saliva	*Acanthomia spp.*	Coreidae	Africa
Larvae roll leaves	*Lamprosema spp.*	Pyralidae	India, S.E. Asia
Defoliate	*Epilachna varivestis*	Coccinellidae	U.S.A., Mexico
adults eat leaves	*Ootheca mutabilis*	Chrysomelidae	E. Africa, Nigeria
scarify foliage	*Tetranychus spp.*	Tetranychidae	Cosmopolitan
larvae bore stem	*Ophiomyia phaseolii*	Agromyzidae	Europe, Africa, India, S.E. Asia, Australia
adults eat pollen	*Coryna spp.*	Meloidae	Africa, Asia, S. America
infest flowers	*Taeniothrips sjostedti*	Thripidae	Africa
adults eat flowers	*Mylabris spp.*	Meloidae	Africa, India, S.E. Asia
adults eat flowers	*Epicauta spp*	Meloidae	Africa, Asia, S. America
infest flowers	*Apion spp.*	Apionidae	Cosmopolitan
larvae bore pods	*Maruca testulalis*	Pyralidae	Pantropical
larvae bore pods	*Heliothis armigera*	Noctuidae	Cosmopolitan in Old World
larvae bore sown seed	*Delia platura*	Anthomyiidae	Cosmopolitan
attack ripe seeds	***Acanthoscelides obtectus***	**Bruchidae**	**Cosmopolitan**
attack ripe seeds	***Callosobruchus spp.***	**Bruchidae**	**Cosmopolitan in warmer regions**
attack ripe seeds	***Zabrotes subfasciatus***	**Bruchidae**	**Cosmopolitan**

Pests damaging ripe seeds in pods or in storage shown in bold

duration pigeon peas (at IITA, Ibadan) regularly give zero yield if not protected by insecticides, whereas if protected, yields of 1500 kg/ha can be obtained; and tall, long-duration varieties may increase yields 5 × with insecticides compared with unprotected crops. In general, grain legumes can be said to increase yields 2× to 10× when protected by insecticides during growth. Insect pests are likely to be the most important limiting factor on legume yields (Singh and van Emden 1979).

Post-harvest losses due to insect damage are easier to assess, and range from 25 to 40% of total losses, depending on country etc., and are the major cause of loss (see Table 3) (FAO 1977). Infested seeds can be seriously damaged in storage with 80% of individual seeds damaged within 6-8 months. Bruchids at any stage of life cycle in the seed render it unpalatable, the emergence holes render processing difficult, and the larvae deposit high levels of uric acid in the seeds (Southgate 1979).

Table 3. Post-harvest losses of pulses (Grain legumes not including groundnuts or Soybeans); by circumstance. % loss of commercial value due to unpalatability, unsaleability, *etc*

FAO 1977

Region and Country	Total % Post-Harvest	Broken Grains	Insect Damage	Mould Damage	Rodent Damage
AFRICA					
Nigeria	1.4		30-70		
Swaziland			6.8-24.6	0.2-14.5	0.2
Uganda	10.3-19.0	4.0-6.4	0.7-2.0	5.6-10.6	
LATIN AMERICA					
Nicaragua	10-35		63		
Average	15.5-28.5				

Laboratory trials involving drilling holes to remove 1, 5 or 10% of seed weight of seeds of *Mucuna andreana* to mimic the action of *C. maculatus* have been done, with the result that the seedling was less able to recover from artificial herbivory (Janzen 1977c), which showed that this artificial herbovory decreased seedling viability on germination. Of the less economically important legumes, seed predation of *Bauhinia*

pauletia (Leguminoseae) by *Gibbobruchus cristallis* (Bruchid) resulted in loss of viability of 45% of seed crop (Janzen 1978a). Of 81 fruit crops of *Pithecellobium saman* in Pacific coastal lowlands, approximately 50% of ovules and 22% of full-sized seeds have been reported aborted. *Merobruchus columbinus* (Bruchid) was found in all samples, and a total of 43% of seeds were killed by insect attack of which only 1-3% was attributed to *Stator limbatus*, the remainder being attributed to *M. columbinus* (Janzen 1977b). In many cases the viability of more than 70% of seeds was destroyed in storage by the bruchids, though studies have not been widely reported in non-economic legumes. However seeds protected from stress are often able to germinate in spite of bruchid damage, e.g. both *Pisum sativum* and *V. unguiculata* showed a negative correlation of germination and seedling survival with the number of emergence holes, but some germination in spite of damage. Seed size plays a part here, since a single larva of *C. maculatus* will consume approximately a quarter of a cotyledon in a large-seeded *V. unguiculata*, whereas it will consume the whole of both cotyledons in small-seeded *P. radiata*, thereby removing all chance of germination (Southgate 1979).

Economic damage to legumes by insect pests, in particular by bruchids, has been measured as indicated above. However there is damage that is not possible to quantify. Many legumes will grow in arid and nutritionally deficient environments, where other plants will not survive. They are often very important ecologically, both to colonise land (e.g. after a volcanic larva flow), and to limit the spread of desertification. Bruchids may limit the spread of legumes, thus preventing this valuable activity.

On the converse side, because of their colonising ability some legumes are weeds on agricultural land. Bruchids could be used to control these, although their proximity to a crop would mean care would have to be taken to control possible migration of the pest to a grain crop (Southgate 1979).

Introduction 2
Physical barriers

Seed resistance to a pest can be defined as factors present in the seed or pod which by their presence reduce or prevent damage to the seed by this pest.

Resistance to the Old World (*C. maculatus* and *C. chinensis*) and New World (*A. obtectus* and *Z. subfasciatus*) bruchids in Legume seeds can be classed into six categories, depending on the stage of larval development at which development is terminated, as follows:

0- adults emerge; i.e. no resistance

1- larvae develop but die when they reach pupation, or mature to adults and then fail to emerge; this results in seed damage but there is no growth of pest population which would result in wider spread damage of stored product

2- larvae die as they penetrate through the testa into the cotyledon; the result is cosmetic damage only

3- eggs laid, however larvae die before penetrating the testa; cosmetic damage results from eggs attached to the testa

4- eggs laid but development arrested before hatch

5- no eggs laid

(this classification is compiled from Simmonds *et al.* 1989)

Some factors involved in determining why development of the bruchids is arrested at each stage are given below; physical barriers would result in resistance stages 2 to 5, whereas biochemical factors could account for all stages 1 to 5.

2.1 ENVIRONMENT

The environment in which the *Phaseolus* grows can affect its pest relationships. For instance, *A. obtectus* does not infest stands of *P. vulgaris* where it is grown under a canopy of another crop e.g. corn (maize), as the gravid females are unable to locate the host plants (Labeyrie 1981). In the converse case *Gibbobruchus cristicollis* attacks *Bauhinia pauletia* (Leguminosae) trees in a deciduous forest; however, as surrounding trees are removed, less damage to the seeds is observed, and when the legume trees are in the open and surrounded by grass pasture there is no damage by this pest (Janzen 1978a).

Another protection strategy is to produce seed with such a long intervening period that many pest individuals have died in the intervening period. This strategy is illustrated by the legume *Ateleia herbert-smithii* (Sophoreae), which normally produces one seed crop in three years: natural predators of the pest *Apion* (*Apionidae*, Coleoptera weevils) reduce the population to a level at which it does not devastate the following seed crop. At times when a seed crop is produced within two years of the previous one, devastation of the crop is far greater by this pest, suggesting that a three year gap reduces survival of adults and limits damage to the seed crop (Janzen 1981).

Other defences, e.g. rapid dehiscence of the pod, special characteristics of the husk, synchronisation of seed set, special seed characteristics, all probably evolved to reduce pest damage, have mostly been circumvented by opportunistic evolution on the part of the bruchids (Center and Johnson 1974).

2.2 TESTA

The testa is the hard tegument surrounding the seed, and it has a number of functions to fulfil. Perhaps the most important of these is to prevent seed dessication during the quiescent period, when conditions may not be favourable to seedling growth. Since many Leguminosae use vertebrate dispersal agents, which eat the fruit, pass the seed through the gut, and deposit the seed some distance from the parent plant (and in an environment favourable for growth), the testa also needs to be resistant to mammalian gut enzymes to preserve the seed (Janzen 1981). This dispersal mechanism is so

widespread that many seeds require the stimulation of being in the mammalian gut before they germinate. Both of the above protective features are mainly physical attributes of the testa, its hardness and impermeability.

Toxins may also be concentrated in testa, e.g. azetidine-2-carboxylic acid is found in *Delonix regia* and *Schizolobium parahybum*, and has been shown to be toxic to *C. maculatus*. Larvae develop in testa-free cotyledon meal, but *C. maculatus* is not normally a pest of this species in the wild, suggesting an important role for the toxin in the testa (Janzen 1977).

The testa is also often an important feature of the seed for host recognition by the pest.

2.2.a Testa Hardness

The testa itself presents a mechanical barrier to pest penetration (Stamopoulos and Desroches 1981). In addition, physical features of the testa may be important for recognition; very smooth or very rough seeds are not liked for oviposition, although *A. obtectus* may lay in the troughs on very rough seed coats (Nwanze *et al.* 1975; Nwanze and Horber 1976; Simmonds *et al.* 1989). The egg (normally cemented to the testa to aid larval penetration) may not be cemented to the seed coat properly on some varieties, e.g. *Z. subfasciatus* cannot penetrate *P. vulgaris var. aborigineus* (Simmonds *et al.* 1989), and macrosclerids in the testa of some rough varieties of cowpea prevent penetration by *C. maculatus* (Nwanze and Horber 1976).

The mechanical barriers have different effects on different species of bruchid pest. Different species of Bruchid are able to drill through different hardness of testa e.g. *C. maculatus* normally penetrates the very thin testa of *Vigna unguiculata*, wheras *Mimosestes sallaei* drills through the thick *Acacia farnesiana* seed coat (Janzen 1977). Different species of bean have different hardnesses of seed coat: *P. vulgaris*, *P. coccineus* and *L. purpureus* have roughly the same physical hardness, and roughly twice as hard as that of *P. lunatus*. Moisture content of the seed affects testa hardness, with seeds of 6% m.c. roughly 18x harder than those with 42% m.c., though in the comparison of the testa hardness of different species all seed moisture contents were the same.

On the other hand *A. obtectus* larvae penetrate *P. vulgaris* and *P. coccineus* more than *L. purpureus*, and virtually do not attempt to penetrate the testa of *P. lunatus*, suggesting that in addition to testa hardness stimulants or depressants to larval penetration behaviour are also present (Thiery 1984). Some species have an extra thick testa, e.g. *P. acutifolius* and *P. anisotrichus* (0.6mm compared with the more normal 0.06-0.1mm). This extra thickness prevented penetration by four species of bruchid in a laboratory trial (Simmonds *et al.* 1989).

Although the testa may contain toxins (see above) this may have no effect on the larvae penetrating it as the testa may be bypassed by the larva, passed undigested through the gut or avoided by penetrating through a crack in the testa. That the testa of even susceptible varieties, e.g the commercial haricot bean, is toxic to the major pests *A. obtectus* and *Z. subfasciatus*, is easily demonstrated by including the testa in artificial diets (Stamopoulos and Huignard 1980).

2.2.b Deterrence and Recognition

Before the pest species oviposits, some form of recognition is likely to occur to determine if the eggs are being laid on seeds in which the larvae will be able to develop (Nwanze *et al.* 1975). Host selection is probably sensorial (Applebaum 1964) and is likely to encompass a variety of stimuli, including *physical* (seed hardness and curvature, roughness of coat, colour), and *chemical*, which **includes** attractants (that is, chemicals the presence of which the pest has evolved to be attracted or stimulated into oogenesis and oviposition by) and **deterrents**. In general, the presence of toxins and deterrents in the seed surface are easy to demonstrate in the laboratory, but the absence of recognition signals or feeding excitants is very much less easy (Kogan and Paxton 1983). Plant varieties or species resistant to a particular bruchid pest species on the whole exhibit reduced egg lay and reduced larval penetration on the seeds (Goméz *et al.* 1985); and in the case of *P. wrightii* this has been attributed to the lack of chemical stimulus for oviposition (Simmonds *et al.* 1989). In other cases the absence of egg lay has been attributed to lack of recognition by the pest species. However in laboratory trials using *P. filiformis* chemical deterrents are associated with the surface of the testa, as evidenced by gravid females appearing to avoid the seeds (Simmonds *et al.* 1989).

2.3 SEED SIZE

Bruchid predation may have been a limiting factor on the evolution of large-seeded varieties. Large-seededness confers on the seedling the ability to grow more vigorously initially and compete in a densely populated forest. Each large seed contains a large amount of energy compared with a small seed, so in a single seed crop there will be fewer large seeds, or more small seeds. For this reason damage by a pest has the potential to be more devastating on the next generation of a large-seeded variety than on a small-seeded variety; e.g. *Tachigalia versicolor* (Caesalpinieae) (large-seeded) is damaged by *Amblycerus* (Janzen 1981).

Studies on the effects of seed size or numbers of seed per pod on the rate of attack by bruchids (Varaigne-Labeyrie and Labeyrie 1981), on the number of eggs laid on a seed, or on the emergence of adults (Simmonds *et al.* 1989), shows that there is no correlation between any of these parameters. However a single large seed may contain the energy which could feed many smaller seeds to a lesser extent, so the destruction of one larger seed by pest attack is all the more disastrous (Simmonds *et al.* 1989).

Introduction 3 & 4
Biochemical Basis of Resistance

There are two levels of biochemical resistance observed in legume seeds, general resistance which prevents infestation by a wide range of pests, and specific resistance targetted at specific pest species, in particular those able to counteract the general resistance chemicals. The former is universal in legumes, whereas the latter may be demonstrated in a few wild types of a particular species, these wild types exhibiting resistance to an insect which is normally a pest of this particular species.

Many of the biochemicals involved in resistance to pathogens and pests are products of secondary metabolism. The definition of a product of secondary metabolism is that the product is not essential to the metabolism of the cell (Swain 1977), since any products of primary metabolism (apart from excretion products such as oxalic acid) are likely to be metabolised by the pest (or pathogen), as this organism will have similar essential biochemical pathways to those of the host. Secondary compounds accumulate in the seed, which is not able to respond defensively to damage since it is in a quiescent state; and these secondary compounds can be considered to have a role specific to the seed since they often disappear soon after germination. There are probably 400,000 secondary compounds used as defense in plants, in the same order of magnitude as the number of different plant species (Swain 1977).

Nitrogen-containing defenses (e.g. non-protein amino-acids, alkaloids, lectins, and protein-based enzyme inhibitors) may have a dual role, serving also as a nitrogen store to be used by the growing seedling. Other defenses which do not contain nitrogen, such as tannins or phenolics, and substances which can not be metabolised readily, such as lignin, cannot serve the dual purpose, and can be thought of as more "expensive" to the seed energetically (Janzen 1981): on the other hand, when nitrogen is in short supply e.g. in an environment which is poor nutritionally, defences which do not tie up nitrogen can be "cheaper" in scarce resources (whilst being more "expensive" in terms of energy, which is not a limiting resource in these environments) (Fagerström 1989).

At the present time it has been difficult to ascribe the resistance of a particular species to one particular substance, since although many substances are known to be

toxic to the pest when fed in artificial diets the seed itself often exhibits higher levels of resistance than would be expected. In particular Simmonds and co-workers have found no correlation between resistance of *Phaseolus* to bruchid attack and content of individual lectins, saponins, or trypsin inhibitors, and this effect has been ascribed to possible synergy, or to resistance compounds not yet detected (Simmonds *et al.* 1989).

The presence of both protein and non-protein antimetabolites, and their specific action against the main bruchid pest species, differs between Old World accessions and New World accessions: 70% of accessions exhibited toxicity to the Old World bruchids (e.g. *C. maculatus* and *C. chinensis)*, whereas only 20% exhibited toxicity to the New World bruchids (e.g. *A. obtectus* and *Z. subfasciatus*). This difference is further brought out in inheritance studies, as resistance to Old World bruchids tends to be dominant, (Simmonds *et al.* 1989), whereas to New World species it is often recessive.

Introduction 3
Non-Protein Antimetabolites

3.1 ALKALOIDS

The presence of the natural insecticides *alkaloids* has been associated with insect resistance, but their high toxicity to mammals (Fellows 1989), and their effect on taste, making the meal bitter and unpalatable (Nowacki 1980), has minimised their value for incorporation into food crops. These compounds are particularly widespread in the Leguminosae: approximately 60 genera in the subfamily **Papilionoideae** contain alkaloids (Kinghorn and Smolenski 1981).

Many alkaloids are sugar analogues, consisting of sugar rings containing nitrogen, but highly hydrophobic (Fellows 1989). They are normally considered to be derived from amino-acids (Levinson 1976). These inhibit glucosidases, with different alkaloids affecting glucosidases or mannosidases of different animals e.g. castanospermine is inhibitory to mammalian α-glucosidase, DMDP[1] to *C. maculatus* β-glucosidase, and swainsonine to mannosidases (Fellows 1989). As a result they can impair the function of the nervous system, and may also inhibit RNA synthesis (Levinson 1976).

Some insects have evolved ways of dealing with alkaloids, for example nicotine from *Nicotiana tabacum* is disgorged unchanged through the gut by *Manduca sexta* and *Heliothis viriscens*, and cocaine is excreted unchanged by *Erythoxylum coca*. In *Eloria noyesii* nicotine from *N. tabacum* is stored unchanged as a predator repellant (Blum 1983).

As well as their perceived role in preventing predation (in plants containing them, castanospermine inhibits aphid feeding, and DMDP inhibits *Spodoptera* feeding (Fellows 1989)) these compounds may have a role in the regulation of protein synthesis in the

[1] 2,5-dihydroxymethyl-3,4-dihydroxy pyrrolidine, an analogue of β-D-fructofuranose

plant (Swain 1977). However the former is probably their major role since alkaloids are often secreted into the ancillary structures e.g. vacuoles, bark, leaves and fruit (Levinson 1976).

3.2 NON-PROTEIN AMINO-ACIDS

Non-protein amino-acids are small molecules of amino-acid structure $NH_2-CH-COOH$ (with *side chain* attached to the CH), which for chiral reasons or because their side chain is different, are not one of the 20 amino-acids found in functional proteins, nor one of the biochemical intermediaries. Their toxic effects are caused by their mistaken incorporation into proteins as substitutes for normal amino-acids, which upsets the normal folding of the protein rendering it non-functional.

Non-protein amino acids have been associated with a protective role in legume seeds since their presence at high concentrations is associated with freedom from insect attack (Rehr *et al.* 1973). In trials several non-protein amino acids were lethal to *C. maculatus* at concentrations of 0.1% in artificial diets (Janzen *et al.* 1977), whilst certain amino-acids may accumulate in seeds to very high concentrations e.g. 5-hydroxy-L-tryptophan reaches 14% dry weight of *Griffonia simplicifolia* seeds, canavanine 7-10% dry weight of *Dioclea megacarpa*, and L-3,4-dihydroxyphenyl alanine 8% dry weight of *Mucuna multisiana* (Bell 1976). They are widely present in the Leguminosae, especially Papilionoideae in which canavaline is found in 60% of spp (Nowacki 1980). Evidence exists that they are present as a defensive mechanism, although they may also be present as a nitrogen store. 40% of seed canavanine in *Dioclea megacarpa* (Janzen 1981) and 60% of canavanine in *Medicago sativa* are used up during germination (Bell 1976): the remainder may be exuded by the seedling to reduce inter-specific seedling competition immediately after germination (Bell 1981). The concentration in the seed will be limited by how much extra energy is required to transform them into a usable form of amino-acid at germination, balanced by the minimum concentration required to confer resistance to predation. Larger seeds, which tend to rely on protection because of the high investment of energy into each one, contain higher concentrations of non-protein amino acids than smaller seeds which may escape predation by their larger

numbers, for the same total energy cost to the plant.

In most cases non-protein amino-acids are a general defensive mechanism (Rosenthal and Bell 1979), and this is perhaps best illustrated by the specific tolerance exhibited by certain pests of legumes, e.g. *Caryedes braziliensis* is tolerant to canavanine, which is present in *Dioclea megacarpa* at 7—10% of seed dry weight; the bruchid has evolved a unique enzyme mechanism to recognise the abnormal amino-acid, and to convert it to useful amino-acids (Rosenthal *et al.* 1976; Rosenthal *et al.* 1977; Janzen 1981), though it has been noted that free amino-acids present at high concentrations (up to 5%) in seed meal may be toxic whether they are normal or non-protein forms (Janzen *et al.* 1977).

3.3 TERPENOIDS

Although a few specialist bruchids do develop in host seeds containing terpenoids (Johnson 1981), several of these complex isoflavonoids are known to exhibit insecticidal properties, and may therefore play a role in legume seed protection against the non-pest species; their major effect is to cause a dramatic decrease in oxygen uptake. Four rotenoids have been isolated from the seeds of the legume *Lonchocarpus salvadorensis* which appears to be attacked by very few of the indigenous bruchids in Costa Rica (Birch *et al.* 1985). These rotenoids, tested at physiological concentrations in artificial diets (0.3 to 0.003% DW), were either lethal to *C. maculatus* or at least detrimental (Birch *et al.* 1989).

Green orange peel also contains terpenoids which effectively prevent insect and mammal predation before ripening (Kogan and Paxton 1983), and milkweed (*Asclepias humistrata*) contains steroids which upset the metabolism of the sucking insect *Cycnia inopinatus* (Blum 1983).

Saponins are steroidal glycosides, and are also found in various legume seeds. Various saponin fractions have been demonstrated to be toxic to *C. maculatus* (Applebaum *et al.* 1965), or to affect the palatability and hence reduce intake (Nowacki 1980). Similarly sapogenins, whilst not affecting larval development in *C. chinensis*, have been demonstrated to be oviposition deterrents for this insect (Applebaum *et al.* 1965), or to cause hormone imbalance and upset the membrane permeability of the

insect gut (Applebaum and Birk 1972).

The presence of saponins at high concentrations in seeds of some *Vigna* species is associated with resistance to the bruchids *C. maculatus* and *C. chinensis*, however in many seeds of this and *Phaseolus* species saponin levels are low, even in seeds resistant to attack, suggesting a secondary role (Birch *et al.* 1985).

3.4 POLYPHENOLS

Tannins are probably the most important group of plant secondary defensive compounds (Swain 1977). Acting generally they complex with proteins and inactivate enzymes (by *H*-bonding from the phenolic -OH to N-containing amino-acid side chains) (Powers and Whitaker 1977; Swain 1977), and are often produced in response to predatory attack (Kogan and Paxton 1983).

The *condensed* types, more effective than the *hydrolysable* types and also termed pro-anthocyanins, are widespread in all higher plants (Swain 1977). In legume seeds tannins are confined to the region near the testa and to the testa itself: however many bruchids bypass this level of defense by passing the testa through the gut undigested or boring through the testa without ingesting (see section 2.2.a, page I.31). Pests of a particular species of legume may not develop on the bean meal when the testa is mixed in with the meal, although they are able to enter the cotyledon and develop normally having passed through the testa and into the intact seed (Stamopoulos and Desroches 1981). Tannins may also be overcome by elevated gut pH, hydrolysis by gut enzymes, or by adsorption to the peritropic membrane, although none of these tolerance mechanisms have been documented for legume pests (Becker 1984).

3.5 CYANOGENS

Cyanogenic glycosides release the simple toxin *hydrogen cyanide* when subjected to hydrolysis, e.g. from predator gut enzymes. Wild Costa-Rican lima beans contain up to 3.45% linamarin, which is a cyanogenic glycoside (Janzen 1981), and cyanogenic

glycosides (cyanogens) are thought to be a defensive mechanism, although the contents of these cyanogenic glycosides vary enormously between individuals, and in *P. vulgaris* coloured seed generally contains higher concentrations than white (<2mg/100g) (Nowacki 1980).

Another legume which makes use of cyanogens as a means of defence is the *Acacia*, in the mature plant. Those species which do not contain cyanogenic glycosides have hollow thorns and a symbiotic relationship with ants which protect the plants from insect predators; if the ants are removed from these plants pest damage is severe, suggesting both a protective role for the ants and for the cyanogenic glycosides in other species (Janzen 1981).

3.6 POLYSACCHARIDES

A wide variety of polysaccharides are present in legume seeds, and they have not been well characterised. What few studies have been done indicate that some polysaccharides present in particular seeds are toxic to non-pest insects, and may act (probably synergistically, given the number of other factors also present) to cause pest resistance.

In particular, pectosans contained in *P. vulgaris* seeds have been implicated in resistance against *C. chinensis* (Ishii 1952), and a heteropolysaccharide present in resistant seeds at 1% prevents *C. chinensis* larval development, although at this concentration it does not affect a pest of *P. vulgaris*, *A. obtectus*. At 2% in artificial diets it also caused *A. obtectus* to fail to develop, and the difference in antimetabolicity between pest and non-pest has been ascribed to differential digestion of the hetero-polysaccharide, resulting in an inactive form (Applebaum and Guez 1972). Similarly lignin extracted from the tegument of *P. vulgaris* has been shown to be toxic to *A. obtectus*, though its role in conferring resistance in the seed is probably minimal (Stamopoulos 1988).

The crude non-starch carbohydrate extract (approximately 4% DW of seed meal) from a line of *P. vulgaris* which is resistant to *A. obtectus* is able, incorporated into artificial diets at this physiological concentration, to confer the greater part of the observed wild-line resistance to this insect (Gatehouse *et al.* 1987). This confirms that

carbohydrates, and in particular polysaccharides, may confer both general resistance, i.e. to non-pests, and specific resistance to particular pest species.

3.7 FLAVONES AND ISOFLAVONES

Whilst flavones are found in many Papilionoideae, their role in combatting insect predation is unproven (Kogan and Paxton 1983). One role proposed is as a response to pathogenic mould attack, 'though these compounds are very toxic to mammals by their œstrogenic effect (Nowacki 1980). However their main roles in the plant (rather than in the seed specifically) are in protection of the seedling (Kogan and Paxton 1983), as growth regulators, and as insect pollinator guides (Swain 1977).

Introduction 4
Protein Antimetabolites

Protein antimetabolites which confer resistance to insect pests are found in the cotyledons of beans. Using proteins as a means of natural resistance to insect pests has an important advantage to human cultivators: heat (cooking) can be used to denature and inactivate protein antimetabolic activity (Liener 1980) before human consumption, wheras of course the insects are not able to denature the proteins in this way; and since proteins are primary gene products the genetics for introducing the means of resistance are simpler than for the introduction of a whole biochemical pathway to manufacture a secondary compound conferring resistance.

4.1 LECTINS

Lectins are present in *P. vulgaris* landraces or commercial cultivars at varying levels, and these landraces may be categorised according to dryweight content of lectin into the ranges low (1.8% of seed meal), medium (2-2.5%), or high (3.5-3.6% of seed meal, or up to 15% of total protein in the cotyledons) (Pusztai *et al.* 1979). An example of a variety containing high levels is the commercial cultivar *Processor*. The lectin in the seed is thought to have a role as a storage protein; in *Lens culinaris* the lectin appears in the seed cotyledons as they mature and dry, and is all used up within 2-3 weeks of germination (Howard *et al.* 1972). In cultivars of *P. vulgaris* containing no lectin (absence of lectin is a single recessive allele) the major storage protein *phaseolin* compensates in quantity for the absence of lectin, and the absence of lectin does not appear to affect yield or a number of other agronomic parameters (Osborn and Bliss 1985). Individuals which are lectinless may also occur in landraces which are normally considered to contain lectin (Brücher 1968), and there may be considerable variation in the levels of lectins present between individual seeds.

There is some evidence that the lectin family of genes, which includes members encoding proteins that are carbohydrate-binding (the traditional "lectin"), and also proteins which, whilst encoded for by a very similar sequence, do not exhibit

carbohydrate-binding properties, are primarily concerned with protein-based resistance to insect attack in *P. vulgaris*; this family of genes has been shown to encode **PHA** (the 'original' lectin in *P. vulgaris*, which is probably a factor in resistance to *C. maculatus*, many other non-pest species, and vertebrates), **arcelin** (a factor causing wild-line resistance to one of the major pest species *Z. subfasciatus*) (Osborn *et al.* 1988), and the mammalian α-amylase inhibitor (termed in this work **CBAI**) (which is active against both mammalian and *C. maculatus* enzyme) (Moreno and Chrispeels 1989). Proteins encoded for by this family of genes have also been detected in the maturing bean seed but their function not resolved in every case (Cerrotti *et al.* 1989).

Agglutinating lectins, the phytohæmagglutinins, are thought to have a role in insect resistance, as inclusion of *P. vulgaris* lectin in artificial diets at 0.5% prevents the development of *C. maculatus* (Janzen *et al.* 1976). The lectin in the diet binds to the gut epithelial wall and may disrupt it in this insect, and may also disrupt adipose tissue at pupation (Gatehouse *et al.* 1989); this mechanism is very similar to that observed in mammalian guts, in which the lectins bind to and disrupt the gut epithelial walls (King *et al.* 1980). Lectin binding also upsets the balance of production of digestive enzymes causing lethal nitrogen losses through the gut. The lectins are heat-labile, e.g. the lectin of the navy bean can be rendered ineffective and non-binding (hæmagglutinating) by autoclaving (121°C for 5 mins) (Kakade and Evans 1965).

Some reports of undigested starch in rat faeces, which have led workers to believe that the *P. vulgaris* α-amylase inhibitor has a major part to play in the low effective nutritional quality of raw beans in mammalian diets (Jaffé *et al.* 1973), could also be explained by prolonged lectin exposure causing disruption of the digestive processes in the gut (Powers and Whitaker 1977). Other protein components of the seed may also have a part to play, and the *P. vulgaris* α-amylase inhibitor may be important for its role as an enzyme inhibitor. The Kidney bean amylase inhibitor, when fed at physiological concentrations (0.5%) in artificial diets free of lectin, prevents the development of non-pest species *C. maculatus* and *C. chinensis* (Ishimoto and Kitamura 1988; Ishimoto and Kitamura 1989), and conversely some species of both *Vigna* and *Phaseolus* contain high levels of lectins but are infested by bruchids (Birch *et al.* 1985).

The species of bruchids which are able to develop on *P. vulgaris* show tolerance to the seed lectin, e.g. *A. obtectus* does not appear to digest the lectin itself, but the lectin does not bind to the gut epithelium, so presumably no disruption occurs

(Gatehouse *et al.* 1989).

Lectins may have other roles in the seed. They may have a role in embryogenesis (e.g mitogenic stimulation in *Lens culinaris*), and their carbohydrate-recognition properties suggest that they may have a post-translational transport role in the cell (Fellows 1989). Other lectins may not have this role e.g. in the pokeweed (*Vicia cracca*) lectins are found throughout the plant, and although the pokeweed mitogen is known to affect other species it has no deleterious effect within *V. cracca* (Howard *et al.* 1972).

4.2 ARCELIN

Seed protein in *P. vulgaris* constitutes between 19 and 22% (dry weight) of the seed. The protein groups making up this whole are just one or two major storage (probably) protein groups making up around half of the total, with all of the remaining biochemically important proteins making up the remaining half. In cultivated, bruchid-susceptible seeds phaseolin is 36-46% of protein, and lectin a further 11-13% (Osborn and Bliss 1985; Romero Andreas *et al.* 1986); lectinless phenotypes exist which contain no lectin and up to 61% of the protein as phaseolin (Osborn and Bliss 1985). A recently identified protein family named **arcelin** has a similar amino acid sequence to the PHA-lectin, and is also thought to play a role in insect resistance in *P. vulgaris* (Osborn *et al.* 1986); arcelin-containing phenotypes, which contain 12-14% phaseolin, and high levels of arcelin, have a similar overall total protein content, seed weight and reproductive fitness compared with normal beans (Romero Andreas *et al.* 1986).

The arcelin proteins have not been found in cultivated varieties of beans, and exist as four major variants, divided geographically. In each case (as with lectin and phaseolin) expression is controlled by a single dominant gene (Osborn *et al.* 1986). There is no genetic linkage with phaseolin inheritance, and relative levels of expression of these two proteins may be determined competitively; however arcelin expression is tightly linked to lectin expression (as determined in F_2 back-cross experiments) *(ibidem)*.

The presence of arcelin variant 1 has been shown to confer resistance to bean attack by *Z. subfasciatus*, and arcelin 1 has been shown to be an insecticidal factor in feeding trials (Osborn *et al.* 1988). Similarly the other arcelin variants have been

associated with insect resistance in the wild accessions in which they were detected (Osborn *et al.* 1986). Back-crosses into cultivated lines indicate that both arcelin 1 and arcelin 2 variants decrease % *adult emergence* and *adult weight*, and that all arcelin types increase the life cycle duration of *Z. subfasciatus* (Harmsen *et al.* 1988). These observed differences may be due to different mechanisms by which the arcelin types operate, or different levels of expression in the back-cross lines, since arcelin 1 has to be present at 10% in artificial diets to ensure significant levels of *Z. subfasciatus* resistance (Osborn *et al.* 1988).

4.3 CARBOHYDRASE INHIBITORS

Carbohydrase inhibitors, in particular inhibitors of the major animal carbohydrase α-amylase, have been detected in a number of varieties of *P. vulgaris*, and also at lower levels in *P. lunatus*, *P. coccineus* and *P. aborigineus* (Jaffé *et al.* 1973; Nagahiro 1981), and many of these are immunologically similar (Pick and Wöber 1978b). Perhaps the best characterised of the grain α-amylase inhibitors is that of wheat, which has been shown to be active against both *Tribolium confusum* (a wheat pest) and *C. maculatus* (not wheat pest) amylase enzymes *in vitro*, but only against *C. maculatus* enzymes *in vivo* (Gatehouse *et al.* 1986). In a similar way the RKB (*P. vulgaris*) α-amylase inhibitor inhibits the larval gut amylases of *C. maculatus* and *C. chinensis* both *in vitro* and *in vivo* (at the physiological concentration of 0.5%), and neither of these are pests of *P. vulgaris*; whereas it has no effect on larval gut amylase of *Z. subfasciatus*, which is a pest (Ishimoto and Kitamura 1989). *Z. subfasciatus* has two amylase enzymes, AII appears to be the enzyme found in other bruchids and is inhibited normally; and AI, detected in small quantities on a *V. unguiculata* diet, may be different (Lemos *et al.* 1990). The effect on *Callosobruchus* was independent of hæmagglutination activity, and Ishimoto and Kitamura suggest that the amylase inhibitory activity is the major factor involved in preventing predation on *P. vulgaris* by *C. maculatus* (Ishimoto and Kitamura 1988). The RKB α-amylase inhibitor does not inhibit plant α or β-amylase (Powers and Whitaker 1977; Pick and Wöber 1978a), nor *Bacillus* or *Aspergillus* α-amylases, but is inhibitory to the mammalian amylase (Pick and Wöber 1978a).

There is other evidence to suggest that these α-amylase inhibitors play a role in resistance of the seeds of *P. vulgaris* to bruchid predation: whilst *C. maculatus* α-

amylases have pH optima between pH 5.2 and 6.0 (Campos *et al.* 1989), mammalian α-amylases have optima around pH 7.0. Both the cranberry bean amylase inhibitor and that of white kidney bean have a greater inhibitory effect (form more stable complexes with enzymes) at pH 5.5 (Kotaru *et al.* 1987; Marshall and Lauda 1975). The amylase is also only produced during the final stages of seed maturation (Pick and Wöber 1978b). The α-amylase inhibitor from black bean (*P. vulgaris*) is not digested by rat proteases, both *in vitro* and *in vivo*. In fact this protein, probably present to reduce predation, has been used to counter diabetes sugar loading in rats because of the way in which it slows digestion of polysaccharides (Menezes and Lajolo 1987).

The *P. vulgaris* α-amylase inhibitor has a normal molecular structure as a tetramer (49k) of 14k subunits, containing 8.6% carbohydrate (Powers and Whitaker 1977), or 9-10% (Marshall and Lauda 1975) carbohydrate. The carbohydrate appears to be less important to its activity, since 70% of the carbohydrate can be removed with endo-β-N-acetylglucosaminidase H, with no effect on activity, wheras pronase treatment renders it inactive. Treatment of the amino-acids has also yielded interesting results; oxidation of a single tryptophan side-chain reduces inhibitory activity by 50%, and histidine is important to activity (Wilcox and Whitaker 1984).

Sequence analysis of the α-amylase inhibitor indicates that it is derived from a PHA-like gene (Moreno and Chrispeels 1989), further implicating this gene family as being involved in the expression of insect resistance.

4.4 PROTEASE INHIBITORS

Protease inhibitors, active against mammalian proteases (trypsin, chymotrypsin, etc) are found in many species of *Phaseolus* and other Leguminosae. The levels of protease inhibitors are highest in *P. vulgaris*, but are also high in *P. lunatus*, *P. coccineus*, and *P. aborigineus* (Jaffé *et al.* 1973). However their importance in preventing predation is less certain: although the trypsin inhibitor is present in navy beans and rats fed on a diet containing these beans fail to grow, this effect is more likely to be due to the presence of lectins and the amino-acid imbalance (in particular low content of sulphur-containing amino-acids) than to the trypsin inhibitor (Kakade and Evans 1965); protease inhibitors and urease are found in soybeans, but have no effects

on development of larvae of *C. chinensis*. Applebaum and co-workers found low levels of digestive activity in the bruchids (*A. obtectus* or *C. chinensis*) on the proteins casein or azocasein, which require trypsin or α-chymotrypsinogen for digestion, and concluded that these insects do not contain proteases as their larval development was not affected by the inclusion of the trypsin inhibitor found in soy-bean in the diet (Applebaum *et al.* 1965). Since then, three proteases, all cysteine types, have been isolated and characterised from the larval guts of *C. maculatus*. They all have pH optima between pH 5.5 and 6.0, and are inhibited by *Vigna unguiculata* cysteine proteinase inhibitor (Gatehouse *et al.* 1985; Campos *et al.* 1989; Xavier-Filho *et al.* 1987). Many of the proteinase inhibitors in Legume seeds are inhibitors of serine proteases (inhibitors of the more common animal proteases trypsin and chymotrypsin) (Southgate 1979): *A. obtectus* also is known to contain a thiol-type gut protease with pH maxima at pH 5.5 and 6.5 (Wieman and Nielsen 1988); *Z. subfasciatus* also has cystein-type proteases (pH optima 5.5), but in addition has aspartic-type proteases (optimum pH 3.5, inhibited by pepstatin) as a major protease (Lemos *et al.* 1990). Trypsin inhibitors were found in *V. unguiculata* varieties resistant to *C. maculatus*, though also in varieties susceptible to this bruchid, suggesting that they do not affect resistance to *C. maculatus* within the *Vigna* group either (Birch *et al.* 1985; Xavier-Filho *et al.* 1987), though a very high level of trypsin inhibitors in one cultivar was shown to confer resistance (Gatehouse *et al.* 1979). It is likely that the trypsin inhibitors are important in non-specific pest resistance, as inclusion of the cowpea trypsin inhibitor into artificial diets, and expression of the protein in transgenic tobacco plants, confers resistance to a wide range of insects which are not pests of *V. unguiculata* (Gatehouse *et al.* 1990)

4.5 NUTRITIONAL UTILISATION

A number of factors can affect the nutritional suitability of proteins to animal pests, above and beyond antimetabolic effects. Animals need a balance of amino-acids to build proteins for growth, and whilst certain amino acids can be interchanged within biochemical pathways different groups cannot be metabolised in this way. One case of this is that sulphur-containing amino-acids, methionine and cysteine, can be interchanged but because of the need to incorporate the thiol group cannot be formed from any other amino-acid; the amino acid composition of proteins in bean has levels of these amino

acids insufficient to support the growth of rats unsupplemented, and approximately half the equivalent content of the animal protein *casein* (milk) (Kakade and Evans 1965).

Proteins in bean cotyledons may also be complexed with other proteins, or with starch, which may reduce digestibility by restricting access to proteolytic enzymes (Semino *et al.* 1985). The storage protein **phaseolin** has three times fewer potential cleavage sites for the major proteases pepsin, trypsin and chymotrypsin than the animal protein BSA (bovine serum albumin), and when digested from its native state only up to 50% of these sites are cleaved (Romero and Ryan 1978). These same authors found a better correlation between protease digestion *in vitro* and nutritional value *in vivo* than by amino acid composition.

Other authors have found similar protection from animal enzyme digestion in the *P. vulgaris* storage protein phaseolin. Santoro reports a maximum of 66% of phaseolin is digested throughout the gut of rats, and considerably less by any single local group of digestive juices, indicating the combined sequential effect of the sequential sections of the gut have a major part to play (Santoro 1988). Phaseolin is poorly digested by the thiol-type protease from *A. obtectus* gut; however digestion is considerably improved by the presence of reducing agents, and crude gut extract digests phaseolin completely (Wieman and Nielsen 1988).

Introduction 5
Incorporating Resistance into Commercial Lines

A number of strategies can be adopted for the protection of crops from economically damaging insect attack. These can usefully be divided into the *externally applied processes*, and *internal*, or *"Grow with the plant"*, processes. In addition ***"Biological control"*** is the practise of influencing the environment to favour the crop and hamper pest and competitors.

Externally applied processes include the use of insecticides, or the use of antifeedants and pheromones, designed to confuse the pest rather than kill it. These all require continued input of often expensive chemicals, and have additional problems of access to particular parts of the plant, persistence in the environment, and effects of weather conditions on efficacy. *Internal* include both resistance factors found in related species or wild strains, which can be introduced into the plant by **cross-breeding**; and foreign forms of resistance which require **genetic engineering** to introduce them. Legume seeds are inherently, by the presence of a variety of non-specific antimetabolites (e.g. lectins, protease inhibitors) resistant to attack by insects.The rate of attack is not proportional to the presence of chemical barriers, nor to the size of seed, or seeds per pod. It appears to be mainly related to the oldness of the legume in the area, i.e. to how long pest species have had to become accustomed to it (Varaigne-Labeyrie and Labeyrie 1981).

Biological control, applied in the form of management strategies, involves influencing the environment by intercropping, introducing parasites, and harvesting practise. There are a number of parasites of bruchid eggs and larvae, in particular members of 10 families of Hymenoptera, and one family of Diptera. The bruchid larvae are well protected as they pass their whole life cycle within the bean seed. However, the bruchid eggs are relatively vulnerable, since they are usually laid on the surface of the ripening pod e.g. *Caryedon serratus* populations are severely checked (6 adults emerged from 3000 eggs in one study) by the trichogrammatid egg parasite *Uscana semifumipennis*. Other parasites have been documented which attack *Callosobruchus* and *Acanthoscelides* (Southgate 1979).

Pest behavioural aspects may be used to combat attack. *A. obtectus* infestation in seed store often begins by the gravid female laying eggs on the pod in the field in the first instance, so shelling the seed post-harvest will remove the larvae from proximity to the seed by removing the pod and the eggs which it contains. *A. obtectus* will also not settle to oviposit where the bean crop is accompanied by a canopy, e.g. of corn (Labeyrie 1981). Further studies need to be done on the ecology of the Bruchidae to elucidate cultural methods most suitable for effective control, but a number of effective cultural practises involving methods of drying seeds, and storage, are already used by some bean growing cultures (A. M. R. Gatehouse and C. Haines, observations in Colombia 1989).

5.1 CROSS BREEDING

When a species is introduced to an area for the first time, indigenous bruchid species are slow to colonise. Both after the introduction of *Vicia faba* to Japan (Varaigne-Labeyrie and Labeyrie 1981), and the introduction of *P. vulgaris* into Europe (Huignard and Biemont 1981), the pest bruchid came from the original source of material, in the European case 300 years later.

Insect pest damage is often a major selection pressure where it is present, and in centres of origin (e.g. for *P. vulgaris*, Mexico and Colombia) the distribution of landraces of a particular species may remain virtually unchanged over a long period because of this selection pressure (Kaplan 1981). However, where selection pressure from pests is relieved, for instance where a bean species has been introduced into a new geographical location and the pests have not caught up, the plants may undergo rapid phenotypic change to take advantage of the new conditions. This period of phenotypic change is exploited by plant breeders to develop new, and desirable, characteristics for incorporation into cultivated bean lines.

For this reason, the best place to search for landraces resistant to pest species is where resistance has been a long-term selection pressure, e.g. the centres of origin (Gentry 1969). CIAT has been setting up a germplasm and seed bank for Leguminosae in Colombia to this end. Initial findings were that genes conferring resistance in *Phaseolus* were not dominant (Meiners and Elden 1980) and therefore resistance was

not commercially useful (Schoonhoven and Cardona 1982); but results recently have been more promising with respect to *Z. subfasciatus* (Schoonhoven *et al.* 1983), in particular with the confirmation that the components associated with resistance are in the cotyledon rather than in the integument (Osborn *et al.* 1986; Cardona *et al.* 1989). In the case of *P. vulgaris* resistance to *Z. subfasciatus*, genetic inheritance is by a single dominant gene (Osborn *et al.* 1988). A single protein family, known as *"arcelin"*, closely related to PHA lectin ('though lacking the hæmagglutination properties of this protein) has been implicated in conferring resistance to *Z. subfasciatus*, and its antimetabolic effects confirmed in feeding trials. More details on this specific mechanism of resistance are given in Section 4.2 (page I.44), and this gene has been crossed successfully from the wild line into cultivars posessing agronomically useful characteristics, whilst still maintaining *Z. subfasciatus* resistance (Osborn *et al.* 1986).

In a great many cases the wild landraces of a species which have given rise to the cultivated varieties are also susceptible to the pests of the cultivated varieties, and the source for breeding resistance to a particular pest may not be possible to find within the species populations. However within the plant kingdom it is sometimes possible to produce inter-specific crosses within a genus, and this technique has been used to incorporate resistance genes into lines subsequently used for breeding commercially useful varieties.

Particularly successful hybrids arise from species which may have been the original "parents" of the species in question. An example of this is the successful incorporation into *Vigna radiata* (mungbean, or green gram) of resistance to a number of pests derived from *V. sublobata* (Kitamura *et al.* 1988; Fujii *et al.* 1989). The genetic basis appears to be a single dominant gene (Kitamura *et al.* 1988) in *V. sublobata* (a small, non-commercial bean), which confers resistance to *C. chinensis*, *C. phaseolus*, *C. analis*, *C. maculatus* and *Z. subfasciatus*, whilst not altering commercial characteristics of the *V. radiata* commercial varieties (Fujii *et al.* 1989). Potential sources of genes for resistance to bruchids, from plants already posessing agronomically useful characteristics, include *V. umbellata* (rice bean), *V. vexillata* (wild mung), and *P. acutifolius var latifolius* (tepary bean), the last of which has the additional advantage of possessing biochemical pathways for drought tolerance (Birch *et al.* 1985). Resistance of *V. unguiculata* to *C. maculatus* is inherited as the expression of two genes (Xavier-Filho *et al.* 1987).

Within *Phaseolus* possibilities for cross-breeding have been observed, both as naturally occuring hybrids and as a result of laboratory crosses. *P. vulgaris* can cross with *P. flavescens* (Colombian), *P. polyanthus* and *P. coccineus* (Kaplan 1981), though this has not yet been used to incorporate resistance genes.

5.2 GENETIC ENGINEERING

It may not be possible to find genes conferring insect resistance amongst the close relatives of *P. vulgaris*; hence cross-breeding to incorporate resistance may be impossible. Another possibility is to obtain suitable genes from an unrelated plant, and incorporate them into the genome of the target crop by genetic engineering.

However, the possibilities for this are limited, as the factor responsible for resistance has to be a primary gene product, i.e. a protein, to ensure that the DNA is inserted successfully in its entirety: whilst the relatively small amount of genetic material required to code for a protein may transfer, the technology used is not sufficient to ensure the transfer of the series of genes which code for a complete biochemical pathway, such as would be necessary to introduce a secondary compound (Hilder *et al.* 1989). Not all transfers of genetic material produce a predictable result, and fear has been expressed about the interactions of the new genes with the original biochemical pathway and genetic makeup of the plant.

At least 30 species of plants, including both herbaceous and woody dicotyledonous plants, and monocotyledonous plants, have been successfully transformed by genetic engineering, including maize, rice, and 14 other food crops (Gasser and Fraley 1989). Monocotyledonous plants cannot normally be transformed using biological vectors, and foreign genes have been introduced by physical means, or by protoplast regeneration; in dicotyledonous plants tumour-forming bacteria, such as *Agrobacterium tumefaciens* or *A. rhizogenes*, have been used successfully.

These biological techniques are still in their infancy for a great many plant families, and at this stage only two kinds of insect resistance have been introduced by this technique: the *Bacillus thuringiensis* modified endotoxin, and the cowpea trypsin inhibitor (Hilder *et al.* 1990). It was originally thought that insect-resistance conferring proteins would have to be expressed to at least 1% of total protein to be effective, and

concern was expressed that this may affect total yield of the plants (Boulter *et al.* 1989). However the legume trypsin inhibitor CpTI has been successfully incorporated into tobacco plants by genetic engineering, resulting in broad spectrum resistance to lepidopteran pests of tobacco with no yield losses (uninfested transformants compared with uninfested controls) even at expressions of the gene up to 2% of soluble protein in the leaf; expression was promoted using the CaMV promotor with NOS-NEO screening (Cauliflower mozaic virus promotor and kanamycin tolerance) and observed gene expression appeared unrelated to copy number but related to the exact insertion point in the host genome (Hilder *et al.* 1990). Foreign genes are often stably inherited as dominant single copies without yield loss in the progeny, and the transformation is often effective in the field as well as in the laboratory (Uchimiya *et al.* 1989).

Whereas the cowpea trypsin inhibitor gene (CpTI) confers resistance to a wide range of lepidopteran, coleopteran and orthopteran pests, and has no toxicity to mammals (as it is degraded by pepsin in the mammalian stomach) (Hilder *et al.* 1990), the *Bacillus thuringiensis* toxin is more specific in its activity, with different forms of the toxin obtained from different strains of *B. thuringensis* exhibiting an antimetabolic activity to different ranges of lepidopteran, and in some cases coleopteran and dipteran pests as well: the gene for the modified endotoxin which has been used for transformation confers resistance to a range of lepidopterans (Gasser and Fraley 1989), and whilst it is only expressed in host plants at levels around 0.1% of dry weight of tissue it is toxic to the pests at these levels.

5.3 OVERCOMING RESISTANCE

As implied above, the number of pest species which attack a legume species will be broadly related to the oldness of the species in the area (Varaigne-Labeyrie and Labeyrie 1981). As well as the spread of pests from one region to another, pests are able to overcome resistance mechanisms aimed at them, e.g. *A. obtectus* has been shown to be tolerant of the lectin content of *P. vulgaris*, at levels which are toxic to *C. maculatus* (Gatehouse *et al.* 1989), and *Caryedes braziliensis* has evolved tolerance to high levels of the non-protein amino-acid canavanine to make use of the seeds of its host plant *Dioclea megacarpa* (Rosenthal and Bell 1979).

The advantage of multi-component, as opposed to single factor, resistance is that it is more robust to the adaptations of the predators. Multiple mechanisms operating in the same plant give a broad range of resistance to a population of pests which often consists of different strains each one tolerant to one or a few of the resistance mechanisms encountered. The likelihood of pests overcoming resistance is also considerably reduced since this would require concurrent multiple favourable mutation events (Hilder *et al.* 1990).

However this ideal is rarely achieved in practise in a single plant, because of the metabolic cost of insect resistance. A mixed population may have a number of different mechanisms spread amongst different individuals, which would ensure that with given predation at least some individuals escape pest damage: cultivars of cotton (*Gossypium hirsutus* L.) possess different ratios of antibiotic compounds, giving a population tolerance to *Heliothis viriscens* (Fab) (tobacco budworm) (Hedin *et al.* 1983); however preparing populations with this mix of resistance is complicated somewhat by different *in vivo* and *in vitro* results, as in artificial diets many of the compounds (e.g. in cotton gossypol, condensed tannins, flavonoids) are antimetabolic, whereas in the field only the anthocyanin, chrysanthemin and the polyphenol gossypol are effective, and the other tannins have no effect. The differences between *in vitro* and *in vivo* results can be attributed to the presence of other food sources in the field, i.e. low levels of many compounds consumed in a mixed diet with other plants or parts of the plant may have less effect than the compound fed in isolation in a basal diet. Also in the field resistance is often not complete, reaching an upper limit of 80% predator mortality regardless of the high concentration of antimetabolites in the plant (Hedin *et al.* 1983); the significance of this is unclear. In *Phaseolus/ Vigna* many individual factors may cause resistance to specific pests, but it is likely, though unproven, that the field situation is more complex and multiple components are at work, possibly synergistically, in resistant species or lines (Gatehouse *et al.* 1990).

Introduction 6
Conclusions and Introduction to Experimental Work

The foregoing has indicated that the single parameter "resistance" may not be possible to isolate: in particular the compounds involved may have other functions in the plant, affecting physiology, anatomy, ecology, behaviour, etc, and affecting plant resistance to pests apart from the pest being directly observed. This duality of function is especially important with respect to the metabolic cost of maintaining these defensive components, which has to be balanced against the cost (in pest damage) of not having it, competitively, just as the plant's fitness affects its competitiveness with other plants (Janzen 1981).

From the pest point of view many adaptations have occurred to circumvent the defences of leguminous plants, each with an attached cost, for instance in response to toxicity the beetles can either avoid the toxins or develop resistance to them. The advantage is to be able to exploit a foodsource that another insect is unable to exploit, thus avoiding competition pressure (Center and Johnson 1974).

However in all cases the insect (bruchid larva) has a requirement to ingest and digest a source of amino-acids, usually protein, and a source of energy, usually starch. The products of digestion must then be absorbed across the gut wall where they can then be used for the metabolism involved in tunneling and digestion, for growth, and for storage to be used up during metamorphosis to the adult.

These various requirements can be disrupted in a number of ways:

Amino-acid intake can be restricted if the proteins are not digested or partially not digested. This can occur if the proteins are in a form which makes then indigestible, or if protease inhibitors are present in the seeds which oppose the activity of the bruchid proteolytic enzymes.

Energy intake can be restricted by the equivalent means, if the carbohydrate used by the seed to store energy is rendered non-susceptible to hydrolysis to its constituent sugars (e.g. by using a form for which the larva does not have a carbohydrase to digest). Inhibitors of gut carbohydrases, especially the amylase enzyme which is responsible for

the digestion of starch, may be present preventing the carbohydrate source from being made available for metabolism.

Components of the bean cotyledon may also disrupt absorption of the products of digestion. An example of this is the *lectin* component, which has been shown to disrupt the epithelial wall of the gut in mammals, and to bind to the gut walls of bruchids unable to prey on *P. vulgaris*, but the pest species *A. obtectus* appears to tolerate lectin by preventing binding to the gut wall. Lectins (and protease inhibitors) may also cause overproduction of proteolytic enzymes and oversecretion of them into the gut, leading to severe protein nitrogen loss from the animal.

Factors may be present which are actively antimetabolic, by a variety of mechanisms. Examples of these include non-protein amino-acids, which cause the production of non-functional proteins (and at the concentrations present in the cotyledon probably also upsetting osmotic balance in the insect gut), alkaloids and sapogenins which upset the hormonal balance and thus prevent metamorphosis or precipitate development before adequate food stores are laid down, and a wide variety of other components whose antimetabolic nature has been observed but whose mechanisms have not been elucidated.

6.1 AIMS OF THE PROJECT

1- To select sources of resistance to the two major economic pests of the common bean *P. vulgaris* seeds, namely the bruchid species *Z. subfasciatus* and *A. obtectus*;

2- to elucidate the mechanisms of the resistance; and

3- to develop screening techniques to detect the compounds responsible for resistance in both wild-type accessions and the F_1 and F_2 generation seeds of crosses incorporating these selected factors with commercially important features (for instance large-seededness and high yields).

In particular, with reference to point **2** above, the mechanism of the resistance determines how suitable a particular factor is for incorporation into seeds grown for mammalian consumption, and the screening techniques enable resistance factors to be incorporated into cultivars with commercially attractive agronomic characteristics, so that

suitable parents for the next generation of crosses can be easily selected, the mechanism and robustness of inheritance determined, and the influence on other characteristics of the plant assessed.

6.2 THE EXPERIMENTAL WORK

Resistant accessions have been selected by screening seed material from the CIAT *Phaseolus vulgaris* germplasm bank, using insect bioassay technique. Having determined the source of resistance to a particular pest species, factors associated with resistance can be detected by comparing the protein compositions of resistant accessions with those of susceptible accessions on SDS-PAGE. In the first instance I am interested in proteinaceous resistance factors since these are primary gene products and are more easily incorporated into commercial cultivars.

Resistant accessions are also fractionated, in the first instance crudely, and the fractions incorporated into artificial diets (in a basal diet favourable to development of the pest species); the results of bioassays of the crude fractions can be compared with SDS-PAGE analysis of proteins associated with resistance, and used as a basis for further fractionation.

This approach is only successful in as far as the antimetabolic component is a protein and is present at high concentrations. Enzyme inhibition is also examined and the fractions containing inhibitory activity tested in bioassay. A variety of fractionation techniques are used. Interpretation of bio-assay data is not straightforward with either pest species, as resistance appears to be dependent as much on the absence of one component as the presence of another: hence the full effect of the fraction incorporated into artificial diets is masked by the high nutritional background of the basal diet. Proof that resistance is dependent on the absence of particular components is only found by supplementing resistant seed meal with that component and observing the restoration of viability of the meal to the insect.

The eventual aim of the project is to incorporate resistance factors into commercial lines, so screening techniques are developed and tested, and mammalian suitability tested with one of the components responsible for resistance to a particular pest.

Materials and Methods 7
Insect Bioassays and Artificial Diets

7.1 INSECT BIOASSAY, SEEDS OF ACCESSIONS

Bioassay on seeds was performed at **ODNRI** (Slough, Berks and later at Chatham, Kent), and at **CIAT** Colombia. For bioassay of individual accessions 50 seeds were equilibriated to the experimental conditions (25°C, 70% r.h.). Each replicate of the bioassay consisted of 5 beans placed in a 2" × 1" dia. glass tube closed with a porous foam plastic stopper. Ten replicates were set up for each sample. A virgin pair of insects was placed in each tube and left to lay for 48 hours. The adults were removed and the numbers of eggs on each replicate were counted. If oviposition had not occurred the pair was replaced and the procedure repeated. All replicates were left until the emergence of the first adult progeny, from which time adults were removed and counted daily until all had emerged.

From emergence data the eggs laid surviving to adult and emerging (*% survival*), and the median *development period* (time for 50% of total adult emergence to emerge, calculated after Dobie 1978) were calculated.

7.2 ARTIFICIAL DIETS

Diets were prepared from lyophilised material in a base of detesta'd milled haricot bean meal as described previously (Gatehouse and Boulter 1983). Haricot was used for both *Z. subfasciatus* and *A. obtectus*, and in the course of the studies it was shown that the testa from haricot, if left in the meal, is highly antimetabolic. In one or two cases other basal diets were used, for instance to demonstrate that the addition of phaseolin to **G12953** meal (resistant to *Z. subfasciatus* development) overcame resistance, detesta'd **G12953** meal was used as the basal diet.

Materials

teflon molds (cylindrical holes in teflon blocks of internal dimensions 2.5mm × 4mm)

for *Z. subfasciatus* diets were prepared specially by our own workshops, and the glass vial **plastic caps** (to make pellet disks 1mm thick by 7mm across) for preparing *A. obtectus* diets were obtained from SH Scientific

test material was purified as described later, and the basal diet meal (haricot or otherwise) was detesta'd using a razor-blade, milled and passed through a fine sieve, before being lyophilised

1 Material to add to the basal diet was weighed out (for 2.5g detesta'd haricot bean meal) and dissolved in 5ml distilled water. If starch was to be added it was dissolved in water by heating to boiling.

2 The basal diet milled meal was mixed to a liquid paste with this solution and extra distilled water added if necessary, and then freeze-dried.

3 After freeze-drying the flour of mixed basal diet meal and potential antimetabolite was made to a very thick paste with a minimum of distilled water, and packed into the appropriate mould.

4a For *Z. subfasciatus* a cylindrical mould of approximately 0.5cm diameter by 1cm length was used, pellets were coated with gelatin;

4b wheras for *A. obtectus* plastic stoppers were used to make flat disks of 1.3cm diameter by 0.15cm thickness. In both cases 5 pellets could be made from 2.5g of diet.

7.3 INSECT BIOASSAY, ARTIFICIAL DIETS.

Bioassays on artificial diets were performed at **ODNRI**, Slough or Chatham.

For *Z. subfasciatus*, special oviposition chambers were constructed. Crystallizing dishes (8 cm dia. x 4 cm high) were half filled with haricot beans and a fine brass mesh was fixed a few millimetres above; the diet capsules were placed on this mesh. Each diet type was placed in a separate chamber with 40-50 adult *Z. subfasciatus* (0-2 weeks old). The pellets were checked daily for oviposition. If no eggs had been laid the pellets were wiped with tissue and replaced. When 2-5 eggs had been laid the capsules

were transferred individually to glass tubes (2" x 1" dia.). If more than 5 eggs had been laid excess were removed using a scalpel.

Since *A. obtectus* do not attach eggs to the surface of the seed, but rather distribute eggs in the proximity of their hosts and allow the larvae to make their own way, newly hatched first instar larvae of *A. obtectus* were transferred using a paintbrush onto the pellets of meal, and allowed to penetrate if they were able to.

7.3.a Numeric Analysis

Daily emergence counts were taken from the day of the first adult emergence and continued until all the adults had emerged. Hatched egg survival to adult (expressed as a %) was calculated for each replicate, and the mean and standard deviation of the arcsine transformation (ASIN($\sqrt{(\%)}$) taken; and median development period, mean and standard deviation taken of all emergent adults. Where pellets were dissected the number developing to adult was recorded and included in calculations for *hatched egg development* figures, but the *median development period* remained unchanged, based on emerged adults.

7.3.b Chi-Squared Test

The standard statistical test to compare frequencies observed with frequencies

$$\chi^2 = \sum_{j=1}^{k} \frac{(o_j - e_j)^2}{e_j} = \chi^2 = \sum \left(\frac{o_j^2}{e_j} \right) - N$$

expected is calculated as in the equation, where $\mathbf{o_j}$ is the observed frequency in a particular class, and $\mathbf{e_j}$ the calculated frequency for that class based on a particular null hypothesis. **N** is the total frequency, and degrees of freedom are calculated from the layout of the classes, *(no of rows—1)* × *(no of columns—1)* (Campbell 1989).

7.3.c Analysis of Variance

The standard test, also known as **ANOVAR**, assumes that the experimental results are part of a population with normal distribution; hence analysis of variance of percentage data was done on the Arcsin transforms of the percentage values.

Analysis of variance compares a number of population means to determine whether a treatment has an overall effect, or whether the observed effect is due to random variation in the populations; it is an estimate of the contributions of the various factors involved, and their magnitude in relation to the magnitude of the unexplained variation (Campbell 1989).

7.3.d Paired t-test

The paired t-test gives the likelihood that two experimental means are from the same or different populations of results, enabling a statistical estimate to be made whether experimental results are different from each other. The **t-statistic** is calculated

$$\sigma = \sqrt{\frac{N_1 s_1^2 + N_2 s_2^2}{N_1 + N_2 - 2}}$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sigma\sqrt{1/N_1 + 1/N_2}}$$

$$\nu = N_1 + N_2 - 2$$

from the equations given, where the probability is looked up in a table for a given number of degrees of freedom (Campbell 1989).

Materials and Methods 8
Purification of Proteins

8.1 GENERAL METHODS

Reference

Whilst most of the methods used for the purification of proteins were as the result of modifying basic techniques in the light of experience, the mechanisms by which the techniques were effective was studied in order to select suitable parameters for alteration (e.g. flow rates, buffer salts, pH etc). The literature supplied by Pharmacia (*Gel Filtration, Ion Exchange Chromatography*) was studied for chromatographic methods, and general methods were studied in Scopes 1987.

8.1.a Ion Exchange

Charged molecules, e.g. proteins, or polysaccharides containing acid or amide sugars, can bind to a charged resin when in the presence of a suitable buffer which both maintains the charges on the molecule and the resin so they are opposite, and does not include so many ions that binding is competitively inhibited. For the purposes of this work ion exchange involving cation and anion exchange was used, with a salt gradient to elute the proteins from the columns.

8.1.b Gel Filtration

Proteins and carbohydrates are dissolved in a suitable buffer, and passed down a resin which acts as a porous gel with the resultant properties that molecules are separated on the basis of apparent molecular weight, compared with the ideal *globular* protein.

8.1.c Ammonium sulphate precipitation

Macromolecules interact with each other in solution, in some instances to repel and ensure that the molecules stay in solution (like charges repel), and in others to agglutinate and precipitate. In biological systems the majority remain in solution at low concentrations due to repelling between molecules.

However the addition of ions at high concentrations may overcome these repelling forces by neutralising the charges on the molecules, causing the molecules to agglutinate and precipitate out.

One such suitable ionic compound is ammonium sulphate, as it dissolves readily in water to high concentrations without significantly altering the pH of the solution.

8.2 EXTRACTION OF ALBUMIN AND GLOBULIN PROTEINS

Materials

Seeds of G12953 were obtained from CIAT, and seeds of RKB from Suma wholefoods

Sodium *di*-hydrogen phosphate and ***di*-sodium hydrogen phosphate** were obtained from Sigma Chemicals; **Salt** (NaCl) was obtained from May & Baker Ltd (Dagenham, U.K.), and **CM-Cellulose** from Whatman Ltd (Maidstone, Kent)

Sodium phosphate buffer was made from 50mM solutions of sodium hydrogen phosphate and *di*-sodium phosphate mixed in quantities to result in pH 7.0

Methods

Albumin and globulin protein fractions, and a soluble high M_r carbohydrate fraction, were prepared from seeds of *P. vulgaris* (G12953, resistant to both *Z. subfasciatus* and *A. obtectus*, and red kidney bean, a collection of susceptible cultivars).

1 Finely ground seed meals were extracted in 50mM sodium phosphate buffer, pH 7.0, at 4°C overnight (10g/100ml), then centrifuged (9000×g, 4°C, 20 min).

2 The supernatant was dialysed against 25mM sodium acetate buffer, pH 4.6, (5 changes over 24 hours), and

3 centrifuged as above to separate the globulins (precipitate) from the albumins and carbohydrate (supernatant).

4a The globulins were then washed by dialysing against distilled water and lyophilised.

4b The albumins were batch-absorbed onto CM-cellulose at pH 4.6 as described below (Section 8.3, parts 2—4), and eluted using 0.5M NaCl;

5 leaving the carbohydrate fraction in solution. Both fractions were dialysed against distilled water and lyophilised.

8.3 ARCELIN 4

Materials

Haricot beans were obtained from Suma Wholefoods, Gateshead. Meal was prepared by milling the seed in a shear mill and passing the flour through a 1mm sieve

chemicals were obtained from sources as indicated above

S-400 gel-filtration resin was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden)

Method

The major seed storage protein from **G12953** (referred to as *arcelin 4*) was purified by extraction and chromatography on CM-cellulose.

1 30 g seed cotyledons were milled and sieved, and the resulting meal was extracted in 250ml 25mM sodium acetate pH 4.6 at 4°C.

2 The extract was centrifuged at 9,000 × g for 30 min at 4°C and the supernatant batch absorbed on 75g preswollen CM-cellulose.

3 After two washes of the resin with extraction buffer, each wash being removed by centrifugation of the resin slurry at 2,000 × g for 10 min,

4 the resin was packed into a column (2.4cm dia. × 50 cm) and bound material was eluted with a sodium chloride gradient (0—0.5M) in acetate buffer pH 4.6.

Column fractions were assayed by SDS-PAGE as described later (Hames 1981).

5 Arcelin 4 eluted as a single peak at approx 0.22M NaCl; peak fractions were pooled, dialysed against distilled water and freeze-dried. Material which did not bind to the CM-cellulose consisted mainly of heteropolysaccharide and is subsequently referred to as soluble carbohydrate material.

6a Arcelin 4 was further purified by gel filtration chromatography on a column of Sephacryl S-400 (3.6 cm dia. x 100 cm) run in 0.1M sodium borate buffer, pH 7.0, containing 0.1M NaCl.

6b The column had previously been calibrated by chromatography of standard proteins under identical conditions to those used for arcelin 4, and plotting elution volume against log M_r. Chromatography of arcelin 4 gave two peaks, the first of which was pure arcelin 4.

7 Both peaks were pooled, dialysed against distilled water, and lyophilised.

8.4 α-AMYLASE INHIBITOR

Materials

non-bound material was the *soluble carbohydrate* portion which did not bind to cation exchange resin after the *albumin extract* was batch absorbed at pH 4.8

ammonium sulphate and **buffer salts** (e.g. boric acid, sodium hydroxide) were obtained in bulk from BDH

FPLC equipment and **chromatography resins (S-300 Fast Flow gel filtration beads, Q-Sepharose Fast Flow anion exchange and S-Sepharose Fast Flow cation exchange resins)** were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). FPLC equipment consisted of a gradient former, twin syringe-type pumps, injection valve, UV flowcell monitor (set to read A_{280}), and a fraction collector, together with appropriate columns i.e. XK16×80 for gel filtration, XK16×15 for ion exchange

Method

At each stage the inhibitory activity of the purified portions was tested by serial dilution against larval gut homogenate, following the test described for amylase inhibition (page MM.98)

1 *non-bound* material was dissolved in 50mM borate buffer pH 7.0 and ammonium sulphate precipitated to the following saturations of ammonium sulphate:

1a material precipitating at 50% saturation (**AS0-50**)

1b the supernatant of this precipitated at 80% saturation (**AS50-80**)

1c the proteins remaining in solution at 80% saturation (**AS80+**)

2 all cuts were dialysed extensively against distilled water to remove ammonium sulphate, and lyophilised

3 the AS50-80 material, being found to be the most active, was passed through S-300 gel filtration beads (XK16×80, 1ml/min, 5ml per fraction) to resolve a number of peaks. Fractions were run on SDS-PAGE after acetone precipitation and the fractions constituting each peak were pooled

4 peaks were dialysed against distilled water and lyophilised

Ion exchange was also performed on the AS50-80 material, at pH 3.0, 5.0 and 7.0 on S-sepharose (acetate/formate buffer 25mM for pH 3.0, and tris/borate 50mM for the others, using a 0—2M salt gradient) and pH 5.0, 7.0 and 9.0 on Q-sepharose (tris/borate for all of them, with the same salt gradient) without improving resolution of the peaks, nor obtaining higher total inhibitory activity recoveries.

8.5 PPTE

8.5.a Method 1

Materials

non-bound material was the *soluble carbohydrate* portion of G12953 meal which did not bind to cation exchange resin when the albumin protein fraction was batch absorbed at pH 4.8

phenol was obtained in crystalline form from BDH

buffer salts were obtained from BDH or Sigma

Method

1 *Non-bound* material was dissolved in distilled water (700mg in 40ml), and then mixed by shaking at 40°C with an equal volume (40ml) of 95% phenol.

2 The aqueous and phenol phases were separated by centrifuging at 14,000 × *g* for 20 mins at 25°C

3 the aqueous, phenol, and precipitate from the phenol phase fractions obtained were dialysed against 10% acetic acid, 5% acetic, 2% and 1% acetic for 6 hours each, followed by three changes of distilled water.

Phenol is highly soluble in acetic acid, and acetic acid from 10% concentration was used for dialysis as this would remove all traces of phenol from the materials in solution.

In the first extraction, three fractions were produced:

1- material in the **aqueous phase**, on SDS-PAGE entirely carbohydrate

2- denatured protein in the **phenol phase**

3- material precipitated by interaction with phenol

8.5.b Method 2

1 700mg of *non-bound* material was dissolved in 25mM borate pH 7.0 (40ml), and mixed as before with 40ml 95% phenol (at 40°C).

2 Centrifugation gave an *Aqueous phase*, *phenol phase*, and the precipitate as before.

3 the precipitate was resuspended in 10ml 95% phenol (40°C, vortexing) and centrifuged again to give **phenol wash 2** and a precipitate.

4 The precipitate, by now gelatinous, was vortexed with glacial acetic acid (2ml)

to remove last traces of phenol and phenol-soluble material (**Acetic acid wash**)

5 finally all fractions were dialysed as before against successive dilutions of acetic acid and distilled water, and lyophilysed.

Materials and Methods 9
Protein Characterisation

9.1 HÆMAGGLUTINATION

Many plant lectins exhibit hæmagglutination properties, that is they agglutinate red blood cells. The potency of this agglutination can be measured by mixing serial dilutions of the lectin solutions with a set concentration of suspended red blood cells. The minimum agglutinating concentration of a particular protein can be compared with the minimum agglutinating concentration of a standard lectin (e.g. concanavalin A, or PHA E_4).

Materials

96 well Microtitre plates (12 × 8, with U-form bases for hæmagglutination) were obtained from Sterilin Ltd (Teddington, U.K.)

isotonic saline was made up from PBS capsules (pH 7.2) obtained from Sigma

blood was obtained from rabbits, or from willing human volunteers

erythrocytes were precipitated from whole blood by gentle centrifugation (1500g × 10 mins), the serum poured off and the erythrocytes washed twice with isotonic saline and made up to a final concentration of 2% suspension (i.e. making up to approximately the same volume as the original blood sample).

lectin standards and **samples** were produced in the course of protein purification; Concanavalin A was obtained from Sigma, and PHA E_2L_2, and PHA subunits mixed (E_4 . . . L_4) were kindly donated by Dr Pusztai of the Rowatt Research Institute

Method

1 microtitre plates were prepared with 50µl of isotonic saline in each well, except for the first column of wells

2 To each well of the first column 100µl of lectin solution (2mg/ml to 5mg/ml) was added

3 these sample solutions were then serially diluted 1:1 (2× dilution at each step) along the rows by taking 50µl from the first well (containing 100µl) and transferring it to the second well, mixing, taking 50µl from this second well and transferring to the third well, etc to give a 12 step (2048× dilution in the final well) serial dilution of up to eight samples, with 50µl in each well (at the last stage the extra 50µl was discarded)

4 To each well 50µl of 2% solution of erythrocytes was added, mixed thoroughly by swirling, and the plate left at room temperature (19°C) for 1½ to 3 hours.

5 The **end-point** (minimum concentration of lectin which agglutinates the red blood cells) can be assessed visually and this minimum concentration compared with dilutions done at the same time of known standard agglutinins.

9.2 PREPARATION OF ANTIBODIES

Antibodies to purified proteins were prepared in rabbits, according to the following protocol:

Materials

antigenic material was the selected material; in the case of these studies affinity-purified **arcelin 4**, and the **PPTE** (material precipitated after phenol phase separation of the *non-bound* material)

Freunds' adjuvant, both **complete** and **incomplete**, was obtained from Difco

rabbits (New Zealand White) were kept in the Dept of Biological Sciences animal house, fed on commercially available animal feed, and were only used for one experiment involving antibodies, being killed after the final bleed

To prepare the **antigen** solution, selected protein in as pure a state as possible was dissolved in sterile saline to a concentration of 1mg/ml, and emulsified with an equal volume of **Freunds' adjuvant** (1ml for rabbits). The emulsion was made up by adding small quantities of antigen solution to the Freunds' adjuvant, and vortex mixing thoroughly. The emulsion is stable for about 24 hours

Injections: the initial, *sensitisation* injection, used an emulsion made with *complete*

Freunds' adjuvant, whilst the booster injections used the emulsion with *incomplete Freunds' adjuvant.*

Method

1 Injections in rabbit could be subcutaneous in the back (4 × 0.25ml), or intramuscular in the thigh (2 × 0.5ml). The initial injection was by definition **day 0, using antigen emulsified with Freunds' complete**

2 40 days ± 2 days (six weeks) after sensitisation the booster was injected, as before but emulsified with *Freunds' incomplete adjuvant*

3 14 days (2 weeks, day 54) after the booster injection animals were starved for 24 hours and bled 10-20ml from the marginal ear vein. Blood is collected into non-heparinised vacuum tubes

4 Serum was prepared by coagulating the blood (incubation at 37°C for 2 hours) and contracting the clot (overnight on ice), followed by filter-sterilising (filtering over vacuum through 0.22μ filter), and this serum can be stored at 4°C with 0.02% sodium azide as preservative

5 Specificity testing is best done on Western blotting with a variety of materials including the original antigen and like and unlike proteins. Different dilutions of plasma were used (2000× initially, then if the stain was too weak try 1000× or 500×, wheras if too strong (too many different proteins show up) try 10,000× or 20,000×) to determine the optimal concentration to use (best positive to background difference)

6 by examining the antigenic subunits on Western blotting an estimate can be made of the most suitable proteins to include in *primary antibody solution* to "block" and prevent the antibody binding to these proteins where they are on the blot (where these are not the specific proteins which the antibodies were selected to indicate).

9.3.a Basic SDS-PAGE

Denaturing slab gels were used to analyse mixtures of proteins, enabling proteins to be identified according to the subunits observed. Because of the constant charge density caused by interaction of polypeptides with SDS, their migration rate through the gel is inversely proportional to the log(molecular size), i.e. LOG(M_r). Exceptions to this are where the peptides are glycosylated, in which case the branched nature of the resultant molecule retards its passage more than the molecular size would suggest (it correspondingly appears to have a higher molecular size than it actually has); or in some rare peptides which, containing particular amino-acids, do not interact quantitatively with SDS and have a different charge density from that expected.

Separation on an 10cm × 8cm minigel was observed as follows, for 30:1 acrylamide to *bis*- range

subunit size	% acrylamide
100K to 20K	12%
30K to 5K	15%

9.3.b Preparation of Samples

Proteins available in lyophilised form or bean meals ground up were extracted into **2× SDS sample buffer** at the required concentration (2mg/ml of protein); the solution was boiled for 90 sec and cooled before loading on the gel. Similarly solutions of protein at sufficient concentration were combined with an equal volume of **2× sample buffer** and boiled as before. However where the concentration of protein in the solution is too small, for instance in fractions off chromatography columns, special techniques need to be used.

In some cases the concentration of the solution is sufficient to obtain a high

enough concentration of protein by adding 20% (v/v) or **5× sample buffer**, made up the same as **2×** but with the quantities increased accordingly. The other technique, also applicable to protein samples in high salt concentrations e.g. those off ion exchange columns, involves precipitating the proteins and then resolubilising them.

Two techniques were used:

TCA precipitation, where the proteins are precipitated with 60% TCA for 2 hours, the TCA washed off with two changes of ethanol, and the sample redissolved in **2× SDS sample buffer**; and

acetone precipitation, where proteins are precipitated with 80% acetone for 90 mins, dried and then redissolved in **2× sample buffer**

The second was found to be more reliable, since some proteins precipitated with TCA would not then redissolve. The method used is as follows:

Materials for acetone precipitation

acetone was obtained from BDH as the AnalaR reagent, and stored at -20°C

2× SDS sample buffer was made up as described under *Recipes*

Method Acetone Precipitation

1 to 200µl of sample in a 1.5ml eppendorf, add 1.00ml acetone at -20°C

2 mix and leave undisturbed for 90 mins at 4°C

3 centrifuge on a bench centrifuge, 2 mins 13,500rpm

4 pipette off 1.15ml (almost all), taking care not to remove the material from the very bottom of the eppendorf; and place the eppendorf containing sample to dry in a basin of hot water for 20 mins

5 add 50µl 2× SDS sample buffer, vortex mix, and boil for 90 secs before loading onto PAGE gel

9.3.c Recipes for Different Densities of Acrylamide

Materials

Electrophoresis equipment was the twin-minigel apparatus for 10cm × 8cm 0.75mm thick minigels supplied by *Atto Corp* (Genetic Research Instrumentation Ltd, Gene House, Dunmow Rd, Felsted, Dunmow, Essex CM6 3LD). The plates were placed together on their spacers with the rubber seal around the bottom and sides, and held with clamps with the cut-outs facing outwards to ease pouring of the gel mixture.

Acrylamide and ***bis*-acrylamide** were obtained in a premixed form at a ratio of 30:1, in 100ml bottles to make up a 40% solution (Sigma Chemicals). Since a 30% stock solution was required the bottles were made up to 121.4ml by the addition of 84.4ml distilled water, and allowing long enough for the contents to dissolve (minimum 30 mins with rolling).

ammonium persulphate (**APS**) and **sodium dodecyl sulphate** (**SDS**) were obtained from Sigma chemicals

Tris/HCl, ***NNN'N'*-tetramethylethylenediamide** (TEMED), **sucrose**, and **glycine** were obtained from BDH. **Boric acid** and **EDTA** were also obtained from this source

Method

The main gel was prepared according to the recipe (Hames 1981) (for a total of 30ml which allowed an excess for washing),

Ingredient	12% gel	15% gel	stacking gel
Acrylamide/bis 30%	12.0ml	15.0ml	1.0ml
1M tris/HCl pH 8.8	12.0ml	12.0ml	
1M tris/HCl pH 6.8			1.25ml
distilled water	4.0ml	1.0ml	7.4ml
10% SDS in distilled water	240µl	240µl	100µl
TEMED	20µl	20µl	10µl
Ammonium persulphate (freshly made up in 1ml distilled water)	50mg	50mg	25mg

with the acrylamide/tris/water mixture being degassed for 10 mins under vacuum before the addition of the remaining ingredients. This mixture was poured into the gel plates to allow approximately 0.4cm between the bottom of the comb and the top of the main gel to allow adequate stacking in the stacking layer, and the main gel was carefully overlaid with water to ensure a flat and horizontal top surface.

The main gel was allowed to polymerise undisturbed for at least 30 mins, after which time polymerisation could be seen from the distinctive interface between the overlay water and the gel surface. If possible, the main gel was allowed to 'cure' for 15 hours (which decreases the pore size slightly but ensures a more even pore size).

The stacking gel was then prepared, with the addition of 20µl 5% bromophenol blue in ethanol (to dye the stacking gel making the wells more visible), and the overlay water on the main gel poured off (and the surface washed with stacking gel mix) and stacking gel poured on to fill the plates. The comb with former for wells was inserted and the stacking gel allowed to polymerise.

Ingredient	10× Running buffer	2× SDS-Sample buffer
Tris/HCl	30g	0.2M pH 8.8 2.42g
Glycine	141g	
Sucrose		10% 10.0g
SDS	10g	2% 2.0g
Made up to a total volume	1 litre	100ml

Running buffer was made from a **10× stock**, containing 14.1% w/v glycine, 3% tris and 1% SDS (resulting in pH 8.0). Diluted running buffer was poured into the top of the apparatus and into the bottom, ensuring that it displaced air bubbles in both the wells and on the underside of the gel. To ensure the latter, the apparatus was tipped to insure a physical gradient along which the bubbles could be seen to move until none were left under the underside of the gel. This stage is important, as bubbles remaining upset the even flow of current through the gel and cause uneven running.

Each well then had a tiny quantity of 2-mercaptoethanol (β-mercaptoethanol) placed into it (<0.5µl), which ensured that S-S bonds were broken down between subunits, and 10µl of sample (maximum volume of wells 20µl, minimum possible to load approximately 4µl) loaded into each well. Gels were run at constant voltage of 100V (equivalent to approximately 50mA) for 60-120 mins.

Best results were obtained with samples containing low levels of salts, and not highly acidic; where the proteins in the sample may be highly glycosylated better results were obtained by substituting TBE (tris/borate/EDTA buffer, pH 8.0) for tris/HCl buffer in all recipes. In the sample buffer pH 8.8 ensures that the peptides are negatively charged, the SDS maintains an even charge density, and the 10% sucrose ensures that the sample remains in the well for running. Although loadings of up to 5mg/ml protein (50µg protein in the track) were possible to resolve, they resulted in heavy bands. Much lower loadings could be resolved easily, down to approximately 120ng protein in a single band (equivalent to 12µg/ml or 500× less than the maximum).

After the dye front had reached the bottom of the gel the power supply was

switched off, buffer in the apparatus discarded, and the gel plates separated to release the gel. The bottom left-hand corner of the gel was cut with a razor blade to ease identification of the well order, and the stacking gel cut from the main gel, together with any necessary trimming of "flash" gel, before the gel was placed in stain.

9.3.d Visualising Proteins

Materials

Methanol and **acetic acid** were of AnalaR quality, obtained from BDH. **Kenacid blue** was obtained from Sigma

Method

Proteins were visualised by hydrophobic adsorption (to the hydrophobic amino-acid side chains) of Kenacid blue R (0.5% w/v) in 10% acetic acid, 25% methanol, 65% distilled water. The gel was agitated in this mixture then left for a minimum of 90 minutes, and then destained in 10% acetic acid, 25% methanol for at least two changes until the background was clear and the protein bands showed up clearly (Hames 1981). This stain was found sufficient for our purposes although silver staining and amido black staining had been tried as alternatives.

9.3.e Visualising Glyco Groups on Proteins

Materials

Ethanol, **acetic acid**, and **periodic acid** were obtained from BDH. **Sodium borohydride**, **dimethyl sulphoxide (DMSO)** and **danzyl hydrazine** were obtained from Sigma

acetate sheet and permanent marker pens were obtained as normal stationary (Xerox transparent copier paper)

the **transilluminator** was a flat-bed u.v. transilluminator of currently unknown provenance, illuminating at 366nm

Method

1 Gels were fixed after running by immersion in 40% ethanol, 5% acetic acid (aq), for at least 2 hrs.

2 Peptide-linked oligosaccharides were OXIDISED for 2 hours in 0.7% periodic acid, 5% acetic acid, then the gel was rinsed (3× 10 mins in 5% acetic acid (aq) on a shaker)

3 The gel was then REDUCED for 1-1½ hours in 0.5% sodium metabisulphide, 5% acetic acid, and rinsed again.

4 The carbohydrate stain was prepared from two solutions: 60µl concentrated HCl in 100ml dimethyl sulphoxide (DMSO), and 200mg danzyl hydrazine in 100ml DMSO: mixed, added to the gel and shaken at 60°C for 2 hours.

5 Staining was then intensified by placing the gel in 20mg/100ml sodium borohydride in DMSO for 30 mins, and destained in 1% acetic acid (aq)

6 the bands were visualised by ultraviolet light of wavelength 366nm. Best results were obtained by examining the gel both immediately, and after destaining overnight examining it the following morning. In the meantime it was important to protect the gel from ultraviolet light as the fluorescence of the danzyl hydrazine fades rapidly on exposure.

7 Because of the rapid fading of fluorescence, the best method of obtaining a permanent record was to trace the fluorescent bands on clear acetate sheet placed over the gel on the UV trans-illuminator. The gel could then be stained with Kenacid blue to visualise protein bands and the carbohydrate staining overlaid (on the acetate sheet) to identify which protein bands were glycosylated.

9.4 SIZING AND QUANTITATION OF SUBUNITS ON SDS-PAGE

The sizes of subunits on SDS-PAGE were estimated with reference to subunits of known molecular weight. Standard subunits (markers) were obtained in premixed form (SDS VII) from Sigma Chemicals, to give molecular sizes (M_r) as follows: **bovine albumin** 66K, **Egg albumin** 45K, **Glyceraldehyde-3-phosphate dehydrogenase** (rabbit

muscle) 36K, **Carbonic anhydrase** (bovine erythrocytes) 29K, **Trypsinogen** (bovine pancreas) 24K, **Trypsin inhibitor** (soyabean) 20.1K, and **α-Lactalbumin** (bovine milk) 14.2K. The distance migrated down the gel is proportional to the logarithm of the molecular size of the denatured polypeptide chain (assuming that in the presence of SDS all polypeptides carry a similar charge density) and a linear regression can be calculated for each gel using the standard molecular weights of the markers. Sample subunit molecular weights are then readily calculated from their migration and this linear regression.

On a number of gels more than one track of SDS VII markers was run to enable comparisons to be made: however on a single gel the migration distances compared favourably and a single track of markers was considered sufficient for the estimation of subunit sizes. It is known that the amino acids do not bind SDS equally, so the assumption that all polypeptides will carry the same charge density will not be true in some cases. It is also known that the presence of oligo- or poly-saccharide side-chains on the polypeptide chain restrict its passage through the acrylamide sieve, and may make the protein appear to have a greater than expected molecular weight. Careful attention to the acrylamide-*bis* ratio, the presence of mercaptoethanol (which destroys cystein-cystein S-bonds) and to preparation of the samples were used to minimise these effects; none the less these limitations are recognised.

The quantity of protein present in each subunit band was estimated from densitometric scanning of the Kenacid blue-stained gel. The gel (after destaining) was placed on the reading bed of a laser densitometer (Ultroscan XL, LKB 2222-020; LKB, Bromma, Sweden) and covered with a sheet of "cling-film" to prevent it drying and curling. Tracks were marked using the apparatus provided, and laser scans taken giving the maximum density of stain and the integrated *units of density* (width of band × density) for each subunit band.

This method assumes that measurement of density of Kenacid blue stain correlates linearly with the amount of protein present; however differences of staining could be observed in that bands which showed up strongly on PVDF membrane, visualised in normal light by wetting the membrane, (and were estimated to contain 20pmol protein during N-terminal sequencing e.g. WBAI 2) did not show up on Kenacid blue staining, wheras a similar molecular weight band (WBAI 1) showed more strongly with Kenacid blue stain and actually contained less protein (estimated 12pmol). For a

given subunit band, measured density was linearly correlated to amount present up to a limit at which the overloaded bands took on a characteristic shape.

9.5 WESTERN BLOTTING

The presence of a particular protein, or others immunologically similar to it, can be detected in a variety of ways using antibodies: including immunodiffusion, ELISA, and Western Blotting. The last is most useful for identifying the protein which is reacting with the antibodies, since the position of the subunits of the protein from SDS-PAGE is a useful means of identifying the proteins themselves.

The principle of Western Blotting is to separate the subunits as normal by SDS-PAGE, then transfer them by electroblotting onto nitrocellulose membrane; once on the membrane antibodies specific to the protein, raised in rabbit or mouse, are bound to the protein subunits; successively antibodies specific to the host (rabbit or mouse), and conjugated to peroxidase enzyme, are bound to these antibodies; finally the position of the peroxidase enzyme, and consequently of the original antigenic subunits, is visualised using a colourimetric reaction with diamino benzidine (Towbin *et al.* 1979).

Materials

tris/HCl was obtained from BDH, and **sodium chloride** from May & Baker. **6-amino-*n*-hexanoic acid** (AHA) was obtained from BDH. **Di-amino-benzidine** (DAB), **hydrogen peroxide** (20 volumes), and **cobalt chloride** were obtained from Sigma

Marvel (dried milk powder) was obtained from Boots the Chemist (Durham Branch)

Primary antibody was obtained as the serum of blood removed from animals (rabbits or mice) treated to produce antibodies (see previously). The whole blood was gently centrifuged to precipitate cellular material, and 1% sodium azide added to the serum. The serum was then stored at 4°C. For use, a suitable concentration was prepared to maximise the contrast between antigen staining and staining of background or other protein subunits

Secondary antibody was obtained from Bio-Rad Laboratories (Richmond, CA 94804), as the horseradish peroxidase conjugate. The antibody had been prepared in

goat, raised to be antigenic to either rabbit (**GAR-HRP**) or mouse (**GAM-HRP**) antibody proteins (IgG) as appropriate to the primary antibodies

Solutions were made up as follows:

to make 1 litre	**Anode 1**	**Anode 2**	**Cathode**	**10× wash**
Tris	36.33g	3.03g	3.03g	24.22g
Methanol	200ml	200ml	200ml	
AHA			5.25g	
NaCl				90.0g
pH	pH 10.4	pH 10.4	pH 9.4	pH 7.2

Blotting paper was Whatman 3MM filter paper, obtained from Whatman Ltd (Maidstone U.K.) and **nitrocellulose membrane** was obtained from Anderman & Co Ltd (Kingston-on-Thames, U.K.)

the **electroblotter apparatus** was a 15cm×15cm Atto Corp Semi-dry Electroblotter

Method

1 Six squares of blotting paper (approx 12cm×12cm) were soaked in the appropriate buffers as follows: 2 in **anode 1**; one sheet in **anode 2** together with the nitrocellulose membrane; the polyacrylamide gel (immediately after running) was soaked briefly in **cathode** buffer together with the dialysis membrane and three sheets of blotting paper.

2 These were placed each in turn onto the anode electrode of the electroblotter apparatus as follows: 2 in **anode 1**; one sheet in **anode 2**; the nitrocellulose membrane; the polyacrylamide gel; one sheet soaked in **cathode** buffer; the dialysis membrane; two sheets **cathode**. Care was taken to prevent air bubbles being trapped between layers.

3 Electroblotting transferred proteins at a current of 150mA for 60 to 90 mins (1.5mA per cm^2), which was found to be suitable for all proteins tried.

4 After electroblotting the membrane was washed in three changes of 5% marvel (dried milk) in 1× **wash** (made from 10× **wash**) for 5 mins per change. At this stage the membrane is stable and can be left in 5% marvel wash.

5 **Primary antibody** solution was made from suitable antibodies (raised in mouse or rabbit) in 1× **wash**, at a suitable concentration from the antibody serum (500× to 20,000× dilution (in wash solution), depending on the activity of the antibodies), with blocking proteins if appropriate. Approx 10ml is the minimum possible to soak the membrane, though it is easier with 50ml. The membrane was briefly rinsed in distilled water to remove excess marvel, and sealed into a polythene bag with primary antibody solution. Primary binding was allowed to continue for 90 minutes on a shaking waterbath at 42°C.

6 After primary binding the membrane was washed with 5% marvel in wash solution, three changes of 10 minutes each, with agitation.

7 **Secondary antibody** solution used commercially available goat or sheep antibodies conjugated to horseradish peroxidase, specific either to mouse (if the primary antibodies had been raised in mouse) or to rabbit. These were usually made up at 2000× dilution, ie 20µl in 40ml 1× wash buffer, and the membrane sealed in a new bag with this solution and allowed to bind for 90 minutes at 42°C (shaking water bath).

8 After secondary binding the membrane was again washed, and at the same time the substrate prepared. For washing, two washes with 5% marvel in wash buffer was followed by two washes in wash buffer alone.

9 The peroxidase **substrate** must be prepared fresh, and the peroxide only added within a few seconds of applying to the membrane, as the solution discolours considerably within a few minutes. 30mg of **DAB** (diamino benzidine hydrochloride, Sigma) was dissolved in 100ml 1× **wash** buffer, and 3ml of 1% $CoCl_2$ solution added. 100µl of hydrogen peroxide (20 vols) was added and the solution mixed, and allowed to drip from the bottom of a funnel (after passing through filter paper in the funnel to remove any remaining lumps of DAB which had not dissolved) onto the nitrocellulose membrane (this is best done in a darkened room, with enough light to observe the reaction on the membrane surface). Best results were found with the solution being able to cover the

membrane evenly, but flow off to allow fresh solution to fall.

The reaction results in dark red/brown marks appearing where the peroxidase enzyme is present. As soon as these were clearly visible the membrane was thoroughly washed with distilled water, since over a period of time the peroxide solution colours and will discolour the membrane to the extent of making the original bands invisible. The membrane was then dried between sheets of filter paper, and stored away from light in a filter-paper envelope. The blotted membrane was compared with its twin gel stained with Kenacid blue to identify the subunit bands which had immunoreacted.

9.6 ELECTROBLOTTING TO PVDF MEMBRANE PRIOR TO SEQUENCING

In order to separate subunits of a particular protein to enable N-terminal analysis to be performed without the complications of a number of N-termini being present, the subunits were separated by SDS-PAGE and electroblotted onto PVDF membrane (LeGendre and Matsudaira 1989), the individual bands of each subunit cut out, and put into the reaction chamber of the automatic sequencer individually.

Materials

3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) was obtained from Sigma

Immobilon-P transfer membrane (PVDF membrane) was obtained from Millipore

all other materials are as for Western Blotting

Method

The procedure was very similar to that adopted for *Western Blotting*, with the following differences: the transfer buffer (to replace all of **anode 1**, **anode 2** and **cathode** buffers) used was 10mM CAPS with 10% methanol, at pH 11.0. The membrane (PVDF) needs to be pre-wetted in methanol for 2-3 seconds and equilibriated in transfer buffer for 15 minutes, and the gel needs to be equilibriated in transfer buffer for 5 minutes.

Protein bands can be visualised on this membrane by wetting the membrane in 20% methanol and examining it as it dries on a visible light transilluminator - the

protein bands show up as more opaque than the membrane. It is possible to stain the membrane with Kenacid blue without affecting sequencing, but wetting was found sufficiently sensitive.

9.7 N-TERMINAL ANALYSIS

The sequence of amino-acids at the N-terminal of a polypeptide chain can be determined without great difficulty, compared to the difficulties involved in sequencing the whole polypeptide. For the purposes of characterisation of proteins where they are likely to be similar to proteins already sequenced, N-terminal analysis would usually be sufficient to determine which family the protein is from, and how similar it is to known members of that family.

Sequencing is done by a modified **Edman degradation**, and may be done manually or by automatic sequencer. Where the amount of material available for sequencing is severely limited (less than 1nmol protein, such as that obtained from subunit blot to PVDF membrane) the automatic sequencer is the only option. However it is more expensive per residue, and actually slower, than manual sequencing. In both cases the protein to be sequenced needs to be virtually pure or the results will be confusing, and sometimes the only way to separate subunits is by SDS-PAGE and blotting.

Samples for N-terminal sequencing were submitted either as lyophilised material or as the individual subunit bands cut from PVDF membrane after SDS-PAGE and electroblotting. In both cases the automatic sequencer used was an *Applied Biosystems 477A Pulsed Liquid Protein Sequencer*, using an Edman Degradation cycle programme.

Lyophilised material was dissolved in distilled water and approximately 100pmol applied to a glass-fibre filter disk, after which the disk was dried and loaded into the reaction chamber and pre-treated with BioBrene (Applied BioSystems). Each subunit band on PVDF membrane was cut to fit the reaction chamber and the pieces placed so as to ensure an even reaction rate in the vessel. These samples did not need pretreatment. The programmes used to control the cycling of chemicals, temperatures and reactions were standard ones, *NORMAL* for lyophilised material and *Blott 1* for subunit bands from PVDF membrane.

Amino-acids were identified by the resident software from their elution time, quantified by comparison with standards for each amino acid, and compared with their presence in the previous cycle. The software looks for the appearance of new amino acids, and ignores as far as possible carry-over from the previous cycle (termed by the manufacturers *Lag Correction*).

After the software had calculated a "best fit" sequence the *Lag Corrected* data were examined taking into account possible double N-termini, glycine in the SDS-PAGE running buffer (largely removed during blotting and pretreatment) and a poor elution profile which could indicate a glycosylated Asparagine, but for which the software may have selected a different residue; and where necessary (not often) alterations were made. Sequencing, sequence analysis and determination of the most likely N-terminal sequence were carried out by John Gilroy, of Dept Biological Sciences, and he also suggested similarities between the determined sequences and other known ones, facilitating the alignments illustrated in **Results**.

9.8 QUANTITATION OF THE LEVEL OF GLYCOSYLATION

A number of methods of sugar determination were tried to quantify the proportion of native protein which was carbohydrate. Measurement of the change in subunit size from SDS-PAGE was not considered reliable since the saccharide branching would alter the apparent molecular weight. On the native protein, naturally the purity of the starting material would be important, and it is well-known that the oligo- or poly-saccharide side chain could contain a wide variety of sugars.

The simplest non-specific test for carbohydrate is the **phenol/sulphuric acid** test (see page MM.89); however this test is interfered with by protein, in my estimation up to 10%, and therefore is of no use with samples constituting more than about 80% protein (the protein interference becomes so great that it is not possible to say there is carbohydrate present) as is the case for nearly all glycoproteins.

Methods specific to the individual sugars or oligosaccharides require that the material is hydrolysed into monosaccharides. This would tend to result in monopeptides (amino acids) in solution as well, and any non-specific test would be interfered with by this as well. GLC of alditol acetates would be the method of choice, but access to

equipment and supervision in its use was not available at the time that the pure proteins became available.

The method finally used was the **L-cystein-sulphuric acid** assay for measuring neutral hexoses in mono- or poly-saccharides (see page MM.91), with the result obtained multiplied by a factor of 1.6 to account for pentoses and other sugars not directly measured (this factor was based on a brief comparison of the results obtained from the assay on proteins of known carbohydrate content).

9.9 ENZYMATIC DEGLYCOSYLATION OF GLYCOPROTEINS

Because of the branching of the chain where a polypeptide chain is glycosylated, the apparent molecular weight of subunits are much higher than their real molecular weights. In order to determine the real molecular weights, and thus obtain a different measure of the proportion of carbohydrate (glycosylation) of the protein, the glyco-side chains have to be removed.

A chemical method of deglycosylation, with TFMS (described below) was tried, unsucessfully. A number of enzymatic deglycosylation schemes were considered, including **Endo-H**, and **PNGase-F**. The former cuts the glyco-side chain between the sugar attached to asparagine residue in the polypeptide chain and the rest of the sugars, leaving a polypeptide chain with a single sugar residue at glycosylation sites. The latter (**PNGase-F**) removes the glyco-side chain completely, leaving only the polypeptide chain. Therefore enzymatic deglycosylation was done using **PNGase-F**.

Reference Boehringer Mannheim Biochemica technical sheet 0187. B 377. 4. **908 681** 2

Materials

phosphate-buffered saline (PBS) was obtained as pellets to make up 200ml at pH 7.2, from Sigma. **SDS**, **EDTA**, **Triton X-100**,and **β-mercaptoethanol** were obtained from Sigma also

2× denaturing buffer: 0.4% SDS, 1% β-mercaptoethanol, 1% Triton X-100, 20mmol EDTA, made up in PBS

Dilution buffer: PBS pH 7.2

Enzyme: PNGase F (also called Glycopeptidase F, Peptide-*N*-glycosidase from *Flavobacterium meningosepticum*, 100units in 0.5ml). Obtained from Boehringer Mannheim Biochemica (Mannheim, W.-Germany)

Method

1 a sample of protein is made up at 5mg/ml in **2× denaturing buffer** (approx 160µl), and boiled for 90 seconds to denature. The sample is then cooled to 32°C.

2 an equal amount of PBS (**Dilution buffer**) is added to dilute the denaturants, and 5% (v/v) **Enzyme** solution added (ie 16µl to 160µl **2× denaturing buffer** (containing 0.80mg protein) + 160µl **dilution buffer**

3 the sample is incubated for 15 hours to ensure complete deglycosylation. The sample is then run on SDS-PAGE next to an un-deglycosylated sample (both in SDS-buffer) (to check deglycosylation) and molecular weight standards (to measure resultant polypeptide apparent molecular weight)

Where protein samples were not denatured prior to enzymatic action, arcelin 4 particularly, but other proteins to a lesser extent, were not fully deglycosylated compared to boiled and fully denatured proteins.

9.10 CHEMICAL DEGLYCOSYLATION

Chemical deglycosylation of proteins required using a moisture-free oxygen-free atmosphere, and advice and supervision was obtained from Tim Holmes and Jim Lincoln, both of Dept Chemistry, Durham University.

An appropriate atmosphere was made available by inverting a large funnel and blowing nitrogen gas (British Oxygen Company) down through the funnel to fill the space below the funnel. The flow rate was around 30ml/min. To flush out vessels and reaction mixtures after the addition of any substance a pasteur pipette was attached to the nitrogen line, with flow controlled by folding of the flexible tubing.

Reaction vessels were 5ml quartz graduated **Wheaton V-vials** with screw-on caps with teflon septae; these were obtained from Aldrich Chemical Co. (Milwaukee).

Triangular stirrer vanes (teflon) were obtained from Sigma.

Reagents

Trifluoromethane sulphonic acid (TFMS) and **anisole** were obtained from Aldrich, and **diethyl-ether** and **pyridine** from BDH.

A 2ml of TFMS was added to 1ml anisole in a reactivial under nitrogen and on ice.

Method

1 vials were flushed with nitrogen gas, up to 10mg of protein substrate added, and the vials flushed again.

2 under nitrogen (ice cold), 1ml of the TFMS/anisole mixture was added

3 vials were sealed with screw-on caps, and stirred at 30°C for 90 minutes

4 vials were cooled on ice, and 2ml diethyl ether added under nitrogen. The mixture was then shaken to dissolve the reagents in diethyl ether, which made them less reactive with oxygen and moisture.

5 the contents of the reactivial was transferred to a 10ml boiling tube (as addition of pyridine-water to a 5ml reactivial to make a final volume of 5ml resulted in a reaction leaving not very much material inside the reactivial), and 2ml of 50% pyridine in water added. The mixture was vortexed.

6 the diethyl-ether layer was removed, a further 2 ml of diethyl ether added, the mixture revortexed, and the process repeated (in all, 3× 2ml of diethyl ether were used to remove the reagents from the deglycosylated protein).

7 the aqueous phase was then dialysed against 2mM pyridine and lyophilised, and the resultant material taken up in SDS-sample buffer and run on SDS-PAGE adjacent to tracks containing the untreated proteins.

After running the material on SDS-PAGE the gel was stained with danzyl-hydrazine to detect glyco-groups, and with Kenacid-blue to detect the protein subunits. The results were not successful, in that arcelin 4 (and other test proteins) subunits were not identifiable after deglycosylation, and subunits in "deglycosylated" tracks reacted positively to danzyl-hydrazine

Materials and Methods 10
Carbohydrate Analysis

10.1 PHENOL-SULPHURIC ACID ASSAY. GENERAL TEST FOR CARBOHYDRATE

reference: Chaplin 1986 pg 2

Sensitivity 1-60µg glucose in 200µl. Final vol 1.4ml (use 5ml pyrex test-tubes)

Materials

phenol was obtained from BDH in crystalline form. **sulphuric acid** was obtained as the concentrated acid from BDH (H_2SO_4, 18M)

Samples were read on a **UV/Visible spectrophotometer PU8740** by Pye Unicam Ltd (Cambridge, U.K.)

Method

1 to 200µl of samples, standards (containing 0 to 300µg/ml glucose) and controls add 200µl 5% phenol (w/v in distilled water)

2 add 1.0ml conc H_2SO_4 to the surface of the solution rapidly (taking care not to run it down the walls of the test-tube)

3 leave the mixture for 10 minutes. Then shake vigorously

4 leave for 30 minutes. Read absorbance at 490nm

Interference: this method is used to measure hexoses, but is interfered with to a greater or lesser extent by aldoses, ketoses, uronic acids, proteins, reducing agents and heavy metal ions. Where carbohydrate constitutes the bulk of the material it is approximately quantitative.

reference: Chaplin 1986 pg 5

Sensitivity 200ng to 20µg D-glucurono-6,3-lactone in 250µl. Final vol 1.44ml (1.5ml eppendorf)

Materials

Sodium tetraborate ($Na_2B_4O_7.10H_2O$), **carbazole** and sugar standards (glucuronic acid = GluA and galacturonic acid=GalA) were obtained from Sigma Chemicals. Concentrated **sulphuric acid** was obtained from BDH

4Å molecular sieve was obtained from Sigma Chemicals, and was used to dry ethanol to absolute dryness

Reagent A: dissolve 0.95g sodium tetraborate decahydrate in 2ml hot water. Add 98ml concentrated sulphuric acid (ice cold, in an ice bath) carefully and with swirling. Keep this refrigerated

Reagent B: dissolve 125mg carbazole in 100ml absolute Ethanol. This is stable in the refrigerator.

Method

1 200µl of samples, standards (containing 0 to 100µg of GluA or GalA) and controls in 1.5ml eppendorf tubes were cooled in an ice bath for 10 mins

2 1.2ml ice-cold **reagent A** was added with mixing and cooling

3 ependorfs were heated to 100°C for 10 minutes, then cooled rapidly in the ice-bath

4 40µl **reagent B** was added, and the eppendorfs sealed and inverted to mix

5 eppendorfs were reheated to 100°C for 15 minutes, then cooled rapidly to room temperature

6 absorbance was determined at 525nm (green) using the standard blank to zero the

spectrophotometer

Interference: neutral carbohydrates e.g. hexoses up to 10%, pentoses 1%. These both absorb strongly at other wavelengths e.g. hexoses 338nm. Uronic acids do not constitute a major part of the bean carbohydrate so the importance of this interference may be underestimated.

10.3 L-CYSTEINE/SULPHURIC ACID ASSAY FOR HEXOSES

reference: Chaplin 1986 pg 1

sensitivity 0.2 to 20µg glucose in 200µl

materials

sugar standards (glucose, galactose, arabinose, glucuronic acid, pectin) and **protein standards** (bovine serum albumin (BSA) and cytochrome c) were obtained from Sigma chemicals. **cysteine/HCl** was also obtained from Sigma. **concentrated sulphuric acid** was obtained from BDH and made to 86% (v/v) in the department in an ice bath, left for 15 hours to cool

cysteine reagent was made up fresh from 14mg L-cysteine in 20ml of ice-cold 86% sulphuric acid

Method

1 200µl samples, standards (0 to 0.1mg/ml of glucose) and controls were placed in eppendorfs in an eppendorf boiling rack, and left to cool on ice for 15 mins

2 1ml of **cysteine reagent** was added rapidly to the surface of the sample, ensuring good mixing, and the sample cooled on ice

3 samples in the boiling rack were transferred to a boiling water bath and heated to 100°C for 3 mins, after which a green tinge had developed.

4 samples were then cooled rapidly for 90secs on ice, then allowed to reach room temperature for 5 mins

5 the absorbance at 415nm was determined in quartz glass cuvettes, and compared

with a standard curve obtained from calibration with glucose standards

10.4 STARCH DETERMINATION BY UV METHOD

Principle: BCL kit number 207 748

$$\text{Starch} + {}_{n-1}H_2O \xrightarrow{AGS} {}_{n}\text{D-glucose} \qquad \text{pH 4.6}$$

AGS = amyloglucosidase

$$\text{D-glucose} + \text{ATP} \xrightarrow{HK} \text{glucose-6-phosphate} + \text{ADP} \qquad \text{pH 7.6}$$

HK = hexokinase

$$\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{glucomate-6-phosphate} + \text{NADPH} + \text{H}^+$$

G6P-DH = glucose-6-phosphate dehydrogenase

NADPH is produced stochiometrically with respect to the release of glucose, and is measured quantitatively at 340nm.

Materials

Dimethyl-sulphoxide (DMSO) was obtained from BDH. All of the other reagents were obtained from BCL in kit form:

1 citric acid buffer pH 4.6, 6ml containing 84 units AGS and stabilisers

2 triethanolamide buffer pH 7.6, NADP, ATP, $MgSO_4$.

3 hexokinase 200 units, glucose-6-phosphate dehydrogenase 100units, in 0.7ml

Solubilisation

Starch is not very soluble in normal buffers, and is brought into solution in DMSO. The sample of bean (with the testa removed) was first homogenised, then 2mg weighed into a 1.5ml eppendorf with 50µl hydrochloric acid (8M) and 200µl DMSO. The sample was then incubated for 30 mins at 60°C, with frequent agitation (vortex mixing). It was cooled quickly to room temperature, neutralised with 50µl 8M sodium hydroxide, and 700µl McIlvane's buffer pH 4.6 added. 0.2ml of this (freshly prepared) was then used for the assay for starch.

Using disposable 3ml cuvettes:

1	Soln **1**	0.2ml	mix by gentle swirling. Incubate stoppered at 55-60°C for 15 mins
	sample soln	0.1ml	
2	Soln **2**	1.0ml	mix by inversion. Leave at room temperature 3 mins to stabilise. Read $\mathbf{A_1}$ at A_{340}
	distilled water	1.0ml	
3	suspension **3**	20µl	mix by inversion. incubate 10 mins 20-25°C.
			Read $\mathbf{A_2}$ at A_{340}

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \quad [g/l]$$

V=final volume (2.32ml), v=sample volume (0.1ml), MW=molecular weight of substance (MW glucose-MW water = 162.1), d=light path (1cm), and ε= the absorption coefficient for NADPH (@340nm=6.3 $l \times mmol^{-1} \times cm^{-1}$). ΔA is measured as $\mathbf{A_2}$ - $\mathbf{A_1}$, and **c** is the original concentration of starch in the sample, in g/l (mg/ml). $c=0.5970 \times \Delta A$.

Thus the concentration of starch in solution could be determined, and from previous knowledge of the concentration of material in the DMSO extract the concentration of starch in the original material could be determined

Materials and Methods 11
Enzyme and Inhibitor Characterisation

One of the forms of nutritional interaction that resistance factors can take is in affecting the enzymes in the insect gut. The principal enzymes important nutritionally are the carbohydrases and the proteases, and these have been characterised to a limited extent in the two bruchid beetles *A. obtectus* and *Z. subfasciatus*:

Proteases are of a cystein type, not inhibited by inhibitors of mammalian and insect enzymes e.g. soyabean trypsin inhibitor (mammalian and most insect enzymes are serine-type proteases, wheras the main *A. obtectus* and *Z. subfasciatus* enzymes are cystein-type, with some aspartic-type (*A. obtectus* in Hines *et al.* 1990; *Z. subfasciatus* in Lemos *et al.* 1990). Their optimum pH is in the range pH 5 to 5.4 (Lemos *et al.* 1987; Wieman and Neilson 1988; also supported by findings in Gatehouse *et al.* 1985)

Of the carbohydrases the main one is α-amylase, for the digestion of starch into glucose subunits. At least one enzyme in *Z. subfasciatus* is not inhibited by the RKB amylase inhibitor (Lemos *et al.* 1990), which is active against both mammalian enzymes and the closely related *C. maculatus* larval gut enzyme. Optimum pH for this enzyme is pH 5.4 (determined during the course of this work)

Activity of the enzymes is measured by the rate of breakdown of substrate, or rate of release of product (which may be measured by a linked reaction). Activity of inhibitors is measured as the prevention of activity, expressed as a concentration of inhibitor required to inhibit activity by a certain percentage in the case of amylase inhibitory activity.

11.1 PREPARATION OF LARVAL GUT EXTRACT

Materials

DTT (di-thiothreitol) was obtained from Sigma Chemicals

A fine glass tube with matching teflon homogenising plunger, dimensions 0.8cm diameter by 12cm length was used, 'though the source for these is not recorded.

Larvae of either species were collected by breaking open dried beans from cultures of the correct species. For a given dissection, around 60 larvae at approximately larval stage 3 (larval stage 1 is the first stage after hatching, and larval stage 4 the prepupal stage; larval stage 3 ensures that the larvae used are at their largest whilst still actively feeding) were collected by breaking up infested beans. These were placed in finely ground bean meal after removal from the beans, and kept above 15°C for a maximum of 24 hours before dissection.

Dissection

Larvae were dissected under a binocular dissecting microscope by incident light, using two pairs of fine tweezers, in a glass dish set in a bowl of ice. The larva was positioned in a drop of distilled water and the head of the larva held in one pair of tweezers. The other pair (or fine dissecting scissors) was used to cut a slit in the abdomen of the larva, and the body squeezed from the head end to force all of the gut into the drop of water. At this stage care should be taken not to lose the gut contents by rupturing the gut itself. The gut was then cleaned of fat bodies and tracheae, and this was placed directly into a narrow homogenising tube containing 50µl of 1mM DTT in distilled water. When all of the larvae had been dissected, 1mM DTT was added to make up a final amount of 3µl 1mM DTT per dissected gut (nominally 33 guts/100µl; more probably 20 guts per 100µl because of the volume of the gut contents), and the guts in buffer homogenised with a teflon rod. Finally the gut material in buffer was removed using a pasteur pipette into a 1.5ml eppendorf and centrifuged to precipitate the gut wall debris. At this stage the supernatant could be stored frozen without losing its activity noticeably.

Materials

McIlvane's buffer was made up from 0.2M $Na_2HPO_4.2H_2O$ and 0.1M citric acid (both from BDH) as follows:

pH	*0.2M Na_2HPO_4 (ml)*	*0.1M citric acid (ml)*
5.0	51.50	48.50
5.4	55.75	44.25
6.0	63.15	36.85
7.0	82.35	17.65

3mM calcium chloride was added to the final solution as some enzymes are known to require divalent metal ions to function correctly

PBS (phosphate-buffered saline, pH 7.2) was made up as required, in distilled water, using tablets sufficient for 200ml, obtained from Sigma chemicals

Protein substrates were either purified (***phaseolin*** was globulin extract from Red Kidney Bean, and ***arcelin 4*** from G12953) or obtained commercially (***myoglobin*** from Sigma Chemicals)

Bean meals were from seeds supplied by CIAT, detesta'd and milled to a fine flour

Method

Substrates were prepared at a suitable concentration. For pure proteins (e.g. myoglobin, phaseolin) a concentration of 2mg/ml in McIlvane's buffer pH 5.4 was used; for bean meals an extract at 5mg/ml in buffer pH 5.4 (extracted 15 hours 4°C, and centrifuged) proved suitable.

1 120µl substrate (2mg/ml purified protein in buffer pH 5.4; or 5mg/ml bean meal in buffer pH 5.4) was added to 60µl larval gut enzyme preparation (33 guts/100µl 1mM DTT) and 150µl buffer pH 5.4, and incubated at 32°C in a

water-bath

2 20µl samples of the digestion mixture were withdrawn at set time intervals (**time**=0 (immediately after mixing), 20 mins, 40 mins, 60 mins, 90 mins, 2 hours, 3, 4, 6, and 8 (where suitable), 24 and 48 hours after start of incubation, added to an equal volume of 2 × SDS sample buffer, and boiled for 90 secs.

For each protein substrate controls were carried out in the absence of gut enzyme preparation to measure any autolysis under the assay conditions used.

3 Samples were then run on SDS-PAGE in the presence of 2-mercaptoethanol and the subunit bands of the protein under test quantified using a laser densitometer (LKB Ultroscan XL).

4 Densitometric values for each protein were then multiplied by a simple factor to ensure a **time=0** value of **1.00**.

"Remaining protein" was then plotted against time, fitted to the non-linear regression:

$$y\left(\begin{matrix} protein \\ remaining \end{matrix}\right) = A + B \times 10^{-C \times time}$$

where A, B, and C are constants.

The constants were estimated since the equation can be linearised to

$$\underline{LOG(y-A)=LOG(B)+(-C\times time)},$$

and the parameter for **A** estimated by eye for **A**= base value reached of protein undigested at time $\Rightarrow \infty$ (taken as minimum remaining protein). From examination, the value of **B** can be taken as **B=1-A** (since at time=0 remaining protein=1), and linear regression then gives an estimate for **C**. The *real* Sum of Squared Deviance can be calculated, and small alterations made to the parameters (visually), using a computer-drawn graph of the points and best fit line ($r = \sqrt{1-(s_{y.x}^2/s_y^2)}$), to improve the Sum of Squared Deviance gave the final estimates for these parameters. The Half-Life of the digestible protein is calculated from

$$Half\ Life = \frac{LOG(2)}{C}$$

11.3 α-AMYLASE INHIBITION

Since quantitation of amylase activity was not relevant to this study, a suitable concentration of enzyme was chosen to enable inhibition to be studied. The method used was a modification of Bernfeld's (Bernfeld 1955) method for measuring amylase activity, involving incubating enzyme with inhibitor for 30 mins (32°C) to enable complex formation (Gatehouse *et al.* 1986), then incubating with starch solution to allow enzyme activity. Released glucose is then estimated from a colourimetric reation with DNSA in alkali, measured at 490nm. Although the original method uses the absorbance at 540nm, the absorbance at 490nm was found to be more suitable for monitoring this particular reaction since at this wavelength it is more sensitive to release of sugar up to the proposed limit of 20% of substrate concentration.

Materials

dinitro-salicylic acid (DNSA), **glucose**, **mannose** and **hydrolysed potato starch** were obtained from Sigma, and **sodium hydroxide (NaOH)** and **Potassium sodium (+) tartrate (Rochelle's salt)** were obtained from BDH

inhibitor selected materials (mainly protein fractions dissolved at 2mg/ml, also bean meals extracted in buffer at 5mg/ml) were dissolved in an appropriate pH buffer (pH 5.4 for bruchids), and serially diluting with the same buffer either 2× or √10× dilution (see table)

enzyme solution was prepared from the larval gut homogenate, diluted by a suitable amount to release approximately 15% of the substrate under the experimental conditions within 5 minutes, and to show maximally the effects of inhibition. The graph for obtaining a suitable dilution is given below

starch was dissolved at a concentration of 1% (200mg hydrolysed potato starch in 20ml distilled water) by boiling in a microwave oven to dissolve the starch, then cooling in 32°C water bath. This was freshly prepared.

DNSA solution was made of 1g dinitrosalicylic acid dissolved with heating in 20ml 2M NaOH. 30g Rochelle's salt was added and the mixture made up to 100ml with distilled water. This needs 4 hours minimum to completely dissolve but is

then stable

Calibration of Sugars

1 sugars glucose and mannose were made up to appropriate concentrations from 10mg/ml (equivalent concentration to the initial concentration of starch substrate) down to 0.01mg/ml

2 50µl of sugar solution was boiled with 130µl buffer and 100µl DNSA solution for 5 minutes

3 1ml of cold distilled water was added, and the colour allowed to "mature" for 15 minutes

4 the absorbances of the different concentrations of sugars were read at 490nm, 540nm and 610nm (Figure 2-a and Figure 2-b). 540nm and 610nm were the wavelengths used in the literature, and 490nm was found to be the peak of glucose absorbance compared with the blank containing only DNSA and no glucose.

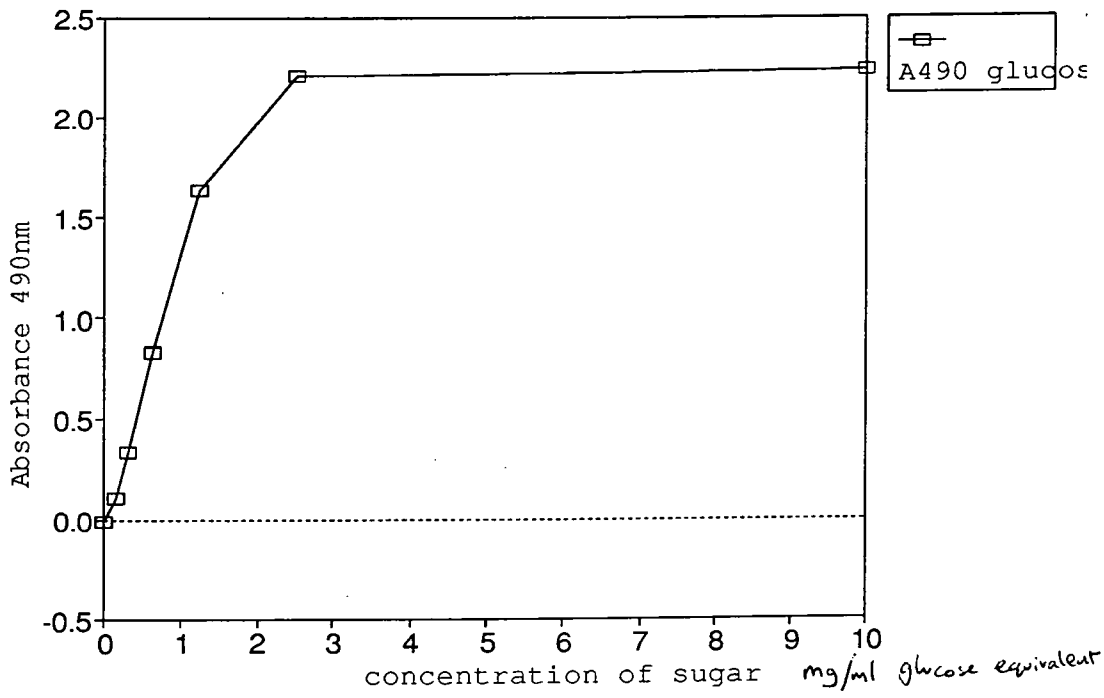


Figure 2-b Calibration of glucose up to 10mg/ml equivalent in the substrate solution, for amylase assay

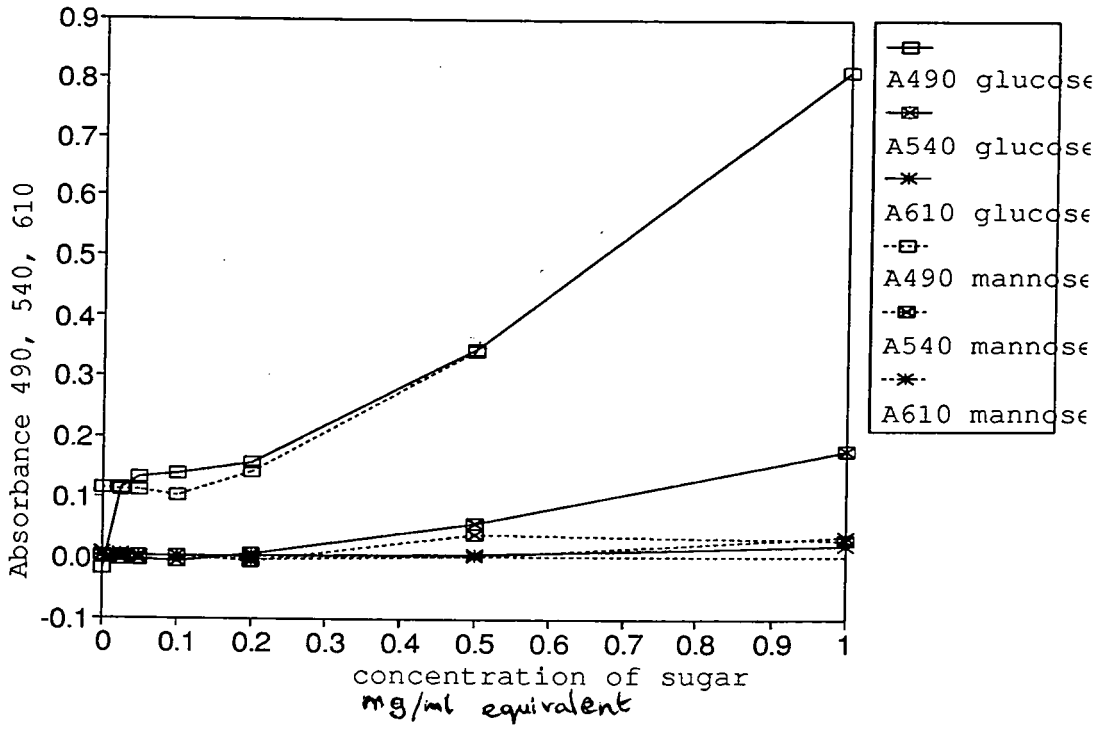


Figure 2-a Absorbance at different wavelengths of standard sugars; calibrating the modified Bernfeld amylase assay

Calibration of Enzyme

1 A series of enzyme dilutions 2× or √10× at each stage were made up from the stock material starting concentration (see Table 4)

2 10µl of enzyme at each dilution was mixed with 80µl buffer of the appropriate pH and equilibriated to 32°C for 5 minutes

3 50µl 1% starch solution was added, the enzyme allowed to digest the starch for five minutes, then the reaction was terminated by the addition of 100µl DNSA solution and boiling for 5 minutes

4 after addition of 1ml cold distilled water and "maturation" for 15 minutes, the absorbance at 490nm was determined

Method of Testing Amylase Inhibition

1 80µl of inhibitor solution (in appropriate pH buffer) was preincubated with 10µl enzyme (at selected dilution) for 30 mins at 32°C. This was to ensure complete

complex formation

2 50µl 1% starch solution was added and incubation continued for exactly five minutes

3 the reaction was terminated with 100µl DNSA solution and boiling for five minutes to develop the colour

4 1ml distilled water was added and the mixture left to "mature" for 15 minutes

5 absorbance was determined at 490nm

Table 4 Optimal pH for amylase enzymes from different sources

Enzyme source	Optimum pH	Dilution used	Activity (mg glu/5 min)
Porcine Pancreatic (mammalian)	7.0	1/600	1.30
Human salivary (mammalian)	7.0	1/20	1.43
Bacillus licheniformis	7.0	1/5000 stock	1.28
Aspergillus oryzae (Fungal)	6.0	1mg/ml, 1/10	1.33
Locusta (Insect)	7.0	5 guts/ml, 1/40	1.31
Z. subfasciatus (Bruchid)	5.4	1 gut/25µl, 1/16	1.25
C. maculatus (Bruchid)	5.4	1 gut/25µl	0.76
A. obtectus (Bruchid)	5.0	1 gut/25µl, 1/3.5	1.12
Barley Malt (plant a and b amylase)	7.0	2mg/ml	1.27

Optimal pH for the amylases from different sources are presented in Table 4, together with the dilutions used and glucose equivalence of activity at this concentration. Glucose equivalence is measured as the concentration of glucose which would give an

A_{490} the same as that observed from the concentration of enzyme, and is therefore given as mg/ml glucose released after five minutes incubation time. A_{490} was selected since this gave the most sensitive results at the selected concentration of glucose, i.e. for release of approximately 10-15% of substrate (1 - 1.5mg/ml). The concentration of enzyme was selected by 2× or √10× serial dilution of enzyme

Serial Dilution	**2×**	**mg/ml**	**√10×**	**mg/ml**
1	1	2.00	1.00	2.00
2	2	1.00	3.16	0.63
3	4	0.50	10.00	0.20
4	8	0.25	31.62	0.06
5	16	0.125	100.00	2.00e-02
6	32	0.063	316.23	6.32e-03
7	64	0.031	1000.00	2.00e-03
8	128	0.016	3162.28	6.32e-04
9	256	0.008	10000.00	2.00e-04
10	512	0.004	31622.78	6.32e-05

and measurement of A_{490} after incubation with 1% starch solution (under standard conditions, see above): where serial dilution does not affect the absorbance the enzyme is working at maximum capacity and 50% inhibition would not affect the rate of release of glucose (on the basis of the glucose equivalents the enzyme was completely digesting the 10mg/ml starch to 10mg/ml glucose at maximum capacity); where serial dilution affects absorbance the highest concentration of enzyme suitable was selected; from the table this can be seen to be equivalent to 15% of starch digested completely (at 2.5mg/ml glucose no further increase in A_{490} was observed, and 1.5mg/ml is at the top of the linear range), a proportion suitable for the stipulation that substrate concentration is not limiting. Selection for optimal concentration is illustrated in Figure 3.

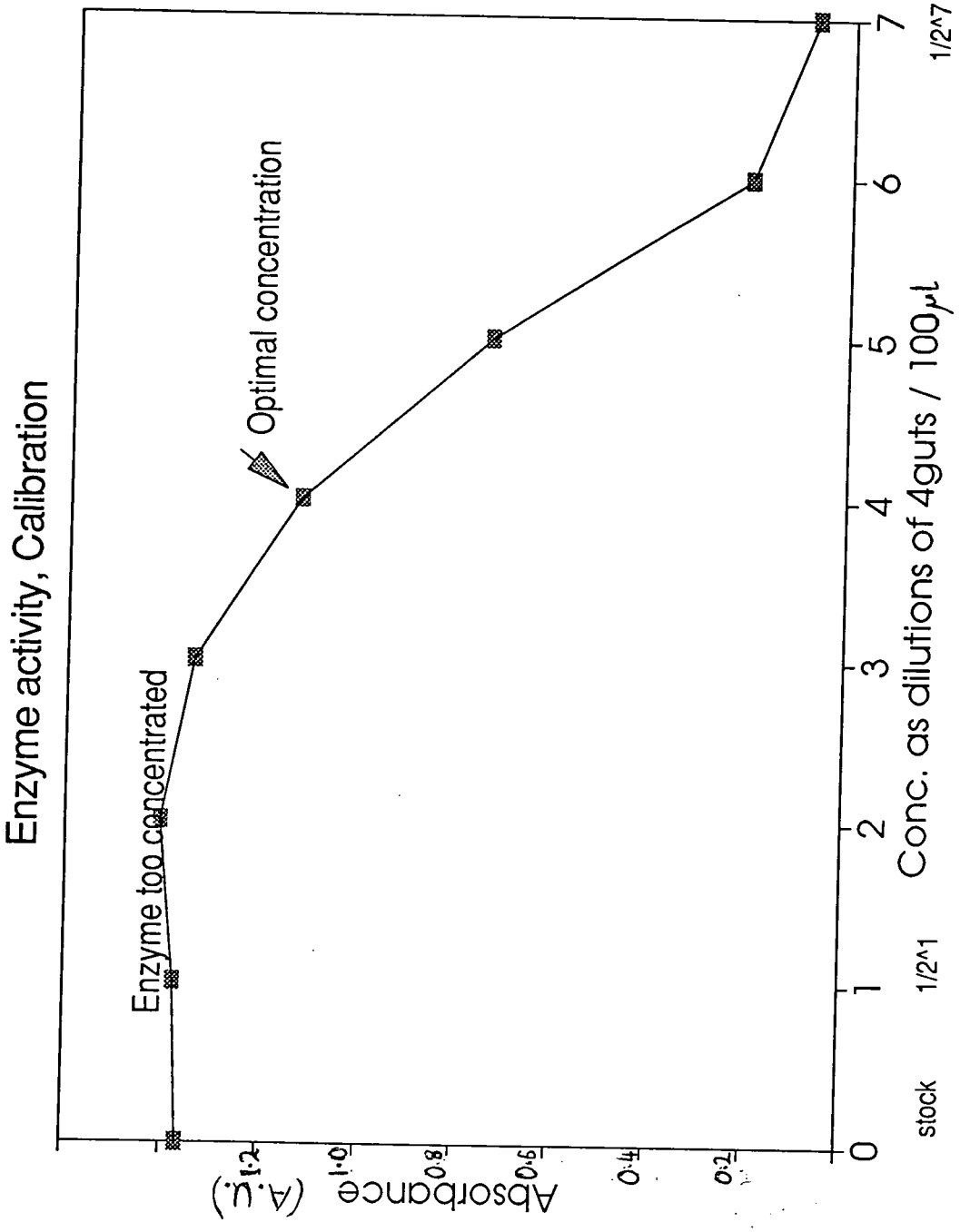


Figure 3 Serial dilution of enzyme under experimental conditions, without inhibitor, to select optimum concentration for measurement of inhibitory activity

Serial dilutions of material to be tested for inhibitory activity were made up in buffer of a suitable pH, and 80µl preincubated (30 mins 32°C) with 10µl enzyme before the addition of 50µl 1% soluble starch. After 5 minutes the reaction was terminated by the addition of 100µl DNSA solution, and the tubes boiled for 5 minutes to bring about the colour reaction. 1ml distilled water was then added and the colour allowed to "mature" for 15 mins before reading the A_{490}.

Although previous methods have read the absorbance at 610nm (which gives a linear response at least up to 10mg/ml), I found that the assay is more sensitive and more linear using A_{490}, at the lower end of glucose concentrations (2mg/ml substrate solution gives 80ng/ml total glucose concentration for 15% of substrate to be released, after dilution with the other solutions making up the assay).

11.3.a Alternative Amylase Assay

A Sigma Chemicals kit (**Amylase 576**) is available to measure enzyme activity, and this measures very selectively using an artificial substrate (**PNPG7** *p*-nitrophenyl-α-D-maltoheptaoside) which is hydrolysed by enzyme not complexed to inhibitor, through a series of reactions[2] to *p*-nitrophenol. This gave good results consistent with the modified Bernfeld method above, and has the advantage of convenience. However it is buffered to pH 6.9 and some of the enzymes precipitate if titrated to lower pHs, and there is some evidence (Marshall and Lauda 1975; Lemos *et al.* 1990) that the enzyme-inhibitor complex may be less active around pH 7.0 than around pH 5.4. Also *Spodoptera* amylase (optimal pH 9.5) failed to have any activity when determined by this kit, so its use was discontinued.

[2] PNPG7 → PNPG3 (**α-amylase**): PNPG3 → PNPG1 (**glucoamylase**): PNPG1 → *p-nitrophenol* (**α-glucosidase**): *p-nitrophenol* absorbs linearly at 405nm

Results 12
Zabrotes subfasciatus Resistance

12.1 BIOASSAY OF ACCESSIONS

A selection of accessions of *P. vulgaris*, obtained from the seed bank at CIAT Colombia, was bioassayed for seed resistance to attack by the larvae of *Z. subfasciatus*. Percent hatched egg survival to adults, and median development periods, are given in Table 5-a.

The bioassay data, % eggs hatched and development period, were transformed to a single unitless number by the equation

$$rf = \sqrt{\frac{\%\ eggs\ hatch}{development\ period}}$$

Resistance levels were then arbitrarily assigned as follows:

Very Resistant	< 0.3
Resistant	< 1.0
Susceptible	> 1.0

Five accessions (G12866, G12882, G12949, G12952 and G12953) were very Resistant to attack by *Z. subfasciatus*; insect survival on seeds of these accessions was less than 15%, and the median development period extended by 25-90% compared with susceptible lines (seeds with very low emergences can be considered to have development times in excess of observation time of 70 days). Four further accessions (G11051, G12923, G12933 and G12954) were also considered resistant to *Z. subfasciatus*: they had survival rates less than 47% (e.g. G11051) or median development periods of more than 40 days, or a combination of the two. The remainder were considered to be susceptible to *Z. subfasciatus* attack.

Table 5-a Screening accessions of *P. vulgaris* for resistance to attack and potential antimetabolites

thesis\results\dfa\vars_f90.wql varieties.dt

Accession	Bioassay		Resistance		Seed Components[1]		
	% surv. to adult	D.P. (dys)	Ratio[2]	Class	PHA	28k	35k
G09989b	60.5	38.1	1.3	S	0.15	0.00	0.00
G10000	78.6	38.1	1.4	S	0.18	0.00	0.00
G10007	50.4	39.1	1.1	S	0.13	0.08	0.00
G11051	36.0	43.1	0.9	R	0.00	0.53	0.00
G12866	11.7	48.5	0.5	R	0.00	0.57	0.00
G12871	70.1	39.1	1.3	S	0.22	0.00	0.00
G12880	65.1	38.2	1.3	S	0.23	0.08	0.00
G12882	11.5	48.9	0.5	R	0.12	0.18	0.35
G12923	73.6	48.3	1.2	S	0.00	0.00	0.56
G12933	75.3	47.0	1.3	S	0.00	0.00	0.54
G12949	5.4	70.1	0.3	R	0.09	0.00	0.68
G12952	0.9	NA	>0.1	R	0.09	0.00	0.60
G12953	0.0	NA	>0.1	R	0.05	0.00	0.66
G12954	21.1	42.0	0.7	R	0.10	0.00	0.57

NOTES: % survival of hatched eggs to adult
Development period measured as median no of days for adult emergence

1 **Seed Components** measured as proportion of total seed protein, estimated from densitometric scanning of Kenacid-blue stained SDS-Page

2 **Ratio** is square root of ratio (% survival / Development period)

Seeds were extracted in **2× SDS buffer** and analysed for protein composition on SDS-PAGE (Plate 1). Bands were identified by their mobility, so that for instance the three bands of phaseolin could be identified in the 45K region, and PHA in the 29K region. The presence of novel polypeptides, in the region 32-36K in some accessions (referred to as *35k* in Table 5-a), and in the PHA region 28-30K in other accessions (referred to as *28k)*, was observed (tabulated in Table 5-a). Accessions were grouped according to the resistance classification into resistant and susceptible, and the various components or bioassay data compared by t-test.

At this stage it was recognised that a team in America had discovered novel proteins of similar subunit sizes whilst investigating the same accessions of *P. vulgaris*. They had named their novel protein family **Arcelin**, types **1** to **4**, and comparison of the SDS-Page mobility and accession number/source has led me to consider that these novel protein subunits are the same as arcelins as follows: *28k* range is equivalent to *Arcelin 2*, *35k* is similar to *Arcelin 4* with some accessions (G12923 — G12933) containing *Arcelin 3* in its place, and those containing both bands (twin bands at $36kM_r$ and $29kM_r$) to *Arcelin 1*. This assignment is illustrated in Table 5-b.

All of the accessions labelled "S" contained normal proportions of PHA and phaseolin (compared with haricot and Red Kidney Bean), and none contained any novel protein bandings *28k* or *35k* (or arcelin variants). On the converse, accessions containing novel proteins have reduced levels of both PHA and phaseolin, and the ratio between arcelin content and phaseolin correlated closely with resistance (Figure 4, labels as per Table 5-b). In this case, *Square Root of the ratio (% survival/Development period)* (a measure of the reduction in increase of population with time) is compared with the simple *ratio of novel protein bands to phaseolin.* This comparison gives a correlation coefficient of **r=-0.839**. As can be seen, accessions containing the variant arcelin 3 are not resistant to *Z. subfasciatus* larval development (based on bioassay data of development period and % hatched eggs emergent as adults).

Seeds of resistant and susceptible varieties were also screened for other potentially antimetabolic compounds. Assay of the inhibitory activity of seed extracts towards larval gut α-amylase activity showed a marked correlation with the presence

Kenacid Blue stained
all bean meals run at 5mg/ml in SDS-sample buffer

Tracks as follows

Gii	phaseolin from RKB	**1**	G09989B (**S**)	**6**	G12882 (**R**)
PHA	*Pv* lectin (E_2L_2)	**2**	G10007 (**S**)	**7**	G12949 (**R**)
A4	arc 4 (affin Purif)	**3**	G12866 (**R**)	**8**	G12953 (**R**)
M	markers SDS VII	**4**	G12871 (**S**)	**9**	G12954 (**R**)
		5	G12880 (**S**)	**10**	G13016 (**S**)

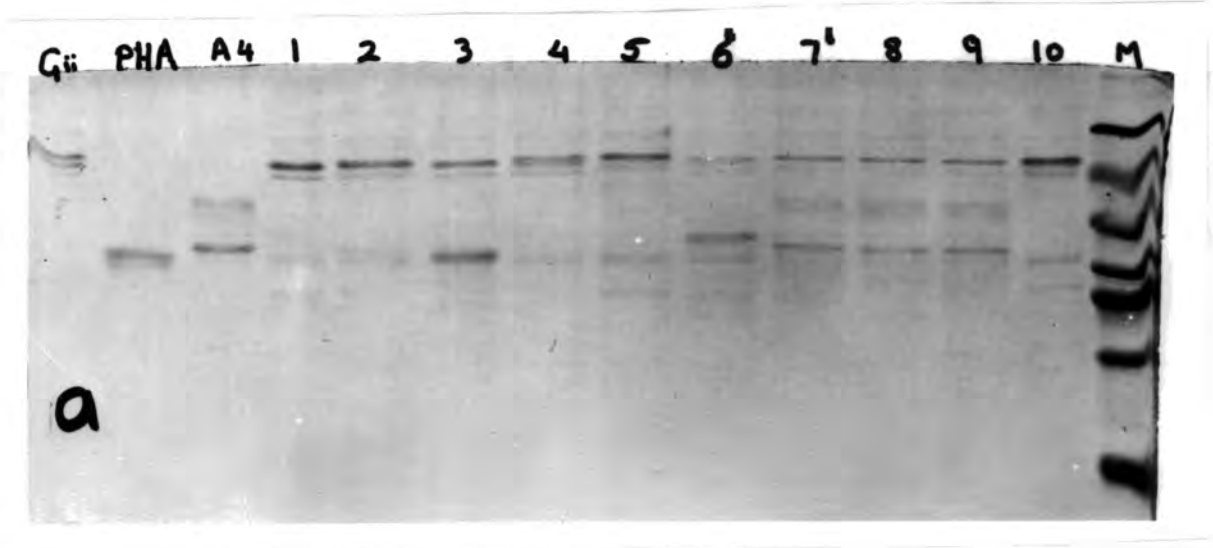


Plate 1 Seed meal of accessions of *P. vulgaris* run on SDS-PAGE to assess protein content. Kenacid Blue stain

Table 5-b Assignment of arcelin proteins and proteins to accessions

Accn	% Surv	Dev Per	Ratio	Arc Type	Protein components		
					PHA	Arc	Gii
G09989b	60.5	38.1	1.3		0.15	0.00	0.85
G10000	78.6	38.1	1.4		0.18	0.00	0.82
G10007	50.4	39.1	1.1		0.13	0.08	0.79
G11051	36.0	43.1	0.9	A2	0.00	0.53	0.47
G12866	11.7	48.5	0.5	A2	0.00	0.57	0.43
G12871	70.1	39.1	1.3		0.22	0.00	0.78
G12880	65.1	38.2	1.3		0.23	0.08	0.68
G12882	11.5	48.9	0.5	A1	0.12	0.54	0.35
G12923	73.6	48.3	1.2	A3	0.00	0.56	0.44
G12933	75.3	47.0	1.3	A3	0.00	0.54	0.46
G12949	5.4	70.1	0.3	A4	0.09	0.68	0.23
G12952	0.9		>0.1	A4	0.09	0.60	0.31
G12953	0.0		>0.1	A4	0.05	0.66	0.28
G12954	21.1	42.0	0.7	A4	0.10	0.57	0.33
G13016	47.1	40.1	1.1		0.15	0.00	0.85

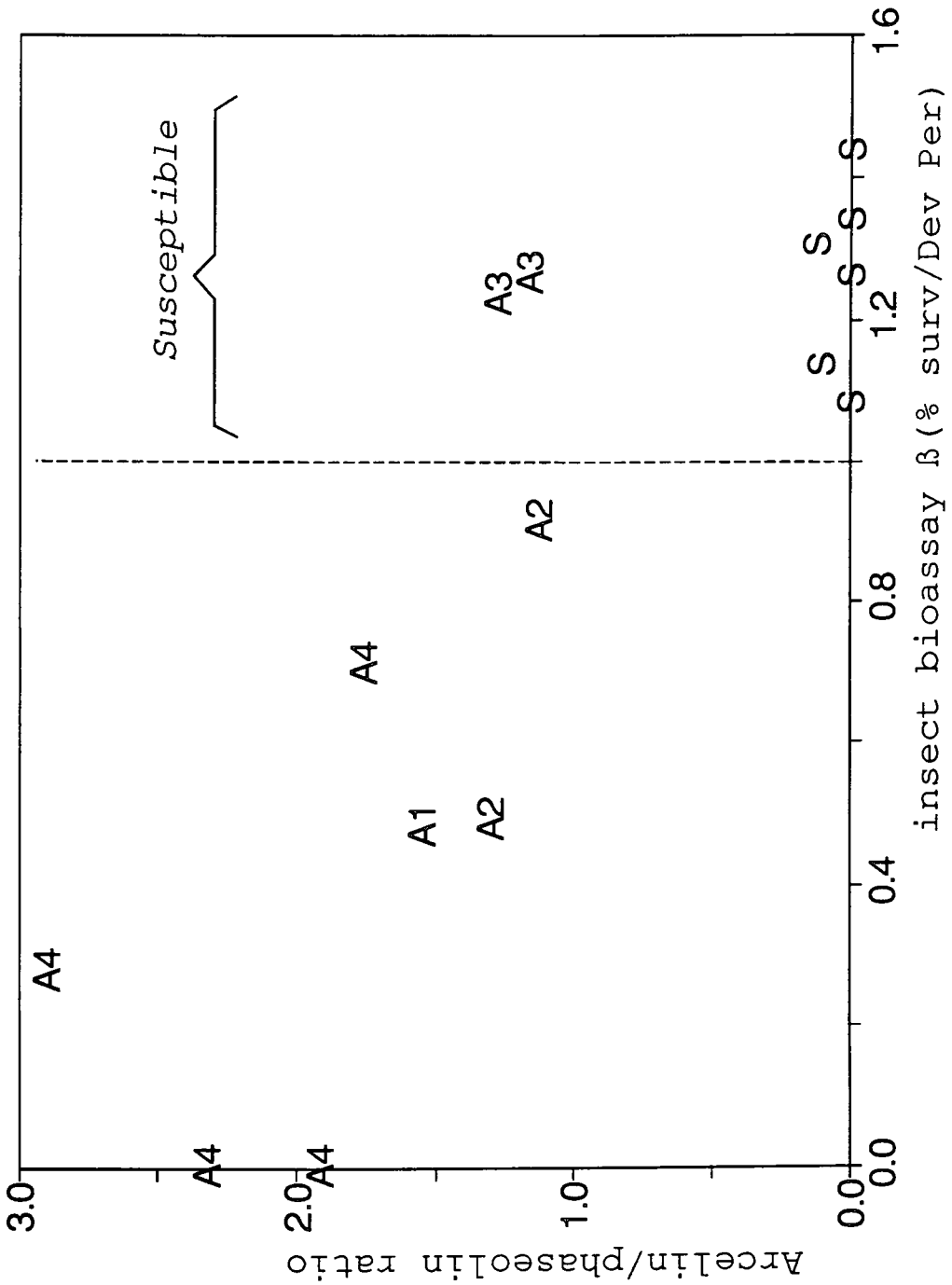


Figure 4 Comparing bioassay data with protein composition of the cotyledon.
S = not containing arcelin
A1, A2, A3 & A4 containing arcelin after Table 5b.

of arcelins type 1, 3 and 4, and possibly because of this association a correlation with resistance (Table 5-c). Resistant accessions (containing arcelin types 1 and 4) showed high levels of inhibition; however so did the two susceptible lines containing arcelin 3 (which incidentally also show a considerably extended development period even though emergence is high); and resistant varieties containing arcelin type 2 did not exhibit inhibition of the pest species enzyme. Colleagues in Japan observed the co-presence of arcelin 4 and inhibition of *Z. subfasciatus* larval α-amylase enzyme, and studied co-inheritance of arcelin 4 and enzyme inhibition over a number of generations, from arcelin 4 and inhibitor (WBAI) parent G12953, crossed with the commercial, low PHA low CBAI (mammalian α-amylase inhibitor) Ofuku-5. The Chi-squared tests of the results (Table 6) demonstrate a very high level of co-inheritance, indicating genetic linkage between these two genes.

Apart from arcelin 3 containing lines, only one susceptible accession showed significant inhibitory activity (57%), whilst the remainder inhibited to less than 40%. Assays of trypsin inhibition, chymotrypsin inhibition, haemagglutination activity, and tannin content showed no correlation with resistance (Gatehouse *et al.* 1985).

12.3 EFFECT OF SEED FRACTIONS ON LARVAL DEVELOPMENT

Experiments involving seed meal, together with analysis of protein components of the seed, suggest that the component of the seed responsible for resistance is to be found in the seed meal, rather than the testa. Accession G12953 (arcelin 4-containing) was then selected to purify out the factors causing resistance. Protein and carbohydrate fractions from resistant and susceptible lines of *P. vulgaris* were added to artificial diets based on detesta'd haricot meal and fed to *Z. subfasciatus* larvae in bio-assays to determine their effects on larval development.

In order to purify the components of the cotyledon, seed meal was extracted in 0.1M borate buffer pH 7.0 at 20g/200ml (overnight at 4°C), and the particulate and insoluble material precipitated by centrifuging. The supernatant solution, containing globulin and albumin proteins and soluble carbohydrate, was dialysed with five changes of 25mM sodium acetate buffer pH 4.8 over 48 hours, and centrifuged to precipitate the globulin proteins. Supernatant and precipitate were then dialysed against distilled water

Table 5-c Amylase inhibitory activity of accessions of *P. vulgaris*

Accession	Arc Type	α-amylase inhibition %
G09989b		25.0
G10000		57.0
G10007		27.0
G11051	A2	0.0
G12866	A2	0.0
G12871		36.0
G12880		23.0
G12882	A1	80.0
G12923	A3	100.0
G12933	A3	100.0
G12949	A4	95.0
G12952	A4	64.0
G12953	A4	100.0
G12954	A4	100.0
G13016		26.0

Table 6 Chi-squared analysis of co-presence of arcelin 4 and WBAI (novel amylase inhibitor)

CHI-SQ ARC4:WBAI

Parent or Generation	no of seeds of each Phenotype		Chi-squared of 3:1 segregation	probability P
	$WBAI^+$:Arc^4	$wbai^-$:arc^-		
Ofuku-5		20		
× **G12953**	20			
F_1	2			
F_2	53	24	1.56	0.2<P<0.3
F_3 (all combined)	60	22	0.14	0.7<P<0.8

$WBAI^+$	WBAI containing (inhibits *Z. subfasciatus* α-amylase)	$wbai^-$	WBAI deficient
Arc^4	arcelin containing	arc^-	arcelin deficient

Data and calculations kindly supplied by Mr Masao Ishimoto of NARC, Tsukuba, Japan

Correlation Chi-squared (χ^2) of the linkage of WBAI to Arcelin 4 was performed with the following values:

F_2 generation 72.4 (highly unlikely to be due to chance)

F_3 generation 77.0 (also highly unlikely to be due to chance).

These were performed using a 2×2 contingency table for presence or absence of Arc 4 with presence or absence of WBAI

Two **null hypotheses (H0)** were proposed:

that there was no single gene effect on the presence or absence of Arc 4:$WBAI^+$ (**H0=nul**), i.e. that the numbers of seeds of each phenotype were equal;

that a single dominant gene controls the presence of the resistant phenotype (**H0=3:1**), i.e. that there are three times as many seeds in the resistant phenotype than the susceptible.

	H0=null	**significance**	**H0=3:1**	**significance**
F_2	10.92	highly unlikely	1.56	likely in 30% of cases
F_3	17.61	highly unlikely	0.14	likely in 80% of cases

and freeze-dried to give a fraction containing the globulin proteins and a fraction containing a mixture of albumin proteins and carbohydrates.

Globulin and albumin seed protein fractions were purified both from the resistant accession G12953, and from commercial red kidney beans (**RKB**, susceptible), and were incorporated into diets at a range of concentrations up to 10% (dry wt/dry wt). Approximate physiological concentrations in these two lines are as follows:

Globulin Proteins	in **RKB**; approximately 10% of seed dry weight (Romero Andreas *et al.* 1986)
	G12953 globulins, measured from extracted fraction as a proportion of starting seed material compose 6% w/w of the seed meal
Albumin Fraction	in **RKB**; approximately 7.5% of seed dry weight (Romero Andreas *et al.* 1986)
	in **G12953**; approximately 15% w/w of seed meal, as estimated from extractions

This suggests that in the commercial strains **RKB** globulins are the major protein component, and in the resistant, **arcelin 4**-containing **G12953** albumins are a more important constituent. The significance of these different ratios *albumin:globulin* is brought out further into the work.

The globulin fractions (containing both phaseolin and PHA) from both resistant and susceptible seed lines had negligible effects on survival to adult (see Table 7-a), and whilst G12953 globulins extended development period slightly (0.9 days) RKB globulins had no effect on median development period, at concentrations up to 10% (the approximate physiological level in RKB). At the physiological levels the albumin fraction from RKB had inconsistent, and non-deleterious effects upon larval development and only extended the development period at 7.5% and 10% inclusion, relative to the control (Table 7-a, analysis of variance in Table 7-b) (NOTE that 5% to 10% inclusion are not significantly different from each other and represent a maximum 5.3 day (17%) extension of development period). The albumin fraction from G12953, on the other hand, was markedly more effective at lower concentrations, with a 7% increase in the development period at 5%

Table 7-a Effects of inclusion of fractions from seeds on development of *Z. subfasciatus*

RESULTS\ZABROTES.SUB\BIOASSAY.WQ1 ALBS+GLOBS

Meal Component	% inclusn	N reps	Dev. Per. (days)	*	% surv. to adult	*
RKB (susceptible variety)						
albumin fraction	0.0	12	31.1	†a	60.3	†a
	2.5	10	33.4	ab	55.3	ab
	5.0	12	33.7	abc	69.6	b
	7.5	10	34.5	bc	65.7	b
	10.0	12	36.4	c	46.3	ab
globulin proteins	0.0	11	31.2	†a	84.6	‡a
	2.5	12	32.2	a	73.6	a
	5.0	12	31.9	a	81.1	a
	7.5	12	31.0	a	67.1	a
	10.0	12	30.7	a	81.8	a
G12953 (resistant variety)						
albumin fraction	0.0	11	32.5	‡a	56.2	†a
	2.5	12	30.5	ab	44.4	a
	5.0	10	34.7	b	34.5	a
	7.5	11	39.8	c	61.5	a
	10.0	11	41.2	c	41.4	a
globulin proteins	0.0	11	33.2	‡a	45.5	†a
	2.5	10	33.9	ab	30.0	a
	5.0	12	34.1	b	47.3	a
	7.5	12	33.5	a	36.5	a
	10.0	4	34.1	ab	40.0	a

* Similar letters indicate means are not significantly different at P>0.05 (Student's paired **t** test). Controls are compared with each other (symbols † and ‡), and levels of inclusion within a single treatment are compared with each other (letters): levels of inclusion between treatments are not compared.

Table 7-b Analysis of Variance of inclusion of protein fractions on development of *Z. subfasciatus* larvae to adult

DEVELOPMENT PERIOD (Days)

	ss	df	MS	F	Prob
Bean	261.48	1	261.48	19.43	.01%
Protein	283.19	1	283.19	21.04	.01%
Conc %	411.07	4	102.77	7.64	.01%
Bean × Prot	19.07	1	19.07	1.42	—
Bean × Conc %	289.40	4	72.35	5.38	.01%
Prot × Conc %	506.32	4	126.58	9.40	.01%
Residual	2732.23	203	13.46		
TOTAL	4502.76	218			

% SURVIVAL Arcsin Transformed

	ss	df	MS	F	Prob
Bean	15.92	1	15.92	3.61	—
Protein	3.58	1	3.58	0.81	—
Conc %	0.79	4	0.20	0.05	—
Bean × Prot	4.12	1	4.12	0.31	—
Bean × Conc %	1.64	4	0.41	0.03	—
Prot × Conc %	4.11	4	1.02	0.08	—
Residual	895.07	203	4.41		
TOTAL	925.24	218			

concentration, and 27% increase in development period at 10% inclusion (Table 7-a). *% survival* was not greatly affected by any treatment. The lack of significant effects on *% survival to adult* is probably due to the variability of the replicates due to inherent problems in bioassay techniques: as explained elsewhere the difficulties in obtaining large quantities of material and the built-in cross-referencing inherent in bioassays of further purified material have led me to consider that these results are a sufficient basis for further work without repetition.

12.4 SEPARATION OF ALBUMIN PROTEINS AND SOLUBLE CARBOHYDRATES

Table 7-a and Table 7-b indicate that components that were actively antimetabolic in bioassay were found in the albumin fraction. This consists of both albumin proteins and soluble carbohydrate, i.e. all components of the seed meal soluble in acetate buffer pH 4.8.

The albumin fraction (supernatant after dialysing borate extract against sodium acetate pH 4.8 buffer, and also obtained by direct extraction of seed meal with 25mM sodium acetate buffer pH 4.8) was mixed with preswollen CM-52 cation exchange resin in the same buffer (50ml); albumin proteins mostly bound to the resin because of their charged amino acids, whereas most polysaccharides are relatively weakly charged and remain in solution.

The resin was separated from the solution by gentle centrifuging (2,000 g × 15 mins) and washed twice with buffer before being packed into a column (2.4cm × 50cm). Material not bound to the resin was termed *Not bound* or *soluble carbohydrate fractions* and contained a little protein amongst the carbohydrate: after dialysis and freeze-drying this was measured to constitute 4% by weight of seeds of both resistant accession **G12953** and of susceptible **haricot**, or **G10019**.

The '*soluble carbohydrate fractions*' were isolated from two resistant accessions, G12953 and G12954, and one susceptible accession G10019. No fraction gave a significant decrease in *% survival* at concentrations up to 10% (approximately 2½ × the physiological level) in bio-assays (Table 8-a, analysis of variance Table 8-b). The material from resistant varieties extended *development period* more than G10019 carbohydrate; overall the effect is significant at 0.1%, however individually the effect is not significant when compared with controls. The variability amongst controls and experimental diets is inherent in bioassay with this particular insect species, but still allows the effects of the fractions tested to be determined.

Examination of albumin and globulin proteins from the resistant accession G12953 by SDS-PAGE showed that the major polypeptides of 32,000 - 36,000 M_r were present in the albumin fraction (Plate 3 track 3). Proteins were eluted from the ion exchange resin using a salt gradient (0 - 0.5M) in the same buffer (pH 4.8 25mM

Table 8-a Effects of carbohydrate material and *arcelin 4* on *Z. subfasciatus* development

RESULTS\ZABROTES.SUB\BIOASSAY.WQ1 NON-BOUND M88

Material	inclus %	N reps	DP days	*	% surv+	*
G12953 non-bound	CTRL	10	40.9	1a	30.0%	1a
	2.5%	10	45.5	a	28.2%	a
	5.0%	10	50.6	a	28.9%	a
	7.5%	10	49.9	a	33.9%	a
G12954 non-bound	CTRL	10	39.9	1b	48.4%	1b
	2.5%	10	46.9	b	15.2%	bc
	5.0%	10	46.9	b	12.3%	c
	7.5%	10	50.5	b	24.5%	bc
	10.0%	10	49.9	b	36.7%	bc
G10007 non-bound	CTRL	10	41.1	1c	22.9%	1d
	2.5%	10	40.2	c	55.6%	d
	5.0%	8	45.5	c	38.8%	d
	7.5%	10	42.0	c	67.6%	d
	10.0%	8	41.9	c	62.2%	d
G12953 Arcelin4, purified from ion-exchange chromatography	CTRL	10	44.9	1d	25.4%	1e
	2.5%	10	45.3	d	9.2%	ef
	5.0%	10	52.8	d	4.1%	ef
	7.5%	10	51.5	d	25.7%	e
	10.0%	10	52.5	d	0.5%	f

Note:

% Emergence figures for each replicate were transformed by arcsin transformation (see numeric methods 7.3.a, page MM.60), and the +mean of transformation calculated before untransforming.

* Levels of inclusion within a single treatments were compared by Student's **t** test (letters): the controls were all similar to each other by this test (†)

Table 8-b Inclusion of carbohydrate material and arcelin 4 on *Z. subfasciatus* development. Analysis of Variance

Non-bound: Development Period

	ss	df	MS	F	Prob
Bean	697.00	2	348.50	16.69	0.001
Conc %	1022.17	4	255.54	12.24	0.001
Residual	2714.59	130	20.88		
TOTAL	4433.77	136			

Non-bound: % SURVIVAL (Transformed)

	ss	df	MS	F	Prob
Bean	1.49	2	0.74	2.87	—
Conc %	0.84	4	0.21	0.81	—
Residual	33.65	130	0.26		
TOTAL	35.97	136			

Arcelin: DEVELOPMENT PERIOD days

	ss	df	MS	F	Prob
Conc %	625.99	4	156.45	6.77	0.001
Residual	1063.51	46	23.12		
TOTAL	1689.30	50			

Arcelin: % SURVIVAL transformed

	ss	df	MS	F	Prob
Conc %	1.65	4	0.41	3.14	0.05
Residual	6.04	46	0.13		
TOTAL	7.69	50			

sodium acetate). Four peaks eluted, with the third the major one consisting of the subunits 33K and 36K: other peaks contained material of PHA size (29K) or larger (43K). The third peak was found to be the active material.

The **arcelin 4** (the third peak) obtained by salt gradient elution from CM-cellulose resin was bioassayed and found to be detrimental to *Z. subfasciatus* larval survival (Table 8-a) at inclusions up to 10.0%; the physiological concentration of this material in the resistant seeds was estimated to be approx. 10%. Survival of larvae to adults was reduced by as much as 85% in diets containing arcelin 4 (Table 8-a).

Examination of this third peak by 2-D gel electrophoresis (non-denaturing/denaturing) indicated that it comprised at least two distinct proteins. These were separated by gel filtration (50mM borate pH 7.0 100mM NaCl, on sephadex S-400 2.4cm × 60cm). The bioassays were repeated with the two peaks obtained from gel filtration, and with the peaks combined in the ratio at which they are present in material purified by ion exchange chromatography; the results of the bioassay are presented in Table 9-. This experiment confirmed the antimetabolic nature of the material purified by ion exchange chromatography, with a 37% reduction in *% survival* observed at 10% incorporation. Arcelin represented 71% of the partially purified fraction from ion-exchange chromatography, and the arcelin at an incorporation level of 7.1% gave a reduction in survival of 42%. "Peak 2" at an incorporation level of 2.9% gave no significant reduction in survival (3%), and arcelin and "peak 2" combined at levels of 7.1% and 2.9% respectively gave results comparable to arcelin alone (reduction in survival 53%). These bio-assay data clearly demonstrate that arcelin is an effective anti-metabolite alone, and that the antimetabolic effects of the material purified by ion-exchange chromatography are due to arcelin alone. Besides its effects on larval survival, arcelin also extended the median development period by up to approx. 30%.

12.5 DISCUSSION

Analysis of protein components of the dry seed by SDS-PAGE reveals the presence of particular subunits which are closely associated with resistance. On the basis of the apparent molecular size of the subunits, and also on the basis of the accessions in which these subunits are observed (in collaboration with CIAT) these subunits are considered synonymous with the subunits observed for the novel protein types named *arcelin* which are present in accessions resistant to *Z. subfasciatus* attack (Osborn *et al.* 1986).

Table 9- Effects of arcelin 4 purified by gel filtration on larval development of *Z. subfasciatus*

Diet	inclusion %	% survival	Median Dev. period (dys)
Crude arc 4 from CM-cell.	10% inclusion	53	36.8
Pure arc 4 (S-400)	7.1% inclusion	48	37.5
peak 2 S-400	2.9% inclusion	81	34.6
Arc 4 + Peak 2	7.1% + 2.9%	39	41.2
CONTROL	haricot meal	83	30.0

Successive purification of the protein which is constituted of these subunits, and its incorporation into haricot-based artificial diets, confirms its antimetabolic nature and again conforms with published observations (Osborn *et al.* 1988), although these authors were working on arcelin type 1 and the material from accession **G12953** appears to be the rather different arcelin type 4. The presence of arcelin in resistant seed accessions also appears to be correlated with reduced levels of phaseolin and PHA, although published work indicates that the presence of arcelin in accessions, and subsequent reduction in compositional levels of phaseolin and PHA, does not affect reproductive fitness (Romero Andreas *et al.* 1986).

The presence of a novel form of amylase inhibitor, active against the *Z. subfasciatus* larval gut amylase *in vitro*, is also associated with resistance, though this may be partially due to its close genetic linkage to arcelin types 1, 3 and 4. Arcelin 4 is not itself an inhibitor of *Z. subfasciatus* larval gut amylase (nor of mammalian amylase)(see Amylase Inhibitor work, wherin fractions bound to CM cellulose do not inhibit. Page R.167, Table 19).

Besides the antimetabolic effect of inclusion of arcelin 4 in artificial diets, inclusion of the non-bound carbohydrate material from resistant accessions also extends development period in line with the possibility that other factors may act in concert with arcelin 4 to give the total observed resistance.

Results 13
Mechanism of Arcelin Resistance

The novel protein arcelin 4 has been implicated in resistance of some wild accessions of *P. vulgaris* to the pest bruchid *Z. subfasciatus*.

Within the Leguminosae, a number of proteins have been characterised and demonstrated to have anti-metabolic properties towards insect pests, through a number of different mechanisms. For example, lectins bind to the gut wall and probably disrupt it; protease inhibitors which are also common in the Leguminosae may interfere with digestion of ingested proteins.

The hypothesis proposed is that *arcelin 4* is not digestible by *Z. subfasciatus* gut proteases, and hence its essential amino acids are unavailable. To test this hypothesis, the digestibility of the protein was assayed relative to a standard protein, myoglobin, and the normal major storage protein of *P. vulgaris*, phaseolin, by incubation of similar amounts of purified proteins with *Z. subfasciatus* gut enzyme preparations. The gut extract from *Z. subfasciatus* contains active proteases with a pH optimum of 5.4.

The degree of digestion was measured by SDS-PAGE analysis of samples from the incubations after varying times of digestion, and determining the degree of hydrolysis at each time point by densitometric scanning of the resulting gels.

13.1 *IN VITRO* DIGESTION OF PURE PROTEINS

In vitro digestibility studies of arcelin 4, phaseolin, and myoglobin were carried out by incubating protein substrate with larval gut homogenate at 37°C, and the remaining undigested protein at set time intervals calculated from densitometric scanning of SDS-PAGE. 37°C was used as a suitable average temperature for a pest of grain stores in the tropics.

Protein remaining was fitted to the non-linear equation

$$y\begin{pmatrix} protein \\ remaining \end{pmatrix} = A + B \times 10^{-C \times time}$$

where **A** is a measure of the protein which will remain undigested at infinite time ($t \Rightarrow \infty$) (*indigestible portion*), and the parameter **C** used to calculate the *half-life*, or time passed for half of the digestible protein remaining to be digested. Parameter **B** is a reflection of parameter **A**.

This is a theoretical equation based on the rate of digestion being proportional to the amount of available substrate remaining, and allows comparisons between different digestion mixtures. Under these experimental conditions it is not possible to give better than relative values, and so far it has not been possible to estimate how much time food spends in the gut. Note this theoretical curve fitted to the data does not imply a mechanism, but is used as an analytical tool.

The half-lives, and values for the parameters, of the proteins under standard assay conditions are given in Table 10, and the amounts of total protein digested after selected time periods illustrated in Figure 5. Myoglobin was completely digested within 3 hours, with a half-life around 18 mins. The plates indicated virtually complete absence of the protein bands, and the computer-generated scanning data gave the same results.

Scanning data for the other proteins, phaseolin and arcelin, do not appear to match the plates. The banding is more visible on the original gels (which were scanned); and at extended time periods in the presence of gut enzymes the sharp definition of the bands appears to be lost, whilst the computer scanner (linear densitometer) records and integrates for a similar total present. Reasons for this are unclear, but repeated readings and repeats of the digestion experiments gave the same results so the computer-generated data were used for analysis purposes.

The phaseolin subunit bands were digested rather slower, and the 40% undigested (as estimated from parameter **A**) illustrates that at least one subunit (from SDS-PAGE, the 43,000 M_r polypeptide; see Plate 2-a, Plate 2-b) was much less digestible, so that although 60% of the protein digested with a half-life of 240 min, the remainder virtually did not digest. Arcelin 4 itself was very resistant to digestion, and the r-value of trying to fit the non-linear equation to the data points of arcelin 4 illustrates that the fit was not very good. However parameters have been estimated, and the best-fit line plotted for comparison.

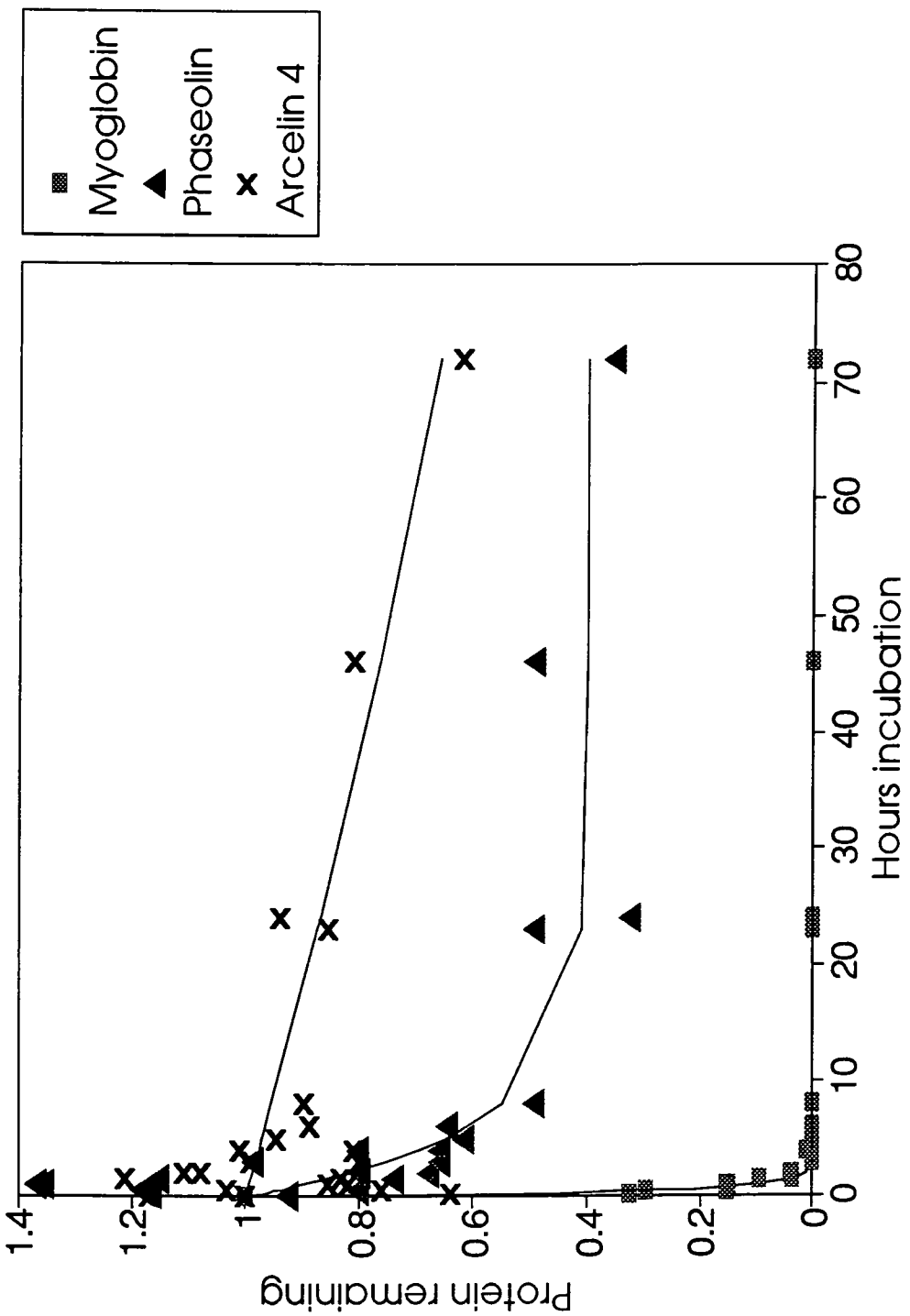


Figure 5 Digestion of purified proteins by *Z. subfasciatus* larval gut proteases, over time

Table 10 Table of parameters for digestion of proteins by *Z. subfasciatus* larval gut homogenate.

A- Pure Proteins at 2mg/ml

\RESULTS\ZABROTES.SUB\MECH_ARC.WQ1 PARAMETERS

	Myoglobin	Arcelin4	Phaseolin
A	0.000	0.000	0.400
B	1.000	1.000	0.600
C	1.003	0.003	0.075
half-life	18.0mins	120hrs	4.00hrs
r value	0.988	0.443	0.812

B- Bean meals at 5mg/ml, with phaseolin in mixture from the larval gut homogenate

\RESULTS\ZABROTES.SUB\MECH_ARC.WQ1 ARC PARAMETERS

	Arc 2	Phas	Arc 3	Phas	Arc 4	Phas
A	0.650	0.555	0.400	0.350	0.600	0.350
B	0.350	0.445	0.600	0.650	0.400	0.650
C	0.022	0.025	0.301	0.100	0.025	0.067
Half-life	20 hrs	12 hrs	24 mins	1 hr	114 hrs	4.5 hrs
r-value	0.407	0.748	0.553	0.779	0.512	0.837

a phaseolin
b arcelin 4 (with phaseolin present from the gut contents)
c myoglobin

In all cases the timing of samples was the same, run in the same order (fully labelled for phaseolin)

0B	no enzyme, start	0m	with enzyme, start	60m	1 hour
72B	no enzyme, 72 hours	20m	20 mins of incubation	90m	1½ hours
end	incubation	40m	40 mins	2h	2 hours
					etc

M markers, from top 66K, 45K (diffuse), 36K, 29K, 24K, 20.1K, 14.2K

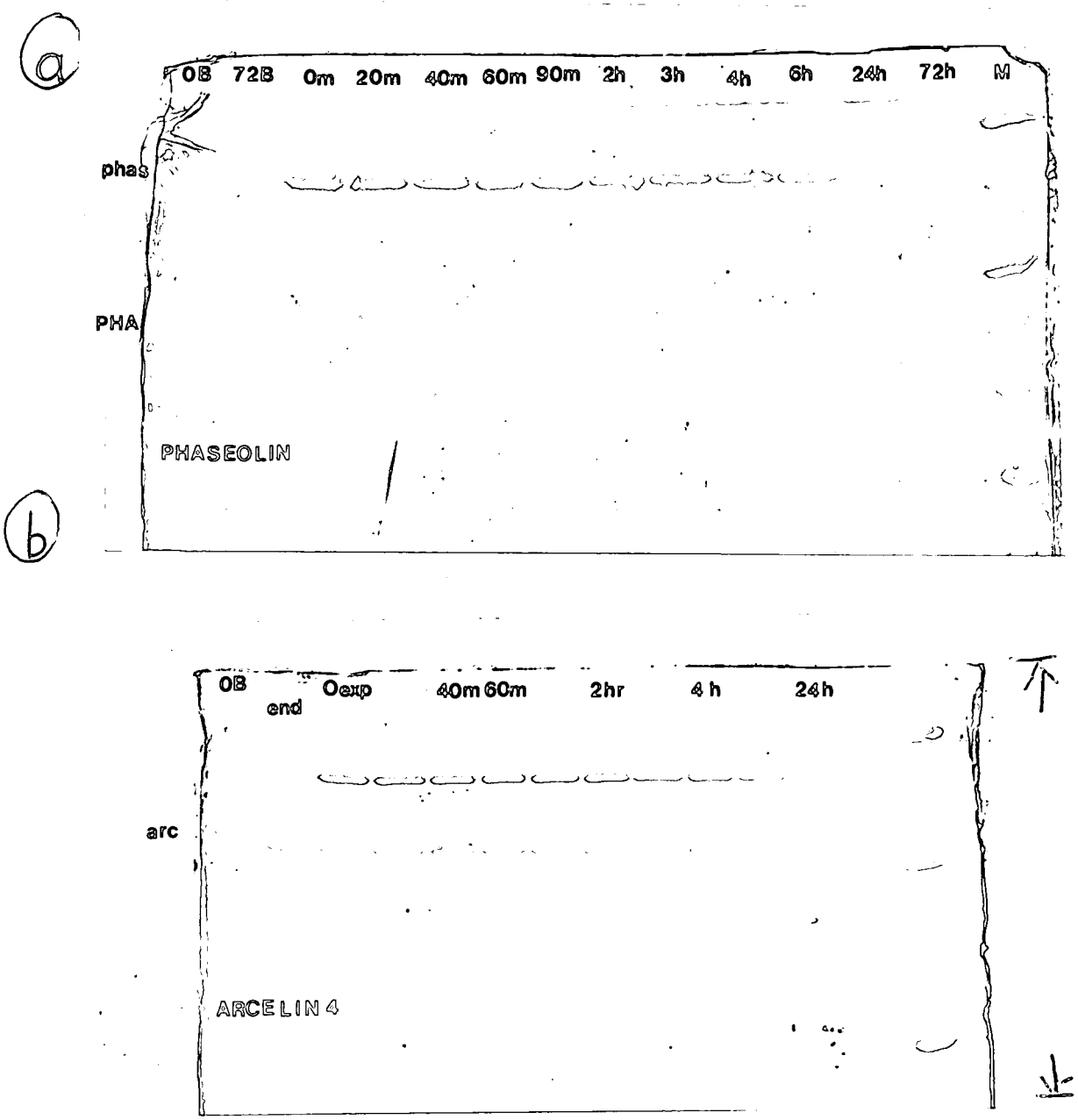


Plate 2 Digestion of proteins *in vitro* by *Z. subfasciatus* larval gut homogenate

a phaseolin
b arcelin 4 (with phaseolin present from the gut contents)
c myoglobin

In all cases the timing of samples was the same, run in the same order (fully labelled for phaseolin)

0B	no enzyme, start	**0m**	with enzyme, start	**60m**	1 hour
72B	no enzyme, 72 hours	**20m**	20 mins of incubation	**90m**	1½ hours
end	incubation	**40m**	40 mins	**2h**	2 hours
					etc

M markers, from top 66K, 45K (diffuse), 36K, 29K, 24K, 20.1K, 14.2K

(c)

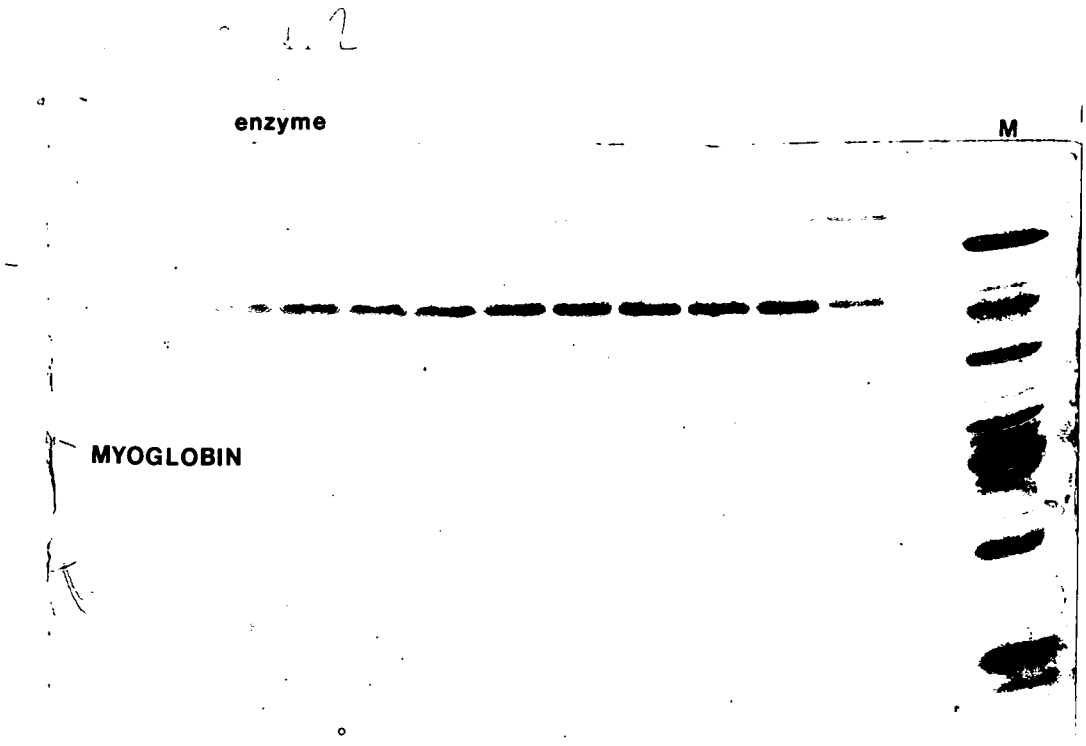


Plate 2 Digestion of proteins *in vitro* by *Z. subfasciatus* larval gut homogenate

13.2.a Arcelin 4 in G12953

Detesta'd bean meal (the testa was removed since the testa contains tannins, and these polyphenols are known to act as enzyme inhibitors and are therefore likely to inhibit the larval gut proteases) at 5mg/ml extracted for 15 hrs (4°C) in buffer pH 5.4, was incubated under the same experimental conditions as the pure protein solutions had been. The variety chosen for test was G12953, as it contains arcelin 4, and since high concentrations of phaseolin are present in the larval gut homogenate this enabled the effects of arcelin 4 on the digestion of other proteins in the mixture to be investigated.

Initial results (Figure 6-a and Table 10) indicated that the rate of digestion of phaseolin was not much altered by its inclusion in a mixture with arcelin 4. The *Half-Life* of the digestible portion was still around 4 hours, and the indigestible portion still around 40% of the whole. From the graph it would appear that 30% of arcelin 4 was digested in this mixture with a half-life around 10 hours, the remaining 70% being indigestible; these parameters were tried and did not improve the r-value (nor greatly affect total digested protein after 24 hours), because of the scatter in the first four hours of digestion, so the previously calculated parameters for arcelin 4 were retained.

13.2.b Other arcelin-containing lines

CIAT also asked us to investigate the mechanisms of action of other lines containing arcelins, since arcelin takes different forms (named form *1* through to form *4*), and the different lines exhibit varying levels of resistance.

In particular we were interested in arcelin 3 since, according to Osborn *et al.* 1988 this shares two to three subunits with arcelin 4, yet our bioassay data suggested that its presence is not associated with resistance in the seed line (see Table 5-c, page R.112). Arcelins 1 and 2 were also of interest since lines containing arcelin 2 do not inhibit larval amylase in *Z. subfasciatus*, and whilst wild lines are heterozygous for the presence of arcelin 1 and do not exhibit high levels of resistance, experimental lines

bred true showed very high levels of resistance, suggesting a more active antimetabolic level of activity than that in the arcelin 4 containing lines.

The rates of digestion of the arcelin and the rate of digestion of the phaseolin are illustrated in Figure 6-b, Figure 6-c and Figure 6-d. Bean meals were prepared from a minimum of 12 detesta'd seeds of each selected accession as follows:

RAZ-2 is a homozygous cross-breed containing arcelin 1, since arcelin 1 is not found homozygous in CIAT accessions;

G12866 is a typical source for arcelin 2; and

G12922 is a typical source for arcelin 3.

Arcelin 1 in mixture with larval gut homogenate showed general lack of digestion of all proteins involved; however the points did not fit an obvious linear regression, nor was it sensible to fit an exponential decay curve to them (Figure 6-b). Arcelin 3 itself was highly digestible, and the phaseolin in this preparation appeared to digest rapidly as well (Figure 6-d). Parameters for arcelins types 2 to 4 are given in Table 10 (arcelin 1 parameters are not included as they were not determined).

13.3 INCLUSION OF ARCELIN 3 IN ARTIFICIAL DIETS

Since arcelin 3 *in vitro* was not resistant to proteolysis, and in bioassay arcelin 3-containing accessions were not resistant to *Z. subfasciatus* development (*development period* was extended, but *% survival* was not reduced), purified arcelin 3 was included in artificial diets to test its antimetabolic effects under the same conditions as had been used for arcelin 4.

Arcelin 3 was purified from the line **G12922** by extracting albumin proteins in 25mM sodium acetate buffer pH 4.8, and binding the arcelin to CM-cellulose, following a similar procedure to that used for arcelin 4, and then included in artificial diets at 10% (again similar to the trials done with arcelin 4) in feeding trials to *Z. subfasciatus*. The results (Table 11) illustrate that arcelin 3 was not antimetabolic to *Z. subfasciatus*, in that there was only a small reduction in survival (not significant at 5% level) and

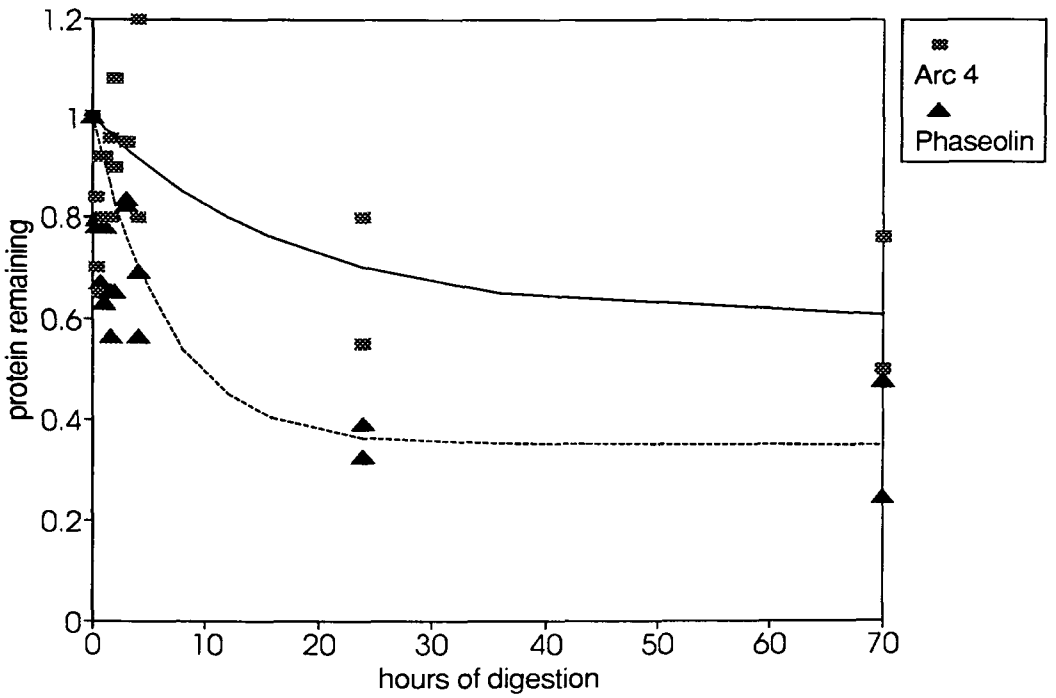


Figure 6-a Digestion of arcelin 4, and phaseolin in the mixture, by *Z. subfasciatus* larval gut homogenate. Bean meal is **G12953**, and phaseolin is present in gut homogenate

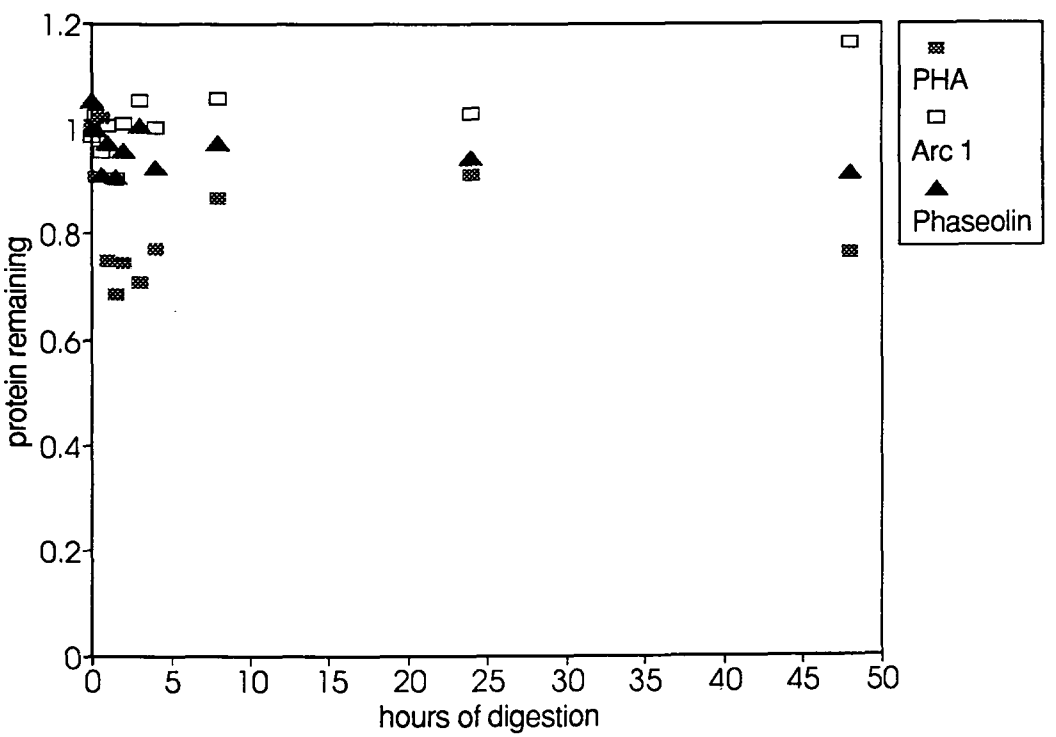


Figure 6-b Digestion of proteins in meal containing arcelin 1 (**RAZ-2**)

Table 11 Effects of *arcelin 3* on survival and development of *Z. subfasciatus* larvae

\RESULTS\ZABROTES.SUB\BIOASSAY.WQ1 ARC3

Diet	reps	% survival	*	Dev Period (days)	*
Haricot Control	6	94.2%	a	43.9	a
Haricot + 10% Arc 3	6	84.3%	a	52.0	b

Few adults actually managed to emerge from the pellets, although on opening the pellets a number of fully developed adults were found inside, both alive and dead (100 days). In view of this % *survival* refers to total number of adults from hatched eggs, wheras *development period* is based on those adults which did emerge

* means have been compared by Student's paired **t**-test, with the % survival values transformed as for %, by arcsin transformation.

increase in development period, certainly not as much as for arcelin 4 under the same conditions.

13.4 OVERCOMING EXHIBITED RESISTANCE OF G12953 BY PHASEOLIN SUPPLEMENTATION

Table 5-b indicates that the accessions containing arcelin and exhibiting resistance also contain low levels of phaseolin (also illustrated in Figure 7). The hypothesis proposed for resistance caused by arcelin 4 is one of non-availability of amino-acids; this would be more pronounced in the absence of phaseolin, which is digestible by *Z. subfasciatus* proteolytic enzymes.

Basal diet made of detesta'd **G12953** meal was supplemented with digestible protein to provide amino acids for larval growth. In a preliminary experiment, acid-hydrolysed casein was used as supplement, included at levels up to 10%. However some factor involved in the inclusion of the casein, whether the casein itself, contaminants present, or the high osmotic pressure generated, reduced larval development to none on diets based on haricot meal (the unsupplemented control gave normal development), presumably having the same effects on G12953, though no comparison could be made since no larvae emerged from the 0% supplemented either with this basal diet.

Therefore **phaseolin**, as RKB total globulins, was used to supplement the basal diet of G12953 meal.

The results (Table 12) also illustrate the effect of inclusion of the testa in artificial diets, with no development of larvae to adult on either susceptible or resistant meals.

Supplementation of basal haricot meal altered *% emergence* beneficially, but did not alter *% survival.* The extra emergent adults may have contributed to the extended development period observed, which amounts to 6.3 days.

Supplementation of resistant accession **G12953** basal diet affected all parameters beneficially, improving *% emergence* from 0%, and improving *% survival* by more than 3×. Although neither *% survival* nor *development period* were as good as for haricot

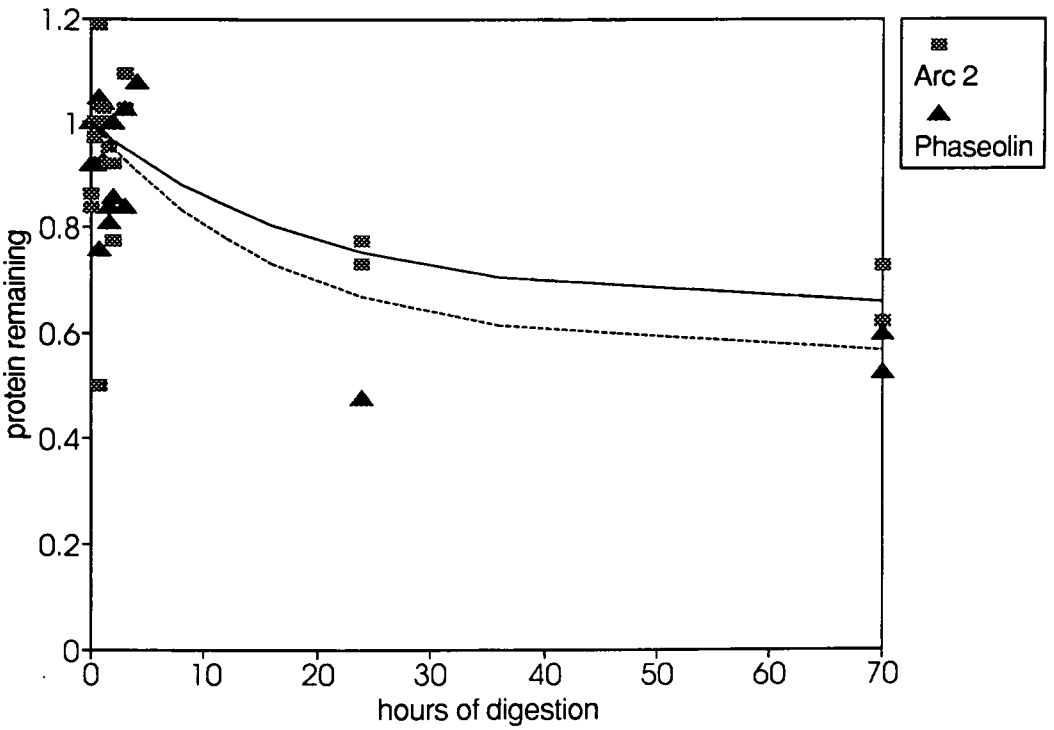


Figure 6-c Digestion of proteins in bean meal containing arcelin 2 (**G12866**)

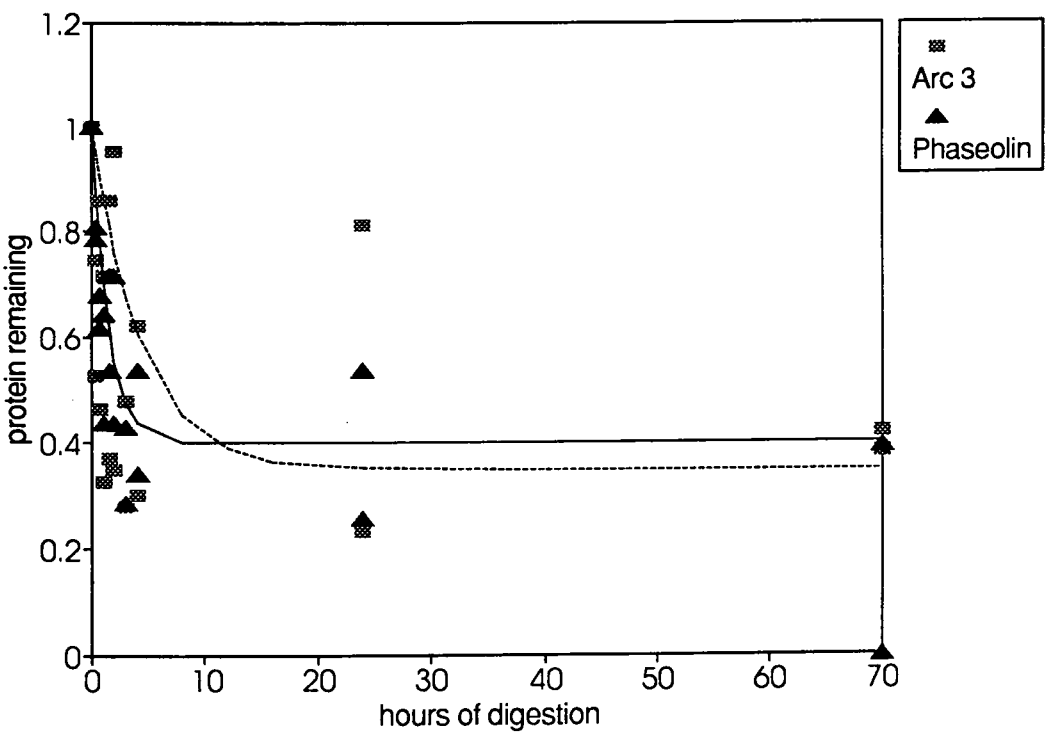


Figure 6-d Digestion of proteins in bean meal containing arcelin 3 (**G12922**)

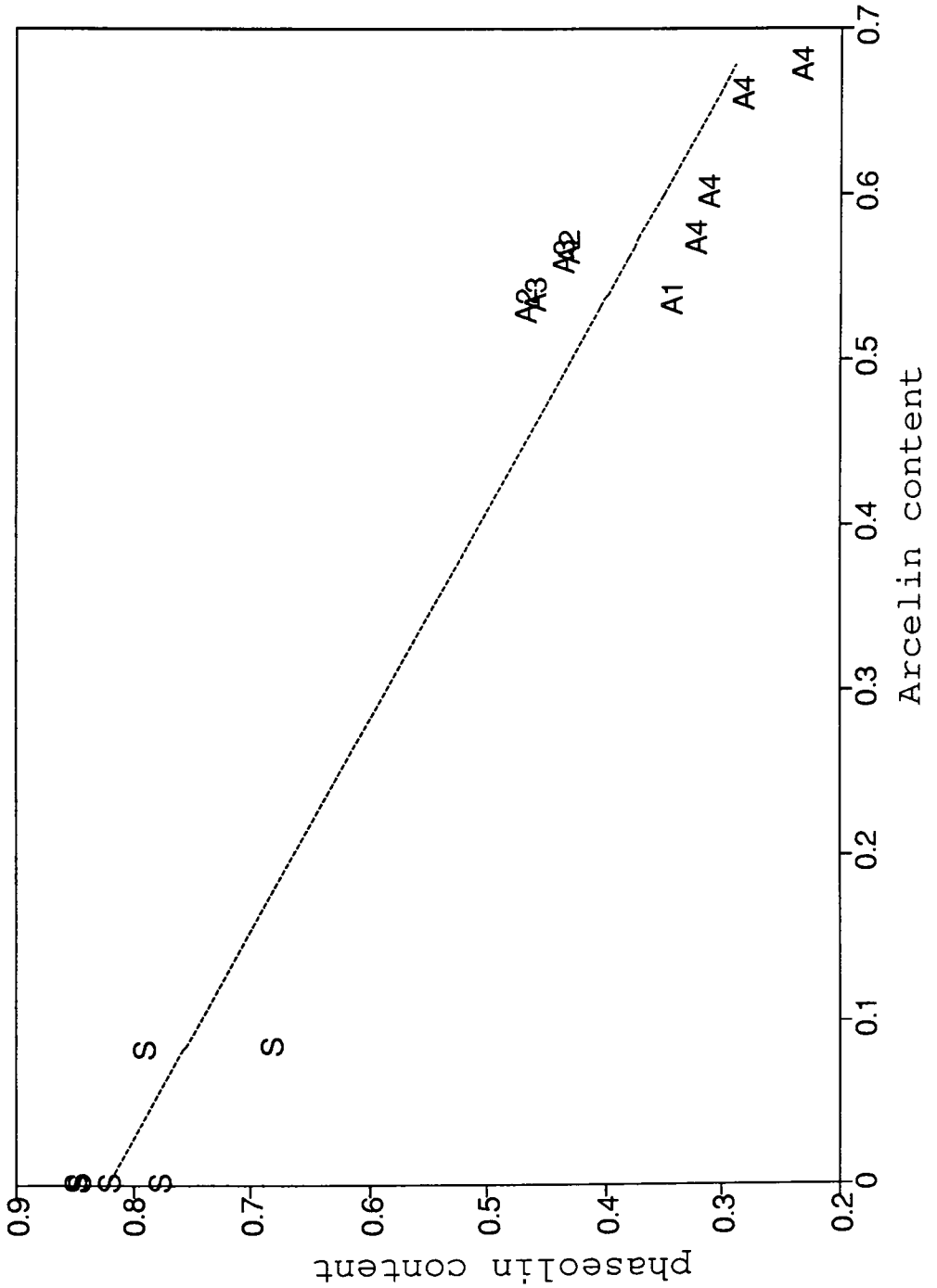


Figure 7 *Arcelin* and *phaseolin* content of different accessions. r^2=0.954

Table 12 Effects of adding 10% phaseolin to G12953 meal on *Z. subfasciatus* development

data from 30 Oct to 20 Nov 1989

Diet	reps	% emergent	% survival	*	Dev Period (days)	*
Haricot inc testa	6	0.0%	0.0%	d	NA	d
G12953 inc testa	5	0.0%	0.0%	d	NA	d
Haricot	6	36.8%	99.2%	a	36.3	a
Haricot + RKB globs	5	45.0%	98.1%	a	42.6	b
G12953	5	0.0%	19.0%	c	0.0	d
G12953 + RKB globs	6	17.4%	64.5%	b	66.8	c

NOTES: Because of the low emergence, the pellets were dissected to count fully developed adults inside the pellets. Failure to emerge may be attributed to failure of the larvae to detect suitable positional signals, e.g. because of pellet coating technique. Figures of *development period* are based on the adults which did emerge; *% emergent* is based on the number of hatched eggs which subsequently emerged as adults (this column is included in the table because it allows comparison to be made with earlier trials and with seed bioassay, at which time the pellets or seeds were not opened to count non-emergent adults); and *% survival* is based on the total number of fully developed adults both emergent and found within the pellets. 3-5 eggs were laid per pellet, and hatch was better than 80% in all cases

* means were compared by Student's t-test and similar letters indicate results not significantly different at $P<0.05$.

basal diet the results showed considerable loss of resistance to *Z. subfasciatus* development.

13.5 INCLUSION OF ARCELIN IN MAMMALIAN DIETS

Having determined a mechanism for pest resistance, it was important to examine if this was a mechanism which could be used sensibly for beans for human and mammalian consumption; i.e. if beans containing arcelin (and resistant to *Z. subfasciatus*) are more toxic to mammals than those without arcelin. For a number of reasons *Arcelin-4* containing seeds were not made available in large quantities, and we were supplied with seeds of the cross **RAZ-2**, which contains *Arcelin 1*. The two forms of arcelin are different in my opinion, but these are the only available data on arcelin toxicity and are included for this reason.

The arcelin-containing cross **RAZ-2** (large-seeded cross of an arcelin 1 containing line with a commercial line — presence of arcelin is dominant) was compared with its susceptible parent, **EMP 175**, which contains normal quantities of PHA and phaseolin. This experimental work was carried out in Rowett Research Institute by Dr Arpad Pusztai, but is reported here because of its relevance to the project and its place in the arcelin story. Data are given without statistics. In all trials bean meals were supplemented to the same extent with amino acids to ensure that nutritional value was not limited by the absence of a single amino-acid where others were present to sufficiency. The feeding trials involved both cooked and uncooked meals, since commercial *P. vulgaris* is toxic to mammals in the raw state (as a dietary protein supplement) because of the disruptive action of PHA lectins on gut epithelial wall, and is nutritionally favourable after denaturation of lectins by cooking.

Rat mean liveweights (averages of four rats) on each diet are illustrated in Figure 8, and various other parameters (total of four rats) of the respective diets in Table 13.

Although the non-protein diet and the diet of 10% uncooked susceptible line follow a similar negative weight gain pattern, previous experiments have indicated that this could be for different reasons. Rats lose protein at a constant rate through enzyme secretion into the gut, and expect to gain more through the action of proteolytic enzymes

From Pusztai's report to CIAT

Diets:

NPC - non-protein diet, for estimation of endogenous protein loss

LA - 10% w/w lactalbumin, ideal protein diet

RAZ-2 resistant, arcelin-containing line

EMP-175 commercial, susceptible line

10% protein in the diet, uncooked bean meal

5% protein in the diet, uncooked, supplemented with 5% lactalbumin protein

10% protein in the diet from **cooked** bean meal

Table 13 Arcelin inclusion as a protein supplement for rats, from Dr A Pusztai RRI

		ideal	RAZ-2 (arcelin containing)			EMP 175 (non-arcelin)		
	NPC	LA	10%	5% (+5% LA)	Boiled 10%	10%	5% (+5% LA)	Boiled 10%
Weight change/10 dy	-73.1	+232.7	+35.9	+100.0	+191.4	-72.6	+7.2	+217.4
% N intake retained[1]	18.5	96.2	79.1	86.0	88.4	63.6	81.0	89.8
N Digestibility (%)	-	100	72.0	83.6	88.6	45.2	73.9	91.9
Biological value	-	98.0	83.3	87.6	86.7	61.7	91.1	92.9
Food Conversion Efficiency	-	0.43	0.10	0.24	0.36	negative	0.03	0.38

[1]% of total N in food which is retained, estimated from total N over 10 days *in feed* - that *recovered in faeces*

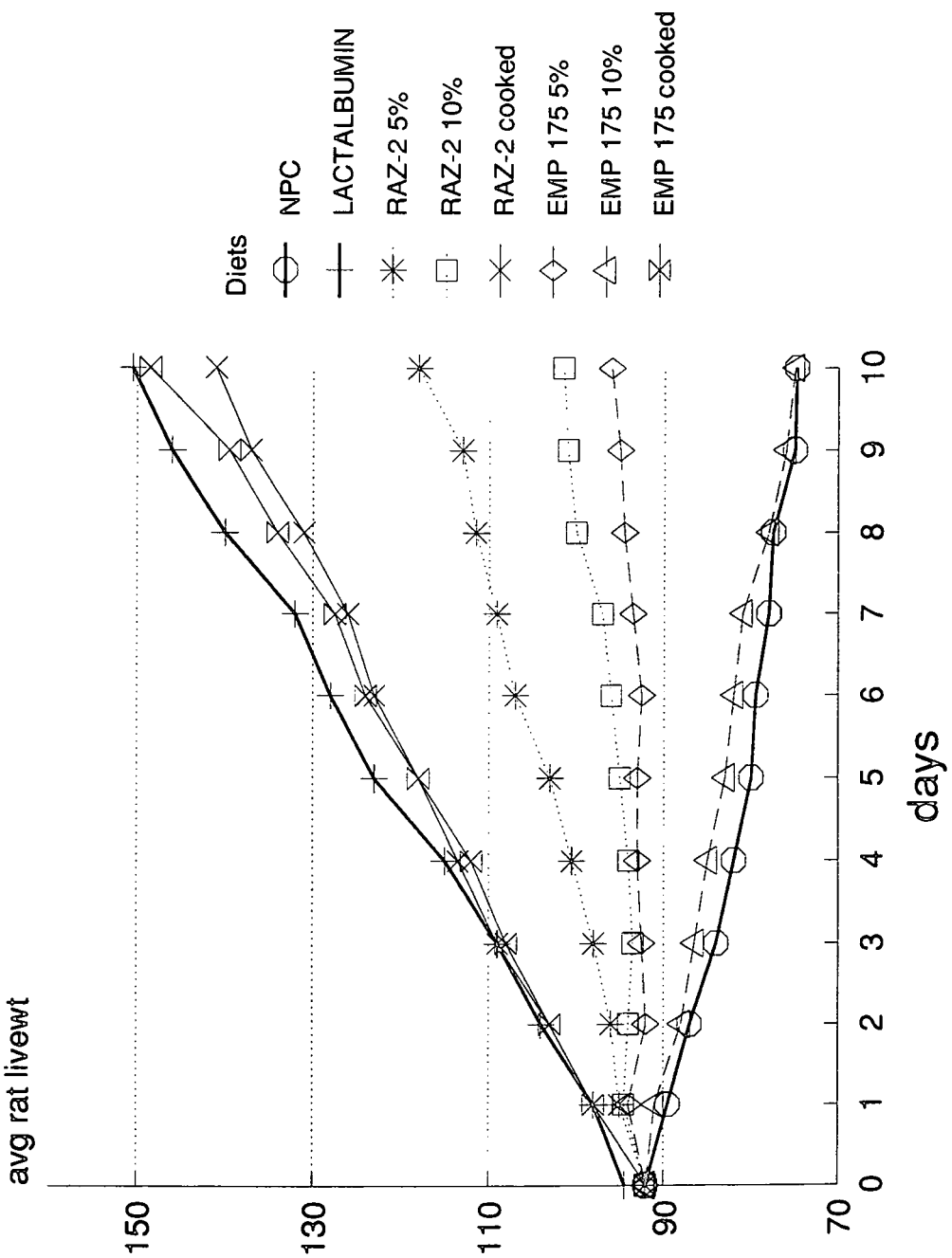


Figure 8 Development of rats fed diets supplemented with arcelin

(amongst those secreted) on proteins present in the diet. Therefore with no protein present in the gut, a certain nitrogen loss, and consequent bodyweight loss, will occur. However lectins (present in EMP 175 meal) are known to disrupt mammalian gut epithelial membranes and to cause oversecretion of pancreatic enzymes (various papers by Pusztai and King, see 4.1 in Introduction), hastening this nitrogen loss in spite of digestion of proteins in the diet. The weight loss on a non-protein diet is usually reversible on suplementation of the diet with protein, wheras the damage done by PHA lectins is often irreversible.

The uncooked meal from RAZ-2 did not exhibit this antimetabolic effect to the same extent, suggesting that RAZ-2 does not contain such high levels of lectins. This suggests that the arcelin containing line (in which arcelin protein largely replaces both phaseolin and PHA lectins) is safer to feed to mammals in an uncooked or partially cooked state as a protein supplement than are conventional *P. vulgaris* cultivars.

Cooked meals not only have protein antimetabolic components deactivated, but also denature the storage proteins (phaseolin amongst them); phaseolin is known to be conformationally difficult to digest in the mammalian digestive system in the native state. In the cooked form it can be seen that the arcelin-containing line was nutritionally very similar to the commercial line (after both have been supplemented with limiting amino acids), and both of these were nutritionally similar to diets made up with ideal milk protein, attesting to its suitability for commercial use as a protein supplement in mammalian diets.

13.6 CONCLUSIONS TO MECHANISM OF ARCELIN RESISTANCE

The hypothesis initially proposed for the antimetabolic action of arcelin 4 was that the protein was conformationally resistant to proteolysis by *Z. subfasciatus* larval gut enzyme, and this, combined with the absence of large quantities of more readily digestible proteins, prevented larval bruchid development through protein starvation.

The presence of proteolytic enzymes and their activity in the *Z. subfasciatus* larval gut is readily demonstrated by digestion of both myoglobin and the normal *P. vulgaris* storage protein phaseolin. The form of the fitted curve was an exponential decay, fitted because digestion rate would probably be substrate concentration dependent,

and with reduced substrate (after some has digested) the digestion rate would be slower. Examination of the data, both visually and statistically (by r^2 value), indicates that this curve fits adequately. However arcelin 4 only digests very slowly if at all, or if the half-life of 10hrs is taken, then this is the half-life for only 30% of the protein with the remainder not digesting under these conditions.

Assuming the relative rates of digestion for the different proteins to be broadly equivalent under the conditions in the larval gut compared with experimental conditions, it is apparent that the arcelin 4 would need to remain in the gut for much longer than phaseolin, and the larva therefore develop more slowly, for sufficient protein to be digested. At this rate of ingestion, energy used for respiration may exceed energy intake.

Arcelin 4 is probably not active as a protease inhibitor, at least not in *Z. subfasciatus* larvae. The rate of digestion of phaseolin is virtually unaffected by being in mixture with arcelin 4, and supplementation of a basal diet of G12953 (low phaseolin, high arcelin 4) with phaseolin overcomes to a great extent the observed resistance of G12953. The result that not all resistance is overcome could be explained in that conditions were not optimal, phaseolin was supplemented at 10% and the ratio of carbohydrate to protein not optimised. However other factors could also be contributing to G12953 resistance, 'though arcelin 4 is probably the major factor.

Different types of arcelin, namely arcelin types 1 to 3, appear to act in a different manner to arcelin 4. Arcelin 1 appears to be active as a protease inhibitor, based on digestibility studies which suggest that neither arcelin 1 nor other proteins in the mixture (phaseolin and PHA subunits were identified) were digested to an appreciable extent. Arcelin 2 appears to limit digestion of phaseolin by its presence, although the effect is less pronounced than for arcelin 1. Both of these arcelin types are associated with resistance to the pest *Z. subfasciatus* in accessions which contain them. Arcelin 3, by contrast, appears not only to be highly digestible itself but also to encourage digestion of phaseolin. All of the arcelin types are the major storage proteins in seeds which contain them (Harmsen *et al.* 1988), largely replacing other seed proteins, so the indigestibility of arcelin 4 is certainly a possible mechanism on this basis, and the digestibility of arcelin 3 may account for the lack of resistance to *Z. subfasciatus* in this group of accessions.

Arcelin 3, according to the Wisconsin team, has only a single subunit different from arcelin 4 (Osborn *et al.* 1986), and yet is readily digestible *in vitro*. This could easily be explained if the single subunit disrupts the conformation of arcelin 3 so that it is open to digestion by larval gut enzymes.

Arcelin-containing lines contain arcelin to such an extent that other seed proteins are much reduced. Thus when fed to mammals, the high levels of arcelin (in this trial, arcelin 1) resulted in lower levels of the antinutritional PHA lectins, resulting in a safer diet for the rats (when fed raw). Given the differences between mammalian and *Z. subfasciatus* gut proteases, it was to be expected that protease inhibitors probably present in the arcelin 1 line would not affect mammalian proteases. Note, however, that as far as we can gather there is no genetic linkage between levels of arcelin and levels of phaseolin, although there is some linkage between arcelin and PHA. My proposed hypothesis is that the arcelin is encoded to form before phaseolin would normally form, and uses up the seed's stores of amino-acids preventing the later laying down of phaseolin stores.

Results 14
Characterisation of Arcelin 4

14.1 MOLECULAR SIZE

Arcelin 4 was purified as previously described, by extraction from milled bean meal in 25mM sodium acetate buffer pH 4.8, binding to cation exchange resin, and elution with a salt gradient. The lyophilised pooled third peak was then taken up in 50mM sodium borate with 100mM sodium chloride buffer pH 7.0, and passed through a gel filtration column of Sephadex S-400 (70cm × 2.4cm). Standards were as per Table 14, with Pk 3 from ion exchange forming the two peaks as shown. This gives approximate molecular sizes for the components as 169K for *Pk 1* (containing the *arcelin 4* resistance-conferring activity, see Table 8-a), and 102K for Pk 2. Repeated batches of arcelin 4 were purified by gel filtration in order to obtain sufficient for those feeding trials, and the elution profiles were consistent.

Subunit sizes can be readily seen from Plate 3 and Plate 4, to be around 33K and 36K.

The molecule was also heat-denatured (in the presence of 0.2% SDS), and enzymatically deglycosylated using Glycan-*N* peptidase (GNP-ase *F* from BCL) for 18 hours at 37°C. Glycoproteins showed a marked change in apparent subunit size after denaturation and enzymatic deglycosylation (Plate 4). However this is due to some extent to deglycosylation removing the branches of polysaccharide chains leaving behind the straight-chain (after SDS-treatment) polypeptide; the polypeptide then runs to a position close to its actual molecular weight position, whereas with polysaccharide branches the polypeptide chains run to a much larger apparent molecular weight than their actual one, since the branches hamper passage through the acrylamide sieve.

The main alternative to enzymatic deglycosylation is chemical deglycosylation with TFMS (trifluoromethane sulphonic acid in anisole). This was tried unsuccessfully with arcelin 4, as on both occasions tried a wide variety of smaller subunits were released which appeared to be glycosylated (i.e. reacted positively with danzyl hydrazine) (Plate 4). In view of the inherent complexities and dangers of working with

Table 14 Calibration and Molecular sizing of arcelin 4 on S-400 column

ARC4 SIZE S-400

Protein	kDa	ml elutn	
Blue Dextran	Void	264.0	LOG(Mr) vs ml r2=0.998
Thyroglobulin	669.0	332.0	
Ferritin	443.0	477.0	
BSA	66.0	536.0	
Cyt c	12.4	710.0	
Peak 1	ml	calc. Mr (K)	
Run 1	476.0	139.0	Avg. Apparent Mr =169.4K
Run 2	454.0	175.5	
Run 3	442.0	199.2	
Peak 2			
Run 1	520.0	87.2	Avg. Apparent Mr =101.5K
Run 2	502.0	105.5	
Run 3	495.0	113.6	

M	SDS VII markers, sizes as indicated	**3**	globulins	**7**	S400 Pk 1*
		4	non-bound fraction	**8**	S400 Pk 2
1	G12953 meal	**5**	CM Pk 1 & 2	**9**	affinity-purified arc 4*
2	albumins*	**6**	CM Pk 3*		
				10	CM Pk 4

* samples further purified to yield arcelin 4
CM peaks eluted from CM-cellulose (cation exchange) by salt gradient
S400 peaks obtained on gel filtration from S400 resin
affinity purified passed through a fetuin-conjugated column to remove PHA

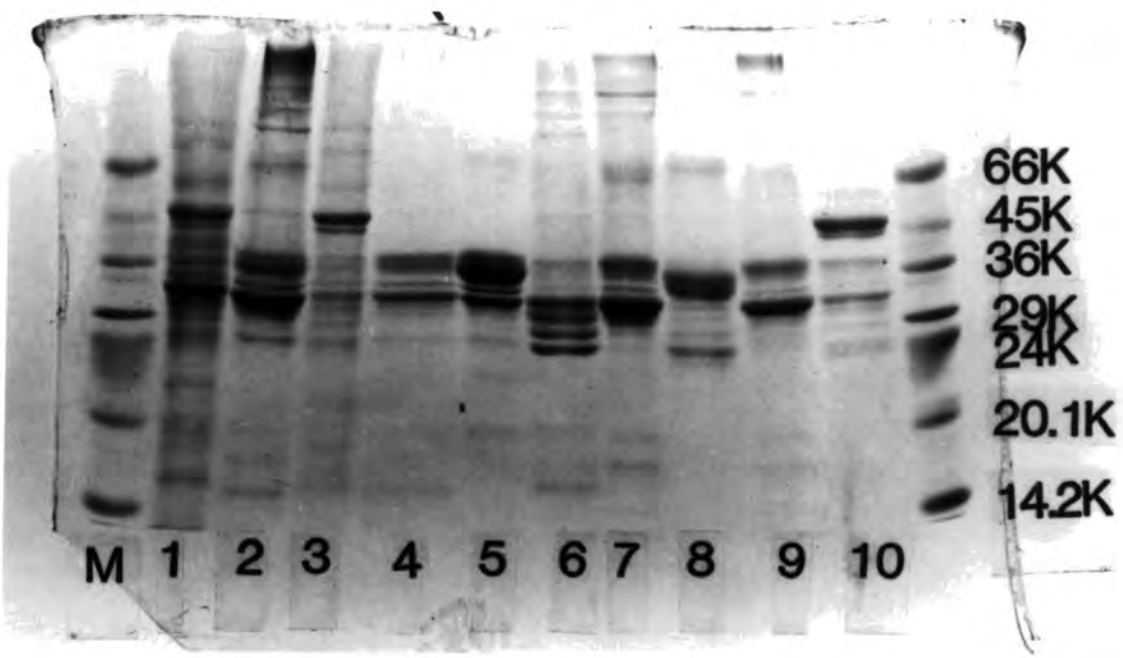


Plate 3 Stages in Purification of arcelin 4

\+ deglycosylated enzymatically with GNPase *F*
\- native protein only denatured with SDS

Gii RKB globulins
PHA *Pv* lectin (E_2L_2)
Pk 1 S400 (gel filtration) Pk 1
Pk 2 S400 Pk 2
arc4 affinity purified arcelin 4
M markers (sizes indicated)

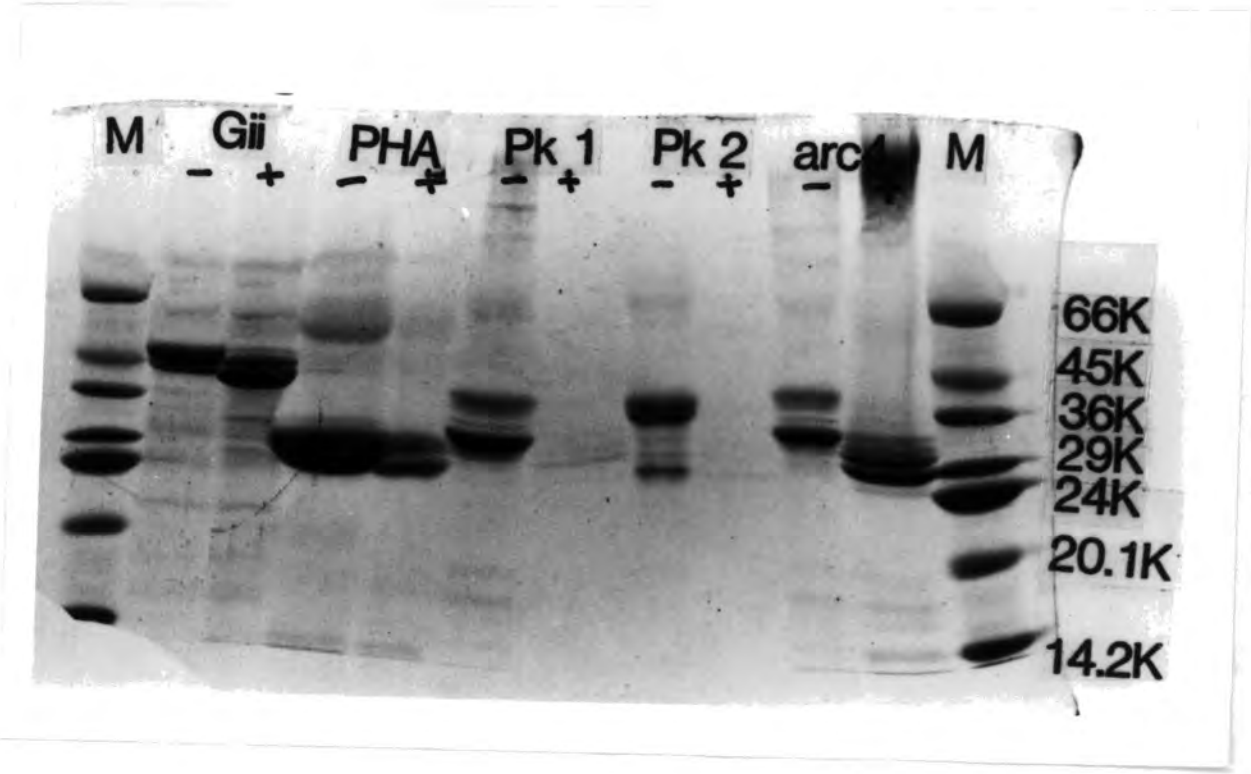


Plate 4 Deglycosylation of arcelin 4 and standard proteins by GNPase *F*

TFMS (see methods section) further trials were not performed, and the enzymatic deglycosylation of the subunits taken as a true measure of polypeptide size.

The exact level of glycosylation was determined by calculating the carbohydrate content of arcelin 4 using a specific carbohydrate assay. The *phenol-sulphuric acid* assay was interfered with too much by the presence of protein (although protein interferes with this assay only to about 10%, where the carbohydrate constitutes 2-5% of the whole this is too much to yield a useful result) and *L-cystein-sulphuric acid* assay (for hexoses) was used instead. This gave results in the order of 10% for carbohydrate content. Enzymatic deglycosylation of subunits yielded polypeptide molecular sizes in the order of M_r 29K and 31K.

14.2 PHYTOHEMAGGLUTINATION ACTIVITY

Arcelin 1 is encoded by a gene similar to that encoding PHA (Osborn *et al.* 1988), and since arcelin 4 is considered to be in the same family as arcelin 1 it can be assumed on this basis to share sequence with PHA. PHA lectins are most noted for their hemagglutination properties, therefore the hemagglutination activity of arcelin 4 was examined by standard procedure using rabbit erythrocytes, and human erythrocytes group A+[3]. Hæmagglutination of erythrocytes was determined by the absence or presence of a blood clot. Plates are not illustrated since this is a standard technique. Arcelin 4 was compared with Con A and with PHA E_2L_2 against rabbit erythrocytes, and with Con A and mixed PHA (E_4 . . . L_4) against human erythrocytes.

Arcelin 4 proved 8 × less effective than E_2L_2 and 64 × less effective than Con A against rabbit erythrocytes, and 4 × less effective than Con A vs human group A erythrocytes (Table 15). Compared with a mixture of PHA (E_4 . . . L_4), arcelin 4 was 128× less effective at hæmagglutination.

Samples were forwarded to Dr Pusztai in RRI to remove any possible PHA lectin contaminant, and to test for residual hæmagglutination. This was done by binding PHA and other agglutinins to fetuin-sepharose in a column in tris/acetic acid buffer 50mM pH 8.0, then eluting the bound material with 50mM sodium acetate pH2.5.

[3] courtesy of Marcelo Valle de Sousa

Proteins are stained with Kenacid blue, with areas of glyco-group danzyl fluorescence marked on over the gel. Pure proteins are run in the left lanes not deglycosylated, and in the right lane treated with TFMS

G12953)
G10019) bean meals
OVO ovomucoid (heavily glycosylated egg protein

OVA ovalbumin
Arc 4 arcelin 4

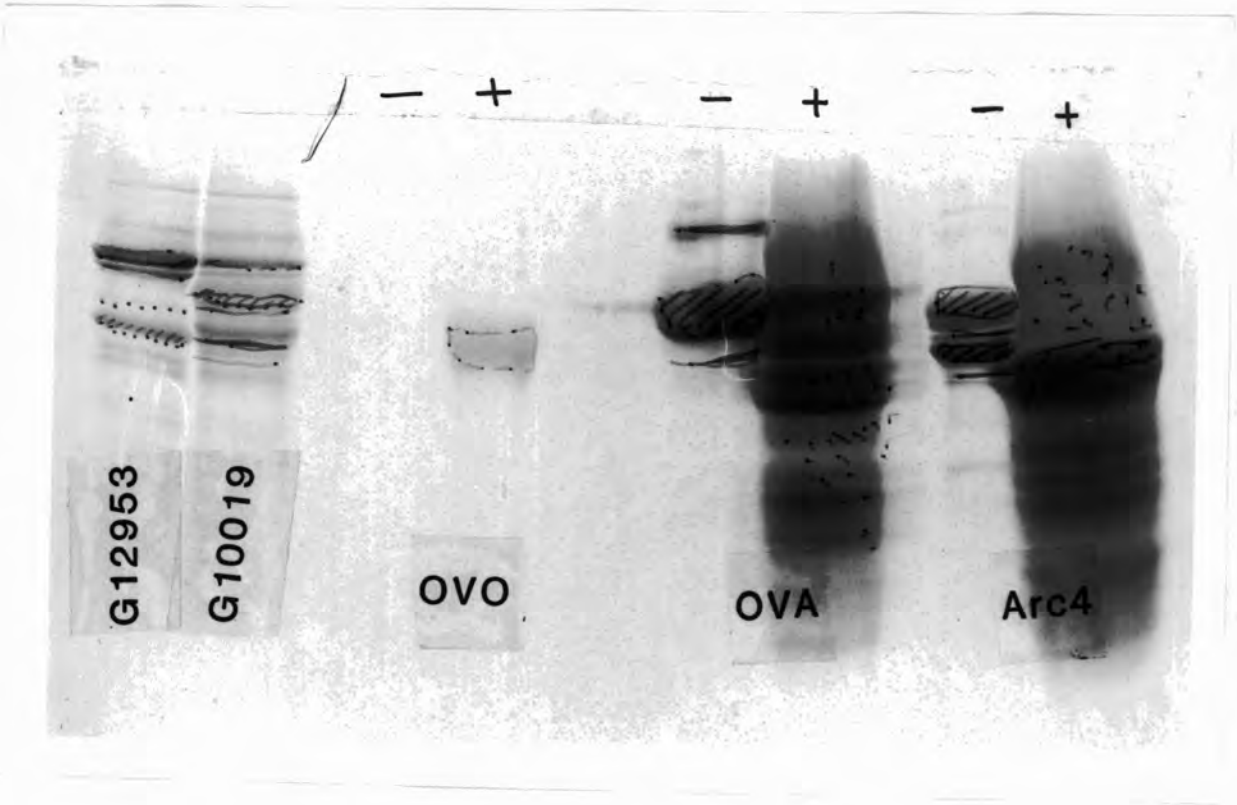


Plate 5 Deglycosylation of standard proteins and arcelin 4 with TFMS.

Table 15 Hemagglutination activity and comparing N-terminal sequences of arcelin 4 subunits and other proteins of the lectin family

ARC4 HEMAGGLUTIN.TB

Protein	rabbit erythrocytes		Human group A	
	serial dil	equiv mg/ml	serial dil	equiv mg/ml
arcelin 4	4	.25	7	.0312
Con A (Sigma)	10	.0039	9	.0078
PHA (E_2L_2)	7	.031		
PHA (E_4 . . . L_4)			14	.000244

ARC4 SEQUENCE: TB

Arc 1	S	N	D	A	S	F	N	V	E	T	F	-	-	-	-	-	N	K	T	N	L	I	L	Q	G	*S*	*etc*
LLP	A	T	E	T	S	F	I	I	D	A	F	-	-	-	-	-	N	K	T	N	L	I	L	Q	G		*etc*
PHA-E	S	N	D	I	Y	F	N	F	Q	R	F	-	-	-	-	-	N	E	T	N	L	I	L	Q	R		*etc*
PHA-L	A	S	Q	T	S	F	S	F	Q	R	F	-	-	-	-	-	N	E	T	N	L	I	L	Q	R		*etc*
Arc4 33K	A	S	E	T	S	F	?	F	T	S	F	-	-	-	D	D	N	K	-	-	L	I	L	Q	G		*n.d.*
Arc4 36k		E/I	E	T	S/T	F	F	F	T	R	S	?	Q	G	D	D	-	-	-	P	L	I	L	Q	G		*n.d.*

Arc 1 : arcelin 1, from Osborn *et al* 1988
LLP : Sequence derived from LLP gene, Hoffman and Donaldson 1985
PHA-E : PHA subunit E sequence
PHA-L : PHA subunit L sequence
Arc4 33K : N-terminal sequence of smaller of two arcelin subunits from SDS-PAGE
Arc4 36K : and of larger of two main subunits

Boxed areas indicate sequence homologies between Arc4 sequences and other illustrated sequences, **?** indicates that the identity of the amino acid is uncertain (probably glycosylated Asn). Otherwise "--" has been inserted to allow alignment
n.d.: sequence not determined
etc : sequence continues as in literature

Peaks eluted sharply with the two subunits of arcelin 4 not binding to the column (SDS-PAGE analysis) and eluting with the pH 8.0 buffer, and then PHA (probably E_4 units) eluting with the pH 2.5 buffer. In this purification 50mg of arcelin was recovered and 14mg PHA, with less than 1mg of additional material; this indicates a 20% PHA contamination, although in normal SDS-PAGE of arcelin 4 the PHA contaminant was not enough to show or stain (e.g. Plate 4).

14.3 N-TERMINAL ANALYSIS AND SEQUENCING OF ARCELIN 4

Purified arcelin 4, prepared by removing the PHA contaminant in a fetuin-sepharose column, was prepared for sequencing. The glycosylated subunits (of apparent molecular weight 33K and 36K) were separated on SDS-PAGE (in two adjacent tracks to increase yield without overloading) and electroblotted onto PVDF membrane in 10mM CAPS buffer. The bands were visualised by wetting the membrane with 20% methanol and viewing with transmitted light, marked with a pencil, and cut out. Pieces of PVDF membrane were stored in eppendorfs at -20°C until needed.

The piece of membrane, containing a single subunit from SDS-PAGE, was placed following standard procedures into the reaction chamber of an ABI automatic sequencer and sequenced by automated Edman degradation, and the computer-determined results interpreted by eye taking into account the presence of glycine in the original gel running buffer (base 1). This procedure was repeated for both subunits and sequences for each were obtained up to 18-20 residues The results, together with comparable known sequences, are presented in Table 15, aligned to indicate similar sequence stretches.

A simple count of sequence differences was done to estimate close similarities. For the sequence illustrated, the *arcelin 4 33K* subunit has 11 differences from the derived sequence of *arcelin 1*, 8 differences from the derived sequence of the *LLP gene*, and also 8 differences from the *PHA-L* subunit. The *arcelin 4 36K* subunit has the characteristic **TSF** (-Thr-Ser-Phe-) and **LILQ** (-Leu-Ile-Leu-Gln-) lectin sequences, but is otherwise difficult to compare. Most noticeable is the **DD** (-Asp-Asp-) insertion, which is only found otherwise in *arcelin 4 33K*.

The level of glycosylation was estimated from L-cystein/H_2SO_4 hexose assay at 10%.

Antibodies to arcelin 4 were raised in rabbits, and used for screening for the presence of arcelin in F_2 seeds and for characterising arcelin itself. The first use for these antibodies was to Western Blot wild-type accessions to determine whether the antibodies were a useful tool for predicting resistance (see Plate 6). The antibodies obviously cross-react with three arcelin types, though with arcelin 1 to a lesser extent than with 3 and 4. At higher concentration of antibody Arcelin 2 does cross-react with these antibodies (Plate 7). Arcelins 1, 2 and 4 are all associated with resistance.

Four populations of F_2 seeds, representing crosses of the resistant accession **G12952** (which is equivalent to **G12953** according to CIAT, and contains *arcelin 4*) with two susceptible cultivars[4] and as each parent (pollen donating or ovule donating), and susceptible accession **G10019** with one susceptible cultivar. Crosses were as follows:

seed population	pollen donating	ovary donating
GH-4	Pijao	G10019
	(susceptible cultivar)	(susceptible accession)
GH-5	Pijao	G12952
		(resistant accession)
GH-13	G12952	A36
	(resistant accession)	(susceptible cultivar)
GH-14	G12952	Pijao
		(susceptible cultivar)

Each population had 30 seeds individually bioassayed, basing resistance classification on a maximum of two eggs on the testa; and after bioassay the individual

[4] susceptible cultivars were **Pijao**: Porillo Sintetico × Mexico II, black seeds, size 20-25g/100 seed; this is a commercial variety in several Latin American countries
and **A36**: E1060 × Calima, red mottled seeds size 35-40g/100seed; a promising CIAT breeding line

Western Blot with Arcelin 4 antibodies (1:20,000)
all bean meals run at 5mg/ml in SDS-sample buffer

Tracks as follows

Gii	phaseolin from RKB	**1**	G09989B (**S**)	**6**	G12882 (**A1**)
PHA	*Pv* lectin (E_2L_2)	**2**	G10007 (**S**)	**7**	G12949 (**A4**)
A4	arc 4 (affin Purif)	**3**	G12866 (**A2**)	**8**	G12953 (**A4**)
M	markers SDS VII	**4**	G12871 (**S**)	**9**	G12954 (**A4**)
		5	G12880 (**S**)	**10**	G13016 (**S**)

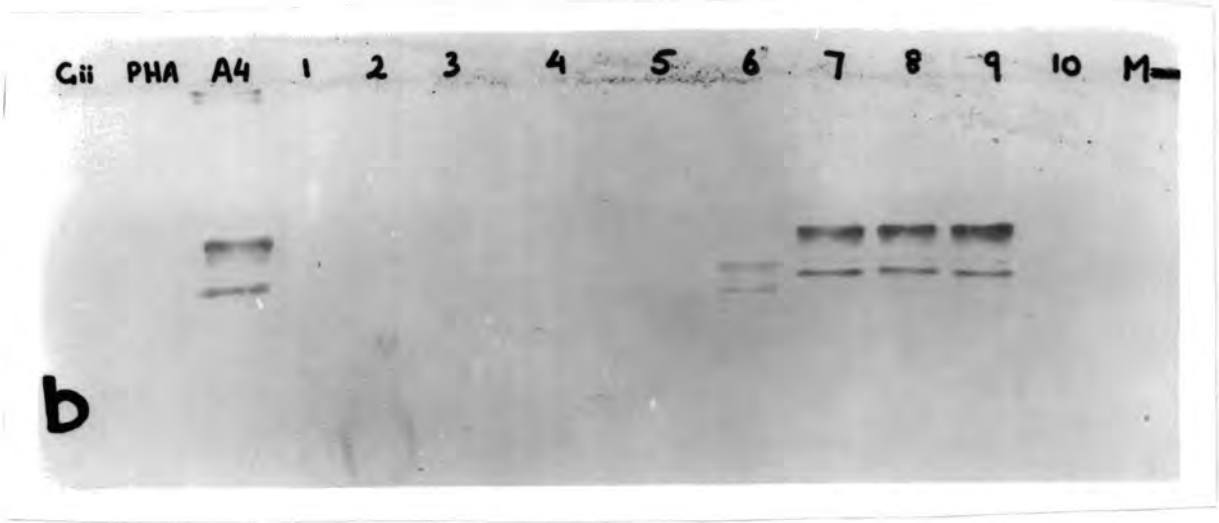


Plate 6 Seed meal of accessions of *P. vulgaris* run on SDS_Page and analysed by Western Blot for the presence of arcelin proteins

seeds were run on SDS-PAGE and blotted against arcelin 4 antibodies.

Seeds were classified according to bioassay data into one of three classes: **R**esistant (no adult emergence from either hatched egg, or development period extended beyond 38 days); **S**usceptible (development period less than 38 days for surviving adults; if only one adult emerged then the other egg failed to hatch or not laid); and **N**ot **C**lassified (**NC**) (either no eggs laid or neither egg hatched). Antibody data gave two classes: **Arc**$^+$ (reacting positively with arcelin 4 antibodies) and **arc**$^-$ (not reacting).

The numbers of seeds in each of the combinations of the two classifications (e.g. **R** and **Arc**$^+$, **NC** and **arc**$^-$) were then tested for each seed population individually (Table 16) by *Chi-squared* (goodness of fit) analysis, based on a 3×2 table (degrees of freedom=2) for a *null hypothesis* that the classes contain equal numbers of seeds. For 0.1% probability that the hypothesis is correct (i.e. 99.9% likelihood of rejecting the hypothesis), this gives a value of <13.81.

GH-5 shows significant deviation from the null hypothesis, that there is no association between the presence of arcelin 4 and resistance to *Z. subfasciatus*, although GH-13 and GH-14 do not. GH-5 has the resistant accession (**G12952**) as the cytoplasmic parent wheras the other two crosses have G12953 as the pollen-donating parent, which may account for the difference.

GH-4 shows significant deviance from the null hypothesis that seeds are equally susceptible/resistant. Since this population did not have an arcelin 4 containing parent it does not have any *arcelin 4*$^+$ antibody reaction, and most of the seeds were susceptible to *Z. subfasciatus* attack.

Taking the seeds with pollen-donating Arcelin 4 combined (**GH-13** and **GH-14**), the *Chi-squared* value is higher than for any individual population of seeds, which indicates that the results from the individual populations serve to reinforce each other rather than cancel each other out, i.e. whilst an individual population may not have shown enough deviation to reach statistical significance the effects within individual populations were real.

Table 16 Chi-squared test on seeds of arcelin+ and commercial cultivar crosses

GH-4	*Arc+*	*arc-*	*TOTAL*
R	0	1	1
S	0	22	22
NC	0	7	7
TOTAL	0	30	30
Chi sq (2x3)		df=2	23.40***

GH-5	*Arc+*	*arc-*	*TOTAL*
R	12	4	16
S	0	8	8
NC	1	5	6
TOTAL	13	17	30
Chi sq (3x2)		df=2	14.39***

GH-13	*Arc+*	*arc-*	*TOTAL*
R	14	5	19
S	2	6	8
NC	2	1	3
TOTAL	18	12	30
Chi sq (3x2)		df=2	5.62-

GH-14	*Arc+*	*arc-*	*TOTAL*
R	11	7	18
S	1	5	6
NC	3	3	6
TOTAL	15	15	30
Chi sq (3x2)		df=2	3.56-

pollen-donating Arc 4 **GH-13 & GH-14**	***Arc+***	***arc-***	**TOTAL**
R	25	12	37
S	3	11	14
NC	5	4	9
TOTAL	33	27	60
Chi Sq (3 x 2)	**df=2**	**chi-sq value**	**8.74***

5%>P>1%

Significance:

- not significant at 10%
- * significant at 5%
- ** significant at 1%
- *** significant at 0.1%

Agarose gel immunodiffusion was used to determine the cross-reactivity of the different arcelin types to the antibodies (Plate 7). 10µl antibody serum, at full concentration in one set, and at 8× dilution in another set, was placed into the central well, and 2× serial dilutions of antigen placed in the wells around in a clockwise direction. For pure arcelin 4 the starting concentration was 2mg/ml, and for seed meals 20mg/ml, extracted in 25mM sodium acetate pH 4.8. The *equivalence position* was taken as the dilution of antigen for which the line of immunoprecipitate was equidistant from the antigen and antibody wells. The concentration of material in this well was calculated from the initial concentration and the number of serial dilutions. The method of agarose immunodiffusion is a standard one and calibration of the pure protein *arcelin 4* is not illustrated. For the seed meals, gels stained with Kenacid blue are illustrated in Plate 7.

For arcelin 4, the *equivalence position* with antibodies was at a concentration of 0.31mg/ml. Comparing this result with that obtained for arcelin 4 containing accession **G12953**, loaded initially at 20mg/ml, gives an equivalence concentration of 5mg/ml meal, i.e. arcelin 4 constitutes approximately 6.25% of the meal. Arcelin 3 cross-reacts very similarly to arcelin 4, as illustrated by the same *equivalence position* of the bean meal (**G12923**). An attempt has been made to affinity purify arcelin 4 antibodies using arcelin 4 (positive binding) and arcelin 3 (negative binding) in Memsep cartridges, but this was not successful as the arcelins are so similar immunologically.

Arcelin types 1 and 2 did not reach equivalent concentrations with full concentration antibody starting at 20mg/ml seed extract, and both required dilution of the antibody 1:8 to resolve suitable *equivalence positions*. After scaling to allow for antibody dilution, **arcelin 1** containing seed meal had an equivalence concentration 12 to 18× that of arcelin 4 bean meal (it is 1/12 to 1/18 times as antigenic to these antibodies), and **arcelin 2** had an equivalence concentration 32× that of arcelin 4.

PHA antibodies were compared with arcelin 4 antibodies to further characterise arcelin 4. Arcelin is clearly related to PHA since PHA antibodies cross-react; however arcelin antibodies do not cross-react with PHA at the concentration (1:20,000) used.

a **antibody serum undiluted**
b **antibody 1:7 diluted (1/8)**
dilutions of extracted bean meal were as follows (numbers refer to *G12882* rosette, antibody serum in centre well)

1	20µl at 20mg/ml	**4**	10µl at 5mg/ml
2	10µl at 20mg/ml	**5**	10µl at 2.5mg/ml
3	10µl at 10mg/ml	**6**	10µl at 1.25mg/ml

Accessions containing arcelin types were as follows: G12882 **arc 1**; G12866 **arc 2**; G12923 **arc 3**; G12953 **arc 4**

The line (or double-line) visualised with Kenacid blue represents a precipitate of antibody and antigen for each seed type

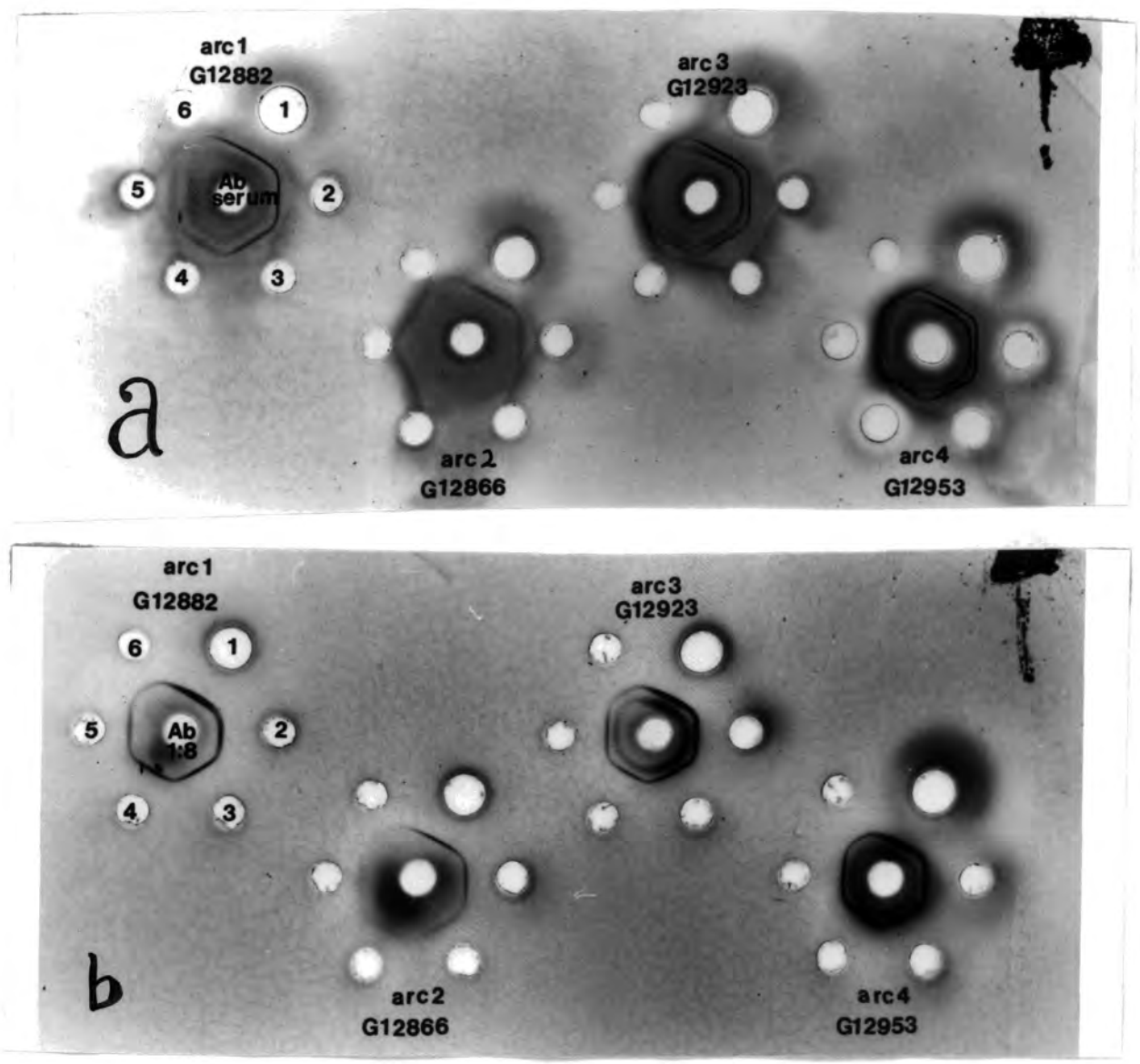


Plate 7 Agarose gel immunodiffusion to compare the four arcelin types

14.6 USAGE OF ARCELIN DURING GERMINATION

Seeds of the arcelin 4 containing accession **G12953** and of a PHA/phaseolin commercial haricot bean cultivar cv. **Processor** were germinated for a number of days and run on SDS-PAGE to determine how the pattern of proteins present in the cotyledons altered during the first 10 days after germination. Two seeds were used for each time period; the seeds were germinated in the dark at 25°C, with periodic watering. Shoots from each variety appeared to grow at the same rate. At each sample time, the pair of seeds from each variety was collected, the shoot and radicle removed, and the cotyledons frozen in liquid air and dried under vacuum, and then ground up and extracted in SDS-cracking buffer before running on SDS-PAGE. The results are presented in Plate 8

Arcelin 4 appears to be mobilised at a similar rate to the conventional storage proteins phaseolin and lectin. Its presence is in such high concentrations in the dormant seed cotyledon (approx 7.1% of the total seed meal = 35% of total protein) suggests that it probably has a role as a storage protein, and its mobilisation after germination confirms this.

Examining the gels suggests that phaseolin and arcelin 4 in **G12953** are almost completely used up by day 7, whereas the equivalents in haricot, phaseolin and lectin, are not fully used even by day 10. This is to be expected since the seeds of G12953 are very much smaller, and since both seeds produce similar length shoots during the first days after germination the food reserves of the smaller seed are likely to be used up faster.

14.7 *C. MACULATUS* RESISTANCE

Arcelin-containing seeds have reduced levels of mammalian antimetabolites, as illustrated by the reduction in detrimental effects on mammals of feeding **RAZ-2** (Table 13 and Figure 8). Studies have shown that PHA lectins in *P. vulgaris* seeds are responsible for seed resistance to the bruchid *C. maculatus* (Gatehouse *et al.* 1985), which is a pest of other commercial Leguminosae seeds 'though not of *P. vulgaris*. Therefore reduced levels of PHA in the potentially commercial line **RAZ-2** could

G12953 (arcelin 4 containing) and *cv* **Processor**
days after initial exposure to germination conditions (0 to 10) asa indicated.
M SDS VII markers (66kDa, 45kDa, 36kDa, 29kDa, 24kDa, 20.1kDa, 14.2kDa)

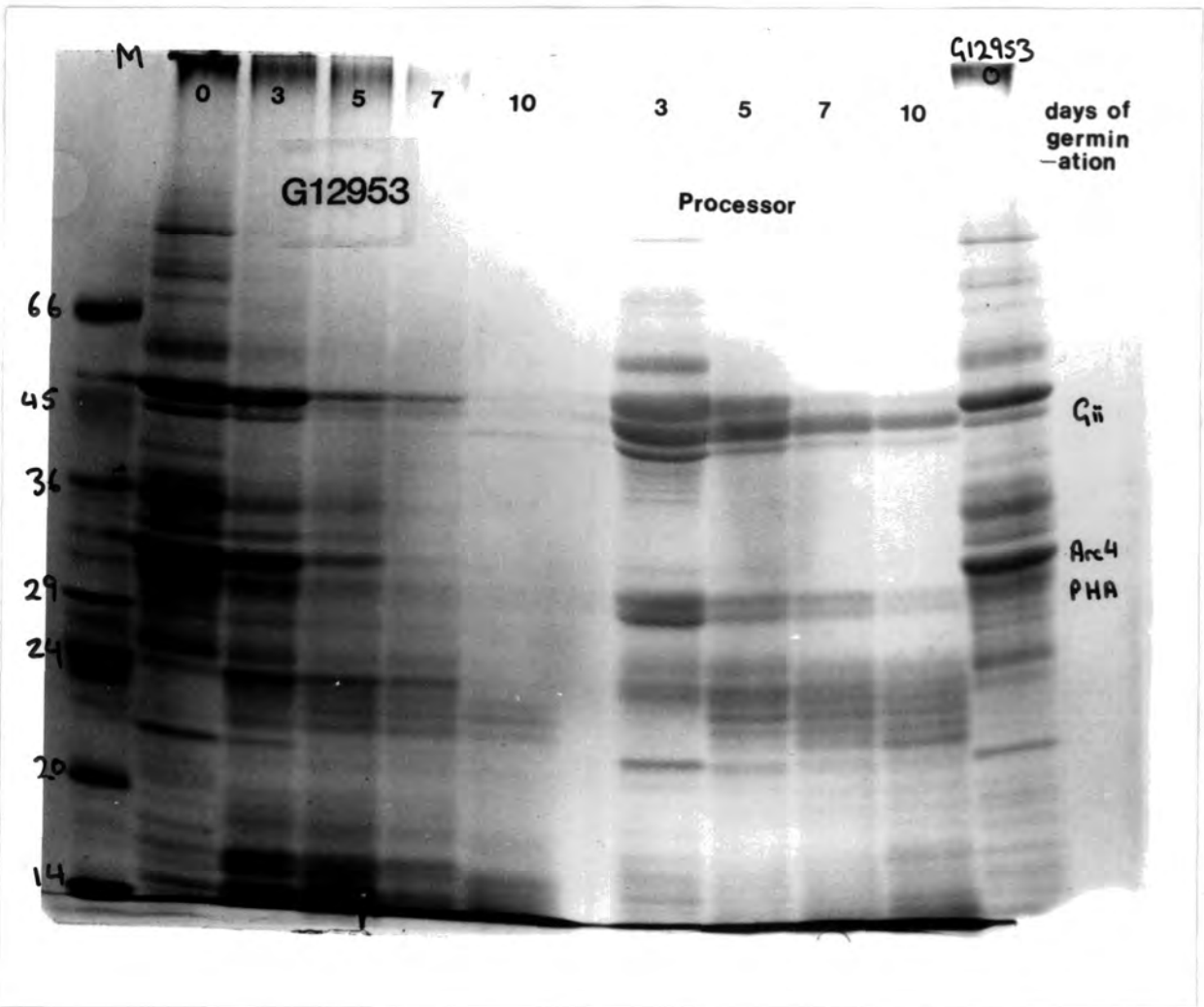


Plate 8 Protein use during germination of arcelin-containing and commercial *P. vulgaris*

possibly lead to reduced resistance to *C. maculatus*.

In view of this danger, bioassays were performed with seeds of crosses containing arcelin (**RAZ-2** containing arcelin1, **G12882** the wild accession containing arcelin 1, and **G12949**, the wild accession containing arcelin 4) and accessions not containing arcelin (**G10019**, a high lectin line; and **EMP-175**, the commercial cultivar parent of RAZ-2), against *Z. subfasciatus* and against *C. maculatus* (Table 17).

C. maculatus eggs hatched and the larvae penetrated the testa, at least to a limited extent (hatch was determined by the presence of frass in the translucent egg). However, no larvae were able to develop to adult, indicating that resistance to this pest is maintained in the selected seed lines in the presence of arcelin 1 or 4.

14.8 CONCLUSIONS, CHARACTERISING ARCELIN 4

Molecular and biological characterisation of arcelin 4 reveals that the molecule, of apparent molecular weight 169K, is composed of glycosylated subunits whose peptide chain apparent molecular weights are 29K and 31K, and whose carbohydrate content is 10%. The apparent subunit sizes of 33K and 36K are consistent with the carbohydrate content.

The concentration of pure arcelin 4 in seed line G12953, as determined by immunological studies as 6.25% (± 3% points) is close to the extraction-measured concentration of 7.1%.

A tetrameric molecule made up of subunits of 29K and 31K + 10% carbohydrate will have a total molecular weight around 138K. This is much smaller than the apparent molecular size estimated from gel filtration. There are a number of possibilities to explain this anomaly:

1- the molecule may in fact be hexameric rather than tetrameric, giving a molecular size (based on apparent subunit size) of 207K

2- apparent molecular sizes based on gel filtration assume that the macromolecule is globular, and make an estimate of molecular size from the diameter of the molecule. Because of the nature of the gel filtration process, an ellipsoid molecule will have an apparent molecular size consistent with the longest

Table 17 Accessions tested in bioassay vs *C. maculatus* (not normally a pest)

VARIETIES VS C.MAC 18/4/90

Variety	*C. maculatus* bioassay		*Z. subfasciatus* bioassay		
	% hatch	% emerge	% hatch	% emerge	Dev Period
G10019	2	0	normally considered susceptible		
G12882	6	0	arcelin-1 containing, resistant		
EMP 175	22.7	0	82.7	98.4	31.56
RAZ-2	16.8	0	84.0	12.4	48.8
G12949	16	0	arcelin-4 containing, resistant		

EMP 175 is the susceptible, commercial cultivar parent of RAZ-2
RAZ-2 is the arcelin-1 containing, large-seeded breeding line being tested for use as a commercial line

diameter of the ellipse, and a rod-shaped protein with an effective globular diameter equal to the length of the rod. These will be in excess of the actual molecular size

3- the tetrameric molecule may be in rapid equilibrium with a dimer of tetramers. Depending on the rate of transition between dimer and tetramer, the two can elute as separate peaks (very slow transition), or as a single peak (rapid transition) with an apparent molecular weight somewhere between the actual molecular weights of the two protein molecules.

The molecule also shows N-terminal sequence homology with both PHA and arcelin 1, with a distinctive **-Asp-Asp-** insertion part way in (after 11 or 13 residues); however the N-terminal sequence is closer to PHA and to the LLP gene than to arcelin 1. The N-terminal sequence is short compared to the number of amino-acids in the whole protein, so it would be unwise to give too much weight to these differences by ascribing evolutionary parentage. However immunocrossreactivity on the whole molecule indicates some sequence similarity, but that both arcelin 1 and arcelin 2 are very different (high concentrations of these are required to immunoprecipitate arcelin 4 antibodies).

Arcelin 4 in the purified state shows no hæmagglutination activity, and both arcelin 4 and arcelin 1 can be purified from contaminating PHA lectin by affinity chromatography of the lectin on fetuin sepharose (arcelin 1 method from Osborn *et al.* 1988). However the presence of arcelin 4 is very clearly associated with resistance to *Z. subfasciatus* attack in the F_2 generation seeds.

High levels of arcelin in seeds of accessions is associated with low levels of both phaseolin and PHA (Table 5-a and Harmsen *et al.* 1986). Mammalian feeding trials using meal from **RAZ-2** (arcelin containing) also confirm that this line in particular, and by inference other arcelin-containing lines, does not contain high concentrations of hæmagglutinins which would normally disrupt mammalian gut epithelial membranes. PHA lectins have also been shown to disrupt *C. maculatus* gut epithelial membranes, hence it is possible that their absence may make the new *Z. subfasciatus*-resistant cultivars susceptible to *C. maculatus*. Bioassay trials reveal that this is not the case, and at this stage it is not possible to determine whether the continued resistance of these lines is due to the low levels of PHA still present in the cultivars, or whether the arcelin

itself or other factors present in the cotyledon ensure that resistance to *C. maculatus* is maintained.

Arcelin 4 is substituting in lines containing it for the major storage proteins in the seed. Since it is observed to be mobilised rapidly during germination and early seedling growth, it is being used by the seed as a major storage protein. Its presence in high enough concentrations to effect resistance by the proposed mechanism will therefore not be detrimental to the development of the seedling.

Results 15
Characterisation of the α-amylase inhibitor WBAI

15.1 PRESENCE OF CBAI VS PRESENCE OF WBAI

Seeds of accessions of *P. vulgaris* wild beans from the CIAT library were tested for inhibitory activity to the pest bruchid *Z. subfasciatus* larval gut amylase enzymes (Table 18).

The hypothesis is proposed that a novel inhibitor, active vs *Z. subfasciatus* larval amylase enzyme and detectable by this activity, is present in some accessions. The novel inhibitor is termed WBAI (Wild Bean Amylase Inhibitor).

Extracts of various accessions were made from 12 seeds (detesta'd, ground together) in citrate/phosphate buffer pH 5.4 at 2mg/ml. Inhibitory activity (expressed as a %) of these extracts on mammalian and *Z. subfasciatus* enzymes were measured under optimal conditions for enzyme activity (pH 7.0 for mammalian, and pH 5.4 for *Z. subfasciatus*, enzymes), and are illustrated in Figure 9. The *P. vulgaris* α-amylase inhibitor described in the literature (Pick and Wöber 1978a; Powers and Whitaker 1977) can be detected by its inhibitory effect on mammalian amylase *in vitro* on extracting meal with buffer pH 5.4, then pre-incubating with enzyme in buffer pH 7.0 for 30 mins (modified Bernfeld assay); for the purposes of this investigation this inhibitor has been termed CBAI (Cultivated Bean Amylase Inhibitor) and its presence determined by measured mammalian amylase inhibition. Further work determined that the CBAI does not inhibit *Z. subfasciatus* gut extract amylase activity to any great extent.

Mammalian inhibitory activity indicates the hypothesised presence of CBAI, and *Z. subfasciatus* inhibitory activity the presence of WBAI. The correlation is high (r=-.905), essentially consisting of two groups of accessions: those which inhibit mammalian enzyme to 80-100% (CBAI containing), and those which inhibit *Z. subfasciatus* larval gut enzyme to 80-100% (WBAI containing).

Although there is no evidence to suggest that the types CBAI and WBAI are

Table 18 *Z. subfasciatus* larval gut amylase inhibitory activity by accessions of *P. vulgaris*, compared with bioassay data

Accn	% Surv	Dev Per (days)	Resist Ratio	amylase Inhib %	Resist
G09989b	60.5	38.1	1.3	25.0	S
G10000	78.6	38.1	1.4	57.0	S
G10007	50.4	39.1	1.1	27.0	S
G11051	36.0	43.1	0.9	0.0	R
G12866	11.7	48.5	0.5	0.0	R
G12871	70.1	39.1	1.3	36.0	S
G12880	65.1	38.2	1.3	23.0	S
G12882	11.5	48.9	0.5	80.0	R
G12923	73.6	48.3	1.2	100.0	S
G12933	75.3	47.0	1.3	100.0	S
G12949	5.4	70.1	0.3	95.0	R
G12952	0.9	—	<0.1	64.0	R
G12953	0.0	—	<0.1	100.0	R
G12954	21.1	42.0	0.7	100.0	R
G13016	47.1	40.1	1.1	26.0	S

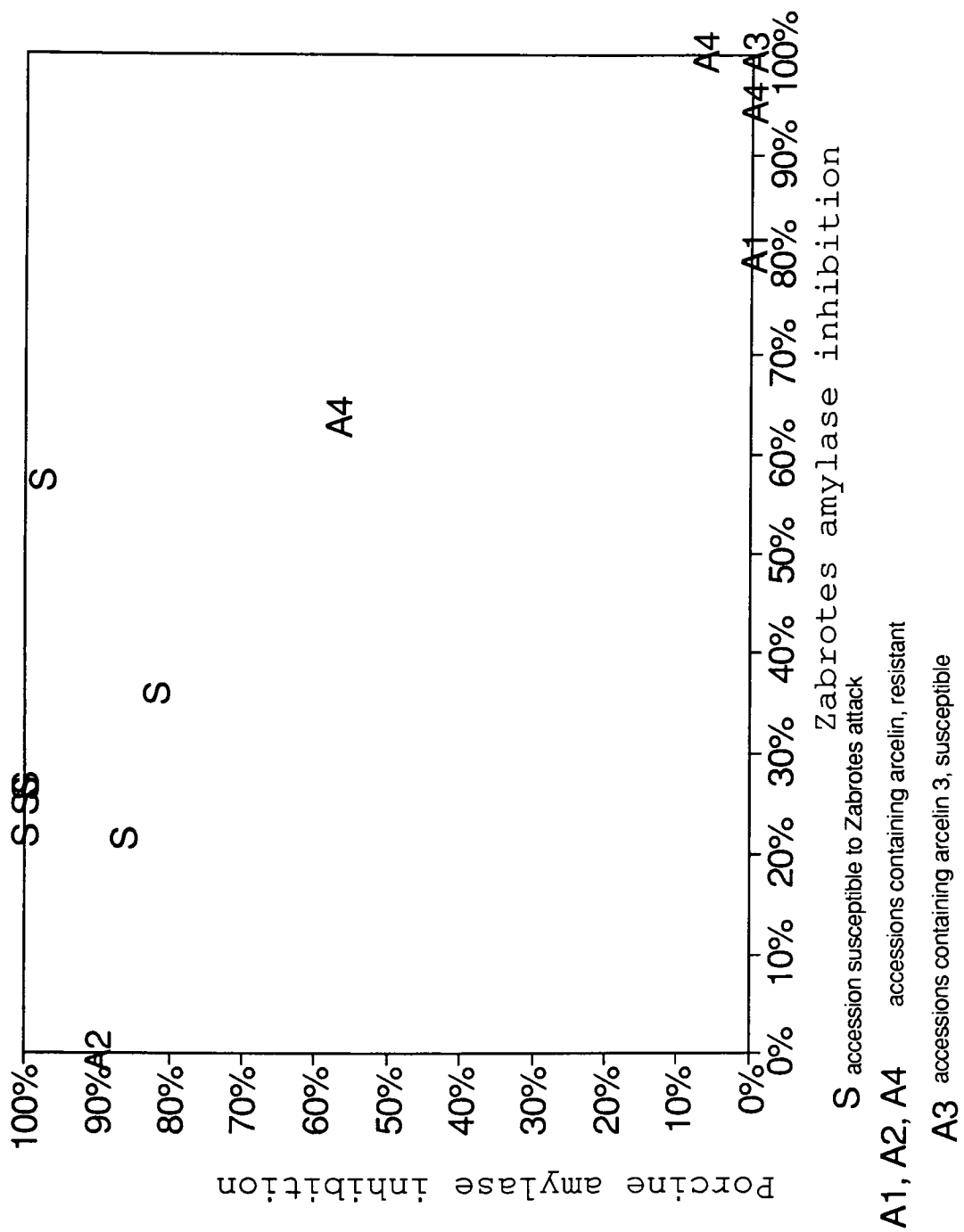


Figure 9 Regression of CBAI (mammalian amylase inhibitor) and WBAI (*Z. subfasciatus* amylase inhibitor) in accessions of *P. vulgaris* (see text)

single entities each, and that other isoenzymes do not exist, within the current investigation no evidence emerges to dispute this schema and since this is the simplest form the single terms will be used to refer to hypothetical single proteins.

Having established the link between WBAI and resistance to the pest, an attempt is made to characterise the nature of the inhibitor, its form, and its similarity and differences to CBAI.

15.2 PURIFICATION

The first stage of purification of the novel amylase inhibitor **WBAI** was to extract all albumin proteins and soluble carbohydrate from the ground bean meal of **WBAI**-containing accession **G12953**. The standard method, (25mM sodium acetate pH 4.8 for 15 hrs 4°C; batch absorption onto cation exchange resin CM-52, and elution with a salt gradient) to separate out portions: *non-bound*, *wash* (the second and third washes of the resin with buffer before packing), and on elution from cation exchange resin *Peaks 1&2* (pooled), *Peak 3* (containing arcelin 4) and *Peak 4*.

The *non-bound* portion was lyophilised and dissolved in 50mM borate pH 7.0, then precipitated to selected % saturations of ammonium sulphate to give three portions: **AS0-50** (precipitated by 50% saturation ammonium sulphate), **AS50-80** (the supernatant of 50% saturation precipitated by 80% saturation), and **AS80+** (the supernatant of 80% saturation). All were extensively dialysed against distilled water and lyophilised.

The amounts recovered at each stage were calculated for an estimated quantity of starting material of 1.00g. 2× serial dilution of inhibitor giving a residual enzyme activity was used to calculate the minimum concentration of inhibitor required to inhibit *Z. subfasciatus* enzyme by 90% ($I_{90\%}$ µg/ml) This was compared with the concentration of bean meal required to inhibit the enzyme under the same conditions (to 90%), 586µg/ml, to give a resultant **Fold purification** (**fold$_{90\%}$**). Simple multiplication of the **fold$_{90\%}$** by the **mg recovery** gives *total recovery of inhibitory activity* in this fraction (**recov$_{90\%}$**), expressed as a % of the total inhibitory activity in the bean meal (Table 19).

The highest **recov$_{90\%}$** was obtained at this stage from the ammonium sulphate cut **AS50-80**. No inhibitory activity was observed in any of the proteins eluted from CM-

Table 19 Extraction of WBAI from G12953 meal; recoveries determined by inhibitory activity

WBAI RECOVERIES AMYLASE.WQ1 RECOV 90

Material	Recov mg	$I_{90\%}$ µg/ml	$fold_{90\%}$	$Recov_{90\%}$
G12953 meal	1000.00	586.43	1.00	100.0%
Non-bound AS0-50	9.84	1192.28	0.49	0.5%
Non-bound AS50-80	43.94	67.07	8.74	38.4%
S-300 void & Pk 1	21.57	NA		
S-300 Pk 2	4.69	28.55	20.54	9.6%
S-300 Pk 3	8.24	216.74	2.71	2.2%
S-300 Pk 4	9.44	1042.48	0.56	0.3%
Non-bound AS80+	48.43	573.07	1.02	5.0%
Non-bound Wash	23.07	42.81	13.70	31.6%
Salt Elutn Pks1&2	6.98	NA		
Salt Elutn Pk3	13.31	NA		
Salt Elutn Pk4	3.37	NA		
Total Non Bound	125.28			75.5%
TOTAL seed Meal	148.94			75.5%

NOTES

$I_{90\%}$: concentration of this material required (µg/ml) to inhibit *Z. subfasciatus* larval gut homogenate to 90%. **none** = no inhibition at 2mg/ml

$fold_{90\%}$: conc required for inhibition / conc of bean meal required for similar inhibition. A measure of concentration factor of the inhibitor

$recov_{90\%}$ is calculated from **$fold_{90\%}$ × mg recovered**

Total Non-Bound is the material from which the ammonium sulphate precipitates are prepared, and contains the greater part of total inhibitory activity

cellulose. A number of different methods were attempted to purify the **WBAI** from **AS50-80**. These included cation exchange, anion exchange and gel filtration. Affinity chromatography was not possible with this material since in order to do so an affinity column containing *Z. subfasciatus* larval amylase would have had to have been prepared, for which an affinity column of an inhibitor specific to the enzyme would have been needed and WBAI is the only known inhibitor of this enzyme. Also in order to obtain suitable quantities of enzyme very large numbers of *Z. subfasciatus* larvae would have been needed.

In all methods of separation, the SDS-PAGE banding was similar, with subunits in the PHA/arcelin 4 range (29K to 36K) and subunits in the CBAI range (14-16K). FPLC gel filtration using S-300 Sepharose Fast Flow (Pharmacia) in PBS buffer pH 7.2 (XK16×100) gave a void volume peak and three following overlapping peaks, of which the second showed the highest inhibitory activity (Table 19) (the fourth peak trailed by some way). Cation exchange on S-Sepharose FF at pH 5.0, pH 6.0 and pH 7.0, and anion exchange on Q-Sepharose FF at pH 7.0, pH 8.0 and pH 9.0 did not improve resolution. Separation on hydroxyapatite in 25mM phosphate pH 7.0 gave separation over the course of washing the column with loading buffer, and all of the inhibitory activity was eluted before the elution buffer was added (equivalent to gel filtration).

Gel filtration was taken as the method of choice and a small amount of WBAI, purified from a succession of batch loadings on this column, was obtained for subsequent analysis.

15.3 BIOASSAY OF AMMONIUM SULPHATE CUTS

The total *non-bound* material, and the ammonium sulphate cuts, were included in artificial diets at physiological and half physiological inclusion levels to *Z. subfasciatus* (Table 20). This was in order to test for the antimetabolic effects of the fraction containing the most purified concentration of inhibitor.

The results indicate that at physiological concentrations the ammonium sulphate cut (**AS50-80**) containing a high proportion of the total seed *Z. subfasciatus* amylase inhibitory activity is not noticeably antimetabolic.

Table 20 Bioassay of haricot based diets with total *non-bound* and *ammonium sulphate cuts* included at physiological and half-physiological levels

Diet		% survival	Development Period
Control		71.4	30.30
Non-bound material	*2.0%*	55.0	31.80
	4.0%	42.0	32.50
AS0-50	*0.562%*	64.3	31.00
	1.124%	65.5	31.10
AS50-80	*0.492%*	68.2	30.75
	0.984%	64.3	31.00
AS80+	*0.946%*	66.6	30.40
	1.892%	61.5	31.75

Whilst some antimetabolic effect is observable from the inclusion of total *non-bound* material, none of the fractions give inhibition of larval development. The results were widely spread but statistical analysis was not performed on these data, as the data were considered not useful in defining a sub-fraction containing antimetabolic material.

15.4 PHYSICAL CHARACTERISATION, MOLECULAR SIZE

The S-300 column used to purify **WBAI** was calibrated using standard proteins of known molecular weight, and the peak corresponding to *Z. subfasciatus* α-amylase inhibitory activity sized (Table 21). The WBAI-containing peak (Peak 2) corresponds to a molecular size of 59.2K, present at approximately 1% of seed meal.

Samples of **AS50-80** were run in two dimensions, on non-denaturing TBE pH 8.8 PAGE *(first dimension)* by tris/HCl pH 8.8 SDS-PAGE *(second dimension)*, which separates different proteins in the first dimension on the basis of a combination of size and charge at this pH, and in the second dimension separates the subunits so that those subunits associated with each protein can be assigned to it (Plate 9).

Borate buffer is used in the first dimension to ensure that heavily glycosylated proteins maintain a single charge, by the borate ion complexing with the sugar side-chains. The spots on the photograph indicate a series of low molecular weight bands (14K to 18K) and a single band (35K) slightly smaller than the main contaminants. The sizes of these subunits were estimated from SDS-PAGE in single dimensions with molecular weight standards. The level of glycosylation of the whole protein was estimated (using the L-cystein/H_2SO_4 assay for hexoses) at 6.06% carbohydrate for **WBAI** (the same assay done on **CBAI**, with the appropriate multiplication factor, gave 8.6% carbohydrate for this protein).

15.5 N-TERMINAL SEQUENCING

WBAI (*Pk 2* from gel filtration) was blotted from 19% SDS-PAGE (to separate maximally in the 10 - 20K region) onto PVDF membrane in preparation for N-terminal sequencing. The bands were visualised by damping the membrane with 20% methanol

Table 21 Molecular weight calibration and N-terminal sequencing of **WBAI**

WBAI SIZE: TB AMYLASE.WQ1 AMY_I STD & PK

Standard	Mr(K)	cm	
α-amylase	200.0	11.8	
alcohol dehydrogenase	150.0	12.2	
BSA dimer	132.0	12.3	
BSA	66.0	14.1	
ovalbumin	43.0	15.6	
SBTI	20.1	18.1	
cytochrome c	12.4	19.8	
LOG(MW) on cm; r-squared		0.989	

Peak	Avg cm	Apparent MW	concentration for I90% (μg/ml)
S-300 void	5.9	void	NA
S-300 Pk 1	13.0	111.7	NA
S-300 Pk 2	14.9	59.2	29
S-300 Pk 3	17.6	23.8	217
S-300 Pk 4	24.5	2.2	1042

CBAI 1	A T E T S F I I D A F — — N K T N L I L Q G	*etc.*
Arc4 33K	A S E T S F ? F T S F D D N K — — L I L Q G	*n.d.*
WBAI 1	A S D T S F N F Y S F — — H E T N L I L Q G	*n.d.*
CBAI 2	S A V — G L D F V L V P V Q P E S K G D	*etc.*
WBAI 2	A V D G L F F A Y Y	*n.d.*
PHA-E	S N D I Y F N F Q R F N E T — — N L I L Q R	*etc.*
Arc4 36k	E/I E T S/T F F F T R S ? Q G D D P L I L Q G	*n.d.*
WBAI 35	I E T S F N F P S F/A N K — D D P L I L Q G	*n.d.*

NOTES: The character "—" is used to show where sequences have been spaced to allow alignment. Two characters in a single box indicates that the exact identity of the particular base was not determined, and "?" represents an unidentified base, probably glycosylated Asn

and allowing to dry whilst examining the blot by transmitted light, and visible bands of protein marked with a pencil.

By this method four bands of protein were detected between 14K and 20K, even though only one band was distinct by Kenacid blue staining, and one thicker band in the region of 35K (bands visualised are sketched onto photograph of gel Plate 10). Overlaying the PVDF membrane with the Kenacid blue-stained acrylamide gel enabled the bands to be identified and cut out. The bands were labelled as indicated.

The bands **WBAI 1**, **WBAI 2** (both from the 10-14 K region) and **WBAI 35** (35K region of the gel) were cut from the PVDF membrane (both adjacent tracks together to maximise available material) and N-terminal sequenced as far as possible (limited by the small quantity of starting material) by an Applied Biosystems 477A automatic protein sequencer (Table 21).

CBAI (purified and supplied kindly by Ishimoto and Kitamura, NARC, Tsukuba, Japan) was also N-terminal sequenced. Initially the protein consisting of two subunits was N-terminal sequenced on the automatic sequencer. This yielded two residues at each position, and the similarity between the sequences, if assigned one of each pair to the encoded sequence of the LLP gene, was recognised. This fit was unlikely to be due to chance and the two subunits of **CBAI** could be considered to have N-termini exactly the same as those reported (Moreno and Chrispeels 1989).

Subsequently the subunits of CBAI were separated on SDS-PAGE and electro-blotted onto PVDF membrane; by this time the team in Japan had reached the same stage and found that the separate N-terminal sequences of the two subunits were identical to our finding. Subunits from N-terminal sequencing were aligned with known N-termini to illustrate three distinct groupings, corresponding to WBAI 1, WBAI 2 and WBAI 35 (Table 21).

WBAI 1 shows sequence homology with **CBAI 1**, and also to the **arcelin 4 33K** subunit sequence. **WBAI 2** shows homology with **CBAI 2**. The **WBAI 35** N-terminal shows similarities to the similarly sized **arcelin 4 36K** subunit, and to **PHA** subunit E. It is especially interesting to note the insertion **DD** (-Asp-Asp-) found in both Arcelin 4 36K and WBAI 35.

The sequence homologies of **WBAI** and **CBAI** are in broad agreement with those found immunologically (Plate 11), in that both the small subunits (10-14K region) and

On the left (**S1**) bulked **AS50-80** material, and on the right a sample (**S2**) further purified by ion-exchange. In each case samples are run in a single SDS dimension to facilitate identification of subunits, and in two dimensions (non-denaturing pH 8 PAGE followed by SDS PAGE).

WBAI subunits (as named for sequencing) marked as follows

<1 WBAI1
<2 WBAI2
<3 WBAI 35K

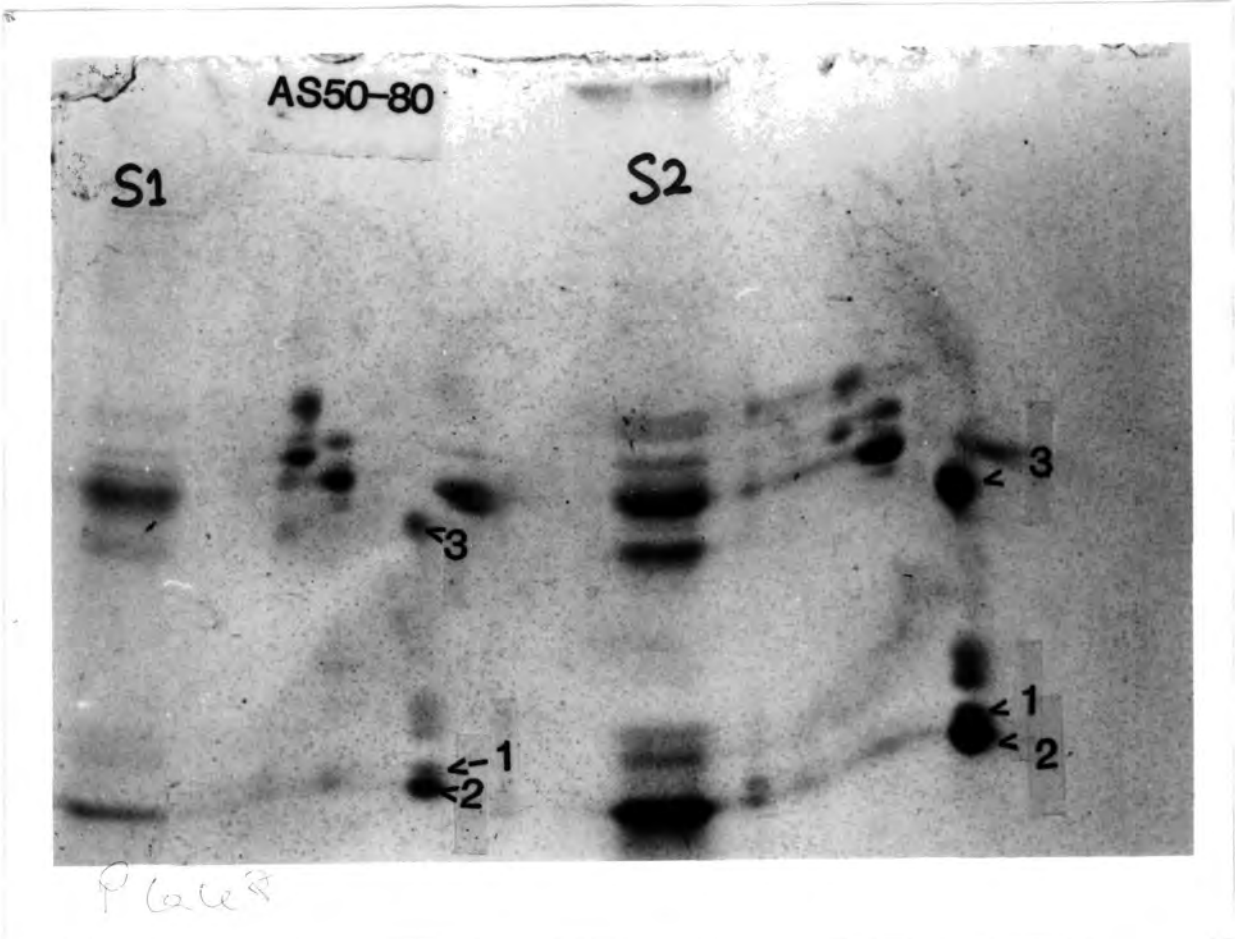


Plate 9 2-D PAGE to separate the proteins and resolve their subunits from **AS50-80**, to resolve **WBAI**

M	markers	**2**	cation exchange Pk 2 (contains purest WBAI)
1	Cation exchange Pk 1	**3**	cation exchage Pk 3

subunits of WBAI labelled as follows

WBAI 35K
WBAI1
WBAI2

other bands are also visible in the 14K region (around WBAI1 and WBAI2)

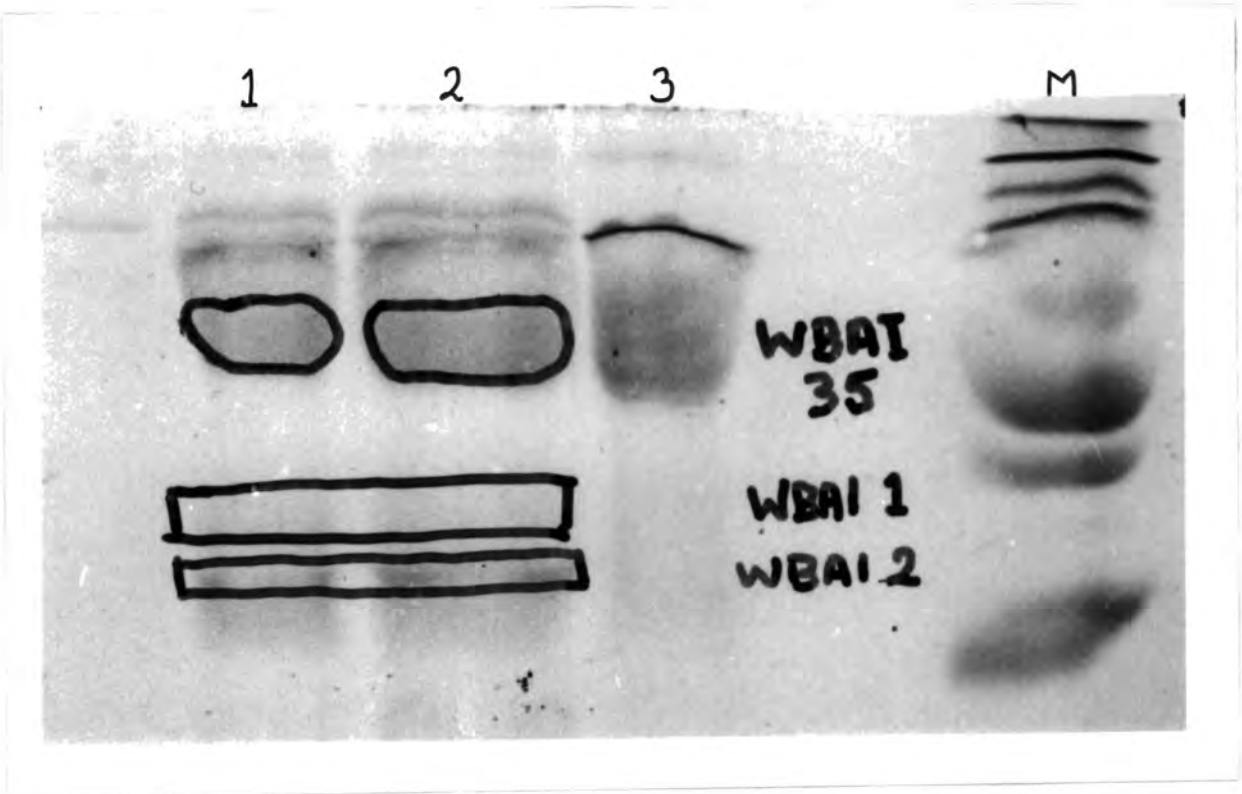


Plate 10 19% SDS-PAGE of WBAI and bands of protein detected on PVDF blot prior to sequencing

a **using CBAI antibodies (1:2000)**
b **PHA antibodies (1:2000)**
c **arcelin 4 antibodies (1:20,000)**

1	PPTE	**4**	AS100+
2	AS80+	**5**	WBAI
3	AS80-100	**6**	AS50-80

markers (which did not react immunologically so are not visible) are in the track after track 6

All samples were at 1mg/ml. The fainter staining with arcelin 4 antibodies is most likely to be due to the greater dilution of serum

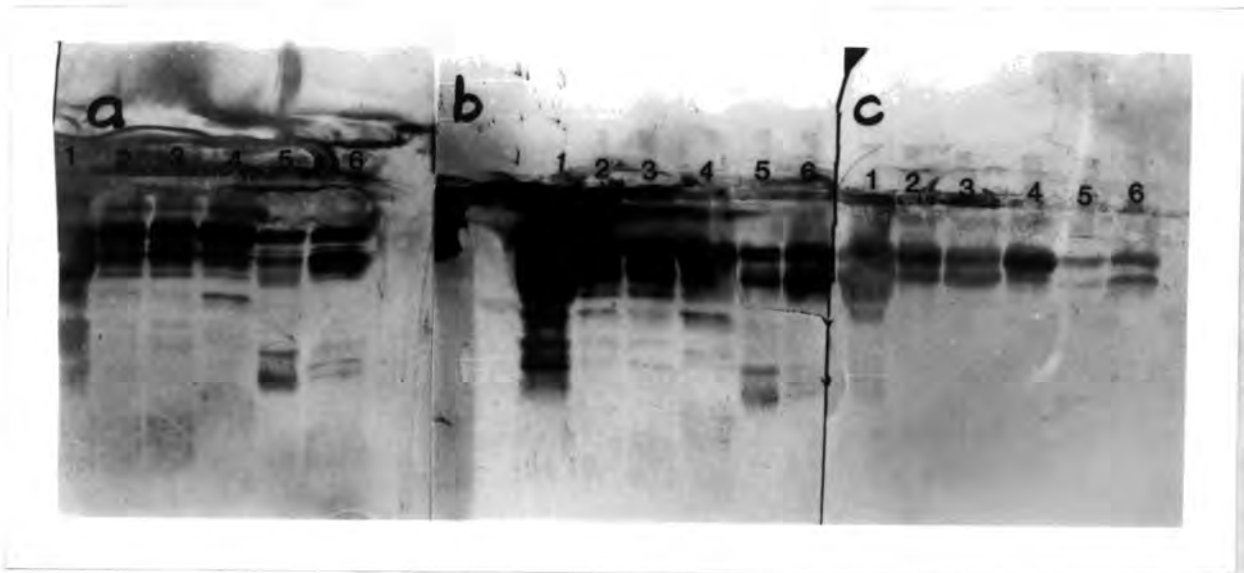


Plate 11 WBAI (novel amylase inhibitor) Western blotted with antibodies to **CBAI** and **arcelin 4**

the larger subunit (35K region) of **WBAI** cross react with antibodies raised to **CBAI** and with antibodies raised to **arcelin 4**. They also react with antibodies raised to PHA, indicating their position in this family.

15.6 ENZYMATIC CHARACTERISATION

Amylase enzymes from a variety of sources were characterised to obtain their optimal pH (either experimentally or from literature), and then 2× serially diluted (at their optimal pH and buffer) to obtain a characteristic curve (e.g. Figure 3) at their optimum pH to enable a suitable concentration of enzyme to be used, giving a similar release of glucose from starch over 5 minutes. At high enzyme concentrations the substrate concentration becomes limiting, so even with high levels of inhibition the inhibitory effects on the enzyme may not be visible. An optimum enzyme activity is taken as the activity (and corresponding concentration) which releases 10-20% of the substrate as monosaccharide over 5 minutes. The eventual optimal concentration of enzyme is also selected as the point on the graph from which dilution of the enzyme causes a roughly proportional reduction in activity, i.e. the enzyme is not in excess and is sensitive to inhibition. Concentration of enzyme used, and pH and buffer used, together with activity under these conditions, are given in Table 22.

The two inhibitors (**CBAI** and **WBAI**) were then compared in their potency at inhibiting these enzymes. $\sqrt{10}$× serial dilutions (every second dilution is 10× more dilute, with the intermediate one 3.1× more dilute than the previous) of each inhibitor were preincubated with the selected concentration of enzyme in the appropriate pH buffer before the addition of starch, and terminated after 5 mins. Plots of the release of glucose with concentration of inhibitor showed a characteristic curve with *excess inhibitor* (no, or virtually no, glucose release) and *too little inhibitor* (glucose release by uninhibited enzyme), and from the border between these two regions the $I_{90\%}$ could be estimated, as the concentration of inhibitor required to inhibit the enzyme to 90% (i.e. only 10% of the sugar released compared with enzyme without inhibitor). The concentration for 90% inhibition was chosen since in some cases small amounts of residual sugar were released even at high concentrations of inhibitor, where the inhibitor:enzyme complex is competitive to the substrate:enzyme complex; $I_{50\%}$ gave similar results, though of course requiring much lower concentrations of inhibitor.

Table 22 Amylase enzymes from various sources: optimal pH, dilution used and activity at this dilution

WBAI enzyme optima AMYLASE.INH\AMYLASE WQ1 OPT PH MM

Enzyme source	Optimum pH	Dilution used	Activity (mg glu/5 min)
Porcine Pancreatic (mammalian)	7.0	1/600 2mg/ml	1.30
Human salivary (mammalian)	7.0	1/20 stock	1.43
Bacillus licheniformis	7.0	1/5000 stock	1.28
Aspergillus oryzae (Fungal)	6.0	1mg/ml, 1/10	1.33
Locust (Insect)	7.0	5 guts/ml, 1/40	1.31
Z. subfasciatus (Bruchid)	5.4	1 gut/25µl, 1/16	1.25
C. maculatus (Bruchid)	5.4	1 gut/25µl	0.76
A. obtectus (Bruchid)	5.0	1 gut/25µl, 1/3.5	1.12
Barley Malt (plant a and b amylase)	7.0	2mg/ml	1.27

Buffers

pH 5.0, pH 5.4, pH 6.0, pH 7.0 McIlvane's citrate phosphate, + 3mM $CaCl_2$

pH 9.5 glycine/HCl

NOTES: Activity is measured as release of glucose over 5 minutes based on an initial addition of starch of 10mg/ml (50µl added to start activity). A figure of 1.0 is therefore equivalent to 10% release of available glucose from the starch

The conventional *P. vulgaris* inhibitor, **CBAI**, was active against mammalian and locust amylases, and also to a lesser extent against *C. maculatus* larval gut enzyme. Conversely the novel inhibitor **WBAI** was active against the bruchid amylases of *Z. subfasciatus* and *C. maculatus*. Neither amylase inhibitor was effective against the amylases from bacterial, fungal or plant sources, and *A. obtectus* amylase activity was not affected by these inhibitors.

Table 23 WBAI and **CBAI** inhibitory activity to enzymes from different sources

WBAI CBAI VS ENZYMES

enzyme source	pH	concentration for I_{90}	
		CBAI	**WBAI**
Porcine Pancreatic	7.0	0.08 mg/ml	none at 2mg/ml
Human salivary	7.0	0.08 mg/ml	none at 2mg/ml
Bacillus licheniformis	7.0	none at 2mg/ml	none at 2mg/ml
Aspergillus oryzae	6.0	none at 2mg/ml	none at 2mg/ml
Locust	7.0	0.02 mg/ml	none at 2mg/ml
Z. subfasciatus	5.4	$I_{50\%}$ at 2mg/ml	0.02 mg/ml
C. maculatus	5.4	0.20 mg/ml	0.20 mg/ml
A. obtectus	5.0	none at 2mg/ml	none at 2mg/ml
Barley Malt (plant a and b amylase)	7.0	none at 2mg/ml	none at 2mg/ml

NOTES

concentration for I_{90} - the concentration of inhibitor required to inhibit enzyme at 90%, i.e. 10% residual activity. Where no inhibitor was obtained at 2mg/ml, the inhibitor was taken as an inactive against that particular enzyme

CBAI α-amylase inhibitor from RKB (Kitamura & Ishimoto 1988)

WBAI α-amylase inhibitor extracted from *Z. subfasciatus*-resistant G12953

15.7 DISCUSSION

Dr Kitamura and Mr Ishimoto extracted **CBAI** from cultivated *P. vulgaris* and kindly supplied 50mg to us together with antibodies to enable comparison to be made. Gel filtration and SDS-PAGE were used to compare the molecular and subunit size of this molecule, together with enzymatic deglycosylation; N-terminal sequencing confirmed that this inhibitor is the same as the previously reported *P. vulgaris* amylase inhibitor (Moreno and Chrispeels 1989).

CBAI is a glycoprotein of native MW 49K, 8.6% carbohydrate, composed as a tetramer of subunits of nearly equal size (118 and 119 residues) MW 15K (Powers and Whitaker 1977). The sequence is very similar to that of PHA, fitting exactly the sequence encoded in the genetic code of Hoffman and Donaldson's *LLP* gene (Hoffman and Donaldson 1985) (Moreno and Chrispeels 1989), which is similar to the PHA-E subunit. Its concentration in the seed is around 1%.

The novel inhibitor **WBAI** is of a similar molecular size, 59.2K, with subunits between 10K and 14K, and another subunit of 35K, suggesting a conformation of a trimer of a pair of small subunits linked to a larger subunit. There appears to be no glycosylation present when measured by enzymatic deglycosylation, although assay gives 6% carbohydrate. Western blots of **WBAI** and **CBAI** vs antibodies antigenic to PHA (E_2L_2), arcelin 4, and CBAI reveal immunological similarities between the WBAI 14K subunit and all of these. The N-terminal sequences compare well. **WBAI1** compares closely with **CBAI1**, the N-terminal end of LLP, and therefore with arcelin 1 and arcelin 4. A simple comparison scoring differences suggests that the amino acid sequence is more similar to **arcelin 4** than to **CBAI**, 'though insertions and deletions in arcelin 4 which make it different from the sequences of PHA and the LLP gene are not repeated in WBAI1. **WBAI2** is similar to the sequence for the second of the two **CBAI** sequences, though not very close. This (**CBAI2**) represents the same as the middle of PHA-E, arcelin 1 etc, for which sequences are available; however it was not possible to compare this sequence with arcelin 4 because no further sequence data is available for this part of the protein.

Further sequence data on the proteins under investigation may have been useful to clarify their evolutionary origins, but there was not enough time in the course of the

investigation to pursue this further.

CBAI is inhibitory to amylases from mammalian and other insect sources, 'though not to all the amylase enzymes of surviving pest species, and has been implicated as a cause of resistance of *P. vulgaris* seeds to *C. maculatus* attack (Kitamura and Ishimoto 1988). To some extent this is borne out, as WBAI retains inhibitory effects against *C. maculatus* enzyme, even though **WBAI** does not inhibit other enzymes which are inhibited by **CBAI**. The lack of inhibitory effect of CBAI on *Z. subfasciatus* (pest of *P. vulgaris*) is probably because *Z. subfasciatus* has evolved altered amylases to overcome its host's inhibitors - proteases in this species are also different and are not inhibited by trypsin and chymotrypsin inhibitors found in *P. vulgaris*.

Since *Z. subfasciatus* has an altered amylase not affected by CBAI a natural progression would be to adapt the inhibitor (or evolve a new one from existing code) to inhibit the *Z. subfasciatus* larval gut enzyme, whilst retaining any previous inhibitory activity necessary for survival.

Resistance and enzyme inhibition appeared to be associated, with the exception of accessions containing arcelin 2 (resistant to *Z. subfasciatus*, but did not inhibit the amylase enzymes) and those containing arcelin 3 (susceptible to *Z. subfasciatus*, but inhibited the enzymes). The specificity if this inhibition (the conventional inhibitor found in *P. vulgaris* inhibits mammalian amylase and *C. maculatus* but does not inhibit *Z. subfasciatus* larval gut amylase) suggests that it is a resistance mechanism. It is possible that **WBAI** is ineffective against non-bruchid enzymes because the inhibitor does not form a stable complex with the enzyme at pHs much greater that pH 5.4 — this has already been reported for **CBAI** which forms a more stable complex with mammalian enzyme at pH 5.4 than at pH 7.0 (Marshall and Lauda 1975), and the same is true of the Cranberry bean amylase inhibitor (Kotaru *et al.* 1987). However if this is the case, it will not affect the activity of the inhibitor/enzyme complex *in vivo*, since the pHs chosen for the enzymes are as far as possible the same as the *in vivo* gut pH.

Some accessions with high levels of one inhibitor retain limited inclusion of the other inhibitor. This effect may have been due to the number of seeds used for the assay. Collected seeds for accessions were neither homogeneous nor homozygous. Amongst the 12 seeds ground there may have been a majority with 100% inhibition of one enzyme, providing an excess of inhibitor sufficient to inhibit the enzyme under the

conditions used ($I_{90\%}$ for WBAI-containing meal was 0.6mg/ml, whereas the seed meal used was assayed at 2mg/ml), whilst one or two seeds may have contained the alternative inhibitor resulting in low levels of inhibition of the other enzyme.

By this hypothesis the presence of **WBAI** and **CBAI** are mutually exclusive, and may be encoded (or their regulator genes encoded) at the same locus. The phenotypic linkage of **WBAI** with arcelin 4, and dominant inheritance of arcelin 4, suggest that the presence of WBAI may be a dominant characteristic. It is important to note that none of the purified fractions from which arcelin 4 is prepared showed any inhibitory activity, and that N-terminal sequencing also illustrates that whilst **WBAI** and **arcelin 4** show some similarities, they are distinct and separate entities.

Results 16
Acanthoscelides obtectus Resistance

A number of accessions of wild *P. vulgaris* from the seed library at CIAT, Cali Colombia exhibit resistance to the development of *A. obtectus*; however *Z. subfasciatus* and *A. obtectus* resistance are not found in all the same accessions (Table 34).

16.1 Effects of Testa Damage on Seed Resistance

A. obtectus larvae penetrate the seed in a different manner from *Z. subfasciatus* larvae: wheras *Z. subfasciatus* females attach their eggs to the testa of the seed, and the larva is able to press against the shell of the egg (*chorion*) to assist in penetration, *A. obtectus* eggs are deposited amongst the seeds and the larvae usually search for cracks in the testa to assist entry through the testa. In order to examine whether resistance to *A. obtectus* is associated with failure to penetrate the testa seeds were bioassayed either with the testa intact or with a portion of the testa removed (Table 24). The results indicate a consistent effect of damage to the testa, with an increase in % survival in the order of 39% and decrease in days of development in the order of 1.4 days (Table 24); however this effect is not consistent across all accessions; there are differences in resistance between intact and damaged seed, but these differences are not greatest for resistant accessions.

Comparisons of bioassay-determined resistance to *A. obtectus* and their protein composition as determined from SDS-PAGE does not appear to yield any correlations. Similarly extracts of seed meal do not appear to contain inhibitors to *A. obtectus* enzymes. This has made determining the factors responsible for resistance to *A. obtectus* difficult.

Table 24 Survival and development of *A. obtectus* on intact seeds or seeds with testa damaged

\ACANTH.OBT\ACANTH.WQ1 testa damage

Accession	Undamaged Testa			Damaged Testa			Anti-PPTE	
	% survival	Dev Per (days)	Ratio	% survival	Dev Per (days)	Ratio		
G09989B	15.0	30.5	0.70	43.0	27.5	1.25		I
G10000	63.0	28.5	1.49	84.0	26.5	1.78		S
G10007	16.0	31.5	0.71	78.0	28.0	1.67		I
G10011	36.0	30.5	1.09	76.0	27.0	1.68		S
G10019	31.0	29.5	1.02	64.0	27.5	1.53		S
G11051	17.0	34.5	0.70	41.0	34.5	1.09		I
G12861	9.0	33.5	0.52	48.0	26.5	1.35		R
G12866	11.0	33.0	0.57	64.0	31.5	1.42		R
G12871	27.0	29.5	0.96	63.0	27.5	1.51		I
G12880	6.0	29.0	0.46	39.0	27.5	1.19		R
G12888	58.0	30.5	1.38	76.0	27.5	1.66	*	S
G12923	51.0	36.5	1.18	96.0	32.5	1.72	*	S
G12933	54.0	33.5	1.27	82.0	33.5	1.57		S
G12949	7.0	46.0	0.39	29.0	44.5	0.81	*	R
G12952	3.0	57.5	0.22	30.0	46.5	0.81	*	R
G12953	1.5	56.6	0.17	32.4	53.4	0.78	*	R
G12954	4.0	49.5	0.28	45.0	47.5	0.97		R
G13016	3.0	31.0	0.32	55.0	27.5	1.41		R
Development Period r^2=0.930, constant=-1.23 dys				**% survival** r^2=0.692, constant=+39.3% points				

NOTES:

Anti-PPTE Reaction on SDS-PAGE to PPTE antibodies preblocked with Arc 4

Ratio Square Root of (% egg survival to adult/Days Development)

Classification of *A. obtectus* resistance with reference to the ratio on undamaged seed

S Susceptible: ratio >1.0

I Intermediate: ratio >0.6

R Resistant: ratio <0.6

16.2 PROTEIN AND CARBOHYDRATE FRACTIONATION

Following a similar procedure to that adopted for *Z. subfasciatus*, bean meals from susceptible (RKB) and resistant (G12953) accessions were fractionated and the fractions included in artificial diets presented to *A. obtectus*. Inclusion of protein fractions (Gatehouse *et al.* 1987 and repeated in Figure 10-a and Figure 10-b) show no significant difference between inclusion of either fraction of the susceptible cultivar, 'though inclusion of either fraction of the resistant accession G12953 have a significantly greater effect than the corresponding susceptible cultivar fraction on larval development period.

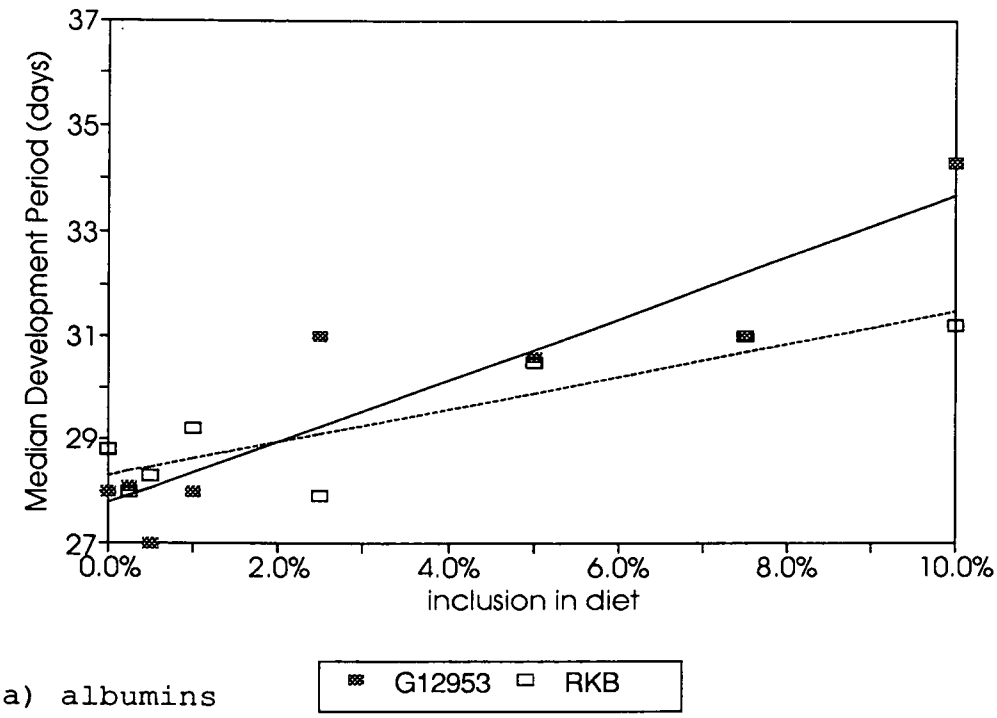
From the same paper inclusion of the *non-bound* (or *carbohydrate*) fraction results in much higher antimetabolic effects (Figure 11-a and Figure 11-b). This non-bound fraction is present at about 4% of total bean meal in G12953 (measured from the recovery during extraction), and the extract is highly gelatinous; its LD_{50} from this data is around 2.5% inclusion. In the susceptible accession G10019, although it constitutes about the same physiological concentration in the cotyledon, the extract is not gelatinous, and does not appear to affect *% survival* or *development period* at concentrations up to 10%.

16.3 SEPARATING THE NON-BOUND FRACTION. PHENOL PHASE PARTITION

Because of the lack of antimetabolic effect of inclusion of the major protein fractions (albumins and globulins) on *A. obtectus* development, and the effect noted with inclusion of *non-bound* material, this material was further frationated by **phenol-phase partitioning.**

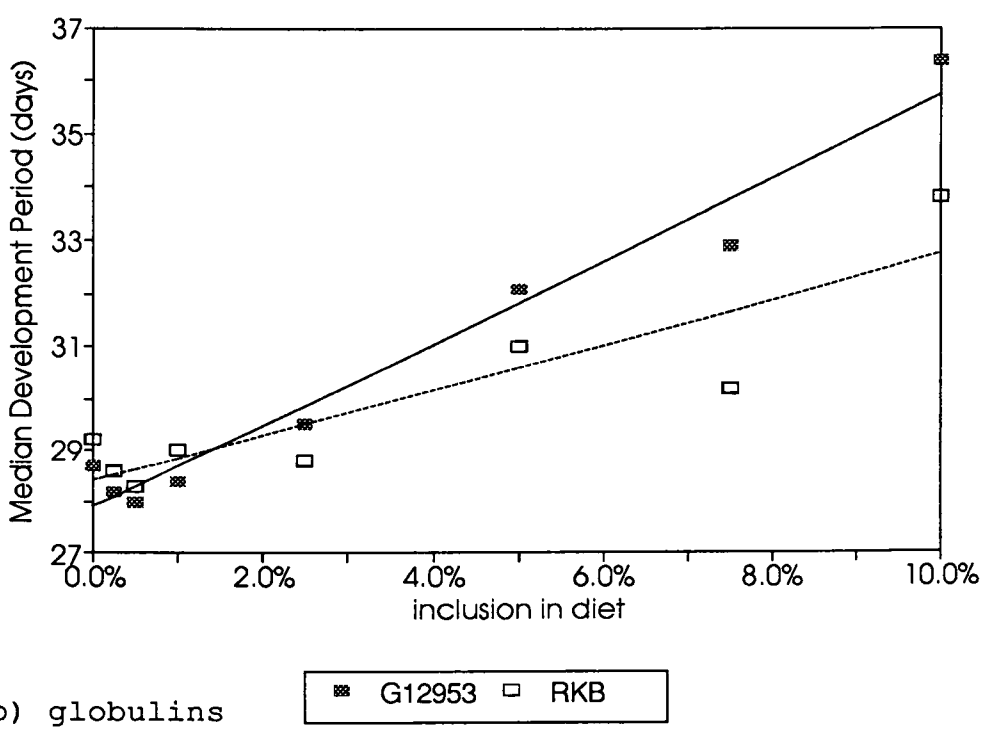
With this method, carbohydrate remains in the aqueous phase, whilst proteins and glycoproteins are highly soluble in the phenol phase, albeit at this stage denatured.

The *non-bound* material was dissolved in distilled water and mixed with phenol by shaking at 40°C. The aqueous and phenol phases were separated by centrifuging at 25°C, and the *aqueous phase* dialysed against 10% acetic acid, 5% acetic, 2% and 1%



a) albumins

Figure 10-a Inclusion of albumin fraction from resistant (**G12953**) and susceptible (**RKB**) beans on development of *A. obtectus*



b) globulins

Figure 10-b Inclusion of globulin proteins from resistant (**G12953**) and susceptible (**RKB**) beans on *A. obtectus* development

acetic for 6 hours each, followed by three changes of distilled water. The *phenol phase* and the *high molecular weight precipitate* (material present as a precipitate underneath the phenol phase) were taken out separately and also dialysed against acetic acid and distilled water. Phenol is highly soluble in acetic acid, and dialysis with 10% acetic acid should remove all traces of phenol from the materials in solution.

In the first extraction, three fractions were produced:

1- material in the **aqueous phase**, on SDS-PAGE entirely carbohydrate

2- denatured protein in the **phenol phase**

3- material precipitated by interaction with phenol. High M_r ppt

16.3.a Extraction 1 in Feeding Trials

Materials purified by phenol-phase partition were included in artificial diets (at 2.5%) presented to *A. obtectus* larvae to identify the fraction containing the antimetabolic fraction. At the time this was done full data was not recorded, only averages of 5 replicates (Table 25). The material giving the highest antimetabolic effect was not the aqueous phase, as expected (as this fraction contains the carbohydrate material), but the insoluble fraction named **High M_r ppt**. This material was virtually intractable in this state, so a refinement of the previous phenol-phase extraction method was used to purify this further.

16.3.b Re-Extraction of Phenol Phase Material

Non-bound material was dissolved in 25mM borate pH 7.0, and mixed as before with 95% phenol. After separation, the precipitate was resuspended in 95% phenol and centrifuged again to give **phenol wash 2** and a precipitate. The precipitate, by now gelatinous, was vortexed with glacial acetic acid (2ml) to remove last traces of phenol and phenol-soluble material (**Acetic acid wash**) leaving the precipitate (**PPTE**), and finally all fractions were dialysed as before against successive dilutions of acetic acid and distilled water, and lyophilysed. The yields of each fraction obtained by this

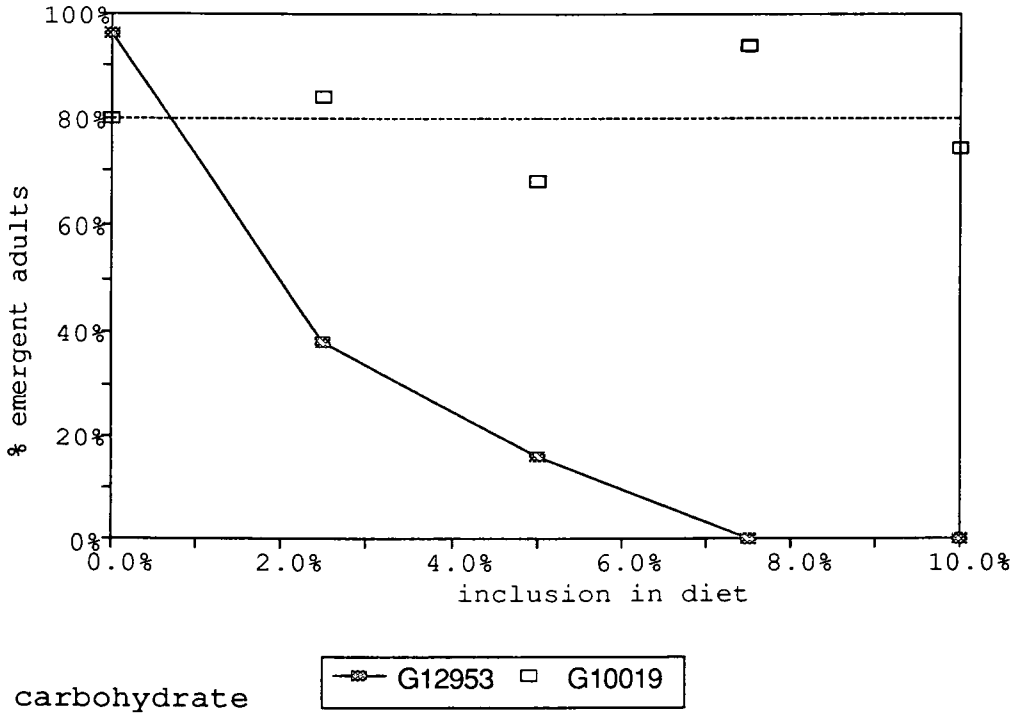


Figure 11-a Inclusion of the *carbohydrate* fraction from resistant (G12953) or susceptible (G10019) accessions vs % survival to adult

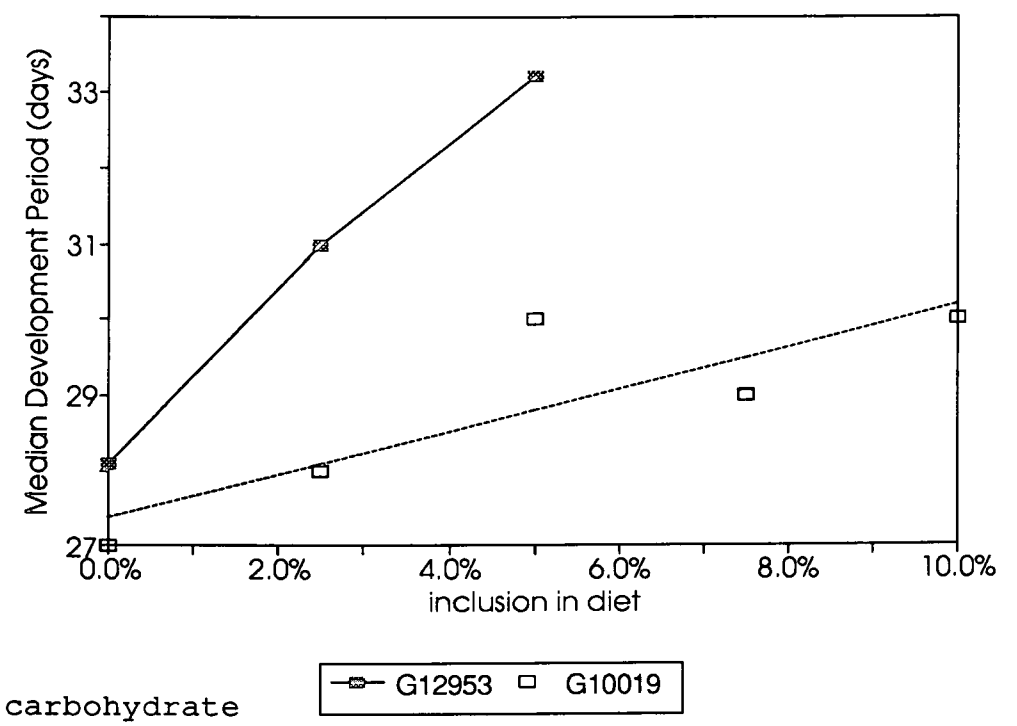


Figure 11-b Inclusion of *carbohydrate* fraction from resistant (G12953) or susceptible (G10019) accessions vs Development Period

Table 25 Inclusion of phenol-phase partition material to *A. obtectus*

Primary separation

Diet	Inclusion	N	% emergent	Dev.Per. (dys)
Control		5	44.0%	30.2
Aq #	2.5%	5	40.0%	32.0
Phe Sol	2.5%	5	68.0%	32.4
High Mr ppt	2.5%	5	none	none

Secondary Fractionation

Diet	Inclusion	N	% emergent	DP (dys)
Control		10	82.0%	34.3
non-bound *carbohydrate*	2.0%	5	44.0%	nd
	4.0%	5	36.0%	27.0
PPTE: "high Mr" material precipitated by phenol after washing	0.5%	5	77.0%	44.0
	1.0%	5	52.5%	41.4
	1.5%	5	45.0%	43.5
AQ sol carbohydrate material	1.0%	5	52.0%	29.3
	2.0%	5	48.0%	nd
Phenol phase	2.0%	5	56.0%	28.7
	4.0%	5	64.0%	31.4
Phenol wash 2	0.2%	5	52.0%	29.0
	0.4%	5	20.0%	nd
PPT wash 10% acetic acid wash	0.25%	5	80.0%	35.5
	0.5%	5	84.0%	37.5

nd not determined because of emergence problems

Table 26 Yields of various phases of *non-bound* material after **phenol-phase partitioning**

Material	% of non-bound	% of cotyledon
AQ soluble	28.6%	1.14%
Phenol phase	53.9%	2.16%
Phenol wash 2	7.2%	0.29%
10% acetic acid wash	1.4%	0.06%
PPTE	8.9%	0.35%

method are given in Table 26. The fractions were run on SDS-PAGE and stained with Kenacid blue to detect protein subunits, and danzyl hydrazine for glyco-groups (oligo or polysaccharide side chains) (Plate 12). Both phenol phases contain proteins showing high levels of glycosylation, and the lack of protein visible in the track of **PPTE** (precipitate of extraction) may be because of the intractability of this material - after freeze-drying, it is hard, lumpy, and virtually insoluble in most solvents including sample buffer. Virtually all of the protein is in the phenol phase, and the large quantity of highly soluble material in the aqueous phase can be assumed to be carbohydrate.

Materials purified by this second extraction were included in artificial diets at approximately physiological and 2× physiological concentration (*non-bound* for comparison was included at half physiological and physiological concentration), Table 25.

The precipitated so-called "high Mr" material caused the highest antimetabolic effect when included in artificial diets at 2.5%: however the secondary purification method, with inclusions of components at up to 2× physiological concentration, failed to give such high levels of antimetabolic effect.

16.4 IMMUNOLOGICAL CHARACTERISATION

Antibodies to **PPTE** were prepared in mice using an emulsion of the intractable PPTE (finely ground) in Freunds' incomplete. These antibodies show high levels of binding to materials of similar subunit size to arcelin 4. In order to minimise this cross-reaction, arcelin 4 was added to the final dilution of antibody (for Western blotting this was 1:2000) at 2mg/ml, and results are given after this pre-blocking.

The accessions to which they show a positive reaction on Western blotting (see Table 24) also cross-react strongly with arcelin 4 antibodies.

Proteins are stained with Kenacid blue, with danzyl hydrazine fluorescence (showing glyco-groups) drawn in on overlay

Standards		Samples (duplicated)	
GII	phaseolin (+ve carbohydrate stain, 43K)	**WS#**	aqueous phase (10mg/ml)
		Ø1	extraction in phenol on first mixing (4mg/ml)
VICILIN	Pea vicilin (no glycosylation)	**Ø2**	extraction by phenol wash of precipitate (4mg/ml)
LEGUMIN	Pea legumin	**PPT**	precipitate (PPTE) (nominally 4mg/ml)
		PPTWASH	extracted in acetic-acid wash of precipitate

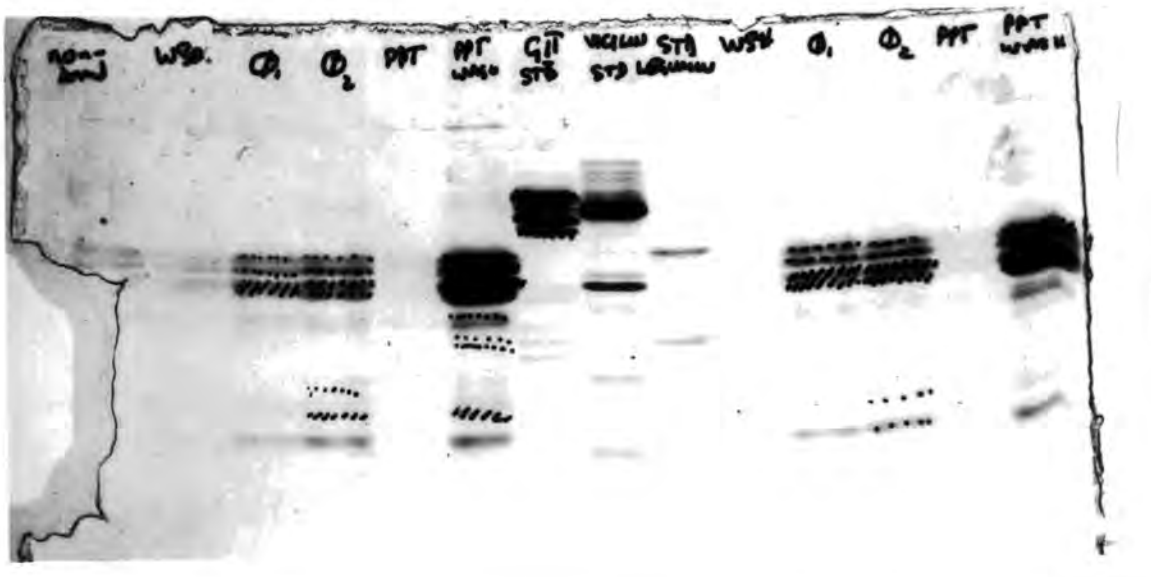


Plate 12 SDS-PAGE and danzyl hydrazine stain of *phenol phase partitioning*

16.5 IS PPTE FROM THE COTYLEDON OR THE TESTA?

For reasons given earlier, it was important to separate antimetabolic effects based on factors in the testa from those based biochemically in the cotyledon. The antibodies obtained were used to detect the presence of antigen in samples of testa cut from the seed, and in samples of cotyledon from which the testa had been removed (Plate 13).

Two samples of G12953 seed were used, one originally supplied in June 1985, and the second, supplied in larger quantities and used to extract the second batch of PPTE, supplied in December 1987. Samples were run on SDS-PAGE, electroblotted onto nitrocellulose, and combined with antibodies (blocked with arcelin 4) at 1:2000.

A certain amount of immunobinding occurs in tracks containing testa only; however the bulk of binding is on tracks of extracts of cotyledon, and from this data it is assumed that most of the PPTE and the resistant portion of *non-bound* or *carbohydrate* material is from the cotyledon.

16.6 AMMONIUM SULPHATE FRACTIONATION

The poor results obtained on fractionating *non-bound* material by the second method of phenol phase partition, the indication that the antimetabolic material may contain protein, and the poor specificity of the antibodies prepared, led to a search for alternative methods of preparation of the antimetabolic material from the *non-bound* fraction.

Total *non-bound* material was fractioned by ammonium sulphate precipitation into four fractions: **AS0-40%**; **AS40-60%**; **AS60-80%**; and **AS80%+**. Western blotting with anti-PPTE antibodies revealed cross-reactivity with the **AS80+** portion, and examination of this on SDS-PAGE revealed similarity between the subunit bands of **Phenol Wash 2** and **AS80+**.

The fractions obtained from ammonium sulphate precipitation were included at physiological concentration (based on recoveries after fractionation, with a total recovery in excess of 80%) and half of this concentration, in artificial diets (Table 27). Whilst the **AS80%+** fraction appears to be highly toxic to the development of *A. obtectus* on

a **SDS-PAGE stained with Kenacid blue**
b **Western blot with preblocked (arc4) PPTE antibodies**. Loading 5mg/ml

1	G12953 Dec 87, no testa	**4**	G12953 Jun 85, no testa
2	" " whole seed	**5**	" " whole seed
3	" " testa only	**P**	PPTE (different extractions)
		M	markers SDS VII

dates refer to the dates accessions were supplied to us

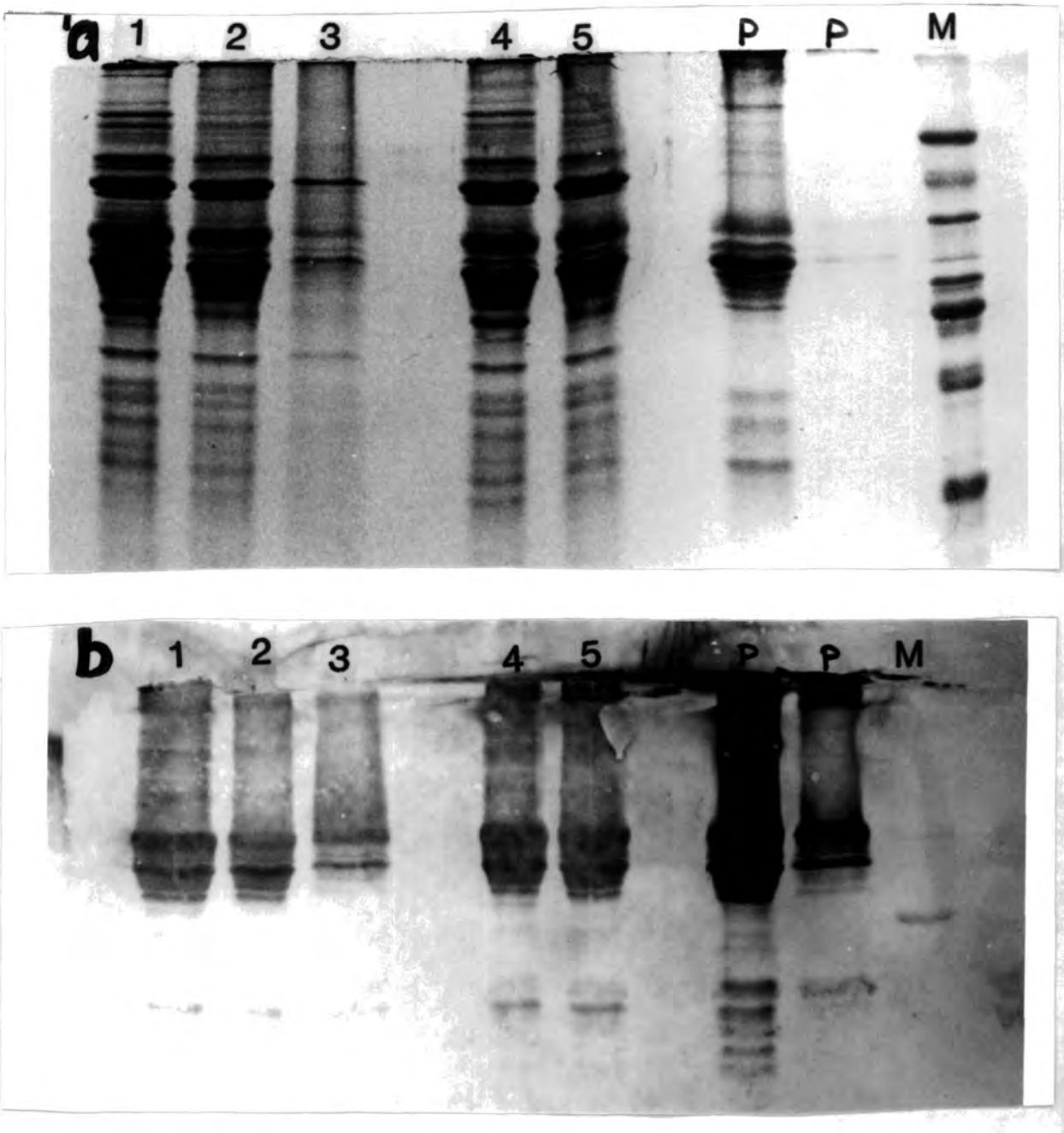


Plate 13 Kenacid-blue staining and Western blotting to determine the location of PPTE in the seed

Table 27 Inclusion of **Ammonium sulphate cuts** vs *A. obtectus*

Material	Inclusn %	N reps	% survival	Dev Per (days)
Control		5	62.4	27.4
non-bound	2.00%	5	60.0	27.9
	4.00%	5	64.0	28.3
AS0-40%	0.55%	5	72.0	28.2
	1.10%	5	68.0	27.4
AS40-60%	0.21%	5	76.0	27.0
	0.42%	5	76.0	28.8
AS60-80%	0.50%	5	76.0	27.9
	1.00%	5	92.0	28.5
AS80%+	0.72%	5	4.0	27.0
	1.44%	5	4.0	35.0

physiological concentration and 2× physiological

these diets, attempts to fraction this into two fractions (**AS80-100%** and **AS100%+**) did not retain antimetabolic effects. The separated fractions **AS80-100** and **AS100+** were found to contain high concentrations of ammonium sulphate, and upon redialysing the material (the first dialysis had been 100ml solution dialysing against 4lt distilled water, eight changes over 3 days) a considerable weight loss was noted together with loss of antimetabolic effect of these sub-fractions.

By this stage there was no time for further feeding trials to be performed to check whether ammonium sulphate (highly toxic to *A. obtectus* larvae) was present in the **AS80+** fraction.

16.7 INHERITANCE OF RESISTANCE

Crosses were made between the resistant accession **G12952** and susceptible cultivars **PIJAO**[5] **(GH-14)** and **A36**[6] **(GH-13)**, and the F_2 seeds bioassayed individually to test the inheritance of resistance.

The resistance classification was based on the first four adult emergences from each seed, although the maximum number of adults to emerge from a single seed was seven (1 of GH-13; five emerged from 27 of GH-13 and from 20 of GH-14). 97 seeds of **GH13** and 92 seeds of **GH-14** were bioassayed, with the classification of seeds being **S**usceptible (first three emergences before day 40, four emergences or greater, or very early (day 35) first emergence); and **R**esistant (less than three emergences or onset of emergence delayed or reduced emergence). Although these figures are arbitrary, they serve to simplify the data.

At this classification the percentage gives approximately 75% not resistant to *A. obtectus*, with 25% resistant. Simple Mendelian inheritance theory suggests that inheritance of resistance to *A. obtectus* is recessive to susceptibility, based on these figures.

---

[5] **Pijao** is a commercial variety used in several countries in Latin America. It is a cross of *Porillo Sintetico* × *Mexico II*, black seed colour and size 20-25g/100 seeds. The cross is called GH-14

[6] **A36** is an advanced breeding line by CIAT which is expected to be introduced commercially. A cross of *E1060* × *Calima*, seed colour red mottled, seed size 35-40g/100 seeds. The cross with **G12952** is GH-13

Genotype	**rr**	**rS**	**Sr**	**SS**
Phenotype	Resistant	not resistant		
Percentage	25%	75%		

Using a different cut-off point for resistance classification, different percentages may have been obtained, but these figures serve to illustrate that *A. obtectus* resistance is recessive. Using these data, the two hypotheses, one that there is no simple Mendelian effect, and the other that the Mendelian effect follows this pattern of 1 resistant to 3 susceptible, were tested by Chi-Squared test (Table 28). For each cross, the Chi squared value exceeds the probability that this segregation is due to chance and is not genetically controlled (**P**>.995 due to some factor), although there are not enough seeds to prove the alternative hypothesis that there is a 1 to 3 segregation. When the values for the two crosses are combined, the Mendelian recessive inheritance hypothesis is virtually proven, at least within **P**0.10 (Chi-squared obtained 0.01587, Chi-squared value required to prove this <0.0158).

16.8 CONCLUSIONS TO *A. OBTECTUS* RESISTANCE

The most obvious feature of *A. obtectus* resistance is that it is not based on the same material as that responsible for *Z. subfasciatus* resistance.

Whilst some accessions exhibit high levels of *A. obtectus* resistance (low *% emergence* and extended *development period*), physical damage to the seed testa reduces this considerably, indicating that whilst biochemical factors are important in conferring resistance, the high levels of resistance found in wild accessions are assisted by physical features of the seeds.

This is because, at least in part, of the method of larval penetration of the seed in the natural habitat. In the wild, *A. obtectus* females lay eggs into the pod next to the seed, or into a groove cut into the pod, and the larvae use their mobility to search for a suitable seed before the testa is fully hardened, and penetrate with the assistance of neighbouring seeds and the walls of the pod. In agricultural storage the seeds will have been handled whilst dry, and a proportion of the seeds will have damaged testae,

Table 28 Numbers of F_2 seeds from crosses of **G12952** with commercial cultivars

GH-13	actual	H0:nul	H0:1/3	H0:nul Chi Sq=19.06186
R	27	48.5	24.25	
S	70	48.5	72.75	H0:1/3 Chi Sq=0.416
Totals	97	97	97	
GH-14	**actual**	**H0:nul**	**H0:1/3**	**H0:nul Chi Sq=27.174**
R	21	46	23	
S	71	46	69	H0:1/3 Chi Sq=0.232
Totals	92	92	92	
Combined	**actual**	**H0:nul**	**H0:1/3**	**H0:nul Chi Sq=45.762**
R	48	94.5	47.25	
S	141	94.5	141.75	H0:1/3 Chi Sq=0.0159
Totals	189	189	189	

NOTES:

R seeds showing some resistance to *A. obtectus*

S seeds showing no resistance

actual number of seeds actually assigned to each classification

H0: nul expected number of seeds falling into each classification, assuming that there are equal numbers of seeds in each classification (there is no genetic effect)

H0:1/3 expected number of seeds assuming **R**esistance is controlled by a single recessive gene, hence 25% of seeds will be resistant, and 75% will be susceptible.

Chi Sq Chi-squared value based on the named null hypothesis. For 1 d.f. the probability (**P**0.05) that the hypothesis is correct is exceeded when the value exceeds 3.84 (i.e. the hypothesis is disproven), and is confirmed for values less than 0.0158 (**P**0.10)

affording an entry hole for the larvae.

Fractionation of the cotyledon material into albumins, globulins and soluble carbohydrate (i.e. not including starch) enabled the antimetabolic factors to be separated from other fractions and detected by inclusion in artificial diets. In this insect the material associated with the highest levels of resistance was the soluble carbohydrate material (*non-bound*), and this was further fractionated to separate neutral carbohydrate from proteins. The fraction at this stage most antimetabolic to larvae had been denatured by interaction with phenol, and a different method of fractionation was tried.

Ammonium sulphate fractionation was tried and gave a fraction which not-only cross-reacted with antibodies raised vs PPTE (an antimetabolic fraction from phenol-phase partition), but also proved antimetabolic to the larvae when included in artificial diets. However at the physiological inclusion rate the antimetabolic effect was not very great, and it is concluded that the high nutritional background provided by the basal diet used (haricot bean meal) masked the full effects of the antimetabolic fractions.

Inheritance of resistance is similarly weak, following the pattern of a single recessive gene carrying the factor for resistance to *A. obtectus*.

Results 17
Possible Mechanisms for *A. obtectus* Resistance

17.1 CHARACTERISING PPTE

A number of methods were used to try to characterise the insoluble fraction obtained after **phenol-phase partition** (PPTE). All met with the problem of its intractability; it does not appear to dissolve in non-destructive solutions.

A range of solutes were tried as indicated (Table 29) and from this and acid hydrolysis it was possible to analyse the carbohydrate and amino-acid composition of PPTE. However since it is not known whether PPTE is a single pure substance or not, indeed it is possible that there is a considerable mixture of substances present, these data are of limited use. Its solubility in DMSO suggests that a complex neutral polysaccharide is present, however in the course of extraction the material was soluble in distilled water, suggesting that it has been modified in some way by interaction with the phenol.

17.2 CARBOHYDRATE COMPOSITION OF RESISTANT AND SUSCEPTIBLE MATERIAL

The *non-bound* material from resistant accession **G12953**, largely composed of soluble *carbohydrate*, was included in artificial diets with deleterious effects on *A. obtectus* larval development (see Gatehouse *et al.* 1987, and Figure 11-a page R.187). The same material from susceptible accession **G10019** did not limit larval development under the same conditions.

The sugar compositions of the *non-bound* material from both G12953 and G10019 (4% of the seed by weight), and of the PPTE extracted from G12953, were determined by GLC of alditol acetate derivatives of the sugars (at RRI) (gross uronic acid content was measured by **carbazole-sulphuric acid** method).

Table 30 shows much higher levels of uronic acids in the resistant accession

Table 29 Solutes used to attempt to dissolve PPTE

Solubility is based on visual assessment of material remaining as a precipitate

Solute	solubility
0.1M NH_3	none after 24 hrs
conc. NH_3 (18M)	none
0.1M NaOH + 0.5M NaCl + 0.05M $NaHCO_3$, pH 10	none
4% SDS in SDS-sample buffer	none
0.1M TriFluoroAcetic Acid	very little
1M NaOH	mostly dissolved after 24 hrs, probably hydrolysed
Pyridine	none
DMSO	mostly dissolved after 24 hrs

(though the level of this sugar is not exceptionally high in the PPTE material), and higher levels of fucose, xylose and arabinose. The high levels of uronic acids may account for the gelatinous nature of the resistant accession *carbohydrate* material in solution, and uronic acids are often associated with arabinose in e.g. pectins.

17.3 SUGARS IN ARTIFICIAL DIETS

Sugar analyses suggested gross differences in the sugar composition of this non-bound fraction, and since this was considered to contain the bulk of the soluble (available) carbohydrate in the seed, the sugars were incorporated into artificial diets at up to twice physiological concentrations and presented to *A. obtectus* larvae (Table 31).

Uronic acids are usually present in the form of polysaccharides, and a number of poylsaccharides containing uronic acids are available commercially, amongst them Poly(galacturonic acid) (*Poly(GalA)*) and pectin (which also contains pentoses e.g. arabinose). These polysaccharides were also included at estimated physiological levels in the artificial diets.

Of the included sugars, only arabinose had any effect on larval development, and no effect was observed with mixtures made up of physiological concentrations of the neutral carbohydrates, and the same with the addition of uronic acids (50:50 mix of galacturonic acid and glucuronic acid). In all cases physiological concentration was based on the concentration in *carbohydrate* fraction, assuming this to be 4% of total bean meal.

This is interesting since arabinose is present at much higher concentrations in hte resistant accession non-bound soluble material than its concentration in susceptible accessions. This effect was not persued beyond physiological mixtures of the sugars because of constraints of time.

Table 30 Sugar composition of *carbohydrate* fraction of resistant **G12953** and susceptible **G10019**

Sugars	total non-bound %		PPTE
	G10019	**G12953**	
Rhamnose	0.17	0.00	0.00
Fucose	0.45	4.27	3.36
Arabinose	7.69	22.91	8.70
Xylose	2.22	7.74	5.24
Mannose	2.31	0.44	0.70
Galactose	3.23	4.83	7.23
Glucose	1.90	1.83	1.29
Uronic acids[*]	1.80	9.70	ND
TOTAL % of Material	19.77	51.72	26.52

NOTE:
Assay by GLC of alditol acetates
[*] uronic acids measured by carbazole-sulphuric acid method

Starch is the major polysaccharide, and energy store, in *P. vulgaris* seeds. It is not normally soluble in acetate buffer, and would not have been measured. Therefore it was possible that some difference in starch structure or content could be contributing to *A. obtectus* resistance.

Starch grains from a range of both resistant and susceptible accessions were examined under a microscope, freshly stained with iodine solution. Size was measured (using video image processing, based on the measurement of area), and a small difference between the sizes in the accessions measured was observed (Figure 12).

Total starch present in resistant and susceptible accessions was measured, using a DMSO extract of seed meal and an enzymatic assay specific to starch. The regression of starch grain size on starch content revealed a correlation r=0.915 (10 accessions). Uronic acid content in an acetate buffer extract was also performed, giving no correlation with starch content (r=0.048 for 13 accessions). The compositions of each accession are given in Table 32.

Resistant accessions (G12949 and G12953) do show a lower starch content than susceptible accessions (average for these resistant accessions is 176mg/g; for susceptible accessions is 296mg/ml): two accessions do not follow this pattern, with **G12942** (susceptible) having low starch content, and **G12880** (resistant) high starch content. This is also reflected in the correlation coefficients of starch content on the resistance coefficient *(square root (% survival/ development period)*, which for all accessions measured (10) is **r=0.444**, whereas for eight accessions excluding the two indicated **r=.739**. This result is also indicated in Figure 13. However the correlation coefficient is considerably higher when the effects of testa are excluded, giving **r=0.851** for the ratio derived from testa-damaged accessions (9 total).

Resistant accessions on average have 30% less starch (as measured) than susceptible accessions.

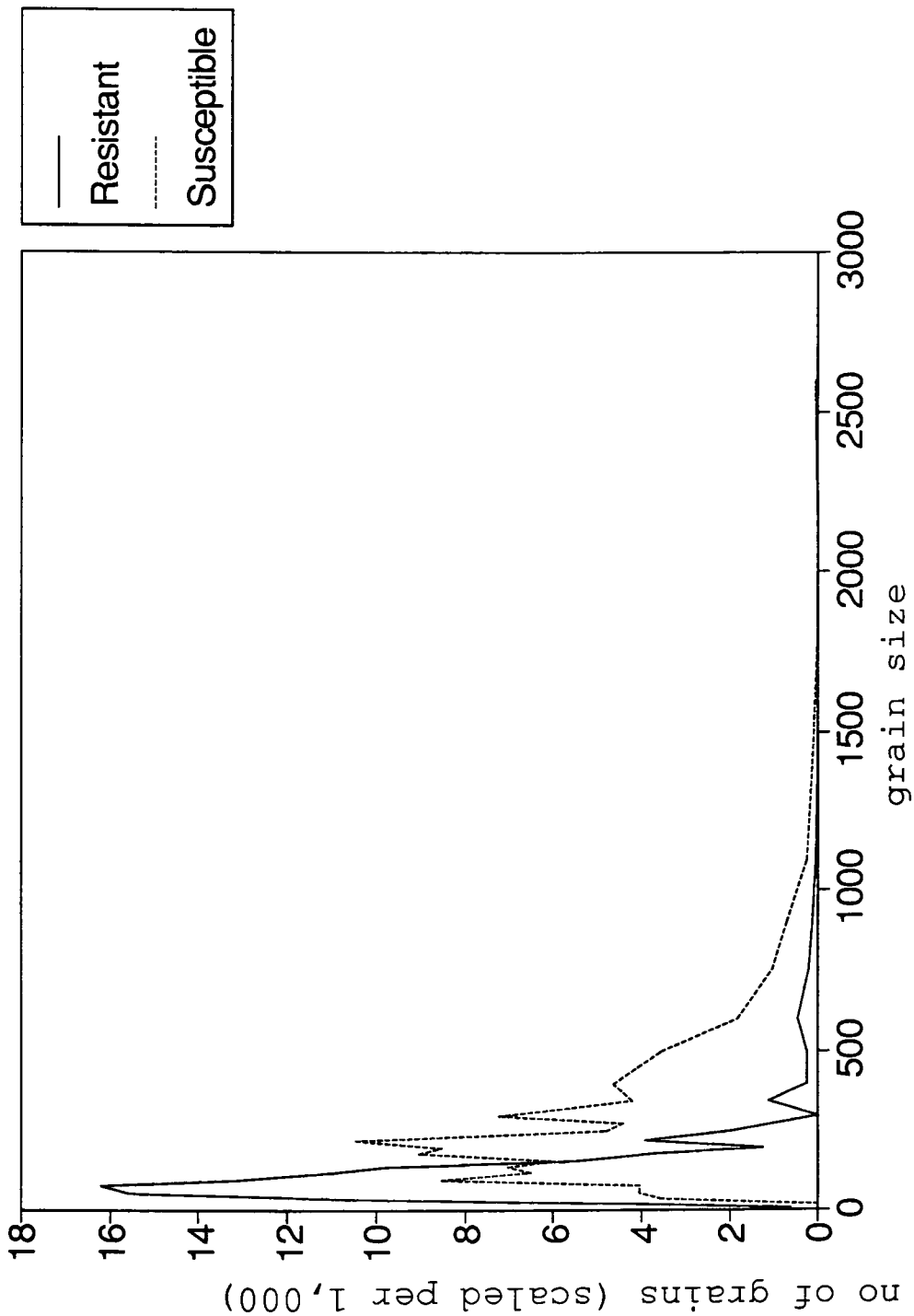


Figure 12 Histogram of starch grain size in *resistant* and *susceptible* accessions

Table 31 Inclusion of pure sugars in artificial diets for *A. obtectus*

Sugar	incln %	N rep	% surv	Dev Per days	Sugar	incln %	N rep	% surv	Dev Per days
Arabinose	CTRL	10	84.0	29.5	Mannose	CTRL	10	88.0	28.5
	0.05	10	48.0	29.5		0.05	10	100.0	28.5
	0.50	10	68.0	30.5		0.50	10	48.0	28.5
	1.00	10	40.0	29.0		1.00	10	88.0	29.5
Galactose	CTRL	10	76.0	29.5	Glucose	CTRL	10	72.0	29.5
	0.05	10	72.0	30.0		0.05	10	88.0	28.0
	0.50	10	80.0	29.5		0.50	10	26.0	28.5
	1.00	10	80.0	30.5		1.00	10	128.0	28.0
Xylose	CTRL	10	68.0	30.5	Glucuronic Acid	CTRL	10	84.0	29.5
	0.05	10	80.0	29.0		0.40	10	100.0	28.5
	0.50	10	64.0	29.5		0.80	10	96.0	29.5
	1.00	10	72.0	31.0	Galacturonic Acid	CTRL	10	96.0	27.5
Fucose	CTRL	10	48.0	29.5		0.40	10	84.0	28.5
	0.05	10	56.0	29.5		0.80	10	72.0	27.5
	0.50	10	68.0	28.5	Poly(GalA)	CTRL	10	80.0	27.5
	1.00	10	88.0	28.5		0.40	10	72.0	27.0
						0.80	10	76.0	27.5
1. Phys conc mixture		10	60.0	32.4	Pectin	CTRL	10	84.0	27.5
2. 2 × Diet 1		10	76.0	32.6		0.40	10	76.0	27.5
3. Diet 1+Uronic Acid		10	80.0	32.1		0.80	10	84.0	28.5
4. 2 x Diet 3		10	64.0	33.3					
6. CONTROL 1&2		10	72.0	32.0					
7. CONTROL 3&4		10	44.0	32.3					

Table 32 starch content and uronic acid content of selected accessions from CIAT bean collection

Accession	Dev Per (days)	% survival	Grain size μ^2	Starch mg/g	Uronic Acid
G09989B	30.5	15.0	nd	316.0	17.6
G10011	30.5	36.0	nd	306.7	18.2
G10017	nd	nd	nd	267.6	21.7
G10030	31.4	61.5	nd	248.2	20.1
G12862	nd	nd	nd	321.1	18.4
G12880	29.0	6.0	265.1	286.0	18.9
G12981	34.8	52.0	163.7	nd	25.2
G12896	nd	nd	535.5	330.8	16.8
G12904	nd	nd	399.9	294.1	15.0
G12942	32.9	90.0	nd	211.6	15.9
G12949	46.0	7.0	64.8	171.2	18.4
G12953J85	56.6	1.5	144.0	181.2	14.5
G12953D87	nd	nd	nd	209.9	16.6
G13014	28.3	69.0	300.2	295.7	19.1
Haricot	28.1	80.0	390.7	335.0	14.9

NOTES:
starch grain size is mean grain area as measured from microscope slide (freshly stained with iodine from dry bean meal to minimise grain swelling), and is measured in square microns μ^2
G12953j85 is seed material supplied from CIAT in June 1985. **G12953D87** was supplied in December 1987, and on bioassay found to be less resistant to development of *A. obtectus*
nd = no data available

17.5 SUPPLEMENTATION OF G12953 WITH STARCH

In order to examine the possibility that lower levels of starch in G12953 meal caused carbohydrate starvation, starch was used to supplement diets based on G12953, and in control diets of haricot.

Diets were prepared from detesta'd milled seeds, and soluble potato starch completely dissolved in distilled water (40mg/ml) by boiling before adding to the meal. Inclusion levels were 3%, 6% and 15%, to test for low levels of unavailability, gross lack of starch, or possible toxic effects of oversupplementation.

The mixed meal was freeze-dried, then as usual a minimum amount of water added and the pellets formed, dried and equilibriated as normal for presentation to *A. obtectus*; and *% emergence*, *total % developed to adult* and *mean development period* determined (Table 33).

Starch supplementation did not overcome resistance, nor did it affect development on haricot meal. The starch inclusion was not detrimental to development, but whatever factors in the G12953 meal make it resistant to *A. obtectus*, supplementation with starch does not overcome them.

17.6 COOKING G12953 TO DESTROY PROTEIN ANTIMETABOLITES

Bean meal (detesta'd) was also autoclaved, both starch supplemented and unsupplemented, and both G12953 and haricot.

Cooking was carried out at 121°C for 5 mins, and was to denature proteins present to destroy any antimetabolic effects from e.g. lectins and protease inhibitors. Starch was added to some diets to overcome any additional carbohydrate shortage in case both *available carbohydrate* deficiency and protein antimetabolite presence were acting in concert.

After autoclaving, the meal was dessicated as usual and made into pellets. However the pellets shrank during equilibriation, and were visibly a different colour. During bioassay no emergence was observed by day 70, and after dissection it was

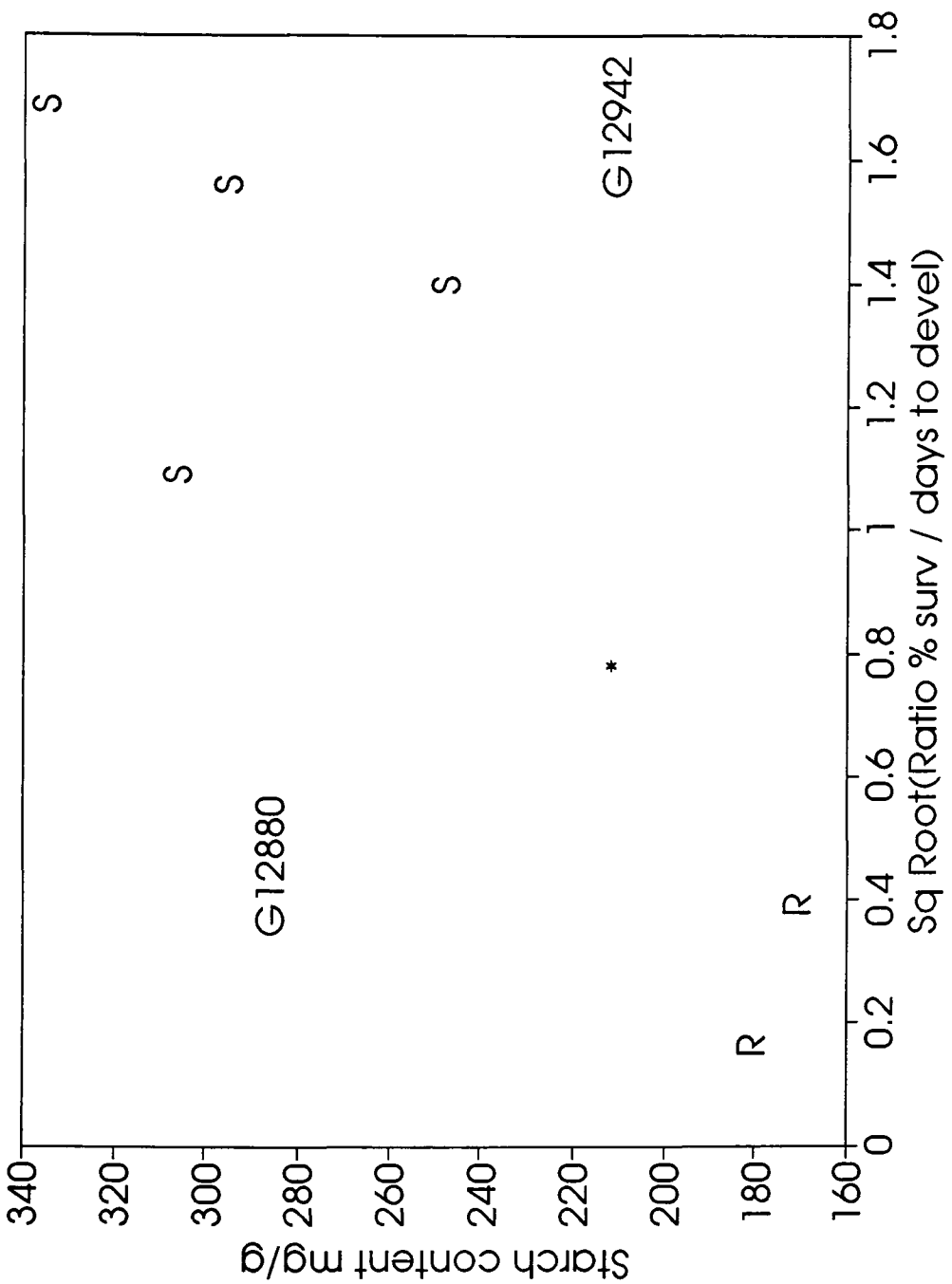


Figure 13 Content of starch of selected accessions against resistance to *A. obtectus*

Table 33 Supplementation of G12953 meal with starch in bioassay vs *A. obtectus*

Diet	Inclusn %	N	% emergent	% develop to adult	Mean Dev Per	SD Dev Per
Haricot	CTRL	24	48%	48%	32.9	3.6
	3%	6	24%	24%	36.8	3.7
	6%	13	52%	64%	33.6	4.4
	15%	10	40%	40%	34.6	1.9
G12953	CTRL	10	20%	28%	54.7	6.3
	3%	6	24%	40%	52.0	3.3
	6%	7	28%	36%	51.7	4.6
	15%	3	12%	16%	52.6	4.2

observed that no larvae had penetrated. Subsequent examination revealed dessicated first instar larvae on the surface of the pellet; presumably cooking had affected the meal making the pellets of both haricot and G12953 impossible to penetrate.

17.7 CONCLUSIONS

The PPTE material was not fully characterised because of the difficulties encountered on attempts to solubilise it. Its sugar composition was determined, and whilst the relative ratios of constituent amino acids were determined since no subunit bands were visible on SDS-PAGE this information was not considered useful.

Sugar contents of the *non-bound* material (soluble carbohydrate mostly) from both resistant and susceptible accessions were compared; *non-bound* from the resistant accession (G12953) contained much higher levels of arabinose and uronic acids (3× and 4× respectively) than the equivalent material from the susceptible accession. Similarly some resistant accessions had lower starch contents than susceptible accessions, by approximately 40%. Attempts were made to identify a factor responsible for resistance, by feeding individual sugars, mixtures of sugars, and commercially available polysaccharides, in haricot-based artificial diets: no sugars in any combination had antimetabolic effects at physiological inclusion.

Similarly the starch content of resistant meals was supplemented at various levels in case digestible carbohydrate was lacking in resistant meal; again with no major effect on *A. obtectus* larval development. Cooking the meal to destroy the antimetabolic effects of proteins failed to give any response since although the antimetabolites may have been denatured, the meals became too hard for the larvae to penetrate.

Discussion, Chapter 18

The aims of this research project were to

1- select sources of resistance to *A. obtectus* and *Z. subfasciatus*,

2- produce screening methods for parents and F_2 generation seeds for breeding,

and in particular to characterise and investigate the mechanism(s) of this resistance to the major pest bruchids *A. obtectus* and *Z. subfasciatus*. The seed in storage which is damaged by these pests is the common bean (*P. vulgaris*, haricot bean, kidney bean), and the search for factors involved in resistance was made through seed material obtained from the wild, where some seed lines are known not to be damaged by, and not to allow development of, one or other (or both) of these pests in their seeds.

For seeds in storage, the point at which resistance occurs (the prime commercial application of this work) could be **1**- before the larva enters the seed, **2**- whilst the larva is in the seed so that it fails to emerge, or **3**- in reduced fecundity (low bodyweight or immaturity so that breeding and population increase are much reduced).

In the first case, a hard testa, or toxic or repellant substances in the testa, could prevent the larva from entering the seed. Different species of Leguminosae seeds show different testa hardnesses (Janzen 1977; Simmonds *et al.* 1989), and different varieties of *P. vulgaris* contain different substances in the testa. Particularly hard testa may prevent bruchid predation (Simmonds *et al.* 1989), however in many cases bruchids can drill hard seed coats (Janzen 1977), or the testa, although containing toxins active against the larva (Stamopoulos and Huignard 1980), may be bypassed on larval penetration (Hames 1983). In many human agricultural cultures acceptability of the seed for culinary purposes is determined by characteristics of the testa (e.g. colour, texture, hardness) which determine the; for this reason more than biological reasons factors reliant on the testa would probably not be useful in a breeding line.

In the second and third cases, chemicals in the cotyledon can, and already do (in *P. vulgaris*), take a number of antimetabolic forms. Examples of this include **lectins**, which bind to and disrupt gut epithelial membranes (Gatehouse *et al.* 1989), **enzyme inhibitors** (protease inhibitors (Jaffé *et al.* 1973) and carbohydrase inhibitors (Gatehouse

et al. 1986) will be the major inhibitors affecting primary nutrition of the pest), or toxins (eg cyanogenic glycosides (Janzen 1981)). All of these illustrated antimetabolites are toxic to mammals, but are destroyed on culinary treatment (soaking beans, cooking at high temperatures) which are already in use to exploit the high nutritional value of *P. vulgaris* beans (Liener 1980).

Sources of resistance were selected by performing bioassays on seeds from the CIAT wild-line germ banks and breeding banks. By this means, seeds could be classified as having resistance, and seeds from this selection could be contrasted with seeds from accessions classified as susceptible. Sources of resistance to *Z. subfasciatus* were thus easily identifiable. Insect bioassay of accessions with *Z. subfasciatus* and *A. obtectus* (Table 34) illustrate some overlap with accessions resistant to one insect also being resistant to the other.

Promising accessions of wild type material were examined to ensure that the effect was not one of testa — in the case of *Z. subfasciatus*, by ensuring that the eggs hatched normally (i.e. frass could be seen inside the egg), and in the case of *A. obtectus*, by damaging the testa to ensure that the larvae had an entry hole independent of testa. An accession resistant to *A. obtectus* because of factors contained in the cotyledon, where the effect of testa had been minimised by damaging the testa (G12953), was selected to extract out resistance factors. Seeds of this accession were multiplied and used, because of their simultaneous high levels of resistance to both pest species, to investigate resistance factors in *Z. subfasciatus* also.

The most readily apparent feature of resistance exhibited by G12953 is that different factors are responsible for resistance to the two pest species. Arcelin, which is a major factor responsible for resistance to *Z. subfasciatus* (Osborn, Bliss and co-workers 1986, 1988), has very little effect when included in artificial diets presented to *A. obtectus* (the albumin fraction, which contains arcelin, was used in this bioassay). On the converse, the soluble carbohydrate material has only a small effect on *Z. subfasciatus* larval development, and a major effect on *A. obtectus*.

The ranges of the two species do not overlap in the native state (i.e. where the bruchids breed on wild *P. vulgaris*): *A. obtectus* is found in the uplands of the tropics, where the climate is cooler, since high temperatures sterilise *A. obtectus* male adults; *Z. subfasciatus* is found in the warmer valleys. Both species are capable of ingesting

Table 34 Comparing *A. obtectus* and *Z. subfasciatus* bioassay data and resistance factors for accessions of *P. vulgaris*

Variety	*Z. subfasciatus*			*A. obtectus*		
	%Surv	Dev Per	Resistance	% Surv	Dev Per	Resistance
G09989b	60.5	38.1	S	15.0	30.5	I
G10000	78.6	38.1	S	63.0	28.5	S
G10007	50.4	39.1	S	16.0	31.5	I
G11051, A2	36.0	43.1	R	17.0	34.5	I
G12866, A2	11.7	48.5	R	11.0	33.0	R
G12871	70.1	39.1	S	27.0	29.5	S
G12880	65.1	38.2	S	6.0	29.0	R
G12923, A3	73.6	48.3	S	51.0	36.5	S
G12933, A3	75.3	47.0	S	54.0	33.5	S
G12949, A4	5.4	70.1	R	7.0	46.0	R
G12952, A4	0.9	-	R	3.0	57.5	R
G12953, A4	0.0	-	R	1.5	56.6	R
G12954, A4	21.1	42.0	R	4.0	49.5	R
G13016	47.1	40.1	S	3.0	31.0	R

A1, A2, A3, A4 arcelin type of protein present in the cotyledon

lectins and have presumably developed this. It is not clear at what stage *Acanthoscelides* and *Zabrotes* diverged and whether this tolerance to lectins was already parth of bruchid repetoir.

The presence of arcelins of various types is moderately widespread (Osborn *et al.* 1986). *A. obtectus* shows some tolerance to this protein when fed in isolation (Gatehouse *et al.* 1987), although *A. obtectus* is mostly unable to develop on seeds which contain arcelin types. On the converse *Z. subfasciatus* appears unable to utilise artificial diets containing arcelin types 1, 2 and 4 (Osborn *et al.* 1988, and this work).

By a similar token a protein of similar sequence to arcelin is found in arcelin-containing accessions, and has an inhibitory effect on amylase enzymes of *Z. subfasciatus* (Table 5-c page R.112 and Table 18 page R.164), which has no perceptible effect on *A. obtectus* amylase enzyme activity (Table 23 page R.178).

No single factor responsible for seed resistance to *A. obtectus* was isolated in the course of the study, but the ineffectiveness of pure forms of each of the factors responsible for resistance to *Z. subfasciatus* suggests different mechanisms. One possible reason for the differences between the two species could be that *A. obtectus* popluations have overcome selected resistance mechanisms in the arcelin-containing accessions of *P. vulgaris*, and the arsenal of defensive mechanisms has been increased to maintain resistance to *A. obtectus*. *Z. subfasciatus* however, whether because of having less time to evolve mechanisms to tolerate and utilise arcelin, or whether because of lesser selection pressures due to alternative host varieties, has not evolved mechanisms to combat the most obvious factors, and may also have development hindered by the combination of mechanisms which acts on *A. obtectus*.

Resistance to the two species will therefore be considered separately.

Arcelin 4

Arcelin 4, a factor associated with *Z. subfasciatus* resistance, is a protein found in the albumin fraction. Four forms of arcelin are named in the literature (Osborn *et al.* 1986), and all of these expressed as a dominant trait in F_2 generation seeds, with some genetic linkage to PHA. Only one form of arcelin is found in any particular wild-type accession.

The derived sequence of arcelin 1 has been compared with the sequences of PHA, and with the derived sequence of the LLP gene (which probably encodes the amylase inhibitor CBAII), and found to be very similar (Osborn *et al.* 1988; Moreno and Chrispeels 1989). N-terminal sequencing of arcelin 4 subunits was performed, and a good fit was made with both PHA sequences and arcelin 1 (Table 15 page R.149). On the basis of this fit it is likely that arcelin 4 is a separate derivation of the PHA sequence from arcelin 1, rather than an "arcelin" sequence evolving from which the four different arcelin types evolved in their turn.

An attempt has been made to derive a physical structure for arcelin 4. The apparent molecular weight of the glycosylated subunits on SDS-PAGE is 33K and 36K. Deglycosylation (enzymatic, to remove N-bonded poly-saccharide chains) reduces this to 29K and 31K, and the % glycosylation has been estimated at 10% (hexose assay). A total globular molecular size of 170K is larger than would be expected of a tetramer of these subunit sizes; this may indicate that the tetrameric form (the expected configuration based on comparisons with PHA) is in rapid equilibrium with an octameric (dimer of tetramers) form, or of course that the molecule is hexameric. There was not time in the course of this investigation to determine which of the possibilities is the actual one. All of this indicates a family of proteins of similar primary and tertiary structure to the PHA lectin, from which they are assumed to be derived.

Mechanism of arcelin resistance

A lectin-like binding and disruption of the gut epithelial wall might be expected because of arcelin's similarity to PHA. However this is unlikely because at the final stage of purification arcelin can be separated from trace PHA contaminants by affinity binding the contaminant to fetuin: if arcelin itself had hæmagglutinatory properties it wold be expected to bind to the fetuin itself. It is probable that the two pest species have evolved mechanisms to avoid the effects of PHA agglutination; certainly this is observed in *A. obtectus* (Gatehouse *et al.* 1987). Attempts at immunofluorescent microscopy were made but the results remained inconclusive.

Proteins in *P. vulgaris* seeds may also act as enzyme inhibitors. However a careful examination of proteolytic activity of *Z. subfasciatus* larval gut homogenate in and without the presence of arcelin 4 indicated that this particular protein is not a

protease inhibitor, and enzymatic assay revealed that it is also not an amylase inhibitor. The protein **arcelin 4** substitutes for phaseolin as the primary storage protein in accessions of *P. vulgaris* which contain it; to such an extent that accessions containing arcelin of any type have reduced levels of both phaseolin and PHA (also noted by Harmsen *et al.* 1988).

At such high physiological levels the protein could have a major effect on nutrition of the larvae. In the case of arcelin 4, the protein is probably conformationally unavailable to the larva and its gut proteases are unable to digest arcelin 4; addition of phaseolin to a diet based on the resistant meal of accession **G12953** overcame to a great extent resistance observed. This would probably not be the case if a protease inhibitor were present: protease inhibitors in *P. vulgaris* are mostly inhibitors of the serine proteases trypsin and chymotrypsin. Bruchids however have thiol-type proteases (and in the case of *Z. subfasciatus*, aspartate-type proteases) present in the gut which are largely unaffected by these inhibitors. Certainly the presence of arcelin 4 *in vitro* in larval gut homogenate containing phaseolin does not appear to affect the rate of phaseolin digestion. This passive mechanism would account for the relatively limited effect of including arcelin 4 in artificial diets based on haricot, since the haricot base would contain a high level of phaseolin and other readily-digestible proteins.

It is quite likely that other factors contribute to resistance in addition to the presence of arcelin 4 and the absence of phaseolin. In particular the presence of the novel amylase inhibitor **WBAI** is associated with resistance, and its specificity to the larval gut enzymes of the pest species implies a possible role. However the genetic linkage of WBAI to arcelin 4 (determined by cross-breeding) makes it difficult to separate out the two factors. A method of doing so would be to supplement G12953-basal diet with phaseolin and starch/glucose and determine the relative effects of each. However it is likely that total effective resistance is a combination of these factors, and potentially other undetermined factors.

Arcelin types

In contrast to arcelin 4, **arcelin 1** appears to be actively inhibitory to *Z. subfasciatus* larval gut proteases. Digestion of phaseolin and of PHA was slowed until it was virtually undetectable when in a mixture with arcelin 1. Mammals (rats) fed on

a diet supplemented with the arcelin 1 breeding line **RAZ-2** as the major protein component grew better than those fed on *Z. subfasciatus* susceptible cultivar **EMP-175** at the same levels. This suggests reduced levels of active lectins in the meal, and reduced levels of antimetabolites (to mammals) as a result of enhanced *Z. subfasciatus* resistance.

Cooking the meal improved nutritional quality to a level similar both to supplementation with ideal protein, and to that attained by cooking susceptible cultivars for supplementing the diet (bean meals had essential amino acids added to make an ideal mixture). This is in agreement with the result expected if the proteins are conformationally inaccessible to proteolytic enzymes, and/or the antimetabolic factors present were proteins. As noted before, protease inhibitors effective against mammalian proteolytic enzymes are not always effective against *Z. subfasciatus* enzymes, so the converse may well be the case.

The arcelin form **arcelin 3** contains subunits in common with arcelin 4 (Osborn *et al.* 1986) and yet is highly digestible. If the mechanism for resistance to proteolysis of arcelin 4 is due to the conformation of the molecules then the subunits which arcelin 3 does not share with arcelin 4 may be the ones which in arcelin 4 allow it to form a stable and closed molecule; hence the more open nature of arcelin 3 may make it a target for proteolytic attack.

WBAI

Resistance of cultivars and accessions of *P. vulgaris* to *Z. subfasciatus* is not associated with the presence of the conventional *P. vulgaris* amylase inhibitor, termed in this work **CBAI** (**C**ultivated **B**ean **A**mylase **I**nhibitor); in fact there is a negative correlation between the two. This amylase inhibitor is active against mammalian enzymes and against larval gut amylases of the bruchid *C. maculatus*, which is not normally a pest of *P. vulgaris* (Ishimoto and Kitamura 1989). Its role in the seed has been discussed in connection with resistance to pests, and in particular the stability of the enzyme:inhibitor complex at pH 5.5 (mammalian enzyme is most active at pH 7.0, whereas bruchid enzymes more active at pH 5.4) (Marshall and Lauda 1975), and the marked effect on *C. maculatus* of inclusion of purified CBAI in artificial diets indicates a role in connection with resistance to insect pests (Ishimoto and Kitamura 1989).

Furthermore, CBAI shares a very similar amino-acid sequence to PHA lectin (Moreno and Chrispeels 1989), and the importance of PHA in conferring resistance in seeds suggests this role for other members of this genetic family (compare the arcelin types, which also have a PHA lectin-related sequence).

CBAI is not an inhibitor of the amylases of *Z. subfasciatus* nor of *A. obtectus*, which are pests of *P. vulgaris*. This reflects the pattern observed for protein nutrition, in which case PHA lectins and the trypsin inhibitors present in *P. vulgaris* were not effective against the pests *Z. subfasciatus* and *A. obtectus*.

Tightly linked with the presence of arcelin 4 and arcelin 2 is the presence of a novel inhibitor **WBAI** (**W**ild **B**ean **A**mylase **I**nhibitor) which does not inhibit mammalian amylase enzymes but does inhibit the amylases of the pest *Z. subfasciatus*, as well as those of the bruchid non-pest *C. maculatus*. This amylase inhibitor also shows sequence similarities with the PHA gene, and the maintenance of resistance to *C. maculatus* in **WBAI** (CBAI and WBAI are probably not found together in a single seed) confirms the importance of inhibition of the amylase enzyme in resistance to this pest. Its importance in preventing damage by *Z. subfasciatus* has not been possible to test since the high linkage between the presence of arcelin 4 and WBAI makes it difficult to separate the individual effects in the seed meal, and whilst it has been possible to purify arcelin 4 in sufficient quantities for feeding trial bioassay the same has not been possible with WBAI.

A. obtectus

In contrast the antimetabolites responsible for resistance of accessions to *A. obtectus* have not been purified, and the mechanisms not ascertained.

Resistance has been attributed to the *soluble carbohydrate* extract, but to materials precipitated in high saturations of ammonium sulphate, i.e. highly charged molecules. This material in solution is gelatinous, indicating a possible pectin (uronic acid poly-saccharide), and a mechanism of resistance involving disruption of carbohydrate nutrition was proposed, e.g. that a less-common form of poly-saccharide (e.g. an arabinogalacturonan) is present at high concentrations replacing the starch component, and the bruchid larva does not have enzymes suitable for digesting this

material and consequently suffers carbohydrate deprivation.

This hypothesis, that substitution of a less digestible poly-saccharide causes *A. obtectus* larvae to suffer from lack of digestible carbohydrate for energy supplies, is not very likely since resistant accessions contain approximately 40% less starch than susceptible cultivars or accessions, and supplementing artificial diets of G12953 meal with potato starch (which material is readily digested by *A. obtectus* larval gut homogenate *in vitro*) up to 15% (w/w) did not noticeably improve development to adult of *A. obtectus*. However there was a high coefficient of association (**r**) between resistance quotient (a parameter based on larval survival to adult and development period) and starch content. Amylase enzyme inhibition was not marked, probably not at a level to suggest that this was a mechanism of resistance.

Levels of uronic acids were measured in the sodium acetate pH 4.8 extracts of a number of accessions, both resistant and susceptible. This measure might be expected to be an indicator of the levels of uronic acid-containing polysaccharides present. However no association could be made between this measure and biochemical resistance to *A. obtectus*.

A purified fraction from phenol phase partition was associated with resistance, and further purification indicated that this fraction was proteinaceous (phenol phase 2): however its method of purification involved denaturing the protein and it was possible that a covalent complex was formed of the protein with phenol rather than the polypeptide being antimetabolic in the native state (extensive dialysis with acetic acid would have removed any non-covalently-bound phenol).

Resistance to *A. obtectus* is inherited as a recessive trait, controlled by a single gene. Because it is a recessive trait, any outcrossing with pollen from other, susceptible, accessions of cultivated lines of *P. vulgaris* will negate this resistance. The difficulties encountered in the search for factors responsible for resistance to *A. obtectus* may have been symptomatic of this recessive nature — mixing seeds from an accession considered resistant may have mixed seeds actually resistant with a few seeds which were actually susceptible, and similarly accessions considered susceptible may actually contain individual seeds with a genotype resistant to *A. obtectus*. This would result in quantitative rather than qualitative differences between substances present in the seed meals, and with the wide range of compounds present in seed meal the search for

quantitative differences in levels of an unknown substance proved beyond the resources available.

In order to find the exact factors responsible for resistance to *A. obtectus*, a detailed compositional analysis of each seed of a set of seeds where bioassay data is known for each seed would need to be done. Seeds are available from the crosses **GH-13** and **GH-14**; however there was not enough time within the project to complete this analysis.

The factor is likely to be either protein- or polysaccharide-based. Proteins could be denatured by heating, but cooking the whole meal makes the pellets too hard for *A. obtectus* larvae to penetrate; and the protein extracts of globulins and albumins did not themselves confer resistance when included in a rich basal diet. The carbohydrate portion contains some protein, and this material could be cooked to denature what protein it contains. Some means of deactivating polysaccharides before inclusion of *soluble carbohydrate* into artificial diets could be used to prove or disprove polysaccharide involvement in resistance.

Appendix 19
References

APPLEBAUM S.W. (1964) Physiological aspects of host specificity in the Bruchidae - I. General considerations of developmental compatibility. *Insect Physiol.* **110** 783-788

APPLEBAUM S.W., GESTETNER B. AND BIRK Y. (1965) Physiological aspects of host specificity in the Bruchidae - IV. Developmental incompatibility of soybeans for *Callosobruchus*. *J. Ins.Physiol.* **11** 611-616

APPLEBAUM S.W., MARCO S. AND BIRK Y. (1969) Saponins as possible factors of resistance of legume seeds to the attack of insects. *J. Agric. Food Chem.* **17**(3), 618-622.

APPLEBAUM S.W., TADMOR U. AND PODOLER H. (1970) The effect of starch and of a hetero-polysaccharide fraction from *Phaseolus vulgaris* on development and fecundity of *Callosobruchus chinensis* (Coleoptera: Bruchidae). *Ent. exp.& appl.* **13** 61-70

APPLEBAUM S.W. AND BIRK Y. (1972) Natural mechanisms of resistance to insects in legume seeds. *in "Insect and Mite Nutrition"* ed JG Rodriguez, North-Holland pp629-636

APPLEBAUM S.W. AND GUEZ M. (1972) Comparative resistance of *Phaseolus vulgaris* beans to *Callosobruchus chinensis* and *Acanthoscelides obtectus* (Coleoptera, Bruchidae): the differential digestion of a soluble heteropolysaccharide. *Ent.exp.& appl.* **15** 203-207

BAILEY R.W. (1971) Polysaccharides in the leguminosae. *in "Chemotaxonomy of the leguminosae" eds JB Harbourne, D Boulter & BL Turner, Acad.Press N.Y.* pp503-541

BECKER P. (1984) Tannin structure and function. Keeping our perspective. *Am.Nat.* **124** 134-136

BELL E.A. (1976) 'Uncommon' amino acids in plants *FEBS Letts* **64** 29-35

BELL E.A. (1981) I. The physiological role(s) of secondary (natural) products. in *The Biochemistry of Plants vol 7: Secondary Plant Products* eds PK Stompf & EE Conn pp8-14

BERNFELD P. (1955) Amylases, α and β. *In "Methods in enzymology" eds SP Colowick & NO Kaplan. Acad. Press NY* **1** pp149-158.

BIEMONT J.C. AND BONET A. (1981) The bean weevil populations from the *Acanthoselides obtectus* (SAY) group living on wild or subspontaneous *Phaseolus vulgaris* L. and *Phaseolus coccineus* L. and on *Phaseolus vulgaris* L. cultivated in the Tepoztlan region state of Morelos - Mexico. *Series Entomologia* **19** 23-41

BIRCH N., CROMBIE L. AND CROMBIE W.M. (1985) Rotenoids of *Lonchocarpus salvadorensis*: their effectiveness in protecting seeds against bruchid predation. *Phytochemistry (Oxf)* **24**(12) 2881-2884

BIRCH N., SOUTHGATE B.J. AND FELLOWS L.E. (1985) Wild and semi-cultivated legumes as potential sources of resistance to bruchid beetles for crop breeders: a study of *Vigna/Phaseolus*. in *"Plants for Arid Lands"* Roy. Bot. Gdns. Kew pp303-320

BIRCH A.N.E., SIMMONDS M.S.J. AND BLANEY W.M. (1989) Chemical interactions between bruchids and legumes. *in "Advances in Legume Biology" Monographs in Systematic Botany* Miss.Bot.Gdns (in press)

BLUM M.S. (1983) Detoxification, deactivation, and utilization of plant compounds by insects. in *'Plant Resistance to Insects' ACS Symposium Series* **208** Am.Chem.Soc. Wash DC pp265-275

BOULTER D., GATEHOUSE A.M.R. AND HILDER V. (1989) Use of cowpea trypsin inhibitor (CpTI) to protect plants against insect predation. *Biotech.Adv.* **7** 489-497

BRADBEAR N. AND BOULTER D. (1984) The use of enzymic hydrolysis *in vitro* to study the digestibility of some *Phaseolus* seed proteins. *Qual. Plant. Foods Hum. Nutr.* **34** 3-13.

BRÜCHER O. (1968) Absence of phytohemagglutinin in wild and cultivated beans from South America. *Proc.Trop.Reg.Am.Sci.Hort.Sci.* **12** 68-85

CAMPBELL R.C. (1989) Statistics for Biologists, *3rd Ed* 446pp

CAMPOS F.A.P., XAVIER-FILHO J., SILVA C.P. AND ARY M.B. (1989) Resolution and partial characterization of proteinases and α-amylases from midguts of larvae of the bruchid beetle *Callosobruchus maculatus* (F.). *Comp.Biochem.Physiol.* **92b** 51-57

CARDONA C., POSSO E.C., KORNEGAY J., VALOR J. AND SERRANO M. (1989) Antibiosis effects of wild dry bean accessions on the Mexican Bean Weevil and the Bean Weevil (Coleoptera: Bruchidae). *J.Econ.Entomol.* **82**(1) pp310-315

CENTER T.D. AND JOHNSON C.D. (1974) Coevolution of some seed beetles (Coleoptera: Bruchidae) and their hosts. *Ecology* **55** 1096-1103

CERROTTI A., VITALE A. AND BOLLINI R. (1989) Lectin-like proteins accumulate as fragmentation products in bean seed protein bodies. *FEBS Letts.* **250** 157-160

CHAPLIN M.F. (1986) Monosaccharides. *in "Carbohydrate analysis: a practical approach"* eds MF Chaplin & JF Kennedy. IRL Press pp1-36

CREDLAND P.F. (1986a) Effect of host availability on reproductive performance in *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae). *J.Stored Prod.Res.* **22** 49-54

CREDLAND P.F. (1987) Effect of host change on the fecundity and development of an unusual strain of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae). *J.Stored Prod.Res.* **23** 91-98

CREDLAND P.F. AND DICK K.M. (1987) Food consumption by larvae of three strains of *Callosobruchus maculatus* (Coleoptera Bruchidae) *J.Stored Prod.Res.* **23** 31-40

CROWSON R.A. (1960) The phylogeny of Coleoptera. *Ann.Rev.Ent.* **5** 111-134

DECELLE J. (1981) Bruchidae related to grain legumes in the Afro-Tropical area. in *Series Entomologia* **19** 193-197

DOBIE P. (1978) A simple curve describing the development pattern of some beetles breeding on stored products. *J.Stored Prod.Res.* **14** 41-44

DOBIE P., HAINES C.P., HODGES R.J. AND PREVETT P.F. (1988) Insects and arachnids of tropical stored products. Their biology and identification. A training manual. *Storage Dept, Tropical Development and Research Institute, ODA* 273 pages

DUBOIS M., GILLES K.A., HAMILTON J.K., REBERS P.A. AND SMITH F. (1956) Colorimetric method for determination of sugars and related substances *Anal.Chem.* **28** 350-356

EVANS S.V., GATEHOUSE A.M.R. AND FELLOWS L.E. (1985) Detrimental effects of 2,5-dihydroxymethyl-3,4-dihydroxy pyrrolidine in some tropical legume seeds on larvae of the bruchid *Callosobruchus maculatus*. *Ent.exp.& appl.* **37** 257-261

FAGERSTRÖM T. (1989) Anti-herbivory chemical defense in plants: a note on the concept of cost. *Am.Nat.* **133** 281-287

F.A.O. (1977) Analysis of an FAO survey of post-harvest crop losses in developing countries. *AGPP:MISC/27* F.A.O. Rome 53 pages

FELLOWS L. (1989) Botany breaks into the candy store. *New Scientist* 1679 (26 Aug.) 45-49

FELLOWS L. (1989b) Lecture to Biological Society of Durham University

FUJII K., ISHIMOTO M. AND KITAMURA K. (1979) Patterns of resistance to bean weevils (Bruchidae) in *Vigna radiata-mungo-sublobata* complex inform the breeding of new resistant varieties. *Appl.Ent.Zool.* **24** 126-132

GASSER C.S. AND FRALEY R.T. (1989) Genetically engineering plants for crop improvement. *Science* **244** (16 June 1989) 1293-1298

GATEHOUSE A.M.R., GATEHOUSE J.A., DOBIE P., KILMINSTER A.M. AND BOULTER D. (1979) Biochemical basis of insect resistance in *Vigna unguiculata*. *J.Sci.Food Agric.* **30** 948-958

GATEHOUSE A.M.R. AND BOULTER D. (1983) Assessment of the antimetabolic effects of trypsin inhibitors from cowpea (*Vigna unguiculata*) and other legumes on development of the bruchid beetle *Callosobruchus maculatus*. *J.Sci.Food Agric.* **34** 345-356.

GATEHOUSE A.M.R., DEWEY F.M., DOVE J., FENTON K.A. AND PUSZTAI A. (1984) Effect of seed lectins from *Phaseolus vulgaris* on the development of larvae of *Callosobruchus maculatus*; mechanism of toxicity. *J.Sci.Food Agric.* **35** 373-380.

GATEHOUSE A.M.R., BUTLER K.J., FENTON K.A. AND GATEHOUSE J.A. (1985) Presence and partial characterisation of a major proteolytic enzyme in the larval gut of *Callosobruchus maculatus*. *Ent.exp.& appl.* **39** 279-286

GATEHOUSE A.M.R., FENTON K.A., JEPSON I. AND PAVEY D.J. (1986) The effects of α-amylase inhibitors on insect storage pests: inhibition of α-amylase *in vitro* and effects on development *in vivo*. *J.Sci.Food Agric.* **37** 727-734

GATEHOUSE A.M.R., DOBIE P., HODGES R.J., MEIK J., PUSZTAI A. AND BOULTER D. (1987) Role of carbohydrates in insect resistance in *Phaseolus vulgaris*. *J. Insect Physiol.* **33** 843-850.

GATEHOUSE A.M.R., SHACKLEY S.J., FENTON K.A., BRYDEN J. AND PUSZTAI A. (1989) Mechanism of seed lectin tolerance by a major insect storage pest of *Phaseolus vulgaris*, *Acanthoselides obtectus*. *J.Sci.Food Agric.* **47** 269-280

GATEHOUSE A.M.R., MINNEY B.H., DOBIE P. AND HILDER V. (1990) Biochemical resistance to bruchid attack in legume seeds; investigation and exploitation. *in "Bruchids and Legumes: Economics, Ecology and Coevolution"* eds K Fujii *et al.* Kluwer Acad. Publ. 241-256

GENTRY H.S. (1969) Origin of the common bean *Phaseolus vulgaris*. *Econ.Bot.* **23** 55-69

HAMES B.D. (1981) An introduction to polyacrylamide gel electrophoresis. *in "Gel Electrophoresis of proteins" eds BD Hames & D Rickwood IRL Press, Oxford* pp1-91.

HARMSEN R., BLISS F.A., CARDONA C., POSSO C.E. AND OSBORN T.C. (1988) Transferring genes for arcelin protein from wild to cultivated beans: implications for Bruchid resistance. *Ann.Rep.Bean Improvement Cooperative* **31** 54-55

HEDIN P.A., JENKINS J.N., COLLUM D.H., WHITE W.H. AND PARROTT W.L. (1983) Multiple factors in cotton contributing to resistance to the tobacco budworm, *Heliothis viriscens* F. *in "Plant Resistance to Insects" A.C.S. Symposium Series* **208** ed PA Hedin Am.Chem.Soc. Wash DC 347-365

HILDER V.A., GATEHOUSE A.M.R. AND BOULTER D. (1989) Potential for exploiting plant genes to genetically engineer insect resistance, exemplified by the cowpea trypsin inhibitor gene. *Pestic.Sci.* **27** 165-171

HILDER V.A., GATEHOUSE A.M.R AND BOULTER D. (1990) Genetic engineering of crops for insect resistance using genes of plant origin. *in "Genetic Engineering of Crop Plants" eds GW Lycett & D Grierson, Butterworths* pp51-66

HILL D.S. (1983) Agricultural insect pests of the tropics and their control. Cambridge Univ. Press p519

HINES M.E., NIELSEN S.S., SHADE R.E. AND POMEROY M.A. (1990) The effect of two proteinase inhibitors, E-64 and the Bowman-Birk inhibitor, on the development time and mortality of *Acanthoscelides obtectus*. *Entomol.Exp.Appl.* **57** 201-207

HOFFMAN L.M. AND DONALDSON D.D. (1985) Characterisation of two *Phaseolus vulgaris* phytohemagglutination genes closely linked on the chromasome. *EMBO J.* **4** 883-889

HOWARD I.K., SAGE H.J. AND HORTON C.B. (1972) Studies on the appearance and location of hemagglutinins from a common lentil during the life cycle of the plant. *Arch. Biochem. Biophys.* **149** 323-326

HUIGNARD J. AND BIEMONT J.C. (1978) Comparison of four populations of *Acanthoscelides obtectus* (Coleoptera: Bruchidae) from different Colombian Ecosystems. *Oecologia* (Berl) **35** 307-318

HUIGNARD J. AND BIEMONT J.C. (1981) Reproductive polymorphism of populations of *Acanthoscelides obtectus* from different Colombian ecosystems. *Series Entomologia* **19** 149-164

ISHII S. (1952) Studies on the host preference of the Cowpea weevil (*Callosobruchus chinensis* L.). *Bull.nat.inst.agric.Sci.Japan* **1** 185-256

ISHIMOTO M. AND KITAMURA K. (1988) Identification of the growth inhibitor on azuki bean weevil in kidney bean (*Phaseolus vulgaris* L.). *Japan J.Breed.* **38** 367-370

ISHIMOTO M. AND KITAMURA K. (1989) Growth inhibitory effects of an α-amylase inhibitor from the kidney bean, *Phaseolus vulgaris* (L.) on three species of Bruchids (Coleoptera: Bruchidae). *Appl.Ent.Zool.* **24** 281-286

JAFFÉ W.G., MORENO R. AND WALLIS V. (1973) Amylase inhibitors in legume seeds. *Nutr.Reps.Int.* **7** 169-174

JANZEN D.H., JUSTER H.B. AND LIENER I.E. (1976) Insecticidal action of the phytohemagglutinin in black beans on a Bruchid beetle. *Science* **192** (21 May 1976) 795-796

JANZEN D.H. (1977a) The interactions of seed predators and seed chemistry. *Coll. Int. C.N.R.S.* **265** 415-428

JANZEN D.H. (1977b) Intensity of predation on *Pithecellobium saman* (Leguminosae) seeds by *Merobruchus collumbinus* and *Stator limbatus* (Bruchidae) in Costa Rican deciduous forest. *Trop.Ecol.* **18** 162-176

JANZEN D.H. (1977c) Reduction of *Macuna andreana* (Leguminosae) seedling fitness by artificial seed damage. *Ecology* **57** 826-828

JANZEN D.H. AND WILSON D.E. (1977) Natural history of seed predation by *Rosella sickingiae* Whitehead (Curculonidae) on *Sickingia maxonii* (Robiaceae) in Costa Rican rainforest. *Coleopt.Bull.* **31** 19-24

JANZEN D.H., JUSTER H.B. AND LIENER I.E. (1976) Insecticidal action of the phytohemagglutinin in black beans on a bruchid beetle. *Science* **192**, 795-796.

JANZEN D.H., JUSTER H.B. AND BELL E.A. (1977) Toxicity of secondary compounds to the seed-eating larvae of the bruchid beetle *Callosobruchus maculatus*. *Phytochem. (Oxf)* **16** 223-227

JANZEN D.H. (1978) Reduction of seed predation on *Bauhinia pauletia* (Leguminosae) through habitat destruction in a Costa Rican deciduous forest. *Brenesia* **14/15** 325-336

JANZEN D.H. (1981) The defenses of legumes against herbivores. *in "Advances in Legume Systematics"* eds RM Polhill & PH Raven pp951-977

JERMY T. (1984) Evolution of insect/host plant relationships. *Am.Nat.* **124** 609-630

JOHNSON C.D. (1981a) Relations of *Acanthoselides* with their plant hosts. *Series Entomologia* **19** 73-81

JOHNSON C.D. (1981b) Seed beetle host specificity and the systematics of the Leguminosae *in "Advances in Legume Systematics"* eds Polhill R.M. & Raven P.H. pp995-1027

KAKADE M.L. AND EVANS R.J. (1965) Nutritive value of navy beans (*Phaseolus vulgaris*). *Brit.J.Nutr.* **19** 269-276

KAPLAN L. (1981) What is the origin of the common bean? *Economic Botany* **35** 240-254

KING T.P., PUSZTAI A. AND CLARKE E.M.W. (1980) Immunochemical localization of ingested kidney bean (*Phaseolus vulgaris*) lectins in rat gut. *Histochem.J.* **12** 201-208

KINGHORN A.D. AND SMOLENSKI S.J. (1981) Alkaloids of Papilionoideae. *in "Advances in Legume Systematics"* eds RM Polhill & PH Raven pp585-598

KITAMURA K., ISHIMOTO M. AND SAWA M. (1988) Inheritance of resistance to infestation with Adzuki Bean Weevil in *Vigna subolata* and succesful incorporation into *V. radiata. Japan J. Breed.* **38** 459-464

KOGAN M. AND PAXTON J. (1983) Natural inducers of plant resistance to insects. *in "Plant Resistance to Insects" A.C.S. Symposium Series* **208** ed PA Hedin Am.Chem.Soc. Wash DC pp153-171

KOTARU M., YOSHIKAWA H., IKEUCHI T., SAITO K., IWAMI K. AND IBUKI F. (1987) An α-amylase inhibitor from cranberry bean (*Phaseolus vulgaris*): its specificity in inhibition of mammalian pancreatic α-amylases and formation of a complex with the porcine enzyme. *J.Nutr.Sci.Vitaminol.* **33** 359-367

LABEYRIE V. (1981) Ecological problems arising from weevil infestation of food legumes. *Series Entomologica* **19** 1-15

LEGENDRE N. AND MATSUDAIRA P. (1989) "Purification of Proteins and Polypeptides by SDS-PAGE" *in "A Practical Guide to Protein and Peptide Purification for Microsequencing"* ed PT Matsudaira, Acad Press Inc, London pp49-69

LEMOS F.J.A., XAVIER-FILHO J. AND CAMPOS F.A.P. (1987) Proteinases of the midgut of *Z. subfasciatus* larvae. *Arq.Biol.Tecnol.* **30** Abs46

LEMOS F.J.A., CAMPOS F.A.P., SILVA C.P. AND XAVIER FILHO J. (1990) Proteinases and amylases of larval midgut of *Zabrotes subfasciatus* reared on cowpea (*Vigna unguiculata*) seeds. *Entomol.Exp.Appl.* **56** 219-227

LEVINSON H.Z. (1976) The defensive role of alkaloids in insects and plants. *Experientia* **32** 408-411

LIENER I.E. (1980) Toxic constituents of plant foodstuffs. 2nd Edition, Academic Press, New York

LIS H. AND SHARON N. (1973) The biochemistry of plant lectins (phytohaemagglutinins). *A.Rev.Biochem.* **42** 541-547

MARSHALL J.J. AND LAUDA C.M. (1975) Purification and properties of phaseolamin, an inhibitor of α-amylase, from kidney bean, *Phaseolus vulgaris J.Biol.Chem.* **250** 8030-8037

MEINERS J.P. AND ELDEN T.C. (1980) Resistance to insects and diseases in *Phaseolus.* *In "Advances in Legume Science"* eds RH Summerfield & AJ Bunting. Int. Legume Conf. Roy.Bot.Gdns. Kew England pp359-364

MENEZES E.W. AND LAJOLO F.M. (1987) Inhibition of starch digestion by a black bean α-amylase inhibitor, in normal and diabetic rats. *Nutr.Reps.Int* **36** 1185-1195

MORENO J. AND CHRISPEELS M.J. (1989) A lectin gene encodes the α-amylase inhibitor of the common bean. *Proc.Natl. Acad.Sci.USA* **86** 7885-7889

NAGAHIRO M. (1981) The distribution of α-amylase inhibitors in various beans. *Eiyo to Shokuryo* **34**(4) 341-347

NOWACKI E. (1980) Heat stable antinutritional factors in Leguminous plants *In "Advances in Legume Science"* eds RH Summerfield & AJ Bunting. Roy.Bot.Gdns. Kew England pp171-177

NWANZE K.F., HORBER E. AND PITTS C.W. (1975) Evidence for ovipositional preference of *Callosobruchus maculatus* for cowpea varieties. *Environ.Entomol.* **4** 409-412

NWANZE K.F. AND HORBER E. (1976) Seed coats of cowpeas affect ovipostion and larval development of *Callosobruchus maculatus. Environ.Entomol.* **5** 213-218

OSBORN T.C. AND BLISS F.A. (1985) Effects of genetically removing lectin seed protein on horticultural and seed characteristics of common bean. *J.Amer.Soc.Hort.Sci..* **110** 484-488

OSBORN T.C., BLAKE T., GEPTS P. AND BLISS F.A. (1986) Bean arcelin 2. Genetic variation, inheritance and linkage relationships of a novel seed protein of *Phaseolus vulgaris* L. *Theor.Appl.Genet.* **71** 847-855

OSBORN T.C., ALEXANDER D.C., SUN S.S.M., CARDONA C. AND BLISS F.A. (1988) Insecticidal activity and lectin homology of arcelin seed protein. *Science* **240** (8 Apr 1988) 207-210

PICK K-H AND WÖBER G. (1978a) Proteinaceous α-amylase inhibitor from beans (*Phaseolus vulgaris*). Purification and partial characterisation. *Hoppe-Seyler's Z.Physiol.Chem.* **359** 1371-1377

PICK K-H AND WÖBER G. (1978b) Proteinaceous α-amylase inhibitor from beans (*Phaseolus vulgaris*). Immunological characterisation. *Hoppe-Seyler's Z.Physiol.Chem.* **359** 1379-1384

POWERS J.R. AND WHITAKER J.R. (1977) Purification and some physical and chemical properties of red kidney bean *(Phaseolus vulgaris)* α-amylase inhibitor. *J.Food Biochem.* **1** 217-238

PUSZTAI A., CLARKE E.M.W., KING T.P. AND STEWART J.C. (1979) Nutritional evaluation of kidney beans (*Phaseolus vulgaris*): chemical composition, lectin content and nutritional value of selected cultivars. *J.Sci.Food Agric.* **30** 843-848

PUSZTAI A., CLARKE E.M.W. AND KING T.P. (1979) The nutritional toxicity of *Phaseolus vulgaris* lectins. *Nutr.Soc.* **38** 115-120

REHR S.S., JANZEN D.H. AND FEENY P.P. (1973) L-dopa in legume seeds: a chemical barrier to insect attack. *Science* **181** (4094) 81-82

ROBINSON T. (1989) The evolutionary ecology of alkaloids. *in "Herbivores: their interaction with secondary plant metabolites"* eds GA Rosenthal & DH Janzen. Acad. Press. NY, Lond pp413-448

ROMERO ANDREAS J., YANDELL B.S. AND BLISS F.A. (1986) Bean arcelin 1. Inheritance of a novel seed protein of *Phaseolus vulgaris* L. and its effect on seed composition. *Theor. Appl.Genet.* **72** 123-128

ROMERO J. AND RYAN D.S. (1978) Susceptibility of the major storage protein of the bean, *Phaseolus vulgaris* L., to *in vitro* enzymatic hydrolysis. *J.Agric.Food Chem.* **26** 784-788

ROSENTHAL G.A., DAHLMAN D.L. AND JANZEN D.H. (1976) A novel means for dealing with L-canavaline, a toxic metabolite. *Science* **192** (4236) 256-258

ROSENTHAL G.A., JANZEN D.H. AND DAHLMAN D.L. (1977) Degradation and detoxification of canavaline by a specialised seed predator. *Science* **196** 658-660

ROSENTHAL G.A., DAHLMAN D.L. AND JANZEN D.H. (1978) L-canavaline detoxification: a seed predator's biochemical mechanism. *Science* **202** 528-529

ROSENTHAL G.A. AND BELL E.A. (1979) Naturally occurring, toxic nonprotein amino acids. *in "Herbivores: their interaction with secondary plant metabolites"* eds GA Rosenthal & DH Janzen. Acad. Press. NY, Lond pp353-385

SANTORO L.G. (1988) Nutritional utilization by monogastric animals of glycoprotein II (phaseolin), the major 7S protein from kidney beans (*Phaseolus vulgaris*) PhD Thesis Univ Aberdeen

SCHOONHOVEN A.V. AND CARDONA C. (1982) Low levels of resistance to the Mexican bean weevil in dry beans. *J.Econ.Ecol.* **75** 567-569

SCHOONHOVEN A.V., CARDONA C. AND VALOR J. (1983) Resistance to the bean weevil and the Mexican bean weevil (Coleoptera: Bruchidae) in noncultivated common bean accessions. *J.Econ.Ecol.* **76** 1255-1259

SCOPES R.K. (1987) Protein Purification, Principles and Practice. 2nd Editn Springer Verlag NY 321 pages

SEMINO G.A., RESTANI P. AND CERLETTI P. (1985) Effect of bound carbohydrate on the action of trypsin on lupin seed glycoproteins. *J.Agric.Food Chem.* **33** 196-199

SILANO V. (1987) α-amylase inhibitors. *in "Enzymes and their role in cereal technology"* ed James E. Kruger *et al.* Am.Assn.Cereal Chem. pp141-199

SIMMONDS M.S.J., BLANEY W.M. AND BIRCH A.N.E. (1989) Legume seeds: the defences of wild and cultivated species of *Phaseolus* against attack by Bruchid beetles. *Annals of Botany* **63** 177-184

SINGH S.R. AND VAN EMDEN H.F. (1979) Insect pests of grain legumes. *Ann.Rev.Entomol.* **24** 255-278

SOUTHGATE B.J. (1979) Biology of the Bruchidae. *Ann.Rev.Entomol.* **24** 449-473

STAMOPOULOS D. AND HUIGNARD J. (1980) L'influence des diverses parties de la graine de haricot (*Phaseolus vulgaris*) sur la développement des larves d'*Acanthoscelides obtectus* (Coléoptère Bruchidae). *Ent.exp. & appl.* **28** 38-46

STAMOPOULOS D. AND DESROCHES P. (1981) Influence of the tegument of *Phaseolus vulgaris* seeds on the larvae of *Acanthoscelides obtectus* SAY. *Series Entomologia* **19** 165-174

STAMOPOULOS D.C. (1988) Toxic effects of lignin extracted from the tegument of *Phaseolus vulgaris* seeds on the larvae of *Acanthoscelides obtectus* (SAY) (Col., Bruchidae) *J.Appl.Ent.* **105** 317-320

STEPHEN A.M. (1983) Other plant polysaccharides. *in"The Polysaccharides" vol 2 ed GO Aspinall; Molecular Biology Series Acad Press* pp98-193

SWAIN T. (1977) Secondary compounds as protective agents. *Ann.Rev.Plant Physiol.* **28** 479-501

THIERY D. (1984) Hardiness of some fabaceous seed coats in relation to larval penetration by *Acanthoselides obtectus* (Say) (Coleoptera: Bruchidae). *J.Stored Prod. Res.* **20** 177-181

TOWBIN H., STAEHELIN T. AND GORDON J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc.Natl.Acad.Sci.U.S.A.* **76** 4350-4354

UCHIMIYA H., HANDA T. AND BRAR D.S. (1989) Transgenic plants (Minireview). *J.Biotech.* 00419 pp1-19

VAN EMDEN H.F. (1980) Insects and mites of legume crops. *In "Advances in Legume Science"* eds RJ Summerfield & AH Bunting. pp187-197

VARAIGNE-LABEYRIE C. AND LABEYRIE V. (1981) First data on Bruchidae which attack the pods of legumes in Upper Volta of which eight species are man-consumed *Series Entomologia* **19**

WIEMAN K.F. AND NIELSEN S.S. (1988) Isolation and partial characterization of a major gut protease from larval *Acanthoscelides obtectus* Say (Coleoptera, Bruchidae). *Comp.Biochem.Physiol.* **89B** 419-426

WILCOX E.R. AND WHITAKER J.R. (1984) Structural Features of Red Kidney Bean α-amylase inhibitor important in binding with α-amylase *J. Food Biochem.* **8** 189-213

XAVIER-FILHO J., CAMPOS F.A.P., ARY M.B., MACEDO M.L.R., PERES SILVA C., CARVALHO M.M.M. AND SALES M.P. (1987) The biochemical basis of seed resistance to insects. *Arq.Biol.Tecnol.* **30**(1) abs.p45 B28