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The use of isoenzymes
in the study of germination,
development and breeding of legumes

by

Ali A. Al-Helal

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Abstract

Amylase activities and patterns were compared in extracts from mature seeds and from different parts of seedlings at various stages of seed germination of various varieties of different legume species. Apart from soyabean, all varieties had low levels of amylase activities in extracts from mature seeds, while the former had a remarkably high level. Amylase activities and the number of bands increased during seed germination and showed time and tissue specificity. The extracts from cotyledons had the highest activities and the largest number of bands as compared to that of the various parts of embryonic axes.

Amylase activities and patterns had time specific changes during the various stages of seed development of the 7 varieties of different legume species studied. All the different varieties showed changes in zymogram patterns and decreased amylase activity during seed development, except soyabean where amylase activity remained high at all stages.

The different components of amylase were characterised in pea (var. Feltham First). One band of α -amylase activity, restricted to the cotyledons, was present in the middle stages of seed development only, then reappeared at the third day of seed germination. The embryonic axes β -amylase activity decreased as the seed developed, to reach zero level in mature seeds, and reappeared during seed germination. The reverse sequence occurred for the cotyledonary β -amylase.

The pea α -amylase was partially purified using ethanol precipitation, glycogen complex and hydroxyapatite column chromatography. The purified protein had three major bands with few faint bands on the SDS polyacrylamide gel.

The embryonic axes β -amylase of pea was partially purified using the conventional method, ammonium sulphate precipitation, ion exchange chromatography and gel filtration. The purified protein contained two thick bands and many faint bands on the SDS polyacrylamide gel.

The zymogram patterns of phosphorylase, EST, GDH, GOT, LDH, ADH and MDH were investigated during seed development of various varieties of different legume species. The most noticeable changes as the seeds developed were the decrease in activities and number of bands of EST in pea and soyabean, shift in GDH activities between isoenzyme forms in pea (var. English Wonder), increase in GDH activities and number of bands in dwarf French bean and soyabean and increase in activities and number of bands of MDH in pea (var. Feltham First).

The cotyledonary globulin protein of pea (var. Feltham First) was followed on first and two-dimensional SDS polyacrylamide gels during 10 stages of seed germination. The acidic legumin subunits started to break down to subunits of smaller molecular weight from the third day. Convicilin (mol.wt. 70,800), vicilin (mol.wt. 56,000), vicilin (mol.wt. 37,000) and acidic legumin subunits (mol.wt. 42,000) were not detected from the 7, 9, 7 and 7 day of seed germination respectively.

The GOT patterns of pea seeds was used as a marker to study the competition between pollen grains of inbred lines Meteor and no. 1238 in mixtures of various ratios between them. The pollen grains of the latter were significantly more competitive than those of the former. The results also showed the in vivo growth of pollen tubes of inbred line Meteor.

The effect of storage conditions and age of seeds on the EST and GOT patterns was investigated, in broad bean. The only noticeable change was the presence of an additional band of EST in extract from seeds that had been stored for two years at 4°C.

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Ali A. Al-Helal

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INTRODUCTION

1. Isoenzymes

1.1 Definition

Since their discovery in 1938, there has been a growing recognition of the widespread existence of multiple molecular forms of enzymes. This has arisen as a result of the fact that enzymes are proteins of large size, often with additional non-protein components, and most of them are polymers of two or more polypeptide chains (subunits) which may be identical (giving rise to homomultimer forms) or different (giving rise to heteromultimer forms). Therefore, considerable variations can occur producing multiple forms of the same protein with no change in catalytic activities, but having some different properties, such as molecular weight, structure, solubility, net charge, response to inhibitors, immunology and to some extent broad substrate specificity.

Markert and Møller introduced the term isoenzyme, which is synonymous with the term isozyme, to refer to the multiple molecular forms of an enzyme occurring in the same organism, tissue, or within members of the same species, catalyzing identical biochemical reactions. They excluded from this definition the enzymes having wide substrate specificity such as esterase, alkaline phosphatase and peroxidase.

The term isoenzyme is sometimes used to describe any category of



enzyme heterogeneity without any information about the basis of molecular structure and origin. Recent recommendations restrict the use of this term only to those different molecules which have identical catalytic activity, but are encoded by distinct genes. This definition excludes the molecular forms of an enzyme which are produced by post-genetic modification, but not the forms of the polymeric enzyme which are produced by distinct gene loci (Moss, 1979).

1.2 Origin

The origins and development of isoenzymes have been recently reviewed by Markert (1977) and Moss (1979). From their discussions it can be concluded that there are two fundamental mechanisms for generating isoenzymes, genetic mechanisms, and biochemical and physical mechanisms.

1.2.1 Genetic Causes

The multiple forms of an enzyme may have come into existence via the duplication of the structural gene coding for this enzyme during the course of evolution, giving rise to a number of genes capable of producing distinct isoenzymes but catalyzing the same biochemical reaction. Moreover, these genes may undergo mutations producing genes coding for proteins of different structure but catalyzing identical biochemical reactions. Also, the different protein products of these duplicate genes may aggregate to form a polymeric isoenzyme system; homomultimeric isoenzymes composed of several genetically identical

polypeptide chains (subunits) and heteromultimeric isoenzymes composed of several genetically different polypeptide chains. The number of isoenzymes which can be formed for any enzyme, in this manner, is determined by the number of genes coding for this system and the number of polypeptide chains which make up the molecular structure of each isoenzyme. However, this assumption is based on *in vitro* hybridization, but the mechanisms that regulate, *in vivo*, the combination of these subunits (promoters) are obscure.

The isoenzymes may also occur as a result of allelic variation of the same gene locus producing different proteins but catalyzing the same biochemical reaction. Kaplan in 1963 introduced the term allelic isoenzymes to describe these forms. He also reviewed the genetic causes of isoenzymes and he gathered these causes into three groups:

- 1) Duplicate enzymes: Due to different genes but with only slight differences in sequence, immunological, catalytic and physical properties.
- 2) Multiple forms: Due to separate genes and forming hybrids which have significant differences in sequence, catalytic and immunological properties.
- 3) Allelic enzymes: Due to allelic variations of the same gene and not present in all members of a species, as compared to duplicate enzymes which are present in all members of a species.

1.2.2 Biochemical and Physical Causes

The multiple forms of a given enzyme might be produced by several

different kinds of physical and chemical mechanisms, such as the post-synthetic modification of the enzyme, e.g. binding of a variable number of coenzymes, prosthetic groups, amino acid residues, substrates, lipids and carbohydrates. Also, they may arise by aggregation of enzyme molecules with each other or with other non-enzymatic proteins, removing of number of amino acid residues, interaction of the enzyme molecule with anions or cations, as a result of proteolytic activity, or as a result of differences in conformation of the same protein.

Some forms may arise during storage, e.g. Juliano and Varner (1969) reported that an additional band of β -amylase was present in pea seed extract as it was stored at 0°C. However, the same band was also present in the fresh extract of the germinated cotyledons. Furthermore, the separating media in the electrophoresis may affect the results, as reported by Gerbrandy and Varleur (1971) who found that an additional band of phosphorylase activity was present in potato extract when developed on acrylamide gel containing starch as compared to the patterns present in acrylamide gel containing glycogen. However, they attributed this to the separation of one band into two bands.

1.3 Occurrence of Isoenzymes in Higher Plants and their Biological Uses

Since the introduction of the term isoenzymes, a large number of enzymes have been reported to exist in multiple molecular forms in a wide range of plant tissues, and the occurrence of isoenzymes

appears to be widespread among higher plants. The occurrence of isoenzymes in plants have been reviewed by Shannon (1967). Also, Scandalios reviewed the occurrence of isoenzymes in plants with respect to genetic control of the multiple forms (1969) and with respect to development and differentiation (1974).

Although a limited number of isoenzyme systems, among the large number of enzymes that have been reported to be present in multiple forms, were studied in detail, they have proved to be very useful molecular systems for taxonomic, developmental, physiological, biochemical and pathological studies. See Table 1 at the end of the introduction.

Work on higher plant isoenzymes has been directed toward the investigation of a variety of subjects, such as molecular structure, substrate specificity, intracellular location, activity and pattern changes during growth and development, tissue specificity, the specific metabolic role(s) of the individual isoenzyme and their response to inhibitors and growth environments, and as genetic and cultivar markers.

1.3.1 Isoenzymes as Cultivar Markers

Higher plant isoenzymes have been used successfully as cultivar markers. Gates and Boulter (1979b and 1980) reported that they identified 26 cultivars of broad bean by the electrophoretic patterns of non-specific esterase (EST) in extracts from either cotyledons or from pollen grains, and they identified 4 cultivars by the electrophoretic patterns of glutamate-oxaloacetate transaminase (GOT). Also, they used the patterns of EST to investigate the contamination of F_1

progeny and they reported that the patterns of each cultivar were identical at the different growth environments.

It has been reported that the activity and the electrophoretic patterns of β -amylase can be used, to some extent, as cultivar markers in soyabean (Morita and Yagi, 1979) and the authors claimed that there was a correlation between the electrophoretic patterns of β -amylase and the morphological features of the cultivars.

In addition, Gorman and Kiang (1977) showed that several isoenzymes can be used as cultivar markers for soyabean.

1.3.2 Response to Growth Environment

It appears that the different isoenzymes, which show different intracellular location, might show different response to the variation in the growth media, as evidence from the work of Hason-Porath and Pojakoff-Mayber (1969), who studied the intracellular location of malate dehydrogenase (MDH) isoenzymes and its activities in root extracts of pea grown in a saline media. Their results showed that the activity of the cytoplasmic isoenzyme tolerates the presence of NaCl in the growth media, while the activity of the mitochondrial isoenzyme was depressed and the presence of NaCl in the growth media also stimulated the synthesis of a third isoenzyme.

The effect of nitrogen regimes on the electrophoretic patterns of EST, GOT and the total water-soluble protein of broad bean, have been investigated by Gates and Boutter (1979a). From their results it appears that the electrophoretic patterns of EST, GOT and protein of the extracts from seed cotyledons and pollen were not affected by the

nitrogen regime, while those of leaf extracts were.

1.3.3 Tissue Specificity

In several plant species, it has been reported that the relative abundance of the different forms of a given enzyme is not always identical in the different tissue of an organism, and that their distributions vary widely. Moreover, it has been shown that the different tissues might show different electrophoretic patterns for a given enzyme and that some forms are tissue- and intracellular-location specific.

Tissue specificity, intracellular location and relative distribution have been reported for EST (Gates and Boulter, 1979), phosphorylase (Matheson and Richardson, 1976; Gerbrandy and Varleur, 1971; Steup and Latzko, 1979), MDH (Hason-Porath and Pojakoff-Mayber, 1969), glutamate dehydrogenase (Nauen and Hartmann, 1980; McKenzie and Lees, 1981) and leucine-aminopeptidase (Collier and Murray, 1977; Scandalios and Espiritu, 1969) and many others.

Steup and Latzko (1979) studied the distribution and intracellular location of phosphorylase isoenzymes in spinach and pea, and the authors reported that two forms were found in extracts from spinach leaves; one form was exclusively associated with the chloroplasts, while the other was located outside the chloroplasts. Their results showed that two forms were present in pea root extract, while leaf extract had three forms and only two of them were associated with chloroplasts. Also, Gerbrandy and Varleur (1971) showed that one form of the nine isoenzymes which was present in extract from potato

tubers, was associated with the amyloplasts.

The tissue specificity and intracellular location of glutamate dehydrogenase (GDH) was studied in soyabean (McKenzie and Lees, 1981). The zymogram patterns of seed extract had three distinct electrophoretic forms, while the patterns of extracts from other parts of the plant had two distinct electrophoretic forms and one of them was associated with the mitochondria and the other form with the chloroplasts. The authors revealed that the form that was restricted to seed extract was not affected by glutathione and 2-mercaptoethanol, while the other forms were activated by these compounds. In contrast to GDH of soyabean, the 7 GDH isoenzymes of pea shoot are all associated with the mitochondrial matrix and they increased in activity during seed germination (Nauen and Hartmann, 1980).

1.3.4 Activity and Pattern Changes during Seed Development and Germination

The processes of seed development and germination are accompanied by variety of significant metabolic changes, among which are the activity and pattern changes of isoenzymes. Such changes have been reported to occur in several plant species for numerous isoenzyme systems, and are rather widespread among plants (Shannon, 1968; Scandalios, 1974).

The most important reasons for studying the distribution and zymogram patterns of isoenzymes during the course of growth and development, is that they might provide useful informations on the metabolic patterns which associate with each tissue and also they

should provide insight into the possible metabolic role of the individual isoenzyme and into the role which the individual isoenzyme might play in the development and growth of that particular tissue containing it. Moreover, if the isoenzymes distribution is time and tissue specific, the patterns at a particular time may reflect the developmental state of the particular tissue associated with it.

The reason for the age-dependent changes and distribution and the mechanisms which regulate these differential distributions in vivo are unknown, but several possibilities have been suggested (Shannon, 1968; Scandalios, 1969 and 1974; Markert, 1977; Moss, 1979). Among these possibilities are that these changes might be:

- 1) A reflection of changes in the number or activity of cells which contain large amounts of their activities;
- 2) A result of continuous exposure to factors inducing synthesis, such as hormones;
- 3) Exposure to factors that hydrolyze the enzyme, such as proteolytic activity;
- 4) Differential activation and inactivation;
- 5) Production of an inactive latent form;
- 6) Production of an active form from a previously existing latent form;
- 7) Faster rate of turn-over than synthesis or vice versa;
- 8) Differential gene expression which could result from differential transcription and translation.

Among those isoenzymes which were reported to undergo fluctuations during seed development and germination was leucine-aminopetidase (LAP) of

pea. The isoenzymes of LAP have been reported to undergo slight quantitative changes (Collier and Murray, 1977; Scandalios and Espistitu, 1969) and the changes are variety-dependent. Collier and Murray used the insensitivity of one form (the faster moving one) to phenanthroline to estimate the activity of each individual isoenzyme and their results revealed that most of LAP activity was attributable to the faster moving isoenzyme. During seed development, one variety showed continuous increases in LAP activity as the seeds matured, while the other variety showed an increase followed by a decline. Moreover, Scandalios and Espiritu, using electrophoresis coupled with histochemical techniques, showed that one variety exhibited an increase in LAP activity in the first days of seed germination, followed by a decline, while the reverse was true for another variety.

The enzymes which hydrolyze starch also have been reported to show changes during seed development and germination and since they were of particular interest in the research described in this thesis they will be discussed in more detail in Section 2.

1.3.5 Other Uses

Isoenzymes have been widely used as markers in developmental biology. Tanksley et al (1981) used the isoenzyme patterns of nine enzymes to study the genetic overlap between sporophyte and gametophyte in tomato. They studied the isoenzyme patterns in extracts from pollen, at two stages of development, from seeds at various stages of development and from plants (leaf, stem and root) and they concluded that 60% of the structural genes coding for the nine enzymes

in the sporophyte were also found to be expressed in the gametophyte.

An interesting use of isoenzymes in plant breeding is evident from the work of Ellstrand (1984), who used the electrophoretic patterns of five isoenzymes in extracts from mature seeds to study the multiple paternity in radish. Also, Ennos and Clegg (1982) used the ^sesterase zymogram patterns of extracts from seeds and 7 day-old seedling to study the effect of population substructuring on the estimated outcrossing rate in Ipomoea purpurea.

Enzyme polymorphisms have been widely used as markers in population genetics; however no attempt was made here to review this subject although a few examples will be described. Hamrik et al (1979) reviewed the literature on higher-plant enzymes with respect to the variation in genetics of populations and they made the following conclusions:

- 1) The variable substrate enzymes were the most commonly used functional class, while the regulatory enzymes were the least used;
- 2) The preferential use of variable substrate enzyme systems in many plant genetic studies did not result in an overestimate of genetic variation;
- 3) The gymnosperms were more variable than either the monocotyledons or the dicotyledons and the former was more variable than the latter;
- 4) Long-lived woody perennials contained the highest levels of variation, shorter-lived herbaceous perennials and annuals had intermediate levels of variation, and biennials had the least;

- 5) Species with a mixture of asexual and sexual reproduction had more variation than sexually reproducing species;
- 6) Positive association between the amount of outcrossing and genetic variation;
- 7) Wind pollination was associated with high levels of variability;
- 8) Species with high fecundities were highly variable; and
- 9) Cultivated species had slightly higher levels of variation than the noncultivated species.

Isoenzyme analysis, using electrophoretic techniques on either starch or acrylamide, has been used to study various problems in population genetics of forest trees, where other genetic markers are difficult to use or cannot be used. For example, Lundkvist and Rudin (1977) used the isoenzyme patterns of 3 enzymes to detect a genetic variation in populations of a forest tree, Picea abies, while there were great difficulties in estimating the actual genetic variability from morphological markers only. Also, Levin (1976) used the isoenzyme patterns of 13 enzymes to study the breeding system and population genetics of Phlox.

Moreover, in Citrus, plant breeders were faced with the problem of distinguishing asexual, nucellar seedlings from zygotic seedlings and it was often necessary to grow seedlings for five or more years to fruiting in order to distinguish between them, an expensive procedure. Using isoenzyme patterns in extracts from seedling leaf circumvented this problem (Torres et al, 1978b).

Finally, isoenzymes provided a rapid reliable system for studying

the mating system in trees, whereas using dominant markers may take a prohibitively long time (Brown et al 1975 and the references therein).

1.4 Separation of Multiple Forms of Enzymes

Hunter and Markert (1957) introduced the most commonly used technique to investigate isoenzyme heterogeneity. This procedure is based on the coupling of starch or acrylamide gel electrophoresis, which was developed by Smithies (1955), with histochemical staining procedures. This technique has a potential ability to detect small differences in the composition and quantity of isoenzymes in an extract. However, only those variations which produce molecules with different mobilities can be resolved electrophoretically, while the other variations which might occur, producing different molecules but with identical mobilities cannot be resolved by this method. Several other techniques have been used for studying isoenzymes, e.g. isoelectric focussing, ion-exchange chromatography, gel filtration and affinity chromatography.

2. Starch-hydrolyzing Enzymes

The starch-hydrolyzing enzymes are of particular interest in the fields of plant physiology and biochemistry, since they play an important role in plant growth, and particularly in seed germination.

The major enzymes that are known to be involved in starch degradation are: α -amylase, β -amylase, phosphorylase, α -glucosidase (maltase) and debranching enzymes.

2.1 α -Amylase

This enzyme catalyzes the random breakdown of α -(1,4) glucosidic bonds in both linear and branched carbohydrates.

2.1.1 Properties

Swain and Dekker (1966a and b) characterized the enzymes involved in starch mobilization of pea seedlings and they purified α -amylase of germinated cotyledons and reported the following properties for it:

- 1) Its catalytic activity produces a large variety of reducing oligosaccharides of α -configuration with faint traces of low molecular weight oligosaccharides;
- 2) It is heat stable, but loses 23% of its activity when incubated at 70°C for 15 min. in the presence of added Ca^{2+} ;
- 3) Loss of all its activity occurred when dialyzed against EDTA;
- 4) It hydrolyzes starch with complete disappearance of the characteristic starch-iodine colour. Later, different investigators reported more properties for higher plant α -amylase;
- 5) It is the only type of amylase which can attack the intact starch granules (Dunn, 1974);
- 6) It is inactivated at low pH 3.3 (Tanaka and Akazawa, 1970); and
- 7) Only α -amylase, but not β -amylase can hydrolyze β -limit dextrin (Bilderback, 1973) because it can by-pass the α -(1, 6) linkage.

It appears that α -amylases of the different higher plants and the different forms from the same source, might show, to some extent,

different biochemical properties. Tanaka and Akazawa (1970) fractionated α -amylase of barley and showed that one fraction lost 30% of its activity when heated, while the activity of the other fraction was enhanced when heated at 70°C for 30min. Moreover, Jacobsen et al (1980) studied the electrophoretic variants of barley α -amylase and they reported that two forms were stable at pH 3.7 and were not inactivated by EDTA, while the other two forms were inactivated at pH 3.7 and by EDTA. Also, it has been reported that the purified α -amylase of Vigna mungo lost only 50% of its activity when incubated with EDTA and this change was reversible (Koshiha and Minamikawa, 1981). Also, Frydenberg and Nielsen (1965) showed that some forms of amylase in barley are heat-resistant but did not require Ca^{2+} . Nevertheless, Irshad et al (1981) studied the effect of Zn^{2+} and EDTA on the activity of α -amylase of various plants and the authors concluded that plant α -amylase was strongly inhibited by Zn^{2+} and the inhibition was pH-dependent and reversible, and that it could be removed by treatment with EDTA which itself had no effect on plant α -amylase activity.

2.1.2 Occurrence

The activity of α -amylase appears to be lacking in the mature dry seeds of pea, dwarf French bean, Vigna mungo and cereals (Swain and Dekker, 1966b; Shain and Mayer, 1968; Juliano and Varner, 1969; Tanaka et al, 1970; Abbot and Matheson, 1972; Yomo and Varner, 1973; Miyota et al, 1981; Koshiha and Minamikawa, 1983; Onckelen et al, 1977; Gepstain and Ilan, 1970).

However, Davis (1977 and 1979) claimed the presence of α -amylase activity in the axis of mature dry seeds of several cultivars of pea. Also, the author reported that its activity increased during germination and the cotyledons had a negative control on its production. In contrast, Swain and Ekker (1966b) demonstrated that α -amylase activity was restricted to the germinated pea cotyledons, while the axes had no α -amylase activity.

2.1.3 Occurrence of multiple forms

There is a good evidence for the existence of multiple forms of α -amylase in higher plants, such as pea (Yomo and Varner, 1973), barley (Jacobsen et al, 1970; Tanaka and Akazawa, 1970; Bilderback, 1971; Callis and Ho, 1983), rice (Tanaka et al, 1970) and soyabean (Adams et al, 1981a).

2.1.4 Regulation of its Production during Seed Germination

In cereals, the production of α -amylase is known to be regulated by gibberellin (Frydenberg and Nielsen, 1965; Momotani and Kato, 1966; Jacobsen et al, 1970; Tanaka et al, 1970; Bilderback, 1971; Marchylo et al, 1981; Callis and Ho, 1983). Gibberellin (GA_3) that is produced in the embryo diffuses to the aleurone layers and induces the transcription of α -amylase genes (Bernal-Lugo et al, 1981). Also, it has been demonstrated that α -amylase is synthesized de novo in the scutellar epithelium and secreted into the endosperm during seed germination of rice (Myota et al, 1981).

However, there are many contradictions in the literature

concerning the influence of embryonic axis and hormones on the formation of amylase in non-endospermic leguminous seeds during their germination. It is very well known that α -amylase activity increases in the cotyledons during seed germination with a concurrent mobilization of storage starch, but the available information on the mechanisms that control its production are limited and confusing. Furthermore, there is no conclusive demonstration of its de novo synthesis during seed germination.

Varner et al (1963) reported that unknown factors move from pea embryonic axes to the cotyledons, in the first days of seed germination and they stimulate the production of amylase. Thereafter, Sprent (1968b) confirmed that the production of amylase activity in germinated pea cotyledons required the presence of the embryonic axis, but GA had no effect on its production, although high levels of GA retarded its development. Although, it has been reported that amylase was produced in detached pea and dwarf French bean cotyledons (Gepstain and Ilan, 1970 and 1981; Onckelen et al, 1977; Locker and Ilan, 1975), hormones were required for full development of its activity (Gepstain and Ilan, 1970; Locker and Ilan, 1975). In contrast, Yomo and Varner (1973) reported that α -amylase and β -amylase reached full activity in detached pea cotyledons and their formation was regulated by ABA. Also, Parys et al (1983) reported the presence of higher amylase activity in the excised pea cotyledons than the cotyledons of germinated seeds. The authors attributed this difference in amylase activity to the level of reducing sugars which was higher in the latter, and they concluded that the level of reducing sugars may play

a role in the regulation of amylase activity in vivo, in both cotyledons and embryonic axes of germinated seeds.

Moreover, Garcia-Luis and Guardiola studied the effect of GA₃ on the development of amylase activity in germinated pea cotyledons. They first reported in 1975 that GA₃ stimulated the amylolytic activity in the cotyledon extract of intact seedlings. In the second paper (1978) they showed that amylase activity increased in the cotyledons of 9 day-old seedlings when incubated up to 10 days in petri dishes whether GA₃ was added or not. Their results also showed that GA₃ delayed the increases in amylase activity and the GA₃-untreated detached cotyledons had higher amylase activity than the GA₃-treated detached cotyledons. They attributed the stimulation of amylase activity in the cotyledon extract of intact seedlings by GA₃ as an indirect effect by stimulating the transfer of the end products of starch hydrolysis to the growing embryonic axes, preventing them from accumulating to a level that inhibits amylase activity.

The regulation of amylase activity in Vigna mungo has been studied by Koshiba and Minamikawa (1983). The authors reported that the level of α -amylase activity increased in the extracts of seedling cotyledons, up to the 5th day of seed germination and declined thereafter. In the detached incubated cotyledons, the amylase activity continued to increase and reached a level of three times that present in the attached cotyledons. The authors attributed this difference in amylase activity to the breakdown of amylase by the proteolytic activity which was found to be high in the attached cotyledons and very low in the detached cotyledons.

From the literature on the regulation of breakdown of cotyledonary

storage compounds of dicotyledonous plants during seed germination, Chapman and Davies (1983) proposed the most widely accepted hypothesis, namely that the hydrolytic enzyme activities automatically increases in the cotyledons, and the embryonic axes might have no effect on their development at the early stages of seed germination, but at the later stages serve as a sink by removing the hydrolyzed breakdown products and prevents them accumulating to a level that could inhibit enzyme activity by feed-back effects. However, this does not seem to be the case for dwarf French bean cotyledons where the level of endogenous sugars did not regulate amylase activity and synthesis (Gepstain and Ilan, 1981).

Davies and Slack (1981) proposed two hypotheses to explain the role of the embryonic axes in the mobilization of the cotyledonary storage compounds of dicotyledonous plants during seed germination:

- 1) The embryonic axis produces hormones that stimulate the production of hydrolytic enzymes in the storage organ;
- 2) The embryonic axes serves as a sink by removing the end products of hydrolyzed materials from cotyledons.

2.1.5 Activity and Pattern Changes during Seed Development

Most of the studies on amylase in higher plants were directed toward the activity and pattern changes during seed germination and toward the effect of hormones on the production of α -amylase. Studies have not been directed toward the possible changes in amylase activity and patterns during seed development and only limited numbers of reports are available on this aspect, especially in legumes. Such

studies might provide information about the possible role of the individual isoenzyme, particularly if the patterns were tissue specific.

Adams et al (1981a) studied the activity and patterns of α -amylase during seed development of soyabean and in 5 day-old seedlings and their results showed that the two varieties studied had similar changes, showing 8 forms in immature seeds, 6 forms in mature seeds and 7 forms in germinated seeds.

Müntz (1977) analyzed the electrophoretic patterns of phosphorylase and α -amylase in broad bean pods during development and the author's results revealed that one band of amylase activity was present in pod extracts through all the stages of pod development and an additional band was present in pod extracts at the middle stages of pod development only and was coincidental with the rapid degradation of starch, and no changes in phosphorylase patterns were observed.

Bilderback (1971) also studied the patterns of amylase in barley aleurone layer and endosperm during seed development and reported that the maximum activity appeared at 5 and 20 days after anthesis. Four components of α -amylase activity appeared in extracts from the seeds at the early stages, decreased in activity as the seed matured, and were not found in extracts from mature seeds. Another component of amylase has been reported to be present in extracts from seeds at the later stages of development and remains active in extracts from mature seeds.

2.2 β -amylase

β -amylase, as defined by Manners (1973), is the enzyme that catalyzes a stepwise hydrolysis of β -(1,4) glycosidic linkages of starch-type polysaccharides with liberation of maltose, and its action on linear substrates is usually complete, while with branched substrates the action is incomplete because it cannot hydrolyze α -(1,6) glycosidic linkages, so that products are maltose and β -limit dextrin.

2.2.1 Properties

Swain and Dekker (1966a) reported the following properties for β -amylase of germinated pea axes:

- 1) Completely inactivated by heating at 70°C for 15min. in the presence of added Ca^{2+} ;
- 2) Retains full activity in the presence of EDTA;
- 3) Its catalytic activity on soluble starch liberates only maltose with β -configuration; and
- 4) Digests soluble starch with incomplete disappearance of the starch-iodine colour.

However, they did not report the electrophoretic patterns of β -amylase of pea axes and no information is available about these at the present time.

2.2.2 Occurrence and Multiple Forms

β -amylase have been reported to be present in multiple forms in higher plants, in pea (Yomo and Varner, 1973; Juliano and Varner,

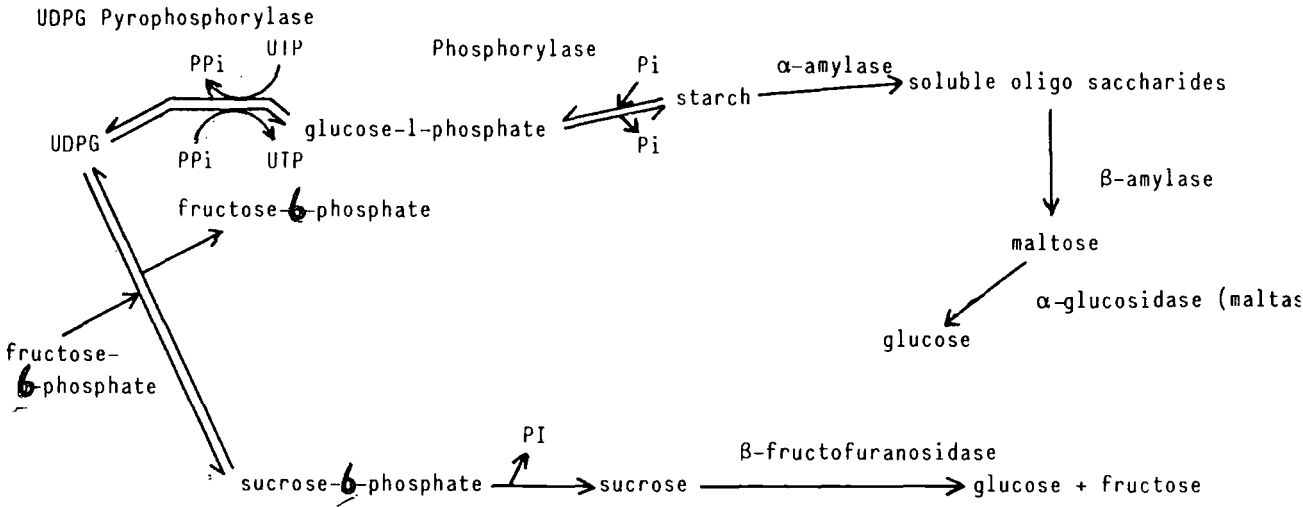
1969; Shain and Mayer, 1968); wheat (Abbot and Matheson, 1972), barley (Bilderback, 1971) and in soyabean (Morita and Yagi, 1979).

β -amylase has been shown to exist in inactive latent forms in barley and can be released by treatment with papain (Bilderback, 1971; Rowsell and Goad, 1962).

2.3 Phosphorylase

Phosphorylase is the enzyme that catalyze the reversible reaction between glucose-1-phosphate and starch.

It appears that there is uncertainty about the in vivo metabolic role of phosphorylase in higher plants. It was first assumed that this enzyme was involved in the synthesis of starch, but now it is known that starch synthetase (UDP or ADP glucose-starch- γ -glucosyl-transferase) is the enzyme catalyzing the synthesis of the unbranched chain of starch (Lelior et al, 1961) and the Q-enzyme (branching enzyme) is the enzyme catalyzing the synthesis of the branch point of starch (Manners, 1973 and the references within). However, recently there has emerged a growing belief that phosphorylase is involved in starch degradation. Swain and Dekker (1966a) proposed two pathways for starch mobilization in pea seedlings, one involving the amylolytic activity (α and β -amylases) and the other involving the phosphorylytic activity. Phosphorylase hydrolyses starch to glucose-1-phosphate, which is utilized by UDPG pyrophosphorylase and β -fructofuranosidase for the production of glucose and fructose, as follows:



Pi: orthophosphate, ppi: pyrophosphate, UTP: uridine nucleotide triphosphate, UDPG: uridine diphosphate glucose.

Abbot and Matheson (1972) also suggested that phosphorylase might function in starch degradation at least in the early stages of seed germination. Moreover, Schilling and Dittrich (1979) went further and suggested that the presence of sufficient orthophosphate (Pi) stimulates the breakdown of starch by the activity of phosphorylase, which is an energy saving reaction, whereas the less efficient breakdown of starch by amylase is diminished.

Although it appears that there is evidence to support the

involvement of phosphorylase in starch breakdown, some investigators still believe that some forms of phosphorylase catalyze the synthesis of starch (Gerbrandy and Verlier, 1971; Tsai and Nelson, 1968 and 1969; de Fekete and Vieweg, 1973). There are two different types of phosphorylase, an unprimed form that does not require primer for the synthesis of polysacchrides and a primed form that requires primer for polysaccharides synthesis. Also, Frydman and Slabink (1973) claimed that the unprimed phosphorylase provides the polysaccharides that are required by starch synthetase for starch synthesis, and also they concluded that phosphorylase acting with branching enzyme could be involved in the first step of the formation of starch granules.

However, in vitro, phosphorylase is able to catalyze the degradation of starch in the presence of high concentration of orthophosphate in the assay media and catalyze the synthesis of starch in the presence of glucose-1-phosphate.

2.3.1 Occurrence and Multiple Forms

Phosphorylase has been reported to occur in higher plants and in multiple forms in many species including pea (Yomo and Varner, 1973; Jacobsen et al, 1970), spinach and pea (Steup and Latzko, 1979), potato, dwarf French bean and broad bean (Gerbrandy and Verlieur, 1971), various plant species (Richardson and Matheson, 1977) and in maize (de Fekete and Vieweg, 1973).

2.3.2 Activity and Patterns during Seed Development and Germination

Matheson and Richardson (1976) studied the activities and the

relative distribution of phosphorylase in pea during seed germination and development and their results showed that one form had a low, but constant level of activity during seed development and increased in activity during seed germination and they assumed that this form might be concerned with starch degradation. The other form had high, increasing levels of activity during seed development and had a constant level during seed germination and the authors assumed that this form might be concerned with starch synthesis. Also, their results showed that phosphorylase activity is cultivar dependent, one variety having three times the phosphorylase activity of the other variety.

2.4 Debranching Enzymes

Debranching enzymes catalyze the hydrolysis of α -(1,6) glucosidic bond of carbohydrates.

There are different types of debranching enzymes and they have been reviewed briefly by Manners (1973). The debranching enzyme that attacks amylopectin and its β -limit dextrin and causes a limited increase in the iodine staining power, but has little or no action on α -dextrins and pullulan, is called R-enzyme. The debranching enzymes that have only little activity towards amylopectin and have high affinity towards α -dextrins and pullulan are called limit dextrinases.

R-enzyme has been reported to be present in pea (Swain and Dekker, 1966a, Shain and Mayer, 1968; Vlodawsky et al, 1971) and in many higher plants, in multiple forms (e.g. Baun et al, 1970; Manners,

1973 and the literature within). Shain and Mayer (1968) studied the mechanisms which regulate its production during pea seed germination and they reported that it was formed by the liberation or activation of a precursor form.

2.5 α -glucosidase

Catalyzes the hydrolysis of maltose to glucose. It appears to be widely distributed in higher plants and occurs in multiple molecular forms (Manners, 1970 and the references within).

3. Germination of Pea Seeds, and the Cotyledonary Storage Protein

3.1 pea Seed Germination

Germination of the seed of the higher plants, as defined by Mayer and Pojakoff-Mayber (1982), is the consecutive number of steps which causes a quiescent seed with a low water content to show a rise in its general metabolic activity and to initiate the formation of a seedling from the embryonic axis.

The process of seed germination in pea is accompanied by several morphological, physiological and biochemical changes. The morphological and the ultrastructural changes that take place have been described for the cotyledons (Bain and Mercer, 1966) and for the embryonic radicle (Yoo, 1970).

The first step in seed germination is the imbibition of water by the seed, which allows the commencement of metabolic activities. The first metabolic change is the increase in oxygen uptake by the seed

(Guardiola and Sutcliffe, 1971; Bain and Mercer, 1966).

The growth of embryonic axis to seedling requires energy, which is derived from the utilization of the energy-yielding compounds contributed by cotyledonary reserved compounds such as starch and protein (Guardiola and Sutcliffe, 1971; Yomo and Varner, 1973; Bain and Mercer, 1966). However, there is a lag of a few days before the main cotyledonary hydrolyzed storage compounds are mobilized to the growing embryonic axes (Bain and Mercer, 1966; Basha and Beevers, 1975; Guardiola and Sutcliffe, 1971). It appears that the growing axes may use its own reserves during the first days of germination (e.g. Davis, 1977 and the references within).

The most apparent physiological changes that take place during pea seed germination are the increase in the cotyledonary enzymatic activities, especially hydrolytic enzymes, which are necessary for the breakdown of the storage products. The development and increase of peptidase, protease and amylase activities have been described for cotyledons (e.g. Guardiola and Sutcliffe, 1971; Beevers, 1968; Yomo and Varner, 1973, Beevers and Splittstoesser, 1968) and for the axes (Swain and Dekker, 1966; Davis, 1977; Murray et al, 1979, Murray, 1979).

3.2 Cotyledonary Storage Protein

The study of pea cotyledonary storage protein has been of great interest to biologists during the last two decades, in their efforts to improve the quality of legume seed storage proteins, which are an important component of nutrition for both humans and animals, but

which are unfortunately deficient in certain essential amino acids.

The reserve cotyledonary proteins of pea are located in distinct membrane-bound organelles called protein bodies (Varner and Schidlovsky, 1963). The mature pea seeds contain three major storage globulin proteins (proteins that are soluble in a dilute salt solution, but not distilled water); legumin, vicilin and convicilin. They are hydrolyzed to amino acids in the cotyledons during seed germination and translocated to the growing axes (Beevers, 1968; Guardiola and Sutcliffe, 1971 and 1972) to provide a source of nitrogen and carbon.

The legumin of pea seeds has been purified and characterized by several investigators (Casey, 1979, Matta et al, 1981; Krishna et al, 1979; Thomson et al, 1978; Croy et al, 1979; Gatehouse et al, 1980). The legumin of pea seeds, of mol.wt. 60,000, consists of six acidic subunits (mol.wt. 40,000) and six basic subunits (mol.wt. 20,000), linked covalently by one or more disulphide bonds (e.g. Croy et al, 1979; Krishna et al, 1979).

Vicilin is a heterogenous protein of mol.wt. 170,000, consisting of a number of protein species of mol.wt. 50,000 and 33,000 and minor components of lower molecular weight (Croy et al, 1980a; Gatehouse et al, 1981).

Convicilin of mol.wt. 290,000 is a tetramer of four subunits of mol.wt.71,000 (Croy et al, 1980b).

4. Pollen Biology

The study of pollen biology has attracted many botanists in recent years, since it has direct application in the field of plant breeding. The physiology of pollen has been reviewed by Johri and Vasil (1961) and the work concerning the regulation of pollen and pollen tube development at the biochemical level in angiosperms has been reviewed by Mascarenhas (1975). Large numbers of articles on pollen biology were presented in a symposium on pollen biology and implications for plant breeding (Mulcahy and Ottaviano, 1983). No attempt was made here to review the biology of pollen, but since the competitive fertilization ability of pollen is of a particular interest in this presentation it will be reviewed briefly.

4.1 Competitive Fertilization Ability of Pollen and its Applications in Plant Breeding

The recent works on the fertilization ability of pollen in angiosperms demonstrated that competition between pollen genotypes exists in maize (Pfahler, 1965, 1967; Mulcahy, 1971, 1974; Ottaviano et al, 1983; Yomada and Murakami, 1983), onion (Currah, 1983), pearl millet (Sarr et al, 1983), Dianthus (McKenna and Mulcahy, 1983), and in alfalfa (Barnes and Cleveland, 1963).

Competition between pollen genotypes, due mainly to the genetic constitution of pollen (Pfahler, 1965, 1967) is expressed as the differential rate of pollen tube growth (Mulcahy, 1971, 1974; Ottaviano et al, 1983; Barnes and Cleveland, 1963), the faster growing

pollen tubes having better competitive characteristics.

The competitive ability of pollen is intensified by style length; the longer the style, the more intensely expressed the pollen competition ability (Ottaviano et al, 1983), and by insect pollination, as compared to wind pollination, because the insects deposit large numbers of pollen on the stigma at each visit and this reduces the variation in the starting times for germinating pollen, while the wind deposits small numbers of pollen grains at a time, resulting in the variation in starting for germinating pollen (Mulcahy, 1979).

The differential fertilization ability of pollen led Mulcahy (1979) to hypothesize that both insect pollination and closed carpels in angiosperms, intensify the male gamete competition, providing a selection at the gametophyte phase and resulting in the exclusion of any haploid genome that does not produce sporophytes with high quality of metabolic vigour. So, according to Mulcahy's hypothesis, the competition between pollen grains results from variation in pollen tube growth rate, while Hoekstra (1983) stated that the competition was associated with pollen vigour, and he hypothesized that the mature ungerminated pollen grain which could shift more of its synthetic process, normally carried out on the stigma or during tube growth, to the maturation stage in the anther, would be able to shorten the lag phase of germination and reach the ovule faster and therefore have a competitive advantage.

The significance of pollen competition is that it allows selection to operate at the gametophyte phase rather than at the sporophyte

phase, and that current investigations on pollen competition show that there is a significant correlation between sporophyte and gametophyte qualities (Mulcahy, 1971, 1974, Ottaviano et al, 1980, 1983). This has important consequences in plant breeding, allowing the possibility of rapid selection of superior sporophytes by selecting correlated gametophyte characters in vitro.

Table 1: Some selected isoenzymes of plants

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Alcohol dehydrogenase</u>			
wheat (<i>Triticum aestivum</i>)	seeds and seedlings	patterns during seed develop. and germ.	Macko et al, 1967
maize (<i>Zea mays</i>)	seeds	simple and compound loci	Schwartz and Endo, 1966
maize (<i>Zea mays</i>)	seedlings	variation and selection	Brown, 1971
peanut (<i>Arachis hypogaea</i>)	seeds and seedlings	patterns during seed develop. and germ.	Cherry and Ory, 1973
<i>Eucalyptus obliqua</i>	seeds	estimation of mating system	Brown et al, 1975
avocado (<i>Persea americana</i>)	mesocarp	genetics	Torres et al, 1978a
maize (<i>Zea mays</i>)	pollen	genetics	Gorla et al, 1983
<u>Acid phosphatase</u>			
<i>Picea abies</i>	seeds	population genetics	Tigerstedt, 1973
<i>Eucalyptus obliqua</i>	seeds	estimation of mating system	Brown et al, 1975
<i>Picea abies</i>	needle and endosperm	population genetics	Lundkvist and Rudin, 1977
<i>Cucurbita</i> spp.	leaf and pollen	genetics	Miller and Mulcahy, 1983

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Amylase</u>			
pea (<i>Pisum sativum</i>)	cotyledons	effect of embryonic axis on its production	Varner et al, 1963
maize (<i>Zea mays</i>)	seedlings	patterns and genetics	Scandalios, 1966
pea (<i>Pisum sativum</i>)	cotyledons	effect of benzyladenine	Sprent, 1968a
pea (<i>Pisum sativum</i>) various	cotyledons various	effect of GA patterns	Sprent, 1968b Gerbrandy and Varleur, 1971
pea (<i>Pisum sativum</i>)	cotyledons	effect of hormones	Locker and Ilan, 1975
Citrus spp.	leaves	polymorphism	Esen and Scora, 1977
dwarf French bean (<i>Phaseolus vulgaris</i>)	cotyledons	effect of hormones	Gepstain and Ilan, 1970
<u>α-amylase</u>			
pea (<i>Pisum sativum</i>)	cotyledons	purification and properties	Swain and Dekker, 1966b
dwarf French bean (<i>Phaseolus vulgaris</i>)	hypocotyl	effect of hormones	Clum, 1967
pea (<i>Pisum sativum</i>)	seedlings	activity and patterns	Shain and Mayer, 1968
maize (<i>Zea mays</i>)	seedlings	allelic expression and physico-chemical properties	Chao and Scandalios, 1971

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>α-amylase (cont.)</u>			
pea (<i>Pisum sativum</i>)	cotyledons	patterns during seed germ.	Juliano and Varner, 1969
pea (<i>Pisum sativum</i>)	cotyledons	possible origin during seed germ.	Swain and Dekker, 1969
rice (<i>Oryza sativa</i>)	grain	patterns and activity during seed germ.	Baun et al, 1970
barley (<i>Hordeum vulgare</i>)	aleurone layer	patterns and effect of GA	Jacobsen et al, 1970
barley (<i>Hordeum vulgare</i>)	embryo-less half seeds	patterns and effect of GA	Tanaka and Akazawa, 1970
rice (<i>Oryza sativa</i>)	seedlings	patterns and effect of GA	Tanaka et al, 1970
pea (<i>Pisum sativum</i>) and wheat (<i>Triticum</i> spp.)	seedlings	activity	Abbot and Matheson, 1972
barley (<i>Hordeum distichon</i>)	grain	effect of sugar level on GA level and enzyme activity	Briggs and Clutterbuck, 1973
pea (<i>Pisum sativum</i>)	cotyledons	patterns and effect of hormones	Yomo and Varner, 1973
pea (<i>Pisum sativum</i>)	embryonic axis	activity during seed germ.	Davis, 1977

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>α-amylase (cont.)</u>			
broad bean (<i>Vicia faba</i>)	pod	patterns during seed develop.	Müntz, 1977
barley (<i>Hordeum vulgare</i>)	aleurone layer	patterns during seed develop.	Rodaway, 1978
pea (<i>Pisum sativum</i>)	embryonic axis	influence of cotyledons on its synthesis	Davis, 1979
green gram (<i>Phaseolus vidissumis</i>)	seedlings	purification and properties	Yamasaki and Suzuki, 1979
<i>Vigna mungo</i>	cotyledons	patterns and activity during seed germ.	Koshiba and Minamikawa, 1980
soyabean (<i>Glycine max</i>)	seeds and seedlings	activity and patterns during seed develop. and germ.	Adams et al, 1981a
<i>Vigna mungo</i>	cotyledons	purification and properties	Koshiba and Minamikawa, 1981
various	seeds	effect of Ca^{2+} on its activity	Irshad and Sharme, 1981
various	seeds	effect of Zn^{2+} on its activity	Irshad et al, 1981

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>α-amylase (continued)</u>			
wheat (<i>Triticum aestivum</i>)	kernels	patterns and effect of GA and seed coat on its synthesis	Marchylo et al, 1981
rice (<i>Oryza sativa</i>)	scutellar epithelium	de novo synthesis	Miyata et al, 1981
okra (<i>Abelmoschus esculentus</i>)	seeds	activity during seed develop.	Bhalla et al, 1983
barley (<i>Hordeum vulgare</i>)	seeds	patterns, purification and effect of GA	Callis and Ho, 1983

β -amylase

soyabean (<i>Glycine max</i>)	seeds	purification and properties	Gertler and Birk, 1965
pea (<i>Pisum sativum</i>)	embryonic axis	properties	Swain and Dekker, 1966a
pea (<i>Pisum sativum</i>)	seedlings	patterns and activity	Shain and Mayer, 1968
pea (<i>Pisum sativum</i>)	cotyledons	patterns during seed germ.	Juliano and Varner, 1969
rice (<i>Oryza sativa</i>)	grain	activity during seed develop.	Baun et al, 1970

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>β-amylase (cont.)</u>			
pea (<i>Pisum sativum</i>) and wheat (<i>Triticum</i> spp.)	seedlings	activity	Abbot and Matheson, 1972
pea (<i>Pisum sativum</i>)	cotyledons	patterns and activity during seed germ.	Yomo and Varner, 1972
soyabean (<i>Glycine max</i>)	seeds	patterns, cultivar markers and relation to morphological features	Morita and Yagi, 1979
green gram (<i>Phaseolus vidissimus</i>)	seedlings	activity and purification	Yamasaki and Suzuki, 1979
soyabean (<i>Glycine max</i>)	seeds	genetics	Hilderbrand and Hymowitz, 1980
soyabean (<i>Glycine max</i>)	seeds	activity during seed develop.	Adams et al, 1980
soyabean (<i>Glycine max</i>)	seeds and seedlings	activity in different varieties	Adams et al, 1981b
okra (<i>Abelmoschus esculentus</i>)	seeds	activity during seed develop.	Bhalla et al, 1983

Branching enzyme

pea (<i>Pisum sativum</i>)	seeds	patterns and activity during seed develop.	Matters and Boyer, 1981
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Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Catalase</u>			
maize (<i>Zea mays</i>)		in vitro hybridization	Scandalios, 1965
wheat (<i>Triticum aestivum</i>)	seeds and seedlings	patterns and activity during seed develop. and germ.	Macko et al, 1966
maize (<i>Zea mays</i>)	seedlings	turnover during seed germ.	Qual and Scandalios, 1971
peanut (<i>Arachis hypogaea</i>)	seeds and seedlings	patterns during seed develop. and germ.	Cherry and Ory, 1973
cotton (<i>Gossypium hirsutum</i>)	cotyledons	activity during seed develop. and germ.	Choinski and Trelease, 1978
cucumber (<i>Cucumis sativus</i>)	seeds	activity during seed develop.	Kindle et al, 1980

Glutamate dehydrogenase

wheat (<i>Triticum aestivum</i>)	seeds and seedlings	patterns during seed develop. and germ.	Macko et al, 1966
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Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Glutamate dehydrogenase (cont.)</u>			
various	seeds	patterns and intracellular location	Yue, 1969
pea (<i>Pisum sativum</i>)	shoot and root	patterns, purification and molecular structure	Pahlich, 1972
broad bean (<i>Vicia faba</i>)	seedlings	patterns and activity	Fawole, 1977
black eye bean (<i>Vigna unguiculata</i>)	seedlings	purification, properties and molecular structure	Fawole and Boulter, 1977
pea (<i>Pisum sativum</i>)	shoot	activity and intracellular location	Nauen and Hartmann, 1980
soyabean (<i>Glycine max</i>)	seeds	patterns, purification and properties	McKenzie and Lees, 1981

Glutamate-oxaloacetate transaminase

maize (<i>Zea mays</i>)	seedlings	estimation of mating system	Brown and Allard, 1970
<i>Pinus sylvestris</i>	needle	genetics	Rudin, 1975
avacodo (<i>Persea americana</i>)	mesocarp	genetics	Torres et al, 1978a
Citrus spp.	seedling leaves	genetic markers	Torres et al, 1978b

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Glutamate-oxaloacetate transaminase (cont.)</u>			
maize (<i>Zea mays</i>)	pollen	genetics	Sari-Gorla et al, 1983
tomato (<i>Lycopersicon</i> spp.)	seedlings	selection	Zamir and Vallejos, 1983

Lactate dehydrogenase

soyabean (<i>Glycine max</i>)	cotyledons	purification, properties and activity during seed germ.	King, 1970
potato (<i>Solanum</i> tuberosum)	tubers	patterns and properties	Rothe, 1974
potato (<i>Solanum</i> tuberosum)	tubers	patterns, purification and molecular structure	Rothe et al, 1978
potato (<i>Solanum</i> tuberosum)	tubers	patterns, purification and molecular structure	Poerio and Davies, 1980
soyabean (<i>Glycine max</i>)	seeds	purification and molecular structure	Jervis et al, 1981

Leucine-aminopeptidase

pea (<i>Pisum sativum</i>)	various	patterns, genetics and activity during seed germ.	Scandalios and Espiritu, 1969
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Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Leucine-aminopeptidase (cont.)</u>			
peanut (<i>Arachis hypogaea</i>)	seeds and seedlings	patterns during seed develop. and germ.	Cherry and Ory, 1973
Lupinus spp.	leaves	relationship among species	Scogin, 1973
<i>Picea abies</i>	seeds	population genetics	Tigerstedt, 1973
mung bean (<i>Vigna radiata</i>)	cotyledons	activity during seed germ.	Chrispeels and Boulter, 1975
pea (<i>Pisum sativum</i>)	seeds and seedlings	patterns and activities during seed develop. and germ.	Collier and Murray, 1977
<i>Picea abies</i>	needle and endosperm	population genetics	Lundkvist and Rudin, 1977
avocado (<i>Persea americana</i>)	mesocarp	genetics	Torres et al, 1978a
pea (<i>Pisum sativum</i>)	embryonic axis	activity of the different forms during seed germ.	Murray et al, 1979

Malate dehydrogenase

wheat (<i>Triticum aestivum</i>)	seeds and seedlings	patterns during seed develop. and germ.	Macko et al, 1966
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Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Malate dehydrogenase (cont.)</u>			
various	various	patterns	Weimberg, 1968
pea (<i>Pisum sativum</i>)	roots	intracellular distribution and effect of saline media	Hason-Porath and Pojakkof-Mayber, 1969
pea (<i>Pisum sativum</i>)	seeds	patterns and effect of saline media	Weimberg, 1967 and 1970
<i>Eucalyptus obliqua</i>	seeds	estimation of mating system	Brown et al,
cotton (<i>Gossypium hirsutum</i>)	cotyledons	activity during seed develop. and germ.	Choinski and Trelease, 1978
broad bean (<i>Vicia faba</i>)	epidermis	activity	Outlaw et al, 1981

Non-specific esterase

maize (<i>Zea mays</i>)		in vitro hybridization	Schwartz, 1964
pea (<i>Pisum sativum</i>)	seedlings	patterns and genetics	Frankeland and Garber, 1965
maize (<i>Zea mays</i>)	seeds	allelic variation	Schwartz et al, 1965
wheat (<i>Triticum aestivum</i>)	seeds and seedlings	patterns during seed develop. and germ.	Macko et al, 1966

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Non-specific esterase (cont.)</u>			
maize (<i>Zea mays</i>)	seedlings	estimation of mating system	Brown and Allard, 1970
maize (<i>Zea mays</i>)	seedlings	isoenzyme variation and selection	Brown, 1971
peanut (<i>Arachis hypogaea</i>)	seeds and seedlings	patterns during seed develop. and germ.	Cherry and Ory, 1973
<i>Picea abies</i>	seeds	population genetics	Tigerstedt, 1973
pea (<i>Pisum sativum</i>)	seeds	patterns	Collier and Murray, 1977
<i>Picea abies</i>	needle and endosperm	population genetics	Lundkvist and Rudin, 1977
broad bean (<i>Vicia faba</i>)	cotyledons, pollen and leaves	patterns and effect of nitrogen regime	Gates and Boulter, 1979a
broad bean (<i>Vicia faba</i>)	seeds	cultivar markers and patterns during seed develop.	Gates and Boulter, 1979b
broad bean (<i>Vicia faba</i>)	pollen	cultivar markers	Gates and Boulter, 1980
<i>Ipomoea purpurea</i>	seeds and seedlings	outcrossing rate	Ennos and Clegg, 1982

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Peroxidase</u>			
wheat (<i>Triticum aestivum</i>)	seeds and seedlings	patterns during seed develop. and germ.	Macko et al, 1966
pea (<i>Pisum sativum</i>)	root	patterns and effect of growth media	Weiberg, 1970
maize (<i>Zea mays</i>)	seedlings	isoenzyme variation and selection	Brown et al, 1971
tomato (<i>Lycopersicon esculentum</i>)	fruit	genetics and population genetics	Rick et al, 1974
<i>Petunia</i> spp.	leaves	patterns and taxonomy	Natarella and Sink, 1975
<i>Citrus</i> spp.	roots	breeding and taxonomy	Button et al, 1976
pea (<i>Pisum sativum</i>)	seedlings	patterns, purification and substrate specificity	Gibson and Liu, 1978

Phosphorylase

pea (<i>Pisum sativum</i>)	cotyledons	activity during seed germ.	Swain and Dekker, 1966
maize (<i>Zea mays</i>)	endosperm	patterns and role	Tsai and Nelson, 1968

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Phosphorylase (cont.)</u>			
pea (<i>Pisum sativum</i>)	cotyledons	patterns and activity during seed germ.	Juliano and Varner, 1969
maize (<i>Zea mays</i>)	seeds	patterns, tissue specificity and activity during seed develop.	Tsai and Nelson, 1969
maize (<i>Zea mays</i>)	endosperm	activity during seed develop.	Tsai et al, 1970
various	various	patterns	Gerbrandy and Varleur, 1971
pea (<i>Pisum sativum</i>)	cotyledons	patterns during seed germ.	Yomo and Varner, 1973
pea (<i>Pisum sativum</i>)	various	purification, properties and activity during seed develop. and germ.	Matheson and Richardson, 1976
broad bean (<i>Vicia faba</i>)	pod	patterns during develop.	Müntz, 1977
various	various	purification, molecular structure and activity during seed germ.	Richardson and Matheson, 1977

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Phosphorylase (cont.)</u>			
pea (<i>Pisum sativum</i>) and	leaves	patterns and intracellular location.	Steup and Latzko, 1979
spinach (<i>Spinacia oleracea</i>)	leaves		
<i>Vigna mungo</i>	cotyledons	patterns during seed germ.	Koshihira and Minawikawa, 1980
<u>Protease</u>			
pea (<i>Pisum sativum</i>)	cotyledons	forms and activity during seed germ.	Beevers, 1968
pea (<i>Pisum sativum</i>)	cotyledons	activity during seed germ.	Guardiola and Sutcliffe, 1971
pea (<i>Pisum sativum</i>)	cotyledons	activity during seed germ.	Yomo and Varner, 1973
pea (<i>Pisum sativum</i>)	cotyledons	activity and substrate specificity during seed germ.	Basha and Beevers, 1975

Table 1 (continued)

<u>Plant(s)</u>	<u>Tissue(s)</u>	<u>Remarks</u>	<u>Reference</u>
<u>Protease (cont.)</u>			
mung bean (<i>Vigna radiata</i>)	cotyledons	effect of embryonic axis on its activity during seed germ.	Kern and Chrispeels, 1978
<u>R-enzyme</u>			
pea (<i>Pisum sativum</i>)	cotyledons and embryonic axis	activity during seed germ.	Swain and Dekker, 1966
pea (<i>Pisum sativum</i>)	seedlings	mechanism of production	Shain and Mayer, 1968
pea (<i>Pisum sativum</i>)	cotyledons	patterns during seed germ.	Juliano and Varner, 1969
rice (<i>Oryza sativa</i>)	grain	patterns and activity during seed develop.	Baun et al, 1970
various	various	patterns	Gerbrandy and Varleur, 1971
<u>Starch synthetase</u>			
pea (<i>Pisum sativum</i>)	seeds	patterns and activity during seed develop.	Matters and Boyer, 1981
<u>Urease</u>			
jack bean (<i>Canavalia ensiformis</i>)	seeds	patterns	Shaik-Metal, 1980

Abbreviations:-

develop.: development

germ.: germination

MATERIALS AND METHODS

1. Biological materials

Seeds of the different inbred lines of pea were supplied by Dr Stig Blixt, Weibullsholm Plant Breeding Institute, Landskrona, Sweden.

Ground meals of F₂ seeds, from the cross between the inbred lines 1238 and 1293, were also obtained from the same source and were stored in sealed containers at room temperature.

The seeds of pea (Pisum sativum L.) Var. Mangetout and the seed meals of the var. Feltham First, at different stages of seed development, were kindly supplied by Dr J. Gatehouse, Department of Botany, University of Durham, South Road, Durham DH1 3LE, England.

The following is a list of the different legume seeds studied.

Pea (Pisum sativum L. var. Feltham First)

Pea (Pisum sativum L. var. English Wonder)

Dwarf French bean (Phaseolus vulgaris, var. Processor)

Dwarf French bean (Phaseolus vulgaris, var. Masterpiece)

Soyabean (Glycine max)

Pigeon pea (Cajanus cajan)

Broad bean (Vicia faba L.)

Black eye bean (Vigna unguiculata)

Jack bean (Canavalia ensiformis)

Bean (Vicia narbonensis)

Scarlet runner bean (Phaseolus coccineus)

Mung bean (Vigna radiata)

Pea (Pisum elatius)

Seeds and seed meals were usually stored in sealed containers at 4°C.

2. Chemicals and reagents

Most chemicals were obtained from BDH Chemicals Ltd, Poole, Dorset, U.K. and were used as supplied.

The following chemicals were obtained from Sigma Chemical Company, Poole, Dorset, U.K.:

- trizma base (tris (hydroxy methyl) amino methane)
- L-malic acid (L-hydroxy succinic acid)
- Potassium iodide
- L-glutamic acid (L- α aminoglutaric acid)
monosodium salt
- o-dianisidine, tetrazotized (fast blue B salt: diazo blue B; fast blue salt BN). Zn Cl₂ complex
- β -amylase (α -1 ~~glu~~-glucan maltohydrolase; E.C.No. 3.2.1.2) from barley
- α -Ketoglutaric acid, free acid
- α -amylase (1,4-alpha-D-glucan glucanohydrolase, E.C.No. 3.2.1.1) from porcine pancreas, type 1-A: DFP treated
- D-glucose-6-phosphate, monosodium salt
- L-aspartic acid
- DL-lactic acid, sodium salt
- pyridoxal-5-phosphate (codecarboxylase)

- α -D-glucose-1-phosphate, dipotassium salt
- α -naphthyl acetate
- β -nicotinamide adenine dinucleotide (β -NAD; β -DPN)
 β -diphosphopyridine nucleotide from yeast
- phenazine methosulphate
- nitroblue tetrazolium (2,2' Di-p-nitrophenyl-5,5'diphenyl-3,3' -
(3,3'-dimethoxy-4,4'diphenylene) ditetrazolium chloride)
- N-acetyl-DL-phenylalanine β -naphyl ester
- azo-albumin (sulfanilic acid-azoalbumin) synthesized from bovine
albumine, fraction v
- fast blue B salt
(diazotized-4'-aminol-2', 5-dimethoxybenzanilide zinc chloride salt)
- α -N-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA)
- agarose
- Coomassie brilliant blue R25°
- amylopectin from potato
- Amberlite IRA-400(CO₃²⁻)
- Dowex-5-(H⁺)

- 3.5 dinitrosalicylic acid
- sodium potassium tartrate tetrahydrate
- S-200 (sephacryl) was obtained from Pharmacia Fine Chemicals,
Uppsala, Sweden
- hydroxylapatite was obtained from micro-Bio Laboratories Limited, 46
Pembride Road, London W11
- L-leucyl- β -naphthylamide HCl, puriss, CHR, A.R. was obtained from
Koch-Light Laboratories Ltd, Colnbrook

3. Seed germination

All seeds, apart from peas, were allowed to imbibe water for 24 hours at room temperature. They were then distributed over alkathene polyethylene beads in a polyethylene tray, lined with a paper towel. The tray was maintained in a dark growth room at 28°C with high relative humidity. The seeds were showered automatically at regular intervals. Prior to harvesting, the seedlings were exposed to light in the laboratories for 2 hours.

The cotyledons of pea seedlings, which were used for total protein studies, were frozen in liquid air and stored at -20°C for 3 days, then were freeze-dried.

4. Growth of plants

The plants were grown in plastic pots containing compost (Levington: John Innes No.2 = 1:3 parts) in the Botanic Garden of Durham University at a minimum temperature of 14°C.

5. Pollination

5.1 Pollination of *Pisum sativum* with mixed pollen samples

Pollen samples of variety Meteor and inbred line 1238 were collected from newly-opened flowers, and stored at -20°C for periods of up to two weeks until required. Weighed subsamples were mixed in various ratios by vigorously shaking tubes containing pollen of the

two genotypes.

Recipient flowers were emasculated before their anthers dehisced and pollen mixtures were applied to the stigmas using a very fine paintbrush. Pollinations were made using replicates of four plants of each genotype. Pollinated stigmas were covered with gelatin capsules for three days, to prevent accidental pollination and to maintain a humid environment for pollen germination. Seeds were collected at maturation (32 days after pollination) and were dried at room temperature.

5.2 Self pollination of Pisum sativum

For self pollinations flowers were emasculated as described above, and then pollinated immediately with fresh pollen. Treated flowers were covered with gelatin capsules, tagged and collected at regular intervals after pollination and were fixed in formalin; acetic acid; ethanol; water (10:5:50:35) and stored at room temperature.

6. Seed collection and storage

The flowers were tagged at the time when they were fully opened and the pods were collected at intervals throughout seed development.

The seeds were dried at room temperature and were stored at 4°C.

The mature seeds of broad bean that were used to study the effect of storage on isoenzyme patterns were collected freshly and stored in sealed containers at different conditions for different periods of time.

7. Seed meals

After removing the seed coat, the seeds were finely ground in a ball mill and stored in sealed containers at 4°C.

8. Solutions

8.1 Buffers

All buffers were stored in sealed dark bottles at 4°C.

8.2 Tracking dye

The tracking dye was prepared by dissolving 10mg of bromophenol blue in 1ml ethanol.

8.3 1% (w/v) Starch solution

1.5g of soluble starch was boiled in 150ml 0.2M sodium acetate buffer pH 5.0 or 0.2M sodium phosphate buffer pH 5.0. As the solution cooled, the volume was brought to 150ml using the same buffer.

8.4 Substrate for phosphorylase

50mg of soluble starch was boiled in 50ml 0.1M sodium citrate buffer pH 6.0 to give a clear solution. As the solution cooled, 0.33g of glucose-1-phosphate was dissolved in the solution and the volume was brought to 50ml using the same buffer.

8.5 Substrate for debranching enzyme

300mg of amylopectin was boiled in 100ml 0.2M sodium acetate buffer pH 5.0. As the solution cooled, the volume was brought to 100ml using the same buffer.

8.6 Agarose solution for second dimensional gel electrophoresis

50mg of agarose was heated with 5ml 0.2M tris/HCl buffer pH 6.8 containing 2% (w/v) sodium dodecyl sulphate (SDS) and this solution was prepared just before starting the second dimensional electrophoresis.

8.7 Dinitrosalicylic acid colour reagent

1.0g 3,5 dinitrosalicylic acid was dissolved in 20ml 2.0M sodium hydroxide. 30.0g sodium potassium tartrate tetrahydrate was added with stirring. The solution was brought to a final volume of 100ml using distilled water. This solution was stored in a sealed bottle and stored at room temperature for no longer than 2 weeks.

8.8 Glycogen reagent

The glycogen reagent was prepared as described by Loyter and Schramm (1962).

1.0g of glycogen was boiled in 50ml distilled water. The solution was cooled and brought to a final volume of 50ml by the addition of distilled water. The solution was centrifuged at 12,000 rpm for 10 min. To the clear supernatant, 2.5g of Amberlite IRA-400(CO_3^{2-}) and 2.5g of Dowex-50(H^+) were added. The mixture was stirred for 30min.

and the resins were removed by centrifugation. The process was repeated by the addition of fresh resins, followed by centrifugation. The glycogen reagent was stored in sealed bottles at -20°C .

9. Electrophoresis

Discontinuous, vertical acrylamide gel electrophoresis was performed according to the method of Davis (1963) and Ornstein (1964).

The separating gel mixture was degassed before adding the ammonium persulphate and TEMED. Then the mixture was poured into the apparatus up to a marked line avoiding trapping any air bubbles. A layer of distilled water was layered on top of the gel by using a syringe. After the gel was polymerized, the layer of the water was removed. The spacer gel mixture was degassed, before adding ammonium persulphate and TEMED. Then the mixture was layered on top of the separating gel and immediately the well former was inserted into the apparatus. As the gel was polymerized, the well former and bottom spacer were removed. The bottom of each well was marked on the glass. Then the mould was fixed in the apparatus using four strong clips. The electrode compartments were filled with the developing buffer and air bubbles were completely removed from the bottom of the gel by using a syringe with a bent needle. A few drops of bromophenol blue were added to the upper electrode compartment and the crude extract loaded on top of the gel in the wells using a $100\mu\text{l}$ syringe with a long needle.

Development was carried out at room temperature and at constant current 15ma for the first 30min., until the enzymes had entered the

separating gel, and then at 25ma. The run was terminated as the tracking dye reached the bottom of the gel. The mould was then removed from the apparatus and the gel removed from the mould.

Usually 40 μ l crude extract was added in each sample well, but when the enzyme was very active only 20 μ l crude extract was loaded.

When the enzyme activity was low 50 μ l or 60 μ l crude extract was loaded.

9.1 Non SDS-acrylamide gel

9.1.1 Separating gel

The 7.5% (w/v) separating gel contained in a total volume of 58.5ml, the following:

acrylamide	4.5g
N,N,N methylenebisacrylamide (Bis)	0.1g
1M tris/HCl buffer pH 8.8	22.5ml
ammonium persulphate (15mg/ml H ₂ O)(freshly prepared)	2.0ml
N,N,N,N tetramethyl ethylene-diamine (TEMED)	20.0 μ l
and distilled water	34.0ml

9.1.2 Spacer gel

The 4.5% (w/v) spacer gel contained in a total volume of 20ml, the following:

acrylamide	0.9g
N,N,N methylenebisacrylamide (Bis)	25.0mg
1M tris/HCl buffer pH 6.8	2.5ml
ammonium persulphate (15mg/ml H ₂ O) (freshly prepared)	0.5ml
N,N,N,N tetramethyl ethylene-diamine (TEMED)	20.0µl
and distilled water	17.0ml

9.1.3 Development buffer

The development buffer was tris/glycine pH 8.3

tris.	3.0g
glycine	14.1g
and distilled water	1L

9.2 SDS-acrylamide gel

9.2.1 Separating gel

The 17% (w/v) separating gel contained in a total volume of 58.5ml, the following:

acrylamide	10.2g
N,N,N methylenebisacrylamide (Bis)	0.1g
1M tris/HCl buffer pH 8.8	22.5ml
10% (w/v) sodium dodecyl sulphate (SDS)	0.6ml
ammonium persulphate (15 mg/ml H ₂ O) (freshly prepared)	2.0ml
N,N,N,N tetramethyl ethylene diamine (TEMED)	20.0µl
and distilled water	33.4ml

The 12.5% (w/v) separating gel contained 7.2g acrylamide instead of 10.2g.

9.2.2 Spacer gel

As in section 8.1.2 except 0.2ml of 10% (w/v) SDS was added

9.2.3 Development buffer

The development buffer was:

tris	3.0g
glycine	14.1g
sodium dodecyl sulphate (SDS)	1.0g
and distilled water	1L

10. Second dimensional gel electrophoresis

The bands of total protein were first separated on 17% SDS-acrylamide gel, under non-reducing conditions, following the procedure in section 8 and thin spacers were used (thinner than the spacers used for the second dimensional gel). Following development the gel was stained in Coomassie blue solution overnight and destained in destaining solution. After that the gel was cut into long strips.

The strip to be analysed by second dimension was incubated in 15ml 0.2M tris/HCl buffer pH 6.8 containing 2% (w/v) SDS and 4% (v/v) 2-mercaptoethanol; the latter was freshly added to the buffer, at room temperature for 1h.

The 17% SDS-acrylamide gel was prepared following the procedure in section 8, except that a layer of distilled water was layered on top of the stacking gel and a 1cm space was left on top of the stacking gel and the well former was not used. After the gel was polymerized,

the layer of the water was removed and the gel strip was placed gently on top of the gel and all air bubbles between the two gel surfaces were removed by pressing the strip with a thin spatula, then a few mls of a hot agarose solution were pipetted over the gel strip and allowed to gel and the gel was developed as in section 8.

11. Enzyme preparation

11.1 Dry seeds

The enzyme was extracted by homogenizing 20mg of seed meal in 100 μ l 0.075M, pH 7.25 chilled sodium phosphate buffer containing 20% (w/v) sucrose in a polyethylene tube, overnight at 4°C. The crude extract was centrifuged for 5 minutes at 500g and the clear supernatant was used for electrophoretic analysis.

In the case of pea F₂ progeny seed meals, 10mg of the meal was homogenized in 100 μ l of the same buffer.

11.2 Pollen

As above.

11.3 Seedlings

The seedlings were divided into cotyledons, leaves, roots, epicotyl, hypoc^otyl and stem and then they were cut into small segments after removing the seed coats from the cotyledons. The tissues were ground in a mortar with the same buffer as above, in a ratio of 1:5 (w/v), and maintained in sealed tubes, overnight at 4°C and

centrifuged as above before use.

12. Total protein extraction

The total protein was extracted by homogenizing the required weight of cotyledon meal in the appropriate volume of 0.2M pH 6.8 chilled tris/HCl buffer containing 20% (w/v) sucrose and 2% (w/v) SDS in a polyethylene tube, overnight at 4°C. The crude extract was centrifuged for 5min. at 500g and the clear supernatant was incubated in a water bath at 100°C for 2min. and used for electrophoretic analysis under non-reducing conditions, while under reducing conditions a 4 μ l 2-mercaptoethanol was added to each 100 μ l extract.

13. Exceptions to this general procedure

13.1 Phosphorylase

13.1.1 Enzyme preparation

20mg of dry seed meal were homogenized in 80 μ l 0.2M tris/HCl buffer pH 6.8 in polyethylene tube for 4h. at 4°C. The crude extract was centrifuged at 500g for 5min. at 4°C. The supernatant was used for electrophoresis after adding one drop of glycerol.

13.1.2 Separating gel

The 10% (w/v) gel contained in total volume of 58.5ml, the following:

acrylamide	6.0g
N,N,N,methylenebisacrylamide (Bis)	27.0mg
1M tris/HCl buffer pH 7.2	22.5ml
ammonium persulphate (15mg/ml H ₂ O) (freshly prepared)	1.5ml
N,N,N,N tetramethyl ethylene-diamine (TEMED)	20.0 μ l
and distilled water	34.5ml

Development was carried out at room temperature for screening lines and at 4°C for studying the isogenic pairs.

13.2 Leucine-amino peptidase and esterase

In addition to the usual procedure, electrophoresis was also performed according to Collier and Murray (1977). The enzymes were separated on 5.5% (w/v) acrylamide gel equilibrated with 0.075M tris/citrate buffer pH 8.6. The development buffer was 0.3M sodium borate pH 8.2.

13.3 Malate dehydrogenase

Besides the usual procedure for enzyme extraction, the enzyme was extracted in the same extraction buffer, containing 2M KCl and 0.02 M EDTA.

13.4 Protease

13.4.1 Enzymes extraction

As described in section 11.1 but using 0.06M tris/citric acid buffer pH 8.6 containing 10% (w/v) sucrose.

13.4.2 Separating gel

The 12% (w/v) separating gel contained in a total volume of 58.5ml, the following:

acrylamide	7.2g
N,N,N,methylene bis acrylamide (Bis)	100.0mg
0.3M tris/citric acid buffer pH 8.6	15.0ml
ammonium persulphate (15mg/ml H ₂ O) (freshly prepared)	2.0ml
N,N,N,N tetramethyl ethylene-diamine (TEMED)	20.0 μ l
and distilled water	43.0ml

No spacer gel was used and the development buffer was 0.3M sodium borate pH 8.6.

14. Staining method

14.1 Enzymes

Following electrophoresis, the acrylamide gels were incubated in 50ml of chilled buffer used for enzyme assay, for 3min. at 4°C for band stabilization.

The gels were incubated in the staining solution for bands development.

Table 2 lists all the enzymes studied with their abbreviation and international numbers.

The standard method for detecting EST, LAP and GOT activities on the gel was that of Gales (1978). The procedure of Collier and Murray was also employed for LAP and EST.

Detection of dehydrogenase activities was based on the method of

Scandalios (1969).

The activities of PER, catalase and amylase were detected on the gel following the procedure of Brewbaker et al (1963), with slight modifications for the latter.

Protease activity was detected on the gel by the method described by Filho and Moreiva (1978).

Table 2: Enzymes studied

<u>Enzyme</u>	<u>Abbreviation</u>	<u>International Enzyme Number</u>
Alcohol dehydrogenase	ADH	E.C.1.1.1.1.
Leucine-aminopeptidase	LAP	E.C.3.4.1.1.
Amylase		
α -Amylase		E.C.3.2.1.1.
β -Amylase		E.C.3.2.1.2.
Catalase		E.C.1.11.1.6
Non-specific esterase	EST	E.C.3.1.1.2
Glutamate dehydrogenase	GDH	E.C.1.4.1.2
Glucose-6-Phosphate dehydrogenase		E.C.1.1.1.49
Lactate dehydrogenase	LDH	E.C.1.1.1.27
Malate dehydrogenase	MDH	E.C.1.1.1.37
Peroxidase	PER	E.C.1.11.1.7
Phosphorylase		E.C.2.4.1.1.
Glutamate-oxaloacetate transaminase (syn. Aspartate aminotransferase)	GOT	E.C.1.11.1.7
Protease		
Debranching enzyme (R-enzyme)		E.C.3.2.1.9

14.1.1 Amylase

The gel was incubated at room temperature for 2h. in 150ml, freshly prepared, 1.0% (w/v) soluble starch in 0.2M sodium acetate buffer pH 5.0. The gel was then washed, twice, in 0.1M acetic acid, and stained in 100ml 0.1M acetic acid containing 0.1g KI and 20mg I₂.

14.1.2 Phosphorylase

The gel was incubated in 50ml, 0.1M sodium citrate buffer, pH 6.0 containing 50mg soluble starch and 0.33g glucose-1-phosphate, for 3h., at room temperature. Then the gel was stained in 100ml solution of 14mM KI and 10mM I₂.

In another experiment, the gel was incubated in 1.0% (w/v) soluble starch in 0.2M sodium phosphate buffer pH 6.0, for 3h. at room temperature. Then the gel was stained in KI, I₂ solution, as for amylase.

14.1.3 Glutamate dehydrogenase

The staining solution was:

NAD	400.0mg
Phenazine methosulphate	2.0mg
nitroblue tetrazolium	35.0mg
Sodium glutamate	300.0mg
0.1M tris/HCl buffer pH 9.0	75.0ml

14.1.4 Alcohol dehydrogenase

The staining solution was:

NAD	40.0mg
Phenazine methosulphate	2.0mg
nitroblue tetrazolium	35.0mg
ethanol	2.5ml
0.1M tris/HCl pH 9.0 buffer	75.0ml

14.1.5 Malate dehydrogenase

The staining solution was:

NAD	40.0mg
nitroblue tetrazolium	35.0mg
Phenazine methosulphate	2.0mg
L-malic acid	660.0mg
0.1M tris/HCl buffer pH 9.0	75.0ml

14.1.6 Glucose-6-phosphate dehydrogenase

The staining solution was:

NAD	40.0mg
nitroblue tetrazolium	35.0mg
Phenazine methosulphate	2.0mg
glucose-6-phosphate	100.0mg
0.1M tris/HCl buffer pH 9.0	75.0ml

14.1.7 Lactate dehydrogenase

The staining solution was:

NAD	40.0mg
nitroblue tetrazolium	35.0mg
Phenazine methosulphate	2.0mg
Sodium lactate	12.0ml
0.1M tris/HCl buffer pH 9.0	75.0ml

14.1.8 Glutamate-oxaloacetate transaminase

The staining solution was:

L-aspartic acid	500.0mg
Ketoglutaric acid	70.0mg
pyridoxal 5-phosphate	10.0mg
fast blue B salt	200.0mg
0.2M sodium acetate buffer pH 5.0	100.0ml

14.1.9 Protease

The staining solution was:

acetyl-DL-phenylalanine β -naphthyl ester (dissolved in 6.0ml DMF)	15.0mg
fast blue B	30.0mg
0.1M sodium phosphate buffer pH 7.2	54.0ml

14.1.10 Catalase

The gel which contained 0.5% (w/v) soluble starch was incubated in 0.5% (w/v) H_2O_2 solution, for 2min. Then the gel was

stained in 0.5% (w/v) KI, for band development.

14.1.11 Peroxidase

The staining solution was:

O-dianisidine	50.0mg
95% (w/v) ethanol	35.0ml
0.2M sodium acetate buffer pH 5.0	14.0ml
3% (w/v) H ₂ O ₂	1.0ml

14.1.12 Non-specific esterase

α -naphthyl acetate (dissolved in ethanol)	15.0mg
fast blue B salt	90.0mg
0.2M sodium acetate buffer pH 5.0	90.0ml

14.1.13 Leucine-aminopeptidase

L-leucyl- β -naphthylamide (8mg/ml H ₂ O)	3.0ml
0.85% (w/v) NaCl	24.0ml
0.2M KCN	3.0ml
0.2M sodium acetate buffer pH 5.0	30.0ml

14.1.14 Total protein

The staining solution was:

Coomassie blue	1g
methanol	1L
acetic acid (glacial)	150.0ml
distilled water	850.0ml

The gel was incubated in the staining solution overnight, but when small volumes of the sample were used, the bands became faint after destaining, so the gel was incubated in a solution of 1:1 staining solution: destaining solution for several days and destained for 15min. before photographing.

15. Effect of heat on amylase activity

To the clear supernatant, CaCl_2 was added to give a final concentration of 5mM. Then the extract was incubated in water bath at 70°C for 15min. and used for electrophoretic analysis.

16. Effect of EDTA on amylase activity

The acrylamide gel, following development, was incubated in $5 \times 10^{-3} \text{ M}$ EDTA solution for 20min., at room temperature and stained for amylase as usual.

17. Measurement of molecular weight

The molecular weights of the different proteins in crude extracts were estimated, on 17% SDS-acrylamide gel, using the procedure described by Weber and Osborn (1969) using the following protein markers:

<u>Marker</u>	<u>Molecular weight (mol.wt)</u>
Phosphorylase b	94,000
Transferrin	76,000
Albumin	67,000
Chymotrypsinogen	25,000
Myoglobin	17,000
Cytochrome C	12,000

18. Destaining

The destaining solution was:

methanol	500.0ml
acetic acid	70.0ml
distilled water	430.0ml

19. Drying the gels

The gels were dried between two sheets of uncoated cellophane in a gel drier.

20. Preparation of material for fluorescence microscopy

Flowers were collected, and stigmas, styles and ovaries excised and fixed in formalin: acetic acid; ethanol; water (10:5:50:35). The gynecia were hydrolyzed for 1h. at 30°C in 1N KOH, then transferred to a solution of 2% (w/v) aniline blue, then stored at 4°C.

The gynecia were mounted in glycerine, gently squashed and examined for the presence of germinating pollen tubes. Microscopy was carried out using a Nikon Diaphot fluorescence microscope fitted with a violet excitation filter block. Photographs were taken on Kodak Technical Pan film (125ASA), processed according to the manufacturer's instructions.

21. Calibration curves for measuring amylase activity

21.1 Semi-quantitative assay

1.0g agarose and 0.5g soluble starch were boiled in 90ml distilled water. As they dissolved, 10ml of 0.5M sodium acetate buffer pH 6.9 was added. A 10ml aliquot was pipetted into a plastic immunodiffusion plate and allowed to set on a flat surface. The plates were covered and stored at 4°C in a plastic sandwich box with damp tissues to maintain humidity.

21.1.1 Assay protocol

10µl holes were punched in the agarose/starch gel using immunodiffusion well punches and template. A 10µl solution containing amylase was pipetted into the hole. The enzyme was allowed to diffuse into the agarose/starch gel on a level surface at room temperature, for 20h. At the end of the period, the amylase activity was visualised on the gel by covering the gel with iodine staining solution (0.1% (w/v) I₂, 1.0% (w/v) KI in 0.1M acetic acid). The diameter of the hydrolyzed area was measured.

The calibration curve was established by using a series of α -amylase (A4268, Porcine amylase, Sigma) concentrations, and 4 replicates were used for each concentration.

21.2 Quantitative measurement

The reducing groups liberated from starch were measured by the reduction of 3,5 dinitrosalicylic acid.

A starch solution was prepared by boiling 1.0g soluble starch in 100ml 0.02 M sodium phosphate buffer pH 6.9 containing 0.006M sodium chloride. The solution was cooled and brought to a final volume of 100ml by adding the same buffer.

21.2.1 Assay protocol

0.5ml of enzyme solution was pipetted into a test tube and a blank was prepared by using 0.5ml sodium phosphate buffer pH 6.9. A 0.5 ml starch solution was added to each tube. The tubes were incubated at room temperature for exactly 3min. with periodic shaking. At the end of the period, the tubes were cooled to room temperature and 10ml distilled water was added to each tube and mixed well. The optical density of the solution was read at a wave length of 540nm on the spectrophotometer and compared with the blank.

The calibration curve was prepared by using a series of concentrations of α -amylase (A 4268, porcine amylase, Sigma) and 3 replicates were used for each concentration.

22. Fractionation with ammonium sulphate

- 1) Solid ammonium sulphate was added, slowly, to a 100ml extract to bring the homogenate to 40% $(\text{NH}_4)_2\text{SO}_4$ saturation. After the addition of all the solid, the solution was stored for 1h. at 4°C
- 2) The solution was centrifuged at 15,000rpm for 20min. at 4°C.
- 3) The precipitate was dissolved in 0.2M tris/HCl pH 6.9 buffer.
- 4) Steps 1-3 were repeated several times to obtain protein precipitation at 50, 60, 70 and 90% $(\text{NH}_4)_2\text{SO}_4$ saturation.

Each fraction was assayed for amylase activity using the semi-quantitative procedure, then dialyzed against 5L of 0.05M ammonium bicarbonate and lyophilized.

23. Purification of shoot β -amylase

23.1 Enzyme extraction

23.1.1 General method

The shoots of 7 day-old pea seedlings, which were cut into small segments, frozen in liquid air and stored at -20°C, were ground in a mortar with 0.2M tris/HCl pH 6.9 buffer. When a large volume of tissue was extracted, a food mixer was used. The homogenate was stirred overnight at 4°C, then strained through cheese cloth and centrifuged at 15,000 rpm for 20min. at 4°C.

23.1.2 As above, but 2-mercaptoethanol (2.0% v/v) was added.

23.1.3 procedure of Swain and Dekker (1966a)

The frozen tissues were ground in a mortar with 0.05M sodium acetate buffer pH 6.0, containing 0.001M EDTA and 2% (v/v) 2-mercaptoethanol. The homogenate was stirred overnight at 4°C. At the end of the period the homogenate was strained through cheese cloth.

23.2 Ammonium sulphate precipitation

The clear supernatant was brought to 70% $(\text{NH}_4)_2\text{SO}_4$ saturation by the addition, slowly, of crystalline ammonium sulphate with stirring. After complete addition of the salt, the solution was stirred for 1h. at 4°C. The solution was centrifuged at 15,000 rpm for 15min. at 4°C. The clear supernatant was brought to 90% $(\text{NH}_4)_2\text{SO}_4$ saturation by the addition of ammonium sulphate (as above). The solution was centrifuged at 15,000 rpm for 15min. at 4°C. The precipitate was dissolved in 0.2M tris/HCl pH 7.4 and dialyzed against 0.01M tris/HCl pH 7.4 overnight at 4°C. The dialyzed solution was either lyophylized or used directly for ion-exchange chromatography.

23.3 Ion-exchange chromatography

The enzyme solution was applied to a DEAE-cellulose column (45 x 1.5cm) equilibrated with 0.025M tris/HCl pH 7.4 buffer containing 0.1% (w/v) sodium azide. The column was washed with the same buffer for 2h. The protein was eluted with the same buffer containing a linear sodium chloride gradient from 0 to 0.5M. The eluate was collected every

20min. at a flow rate of 20ml/h. The fractions containing enzyme activity (as assayed by the semi-quantitative procedure) were combined, dialyzed against 0.05M ammonium bicarbonate and lyophilized.

23.4 Gel filtration

The protein powder containing enzyme activity was dissolved in 0.025M tris/HCl pH 7.4 buffer and applied to an S-200 gel filtration column (48 x 1.5cm) equilibrated with the same buffer containing 0.1% (w/v) sodium azide. The eluate was collected every 20min. at a flow rate of 5ml/h. The fractions containing enzyme activity were combined, dialyzed against 0.5M ammonium bicarbonate and lyophilized.

24. Purification of mature pea cotyledon β -amylase

24.1 Enzyme extraction

7.5g cotyledon powder was treated with ten times its volume of hexane to remove pigments, then filtered. 60ml of 0.2M tris/HCl pH 6.9 buffer was added to the powder and stirred overnight at 4°C. The homogenate was strained through cheese cloth and centrifuged at 15,000 rpm for 20min. at 4°C.

24.2 Ammonium sulphate precipitation

The supernatant was brought to 40% $(\text{NH}_4)_2\text{SO}_4$ saturation and centrifuged (as described before). The precipitate was dissolved in 0.2M tris/HCl pH 6.9 buffer, dialyzed against 0.05M ammonium bicarbonate and lyophilized.

24.3.1 Ion-exchange chromatography

The ion-exchange chromatography was carried out as described in section 23.3.

25. Purification of α -amylase from germinated cotyledons

The procedure of Swain and Dekker (1960) was used until step 9.5 with slight changes. An hydroxylapatite column was used in this study instead of calcium phosphate-cellulose column.

25.1 Enzyme extraction

The cotyledons (from 7 day-old seedlings), which were frozen in liquid nitrogen and stored at -20°C , were ground with 0.025M tris/HCl pH 7.4 buffer (in a ratio of 1/5 w/v) in a mortar or food mixer and then stirred overnight at 4°C . The homogenate was strained through cheese cloth and centrifuged at 15,000 rpm for 20min. at 4°C .

25.2 Precipitation with calcium chloride

0.5M CaCl_2 was added to the supernatant (4ml CaCl_2 /100ml extract) and the mixture was heated with constant stirring for 15min. at 70°C . The solution was centrifuged at 15,000 rpm for 15min. at 4°C . The clear supernatant was dialyzed against 0.01M tris/HCl pH 7.4 buffer and lyophylized. The powder was stored in a sealed bottle at -20°C .

25.3 Alcohol precipitation

50mg samples of the lyophylized powder were added to a 1.25ml

plastic centrifuge tube. The powder was dissolved in 0.67ml 0.025M tris/HCl pH 7.4 buffer. 0.45 ml cold absolute ethanol was added to each tube, with stirring. The tubes were allowed to stand at -20°C for 1h. with occasional stirring. The solution was centrifuged at 3,500 rpm for 15min. at 4°C .

25.4 Precipitation with glycogen reagent

To each clear supernatant, 0.05ml of 0.025M tris/HCl pH 7.4 buffer was added followed by 0.025ml glycogen reagent and 0.05ml of cold absolute ethanol. The mixture was stirred for 10min. at -20°C and then centrifuged at 3,500 rpm for 15min. at 4°C . The glycogen pellet present in each tube was washed with 0.5ml mixture of 40% ethanol and 60% 0.001M sodium acetate pH 5.8 buffer containing 0.005M CaCl_2 . Each pellet was dissolved in 0.0125 ml of 0.01M sodium acetate buffer pH 5.8 containing 0.005M CaCl_2 . The tubes were allowed to stand at 37°C for 1h. then they were combined.

25.5 Hydroxylapatite column chromatography

The enzyme solution was applied to a hydroxylapatite column (1 x 4.5cm) equilibrated with 0.01M sodium phosphate buffer pH 6.5. The column was first washed with 16ml of 0.01M sodium phosphate buffer pH 6.5. The enzyme was eluted with 0.06M sodium phosphate buffer pH 6.5. The fractions were collected every 20min. at a flow rate of 6ml/h. The fractions containing enzyme activity were dialyzed against 0.05M ammonium bicarbonate overnight at 4°C and lyophylyzed.

RESULTS

1. Phosphorylase activity

In gels incubated in 1% (w/v) starch in 0.1M sodium citrate buffer pH 6.0, containing 0.33% (w/v) glucose-1-phosphate and stained in I₂/KI solution, the sites of phosphorylase activity stained dark blue, in a slightly blue background, due to the newly synthesized starch by phosphorylase in the presence of glucose-1-phosphate (see fig. 1); sites of phosphorylase activity were colourless in a blue background in gels incubated in 1% (w/v) starch solution in 0.2M sodium phosphate buffer pH 6.0 and stained with I₂/KI solution (see fig. 8b). This was due to the breakdown of starch by the activity of phosphorylase in the presence of high concentration of orthophosphate.

Phosphorylase activity was not detected in gels which were incubated in 1% (w/v) starch in 0.2M sodium acetate buffer pH 5.0 and stained in I₂/KI solution.

Prolonged incubation of gels in the destaining solution, resulted in fading of the bands. The same result occurred if dried gels were stored for a long time, but they can be restained with I₂/KI solution even after two years.

1.1 Pea seed extract

Zymograms of phosphorylase activity of pea seed extract were most clearly resolved in 10% (w/v) acrylamide gel and tris/HCl buffer pH

7.2 as the main gel buffer. Also development at 4°C gave better stained bands than at room temperature.

Only one electrophoretic band (Rf 0.54) was present for each inbred line. This band appeared to be monomorphic, since there was no variation in its mobility on the gel for the 36 inbred lines examined (see fig. 1).

1.1.1 Pea isogenic lines seeds

The electrophoretic pattern of phosphorylase activity was studied in seed extracts in 12 pairs of isogenic lines. The results indicated that there was no difference in the electrophoretic patterns of phosphorylase activity in these isogenic pairs.

1.1.2 During seed development

One band of phosphorylase activity was present in the seed extract at each stage of seed development of the var. Feltham First investigated. It appeared that there was no change either in the electrophoretic patterns or in the level of enzyme activity, as judged by band intensity, as the seed developed (see fig. 2).

1.1.3 Conclusion

It was concluded from this set of results that insufficient variation exists in the phosphorylase enzyme for it to be useful as a biochemical probe in developmental or genetical studies.

---- represent faint band, barely visible on the gel and not present in the photograph.

— represent faint band, visible on the gel and barely visible in the photograph.


 represent clear band on the gel and photograph. The wider the band the more active the component in the same figure.

Fig. 1

Zymogram patterns of phosphorylase in pea seed extracts.

Track:

No. 1: Inbred line no. 807

No. 2: Inbred line no. 110

No. 3: Inbred line no. 1238

No. 4: Inbred line no. 1263

No. 5: Inbred line no. 181

No. 6: Inbred line no. 1673

No. 7: Inbred line no. 1293

No. 8: Inbred line no. 180

No. 9: Inbred line no. 2162

The gels were incubated in sodium citrate buffer pH 6.0 containing 0.1% (w/v) soluble starch and 0.66% (w/v) glucose-1-phosphate and stained in KI/I₂ solution.

Fig. 2

Diagrammatic zymogram patterns of phosphorylase in extracts from pea (Pisum sativum var. Feltham First) seeds at different stages of seed development.

Track:

No. 1: 9 day-old seeds

No. 2: 11 day-old seeds

No. 3: 13 day-old seeds

No. 4: 15 day-old seeds

No. 5: 17 day-old seeds

No. 6: 19 day-old seeds

No. 7: 21 day-old seeds

No. 8: 32 day-old seeds

Fig. 1

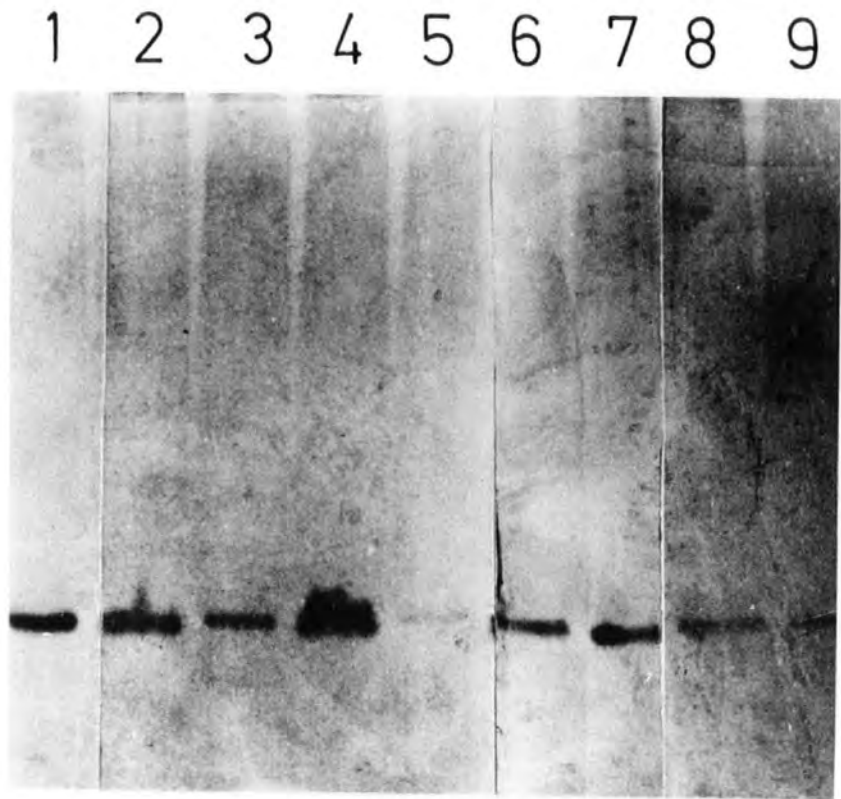
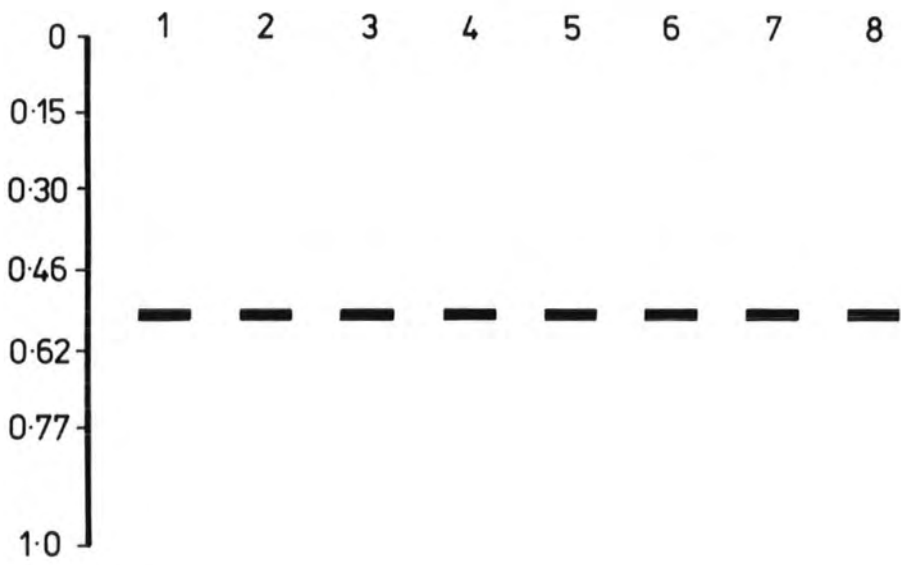


fig. 2



2. Amylase activity

When tested for amylase activity, the gels stained blue due to the starch-iodine reaction. Low concentrations of KI/I₂ solution were found to be advantageous, since a high concentration of KI/I₂ (0.1% (w/v) iodine, 0.5% (w/v) KI), as described by Brewbaker (1968), resulted in a very deeply stained background with poorly resolved bands.

The sites of amylase activity appeared as pink areas due to incomplete breakdown of starch, or white areas as a result of complete breakdown of a starch.

The gels lost their colour after incubation for prolonged periods (about one week) in the destaining solution, but could be restained. The gels also lost their colour if they were stored after drying.

The activities of amylase isoenzymes were studied in extracts of different legume species. Amylase activity bands were detected in the extracts of nearly all the species studied (see fig. 3 for a description of their activities and their relative positions on gels).

2.1 Pea seed extracts

2.2.1 Inbred lines

Two bands of amylase activity were detected for each inbred line of the 20 inbred lines tested. One was a thick, fast migrating band, and the other a thin, slow migrating band (see figs. 4 and 5a), and some lines had a third thin fast moving band, not seen in the photograph.

Fig. 3

Diagrammatic zymogram patterns of amylase in different legume seed extracts.

No. 1: Pea (Pisum sativum var. Feltham First)

No. 2: Soyabean (Glycine max)

No. 3: Broad bean (Vicia faba)

No. 4: Mung bean (Vigna radiata)

No. 5: Jack bean (Canavalia ensiformis)

No. 6: Black eye bean (Vigna unguiculata)

No. 7: Dwarf French bean (Phaseolus vulgaris var. Processor)

No. 8: Dwarf French bean (Phaseolus vulgaris var. Masterpiece)

a) Electrophoretic variation of amylase components

The two major bands, which were detected in each inbred line, were classified as amyl¹ for the less anodic band (the slower) and amyl² for the other (faster). Both isoenzymes were polymorphic and varied in different lines. The amylases were classified according to their migration toward the anode, as F (faster), I (intermediate) and S (slower). Only one variant occurred in any one inbred line (see fig. 4 and 5a).

b) Genetics of amylase

Since availability of plant material was very limited only the mode of inheritance of amyl² was investigated.

A genetic analysis was carried out on the seed meals from the cross between inbred line 1238 and inbred line 1293 to determine the position of the gene responsible for amyl². These two lines were selected because they differ in their amyl² mobilities but also because of the availability of a suitable phenotypic characteristic in the F₂ progeny.

In the F₂ progeny, each plant had either the fast migrating band, the slow migrating band or both bands. When both bands were present, they appeared almost as one large band, since they were very close together. In a few cases the two bands were well separated (see fig. 5b and 6).

From the 71 offspring examined the results show the following ratio:- 22(F):39(F,S):10(S).

Fig. 4

Zymogram patterns of amylase in pea seed extracts.

Track:

No. 1: Inbred line no. 1238

No. 2: Inbred line no. 1263

No. 3: Inbred line no. 2163

Fig. 5

Diagrams show the variants of amylase in pea seed extracts.

a. In different inbred lines

b.1: Normal two bands in F_2 progeny

b.2: Abnormal two bands in F_2 progeny

See text for details

Fig. 4

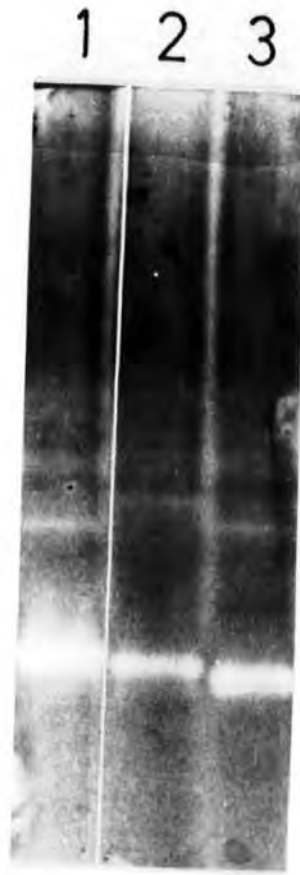


fig. 5

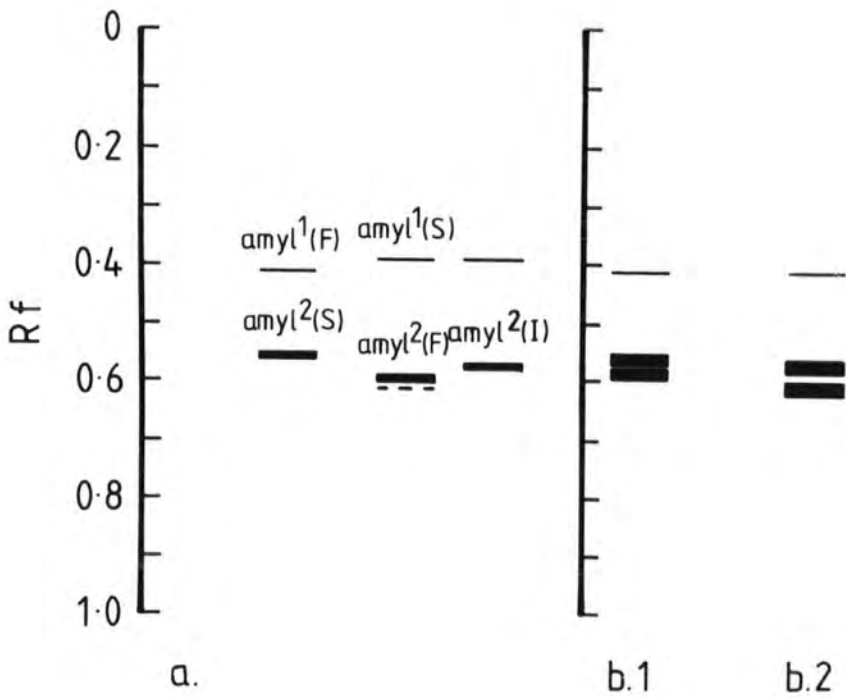


Fig. 6

Zymogram patterns of amylase in pea seed extracts

Track:

No. 1: Inbred line no. 1293

Nos. 2-11: F₂ progeny

No. 12: Inbred line no. 1238

Fig. 7

Diagrammatic zymogram patterns of amylase in pea (Pisum sativum)
seed extracts of different varieties.

Track:

No. 1: Var. Feltham First

No. 2: Var. English Wonder

No. 3: Var. Mangetout

Fig. 6

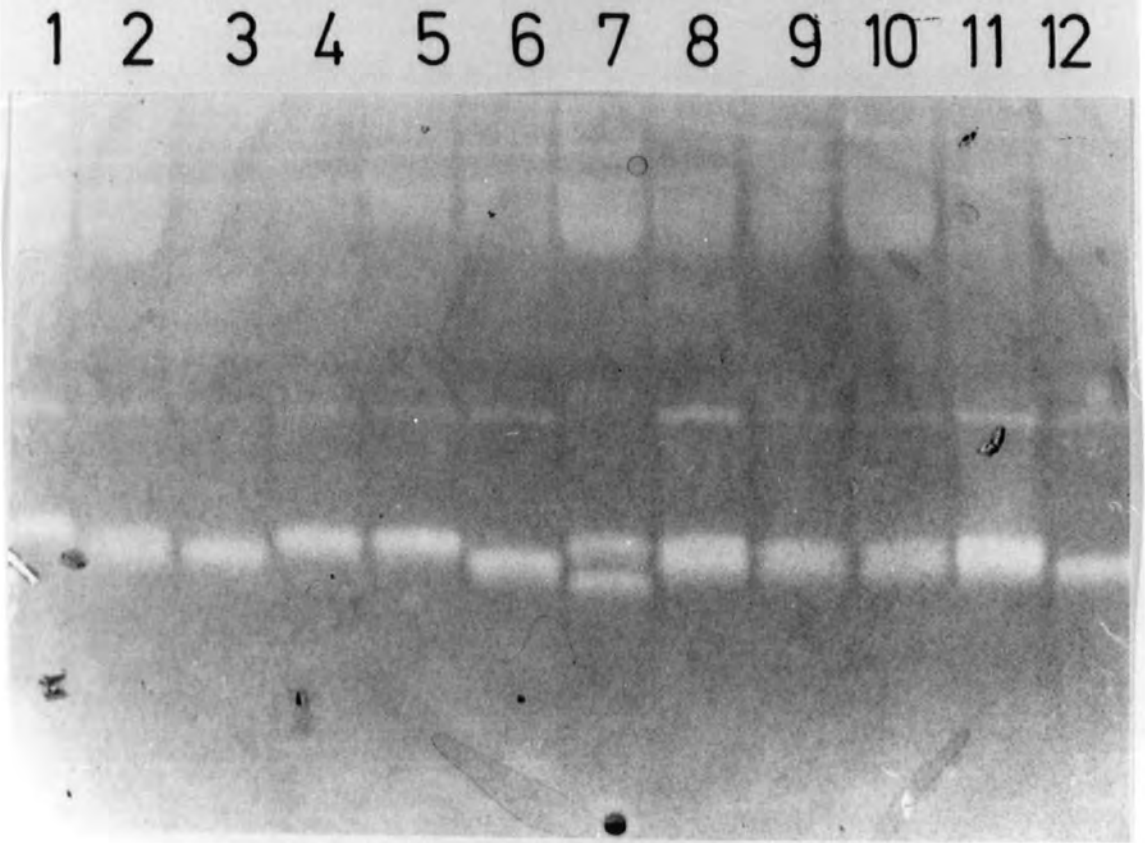
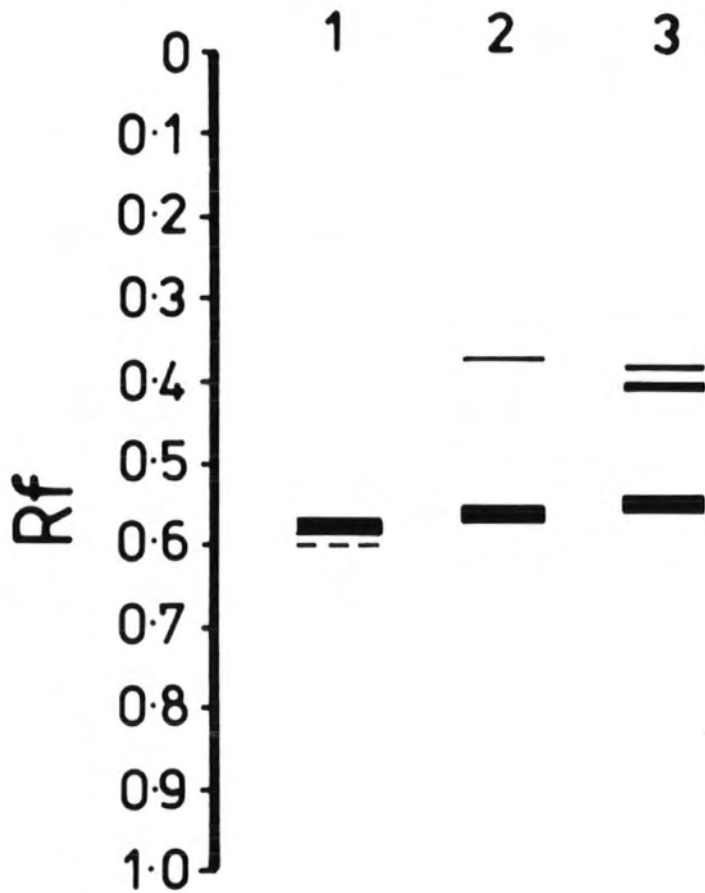


fig.7



2.1.2 Different Varieties of *Pisum sativum*

a) Var. Feltham First

The seed extract consisted of two pink bands and the slow migrating component (Rf 0.58) had the highest amylase activity, while the other component was very faint and barely visible on the gel (see fig. 7).

b) Var. English Wonder

Two distinct electrophoretic, pink bands were present and the fast migrating component was more active than the slow migrating component (see fig. 7).

c) Var. Mangetout

Three distinct electrophoretic bands were detected and the fastest moving component had the highest level of amylase activity (see fig. 7).

2.1.3 Using starch in the separating gel

The activity of amylase in mature seed extracts of different inbred lines, was studied in polyacrylamide gels containing 0.3% (w/v) soluble starch in the separating gel. The gels, following electrophoresis, were incubated in a chilled 0.2M sodium acetate buffer pH 5.0 for 2min. and then were incubated in 0.5% (w/v) soluble starch in the same buffer for 1½h. and then stained in I₂/KI solution as usual.

Three distinct electrophoretic bands were detected in each extract, but only two bands can be seen clearly in the photograph in fig. 8a.

The slowest moving band, which was near the top of the separating

gel (Rf 0.12), was a new (additional), white band and was not detected in gels which had no starch in the separating gel (compare figs. 8a and 4).

The intermediate moving band, which corresponded to amyl¹ in gels having no starch present in the separating gel (see section 2.1.1a), was faint and barely visible on the gel and not present in the photograph due to the low level of its activity and to the deep blue-staining background of the gel.

The fastest moving band, which corresponded to amyl² in gels with no starch in the separating gel was an active component and using starch in the gel increased the distance between the two variants (compare figs. 4 and 8).

The new band was not due to phosphorylase activity, since one band of its activity was detected in gels containing starch incubated either in 0.5% (w/v) soluble starch in 0.2M sodium phosphate buffer pH 6.0, or in 0.5% (w/v) soluble starch in 0.1M sodium citrate buffer pH 6.0 containing 0.66% (w/v) glucose-1-phosphate and both gels were stained in I₂/KI solution (see fig. 8 band c).

2.1.4 Using amylopectin as substrate

The gels were stained slightly red and the sites of amylase activity were pink areas.

Two bands of amylase activity were detected in seed extract of var. Feltham First and they corresponded to the bands which were present in gels incubated in soluble starch solution.

Another deep blue-stained band was present at Rf 0.41 and it was

Fig. 8

Zymogram patterns of starch hydrolyzing - enzymes in pea seed extracts, in acrylamide gel containing starch in the separating gel.

a. The gel was incubated in 0.2M sodium acetate buffer pH 5.0 containing 0.5% (w/v) soluble starch and stained in KI/I₂ solution.

Track:

No. 1: Inbred line no. 1293

No. 2: Inbred line no. 1238

b. The gel was incubated in 0.2M sodium phosphate buffer pH 6.0 containing 0.5% (w/v) soluble starch and stained in KI/I₂ solution. Seed extract of inbred line no. 1293.

c. The gel was incubated in 0.1M sodium citrate buffer pH 6.0 containing 0.5% (w/v) soluble starch and 0.66% (w/v) glucose-1-phosphate, and stained in KI/I₂ solution. Seed extract of inbred line no. 1238.

Note:- The arrows point to phosphorylase bands and all the other bands are due to amylase activity.

Fig 9

Diagrammatic zymogram patterns of starch hydrolyzing-enzymes in pea (Pisum sativum var. Feltham First) seed extracts.

a. The gel was incubated in 0.2M sodium acetate buffer pH 5.0 containing 1.0% (w/v) soluble starch and stained in KI/I₂ solution.

b. The acrylamide gel which contained 0.3% (w/v) soluble starch was incubated in 0.2M sodium acetate buffer pH 5.0 containing 0.5% (w/v) soluble starch and stained in KI/I₂ solution.

c. The gel was incubated in 0.3% (w/v) amylopectin in 0.2M sodium acetate buffer pH 5.0 and stained in KI/I₂ solution.

Note ~~ss~~ This band represents R-enzyme.

All the other bands represent amylase.

Fig. 8

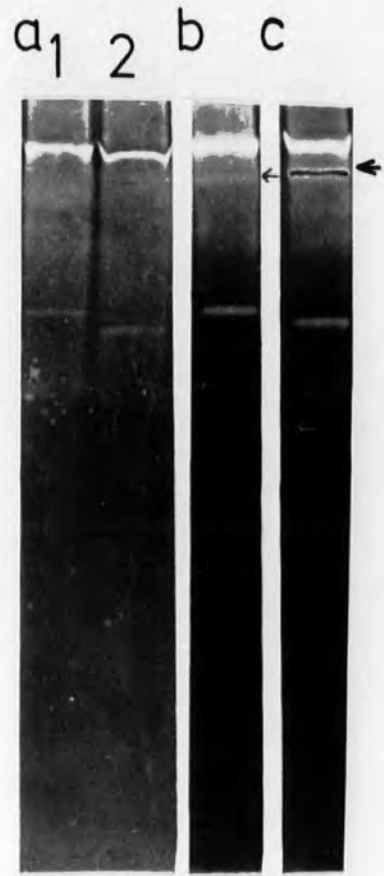
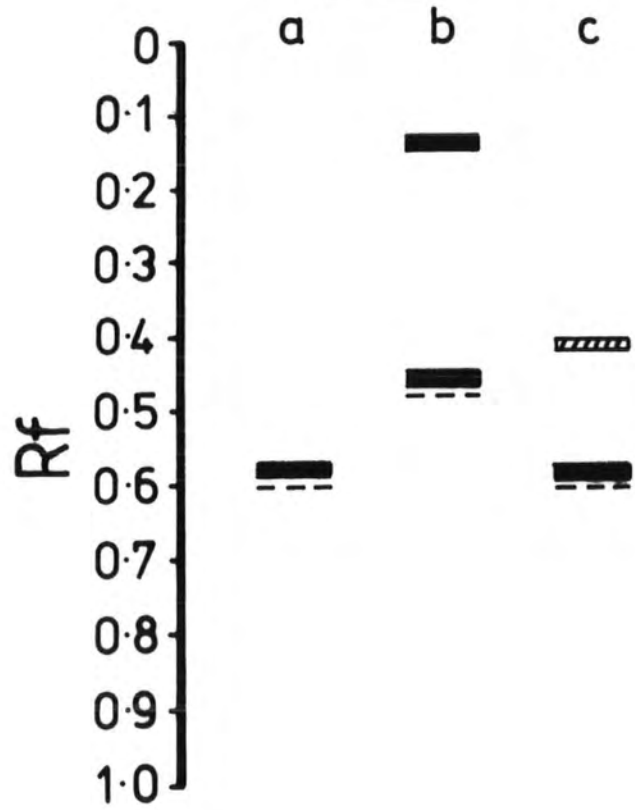


fig.9



due to the activity of debranching enzyme (R-enzyme) (see fig. 9c). The activity of this enzyme was also detected in cotyledon extracts at the various stages of seed germination.

2.1.5 During seed germination

The changes in the electrophoretic patterns and activity of amylase isoenzymes were related to seed germination in two pea varieties, Feltham First and English Wonder (the latter variety having wrinkled seeds). Figure 10 and Figure 12 show the zymogram pattern changes of Feltham First and English Wonder respectively.

2.1.5.1 Var. Feltham First

Zymogram pattern changes were studied in seedling extracts at seven stages of seed germination, up to the ninth day. Extracts of plumule hook, root, stem and cotyledons were compared (see fig. 10).

a) Cotyledon extracts

Extracts from 1 and 2 day-old seedlings, each gave two bands. The slowest migrating component (Rf 0.58) was more active than the other component, which was faint and hardly detected on the gel. These two bands were similar to the bands present in the dry seed extract.

Only one band was present in the extracts from the 3, 4 and 5 day-old seedlings. This band was similar to the slowest migrating band in the dry seed extract.

Extracts from 7 and 9 day-old seedlings, each produced two bands. The slowest migrating band (Rf 0.39) was a new component and well pronounced, whilst the fastest migrating band which was similar to the

band described in extracts from the 3, 4 and 5 day-old seedlings, was faint.

In another experiment, the cotyledons were freeze-dried and the amylase patterns were studied in 40 μ l extracts (1:10 (w/v), cotyledon meal to extraction buffer). As shown in fig. 11, the electrophoretic pattern changes of amylase activity were clearer than those from extracts of fresh materials. The major band of amylase activity at Rf 0.39 (the slowest moving band) was detected from the third day of germination, while, in the extracts from fresh materials, it was detected from the seventh day of seed germination (compare figs. 10a and 11).

b) Root extracts

Extracts from 3, 4, 5, 7 and 9 day-old seedlings each gave one fast migrating component (Rf 0.82) and it appeared to have the same level of amylase activity in the different extracts.

c) Stem extracts

One band was present in each extract from 4, 5, 7 and 9 day-old seedlings and was similar to the band present in the root extracts.

d) Plumule hook extracts

Two bands were detected in each extract of the 5, 7 and 9 day-old seedlings. The fastest migrating band was similar to the band described in the stem and root extracts, while the other band was similar to the fastest migrating band in the cotyledon extracts from 7 and 9 day-old seedlings. It was very faint and barely detected on the gel.

Fig. 10

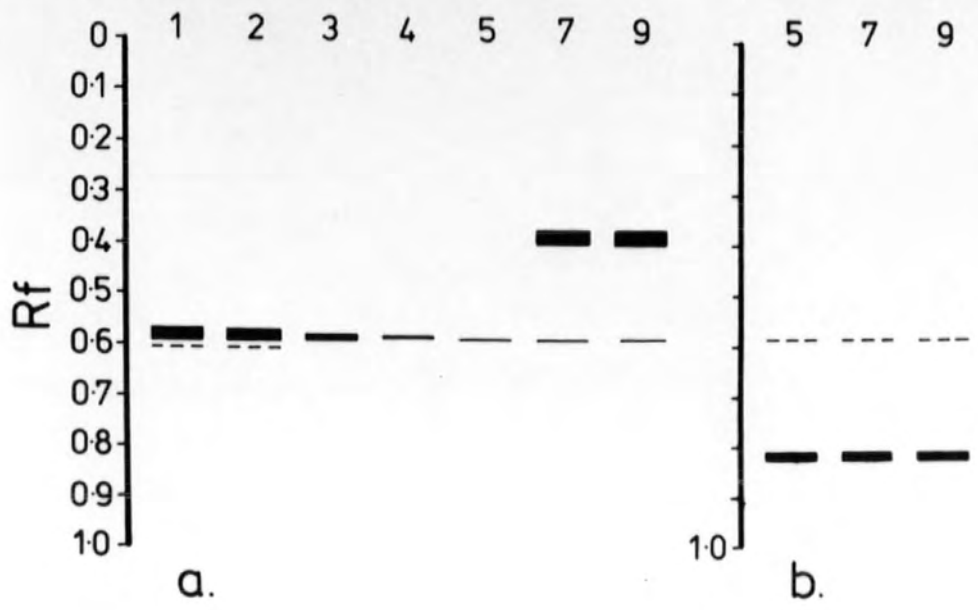
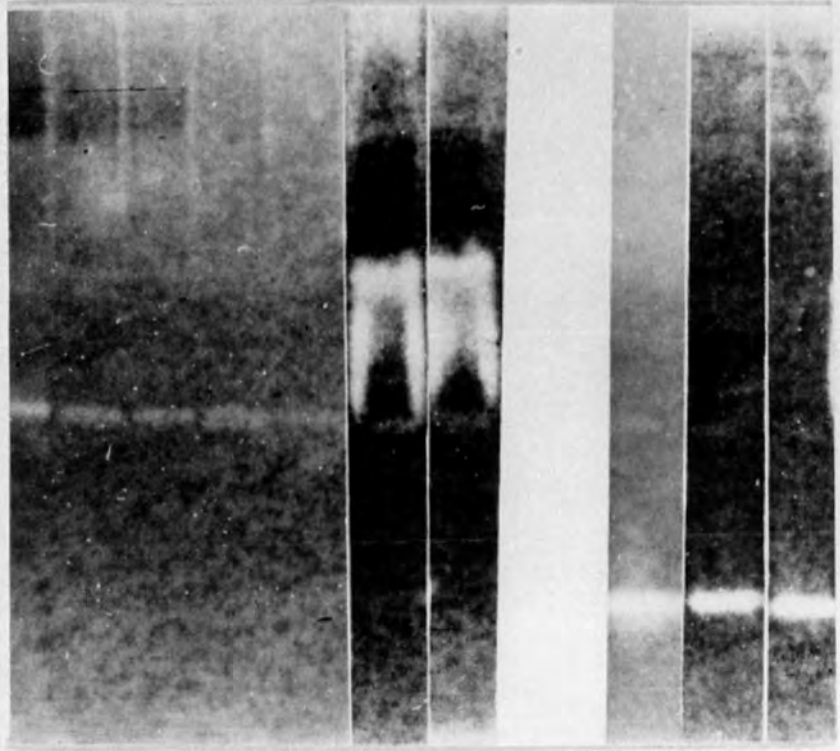
Zymogram pattern changes of amylase in extracts from pea (Pisum sativum var. Feltham First) seedlings at different stages of seed germination.

- a. Cotyledons
- b. Plumule hook
- c. Root
- d. Stem

Numbers are days of germination

Fig. 10

1 2 3 4 5 7 9 ^b 5 7 9



c₃ 4 5 7 9 d₄ 5 7 9

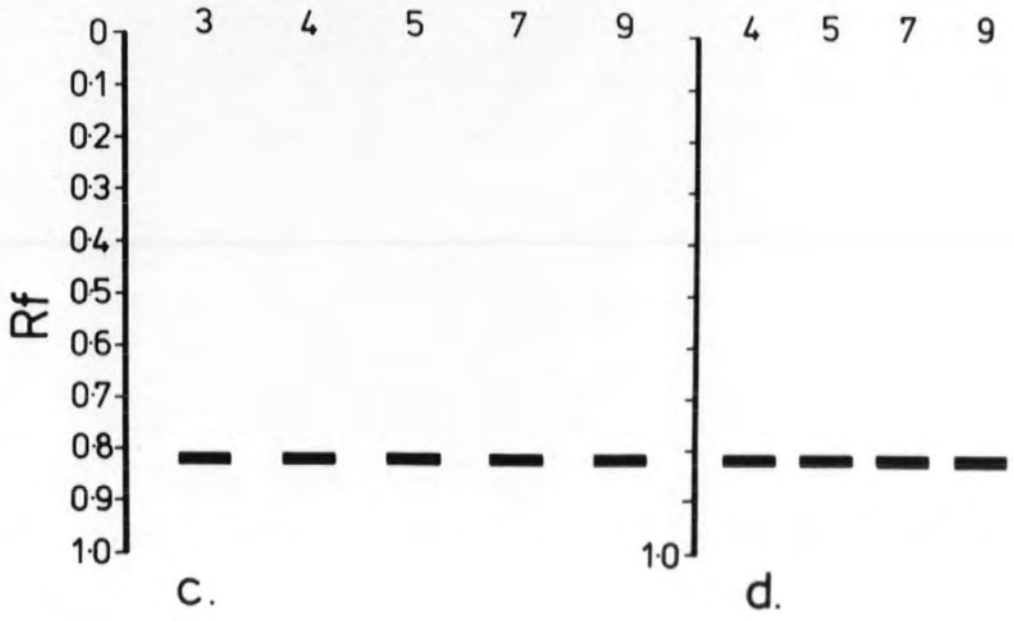
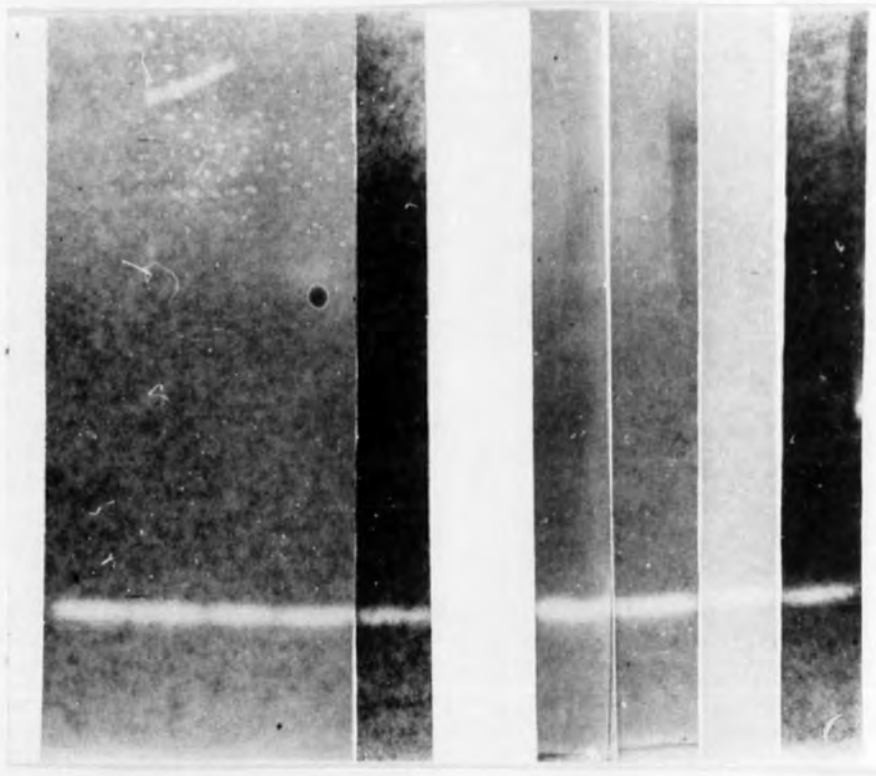


Fig.11

Zymogram pattern changes of amylase in extracts from pea (Pisum sativum var. Feltham First) seedlings at different stages of seed germination (using freeze-dried materials).

Track:

- No. 1: Cotyledons extract of 1 day-old seedlings
- No. 2: Cotyledons extract of 2 day-old seedlings
- No. 3: Cotyledons extract of 3 day-old seedlings
- No. 4: Cotyledons extract of 5 day-old seedlings
- No. 5: Cotyledons extract of 7 day-old seedlings
- No. 6: Cotyledons extract of 9 day-old seedlings
- No. 7: Cotyledons extract of 11 day-old seedlings
- No. 8: Cotyledons extract of 13 day-old seedlings
- No. 9; Cotyledons extract of 15 day-old seedlings
- No. 10: Leaf extract of 15 day-old seedlings
- No. 11: Stem extract of 15 day-old seedlings
- No. 12: Root extract of 15 day-old seedlings

Fig.11



2.1.5.2 Var. English Wonder

Zymogram patterns of amylase activity were studied in extracts from 1, 4, 5, 6, 7, 8, 9 and 10 day-old seedlings. Extracts from plumule hook, stem, root and cotyledons were compared (see fig. 12).

a) Cotyledon extracts

Cotyledon extracts of seedlings up to 7 days old each produced two pink bands. The fastest migrating component was more active and had approximately the same level of activity in all extracts.

A new active component white band (Rf 0.33) was present in the extracts from 8, 9 and 10 day-old seedlings, while the slowest moving band of the two bands which were described above was not present.

b) Root extracts

Zymogram patterns of amylase activity were studied in root extracts from the fifth day of germination onwards.

The extracts from 5 and 6 day-old seedlings each gave two bands. The slow migrating band was similar to the fast migrating band of the dry seed extract. The other component was very active and was the only band present in all the other root extracts.

c) Stem extracts

Zymogram patterns were studied in stem extracts from the seventh day of germination onwards.

One band was present in each extract, except the extracts from 7 and 8 day-old which had an additional faint band similar to the fast migrating band in the dry seed extract.

d) Plumule Hook extracts

The amylase activity was studied in extracts from the seventh day

Fig. 12.

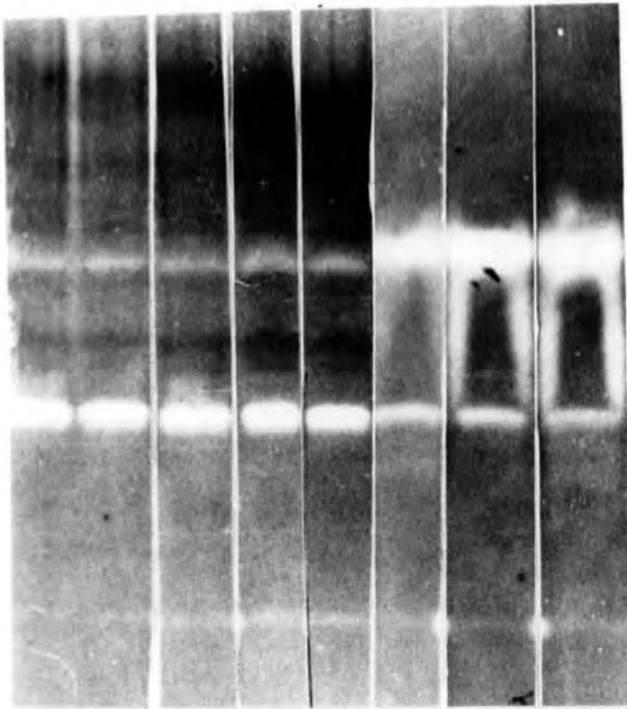
Zymogram pattern changes of amylase in extracts from pea (Pisum sativum var. English Wonder) seedlings at different stages of seed germination.

- a. Cotyledons
- b. Plumule hook
- c. Stem
- d. Root

Numbers are days of germination.

Fig.12

a₁ 1 4 5 6 7 8 9 10



b₇ 7 8 9 10

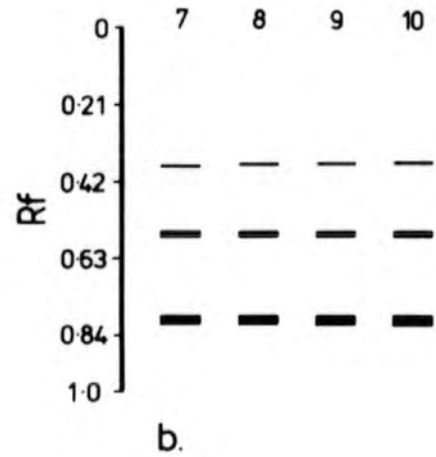
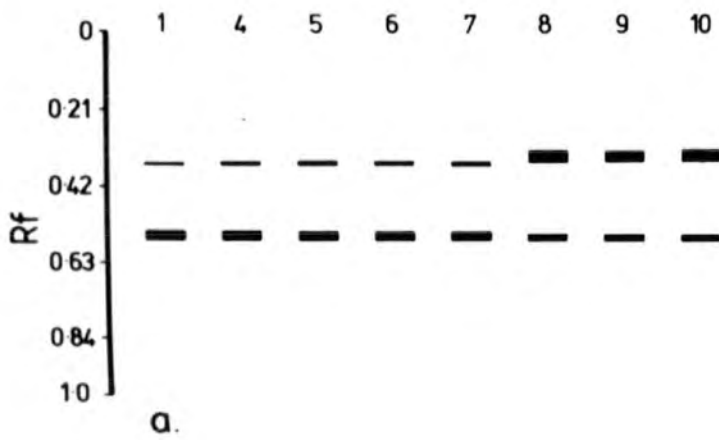
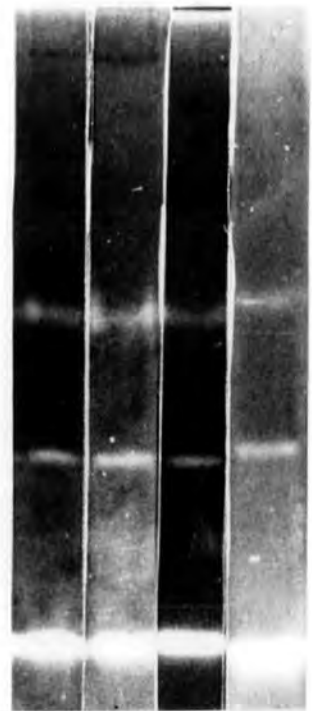
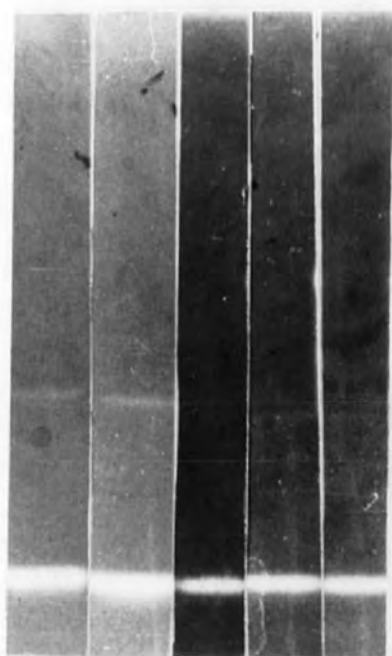
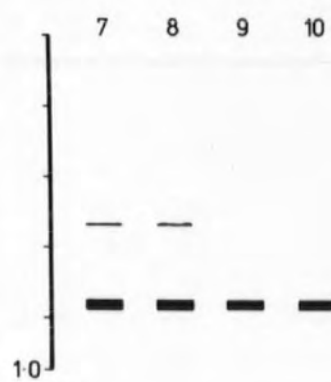
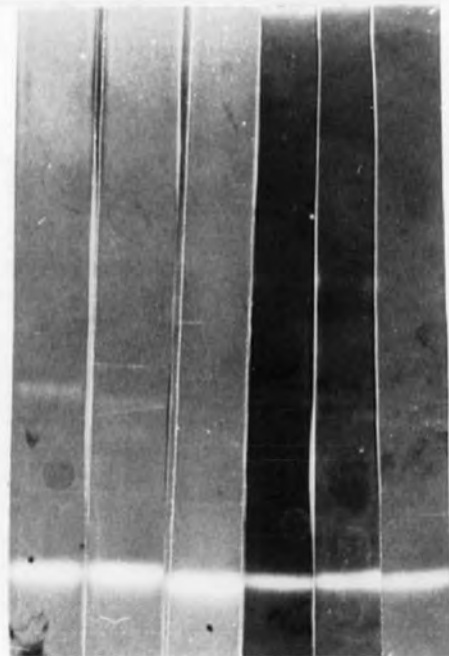


fig.12

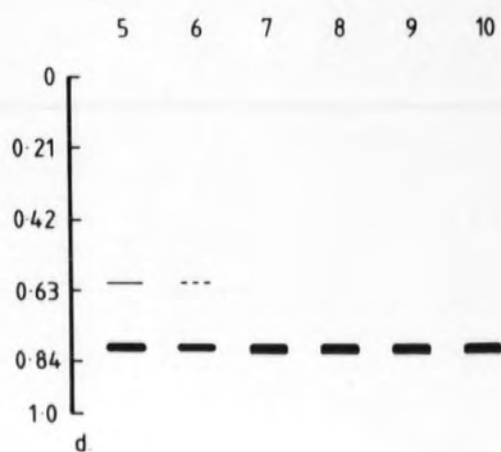
c 7 8 9 10 10



d 5 6 7 8 9 10



c.



d.

of germination onwards. Each extract gave three distinct electrophoretic bands of amylase activity. The fastest migrating component (Rf 0.79) was highest in activity and similar to the band which was described in root and stem extracts. The two slow migrating components were similar to the band which was described in the dry seed extracts with low levels of amylase activity.

2.1.5.3 Different species

Zymogram pattern changes of amylase were related to seed germination in seedling extracts of Pisum elatius.

The electrophoretic pattern changes were studied in seedling extracts of five stages of seed germination, up to the 10th day, and the results in fig. 13 show that the zymogram pattern changes were very similar to the changes which were observed in seedling extracts of the two varieties (Feltham First and English Wonder) of Pisum sativum.

a) Cotyledon extracts

Extracts from 1 and 2 day-old seedlings each gave two pink bands similar to the bands which were observed in the extracts from the different inbred lines and the fastest moving component had the highest level of amylase activity.

The extract from 5 day-old seedlings gave four bands, two pink and two white; both of the latter were new.

The extract from 8 day-old seedlings displayed one white active component, while the extract from 10 day-old seedlings displayed two white bands.

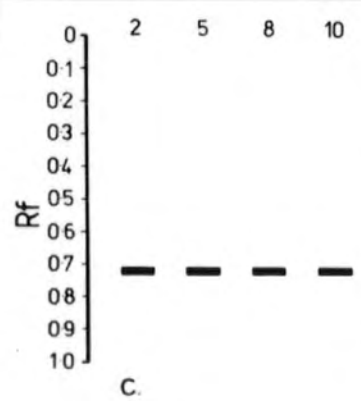
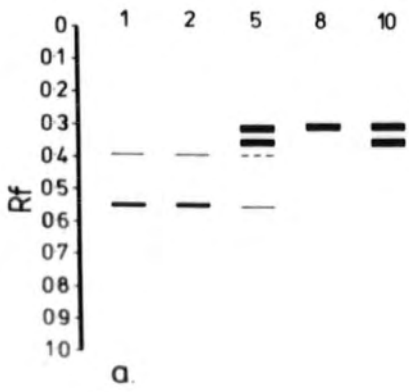
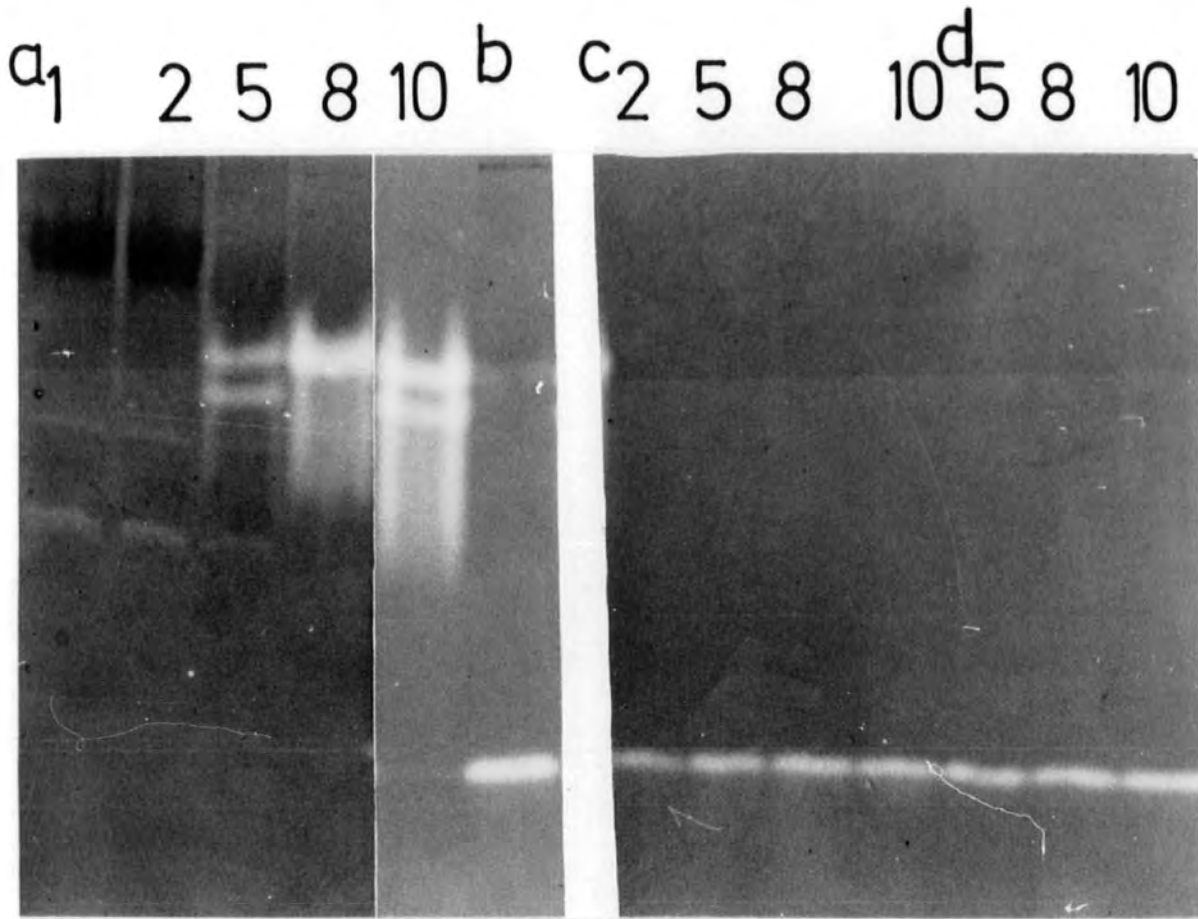
Fig. 13

Zymogram pattern changes of amylase in extracts from pea (Pisum elatius) seedlings at different stages of seed germination.

- a. Cotyledons
- b. Plumule hook
- c. Root
- d. Stem

Numbers are days of germination

Fig. 13



b) Root extracts

The extracts from 2, 5, 8 and 10 day-old seedlings each gave one white, fast moving band.

c) Stem extracts

Extracts from 5, 8 and 10 day-old seedlings each gave one white, fast moving band.

d) Plumule hook

The leaf extracts had one white, fast moving band and one faint, slow moving band.

2.1.6 During seed development

Amylase activity was studied in seed extracts from three varieties of pea (Pisum sativum) at different stages of seed development and the results indicate that the electrophoretic patterns of amylase activity, changed dramatically as the seed developed; the three varieties had nearly the same pattern of changes.

2.1.6.1 Var. Feltham First

As shown in fig.14, the band at position a) in the photograph was a white, faint and wavy band and not present in extracts from the late stages of developing seeds.

The band at position b) in the photograph, was a white, faint band and present only in extracts from 11, 12 and 14 day-old seeds. This band corresponded to the slowest migrating band, which appeared in seedling cotyledon extracts during seed germination; i.e. these bands had the same mobility on the gel (see fig. 15).

Fig. 14

Zymogram pattern changes of amylase in pea (Pisum sativum var. Feltham First) seed extracts at different stages of seed development.

Track:

- No. 1: 7 day-old seeds
- No. 2: 9 day-old seeds
- No. 3: 10 day-old seeds
- No. 4: 11 day-old seeds
- No. 5: 12 day-old seeds
- No. 6: 14 day-old seeds
- No. 7: 15 day-old seeds
- No. 8: 17 day-old seeds
- No. 9: 19 day-old seeds
- No. 10: 21 day-old seeds
- No. 11: 23 day-old seeds
- No. 12: 25 day-old seeds
- No. 13: 27 day-old seeds
- No. 14: Cotyledons extract of 15 day-old seeds
- No. 15: 28 day-old seeds
- No. 16: 29 day-old seeds
- No. 17: 30 day-old seeds
- No. 18: 31 day-old seeds
- No. 19: 31 day-old seed (larger volume of seed extract was used)

Fig. 15

Zymogram patterns of amylase in pea (Pisum sativum var. Feltham First).

Track:

- No. 1: Stem extract from 9 day-old seedlings
- No. 2: Cotyledons extract from 9 day-old seedlings
- No. 3: Extract from 11 day-old seeds
- No. 4: Extract from 14 day-old seeds

Fig. 14

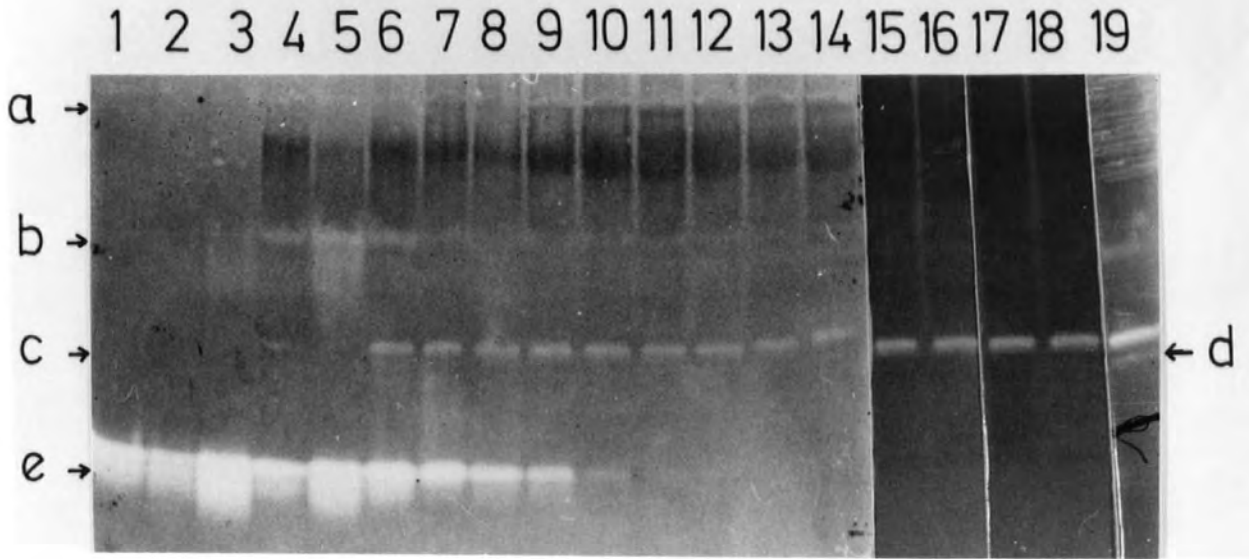


Fig. 15

1 2 3 4



The band at position c) in the photograph, was pink and detected in extracts from the 14th day onwards; it increased slightly in activity as the seed developed.

The band at position d) in the photograph, was pink, faint and barely visible on the gel and is not seen in the photograph except when a large volume of the extract was used.

One white fast moving component at position e) in the photograph corresponded to the fast moving band in stem, root, and leaf seedling extracts, i.e. it had the same mobility on the gel (see fig. 15). This component was detected in extracts at early stages of seed development and decreased in activity as the seed developed and it was completely absent in extracts from the 23rd day. Moreover, this band was not present in the cotyledon extract of the 15 day-old seeds, while it was present in the seed extract, and it was therefore specific to the embryo axes.

2.1.6.1.1 Effect of heat and EDTA on amylase activity

The component at Rf 0.39, coloured white on the gel, which was detected in seedling cotyledon extracts during seed germination and in extracts from 11, 12 and 14 day-old seeds, was inhibited by EDTA but not inhibited when the extract was incubated at 70°C for 15min. in the presence of added Ca^{2+} . This band appeared therefore to have plant α -amylase properties.

The pink components Rf 0.58 and 0.60 which increased slightly in activity as the seed developed and decreased in activity during seed germination, were not affected by EDTA and were inhibited by heat treatment in the presence of added Ca^{2+} . They appeared therefore to

have plant β -amylase properties.

The white, fast moving component at Rf 0.82 which appeared at the early stages of seed development and decreased in activity as the seed developed, was inhibited by heat treatment in the presence of added Ca^{2+} and was not affected by EDTA. It appeared therefore to have plant β -amylase properties, (see fig.16).

The activity of the two pink components which were present in seed extracts of the different inbred lines were inhibited when the extract was incubated at 70°C for 15min. in the presence of added Ca^{2+} and were not affected by EDTA, so they appeared therefore to have plant β -amylase properties.

2.1.6.1.2 Conclusion

From all above experiments it can be concluded that α -amylase was lacking in mature seed extracts and present in extracts of intermediate stages of seed development and then appeared again at late stages of seed germination; it was restricted to cotyledon extracts and is therefore a time-specific protein. The two pink components at Rf 0.58 and 0.60 were β -amylase and increased in activity as the seed developed and decreased in activity during seed germination. The white, fast moving component at Rf 0.82 is β -amylase, decreased in activity as the seed developed and appeared in embryo extracts (root, stem, and leaves) during seed germination.

The two pink bands which were detected in seed extracts of different inbred lines, also had β -amylase properties.

Fig. 16

Effect of heat and EDTA on amylase activity of extracts from 9 day-old pea (Pisum sativum var. Feltham First) seedlings.

a. Effect of 70°C.

Track:

No. 1: Stem

No. 2: Root

No. 3: Plumule hook

No. 4: Cotyledons

No. 5: α -amylase (animal source)

No. 6: Cotyledons

No. 7: β -amylase (plant source)

No. 8: Cotyledons

b. Effect of EDTA

Track:

No. 1: Cotyledons

No. 2: Stem

No. 3: Cotyledons

No. 4: α -amylase (animal source)

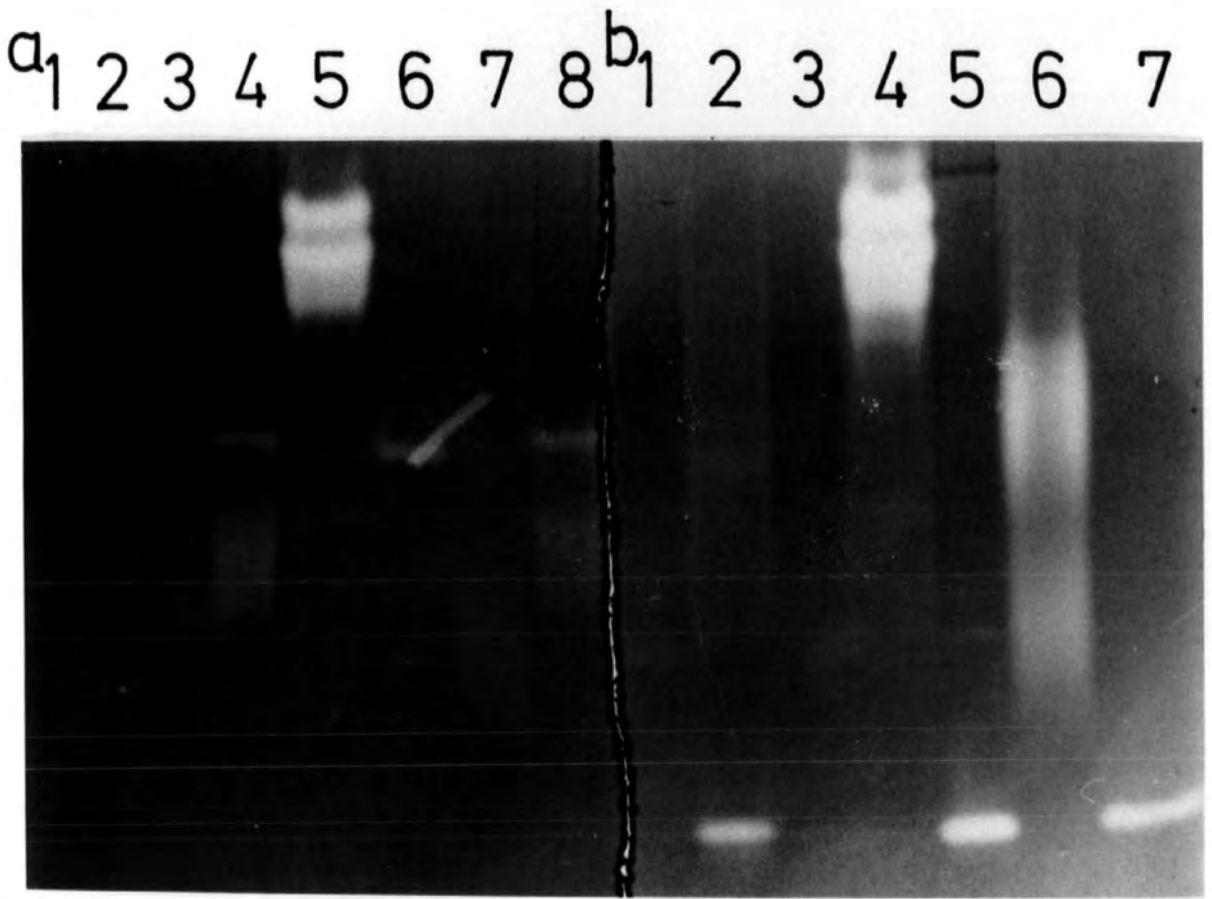
No. 5: Plumule hook

No. 6: β -amylase (plant source)

No. 7: Root

See text for details

Fig.16



2.1.6.2 Var. Mangetout

The zymogram pattern of amylase activity was studied in seed extracts at 13 stages of seed development.

The results in fig. 17 show two faint, slow migrating components at position a) in the photograph. They appeared to have low levels of amylase activity; they were detected from the 13th day of seed development.

One pink component at position b) in the photograph, appeared in extracts from the 10th day onwards; it increased in activity as the seed developed.

The fastest migrating component had a high level of amylase activity in the extracts at the early stages of developing seeds, decreased in activity as the seed developed and was lacking in the mature seed extract. Furthermore, this band was not present in the cotyledon extract of 12 day-old seeds, but it was present in the seed extract, so this band was restricted to embryo axis.

2.1.6.3 Var. English Wonder

The white, fastest moving component, as shown in fig. 18, had a high level of amylase activity in extracts from the early stages of developing seeds and decreased in activity as the seed developed and was completely lacking in seed extracts from the late stages. This band corresponded to the fast moving band in seedlings, root, stem and leaf extracts, i.e. they had the same mobility on the gel (see fig. 19).

The white, slowest moving component was present in seed extracts

Fig. 17

Zymogram pattern changes of amylase of pea (Pisum sativum var. Mangetout) seed extracts at different stages of seed development.

Track:

No. 1: 7 day-old seeds

No. 2: 8 day-old seeds

No. 3: 9 day-old seeds

No. 4: 10 day-old seeds

No. 5: 11 day-old seeds

No. 6: 12 day old seeds

No. 7: 13 day-old seeds

No. 8: 14 day-old seeds

No. 9: 15 day-old seeds

No. 10: 16 day-old seeds

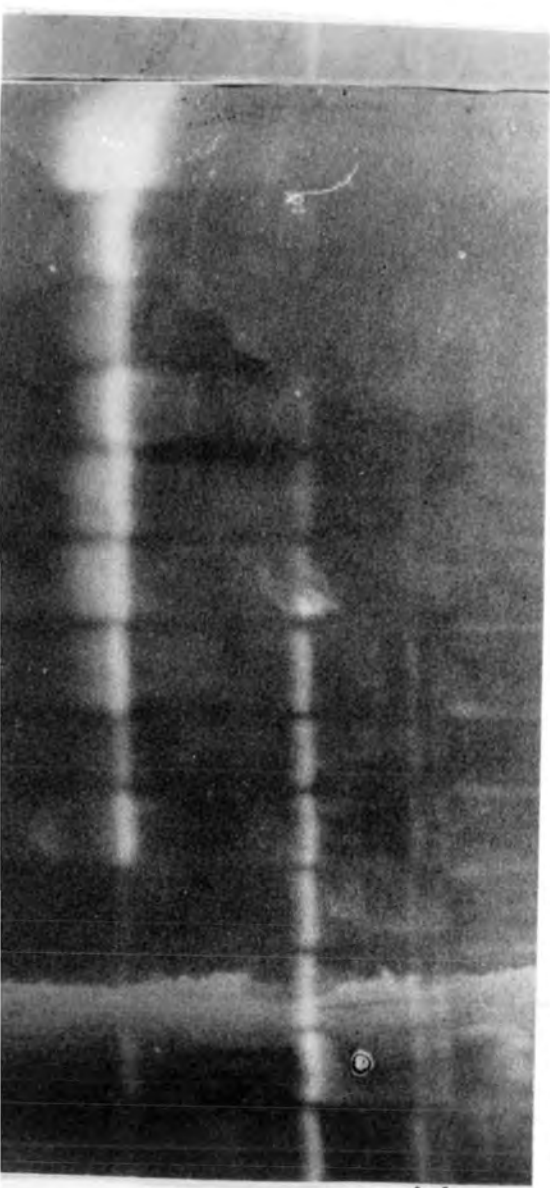
No. 11: 17 day-old seeds

No. 13: Mature seeds

No. Co: Cotyledons extract of 12 day-old seeds

Fig. 17

Co. 1 2 3 4 5 6 7 8 9 10 11 12 13



→ a
→ b

Fig. 18

Zymogram pattern changes of amylase in pea (Pisum sativum var. English Wonder) seed extracts at different stages of seed development.

Track:

No. 1: 10 day-old seeds

No. 2: 12 day-old seeds

No. 3: 13 day-old seeds

No. 4: 14 day-old seeds

No. 5: 15 day-old seeds

No. 6: 16 day-old seeds

No. 7: 17 day-old seeds

No. 8: 21 day-old seeds

No. 9: 18 day-old seeds

No. 10: 19 day-old seeds

No. 11: 20 day-old seeds

No. 12: 21 day-old seeds

No. 13: 23 day-old seeds

No. 14: 25 day-old seeds

No. 15: 26 day-old seeds

No. 16: 27 day-old seeds

No. 17: 28 day-old seeds

No. 18: 30 day-old seeds

No. 19: 31 day-old seeds

No. 20: 32 day-old seeds

Fig. 19

Zymogram patterns of amylase in pea (Pisum sativum var. English Wonder) extracts.

Track:

No. 1: Stem extract from 8 day-old seedlings

No. 2: Cotyledon extract from 9 day-old seedlings

No. 3: Extract from 10 day-old seeds

Fig.18

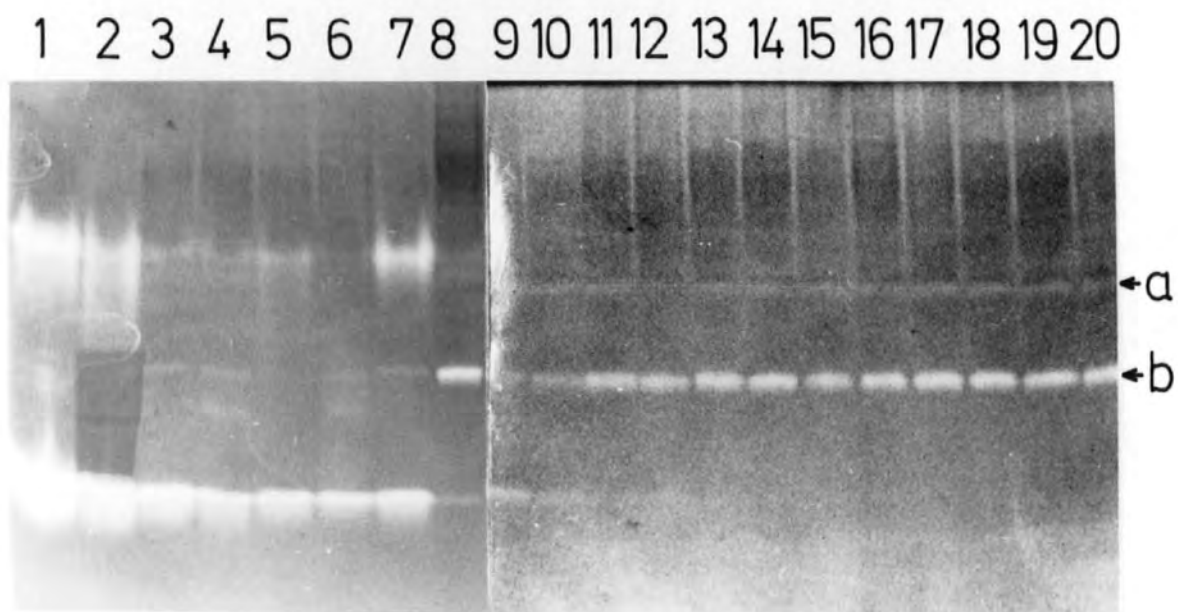
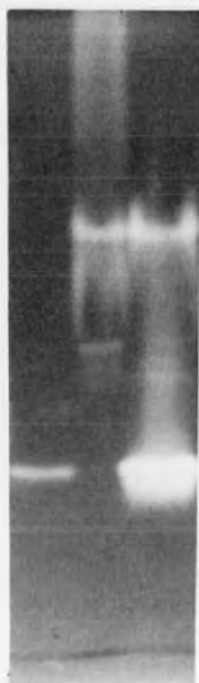


Fig.19

1 2 3



from the early stages of developing seeds only. It corresponded to the slowest moving band (Rf 0.39), which appeared in seedling cotyledon extracts during seed germination, i.e. they had the same mobility on the gel (see fig. 19).

The pink component at position a) in the photograph, had a low level of amylase activity, while the pink component at position b) in the photograph, had a higher level of amylase activity and appeared to increase in activity as the seed developed.

2.2 Soyabean seed extract

The dry seed extract had a very high level of amylase activity and only a small volume of seed extract (20 μ l of 1:25 (w/v) seed meal to extraction buffer) was used. At least four distinct electrophoretic bands were observed. The two intermediate migrating components (Rf 0.44 and 0.51) had high amylase activities and only a small volume (20 μ l) of extract was needed to detect them. On the other hand, the slowest and fastest migrating components (Rf 0.23 and 0.55) had low activities.

2.2.1 During seed germination

The zymogram patterns were studied in stem, leaf, root, hypocotyl and cotyledon extracts from 2, 5 and 8 day-old seedlings.

The results in fig. 20 indicated that the cotyledon extracts had the highest activity as judged by band intensities and the number of bands present.

a) Cotyledon extracts

The electrophoretic patterns of each extract from 2 and 5 day-old seedlings consisted of six distinct electrophoretic bands, while the extract from the 8 day-old seedlings gave at least eleven distinct electrophoretic bands.

b) Root extracts

The root extract from the 8 day-old seedlings gave four distinct electrophoretic bands.

c) Hypocotyl extracts

The zymogram patterns were studied in extracts from 5 and 8 day-old seedlings.

The extracts from 5 and 8 day-old seedlings each gave five bands and the components at Rf 0.43 and 0.47 appeared to be more active than the one at Rf 0.5, and the other two bands were hardly detected on the gel.

d) Shoot extracts

The shoot extract from 8 day-old seedlings gave five distinct electrophoretic bands. The slowest migrating component appeared to have the highest amylase activity.

2.2.2 During seed development

The electrophoretic pattern changes of amylase were related to seed development in extracts at 13 stages of seed development and the results in fig. 21 show that all the extracts had high level of amylase activity and only a small volume of seed extract (20 μ l of 1:25 (w/v) seed meal to extraction buffer) was needed.

Fig. 20

Zymogram pattern changes of amylase in extracts from soyabean (Glycine max) seedlings at different stages of seed germination.

a. Cotyledons

b. Root

c. Hypocotyl

d. Shoot

Numbers are days of germination

Fig. 21

Zymogram pattern changes of amylase in soyabean (Glycine max) seed extracts at different stages of seed development.

Track:

Nos 1 and 2: 21 day-old seeds

No. 3: 25 day-old seeds

No. 4: 27 day-old seeds

No. 5: 30 day-old seeds

No. 6: 35 day-old seeds

No. 7: 36 day-old seeds

No. 8: 37 day-old seeds

No. 9: 40 day-old seeds

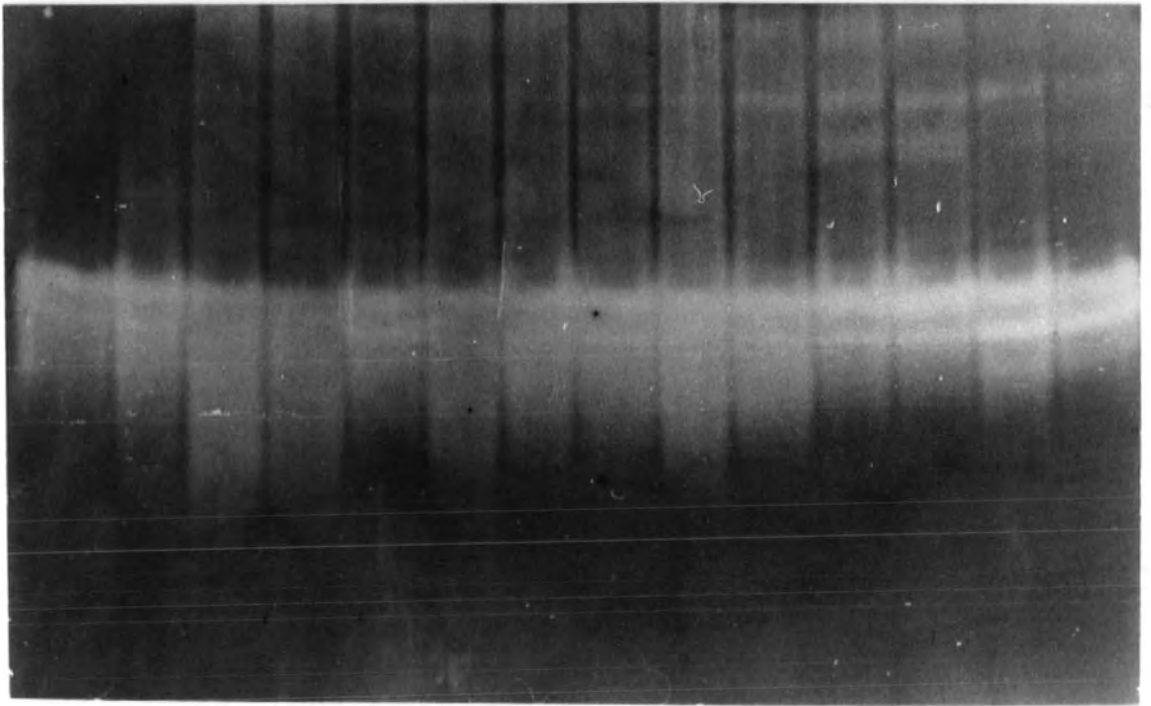
No. 10: 42 day-old seeds

No. 11: 45 day-old seeds

Nos 13 and 14: Mature seeds

Fig. 21

1 2 3 4 5 6 7 8 9 10 11 12 13 14



A total of four distinct electrophoretic bands were present. the two intermediate migrating components had the major levels of amylase activities and they appeared to increase slightly in activity as the seed developed, while the slowest moving component had a low level of amylase activity and was present in extracts at the late stages of developing seeds. The fastest moving component also had a low level of amylase activity and was detected in the mature seed extract only.

2.3 Jack bean seed extract

Amylase activity appeared to be high in the extract from mature seed. Amylase pattern gave three distinct electrophoretic bands. The slowest migrating component (Rf 0.39) had the highest enzyme activity.

2.3.1 During seed germination

Zymogram patterns of amylase activity were studied in extracts from 13 day-old seedlings and fig. 22 shows the zymogram patterns in root, stem, hypocotyl and cotyledon extracts.

The extracts from the different tissues gave a total of three distinct electrophoretic bands.

2.4 Dwarf French bean seed extract

The extract from dwarf French bean (Var. masterpiece) seed appeared to be very low in amylase activity since a large volume (50 μ l) extract was required in order to detect it. The amylase pattern gave two faint bands (Rf 0.42 and 0.44).

The seed extract from the Var. Processor was very low in amylase

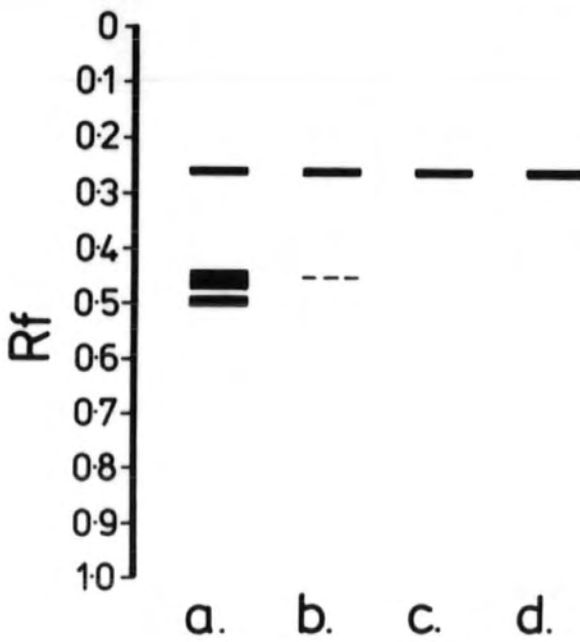
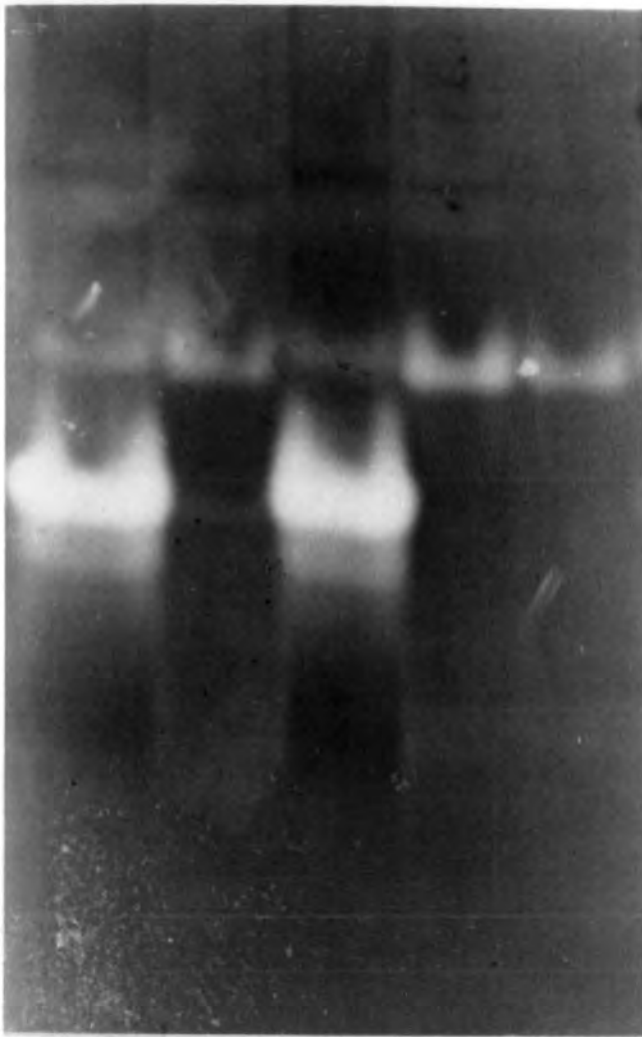
Fig. 22

Zymogram patterns of amylase in extracts from 13 day-old jack bean (Canavalia ensiformis) seedlings.

- a. Cotyledons
- b. Root
- c. Stem
- d. Hypocotyl

a b a c d

Fig.22



activity also; two very faint bands were detected in the extract.

2.4.1 During seed germination

The changes in the electrophoretic patterns of amylase were investigated in relation to seed germination in two varieties. Processor and Masterpiece. The results in figs. 23 and 24 show that both varieties showed the same pattern changes.

2.4.1.1 Var. Processor

Zymogram pattern changes were studied in extracts at five stages of seed germination, up to the eleventh day. Extracts from cotyledons, leaves, root, hypocotyl and stem were compared (see fig. 23).

a) Cotyledon extracts

The extract from one day-old seedlings was very low in amylase activity and three faint bands were present, all barely visible on the gel.

There was a remarkable increase in amylase activity in extracts from the second day of germination onwards. Four bands were present in extract from 2 day-old seedlings and three bands in each extract from 4, 8 and 11 day-old seedlings, and the slowest moving component having the major level of amylase activity (see fig. 23a).

a) Leaf extracts

The zymogram patterns of amylase were studied in extracts from 8 and 11 day-old seedlings. Each extract gave five distinct electrophoretic bands and the two fastest moving components had the lowest level of amylase activities and they were barely visible on the gel.

c) Root extracts

Zymogram patterns were studied in extracts from 2, 4, 8 and 11 day-old seedlings and each extract had one fast moving band.

d) Hypocotyl extracts

One band in extracts from 4 day-old seedlings and two bands in each extract from 8 and 11 day-old seedlings were present.

e) Stem extract

One band was present in each extract from 8 and 11 day-old seedlings.

2.4.1.2 Var. Masterpiece

Zymogram pattern changes were studied in extracts at five stages of seed germination up to the ninth day. Extracts from cotyledons, leaves, root, stem and hypocotyl were compared (see fig. 24).

a) Cotyledon extracts

The level of amylase activity was very low in the extract from one day-old seedlings. Three faint bands, barely visible on the gel, were detected.

The level of amylase activity increased in extracts from the second day of germination onwards. A total of two bands, in each extract from 2 and 4 day-old seedlings, and three bands in each extract from 6 and 9 day-old seedlings, were present and the slowest moving component had the highest amylase activity.

b) Leaf extracts

The zymogram patterns of amylase were studied in extracts from 6 and 9 day-old seedlings. Each extract gave five distinct

Fig. 23

Zymogram pattern changes of amylase in extracts from dwarf French bean (Phaseolus vulgaris var. Processor) seedlings at different stages of seed germination.

- a. Cotyledons
- b. Leaves
- c. Root
- d. Hypocotyl
- e. Stem

Numbers are days of germination.

Fig. 23

a₁ 2 4 8 11 b 8 11 c 2 4 8 11 d 4 8 11 e 8 11

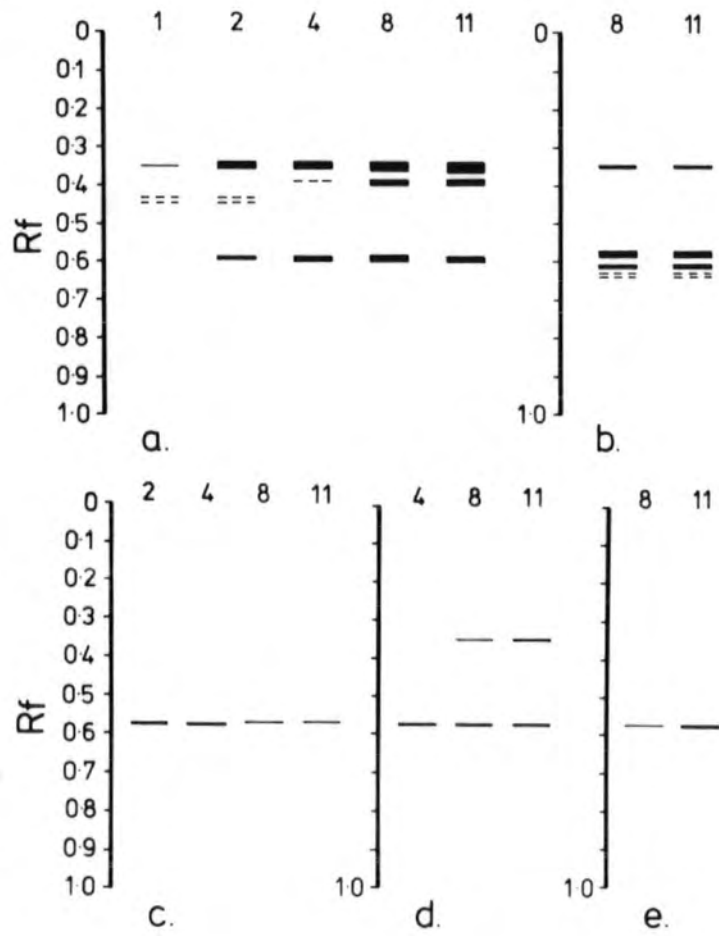
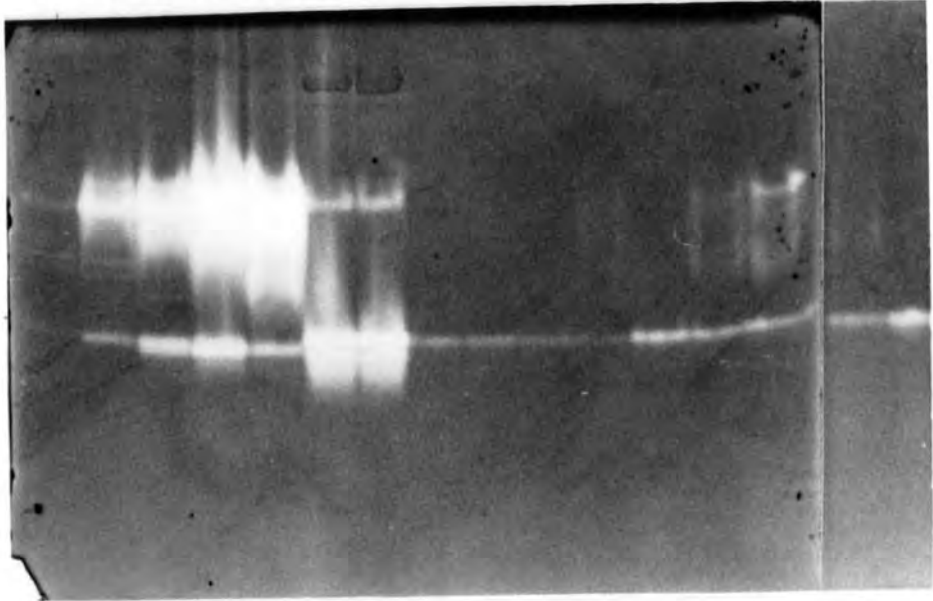


Fig. 24

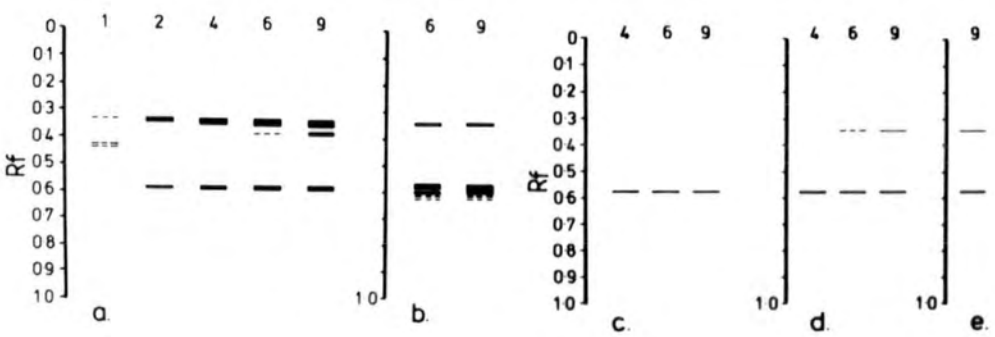
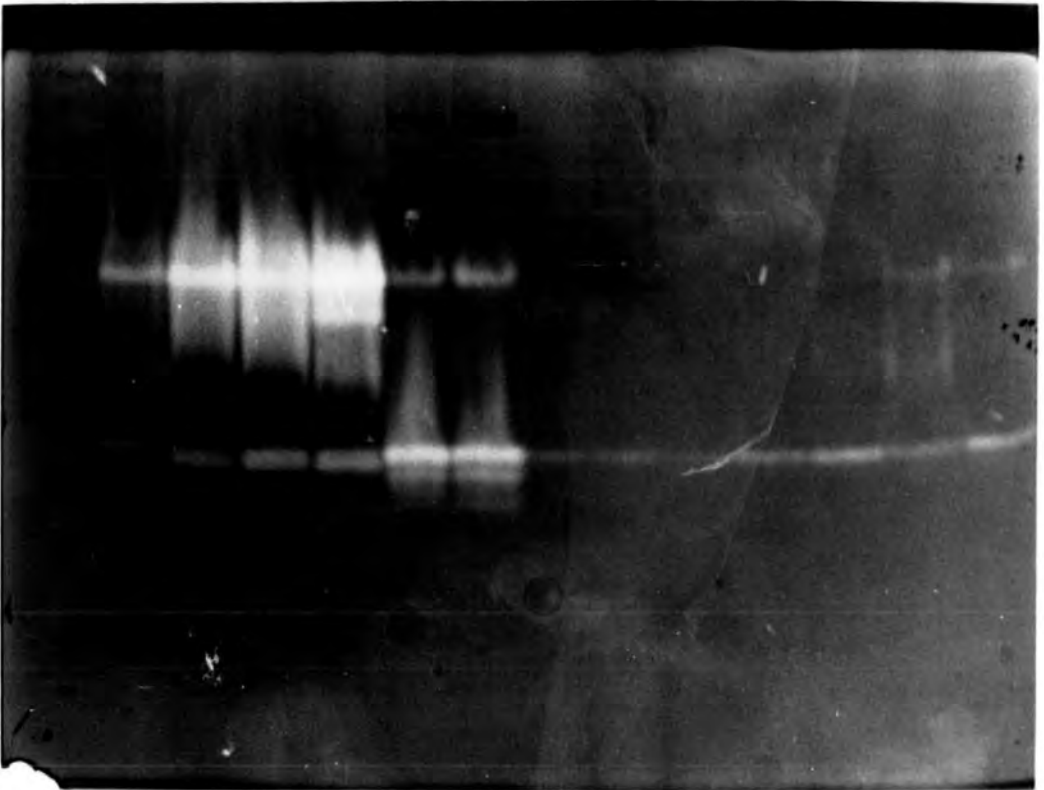
Zymogram pattern changes of amylase in dwarf French bean (Phaseolus vulgaris var. Masterpiece) seedling extracts at different stages of seed germination.

- a. Cotyledons
- b. Leaves
- c. Root
- d. Hypocotyl
- e. Stem

Numbers are days of germination.

Fig. 24

a₁ 2 4 6 9 b₆ 9 c₄ 6 9 d₄ 6 9 e₉



electrophoretic bands and the two fastest moving components had the lowest activities and were barely visible on the gel.

c) Root extracts

Zymogram patterns of amylase activity were studied in extracts from 4, 6 and 9 day-old seedlings and one band was present in each extract.

d) Hypocotyl extracts

One band was present in extract from 4 day-old seedlings and two bands in each extract from 6 and 9 day-old seedlings.

e) Stem extract

One faint slow moving band and one fast moving band were present in extract from 9 day-old seedlings.

2.4.2 During seed development

The electrophoretic pattern changes of amylase were studied in relation to seed development in extracts from two varieties of dwarf French bean and the results in figs. 25 and 26 show that amylase patterns exhibited changes as the seed developed, both varieties having the same pattern of changes. In both varieties, seed extract at the early stages of developing seed, each gave three distinct electrophoretic bands, while the extract at the late stages each gave two distinct electrophoretic bands. The two fastest moving components were faint and had low levels of amylase activities in all extracts. On the other hand, the slowest moving component had a high level of amylase activity, especially in the extracts from the var. Processor and was present only in the extracts at the early stages of developing seeds.

Fig. 25

Zymogram pattern changes of amylase in dwarf French bean (Phaseolus vulgaris var. Processor) seed extracts at different stages of seed development.

Track:

No. 1: 23 day-old seeds

No. 2: 24 day-old seeds

No. 3: 25 day-old seeds

No. 4: 26 day-old seeds

No. 5: 27 day-old seeds

No. 6: 28 day-old seeds

No. 7: 30 day-old seeds

No. 8: 33 day-old seeds

No. 9: 35 day-old seeds

No. 10: 43 day-old seeds

No. 11: 45 day-old seeds

No. 12: Mature seeds

Fig. 26

Zymogram pattern changes of amylase in extracts from dwarf French bean (Phaseolus vulgaris var. Masterpiece) seed extracts at different stages of seed development.

Track:

No. 1: 20 day-old seeds

No. 2: 27 day-old seeds

No. 3: 28 day-old seeds

No. 4: 30 day-old seeds

No. 5: 32 day-old seeds

No. 6: 33 day-old seeds

No. 7: 38 day-old seeds

No. 8: 42 day-old seeds

No. 9: 46 day-old seeds

No. 10: Mature seeds

Fig. 25

1 2 3 4 5 6 7 8 9 10 11 12

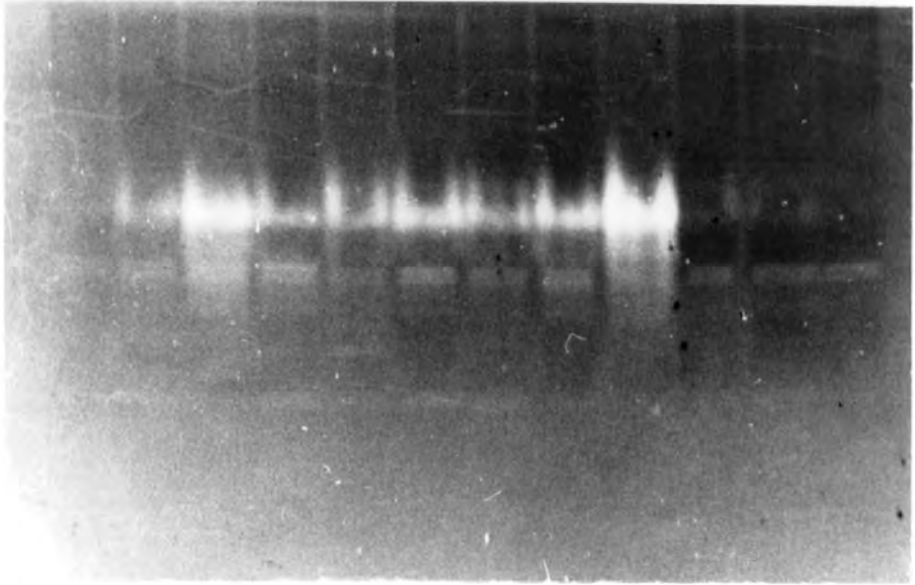
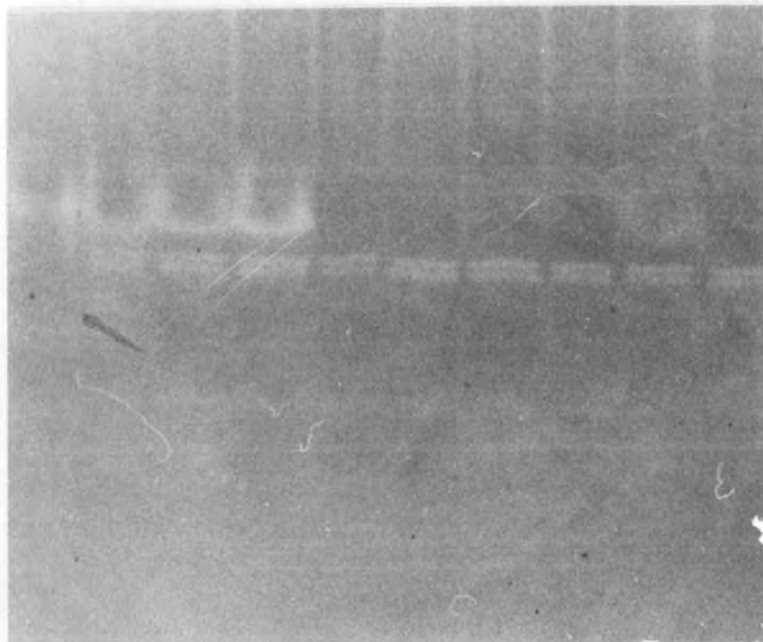


Fig. 26

1 2 3 4 5 6 7 8 9 10



2.5 Scarlet runner bean

Amylase activity was almost absent in the extracts from mature seeds, since only one very faint band, barely visible on the gel, was present.

2.5.1 During seed germination

Zymogram pattern changes were studied in seedling extracts at three stages of seed germination and extracts from cotyledons, root and stem were compared. Fig. 27 shows the zymogram patterns of the different extracts.

a) Cotyledon extracts

Amylase activity was very high in cotyledon extracts and the extract from 7 day-old seedlings gave one slow moving band and two fast moving bands, while the extract from 9 and 12 day-old seedlings, each gave two slow moving bands and one fast moving band. In all extracts the slowest migrating component was well resolved and had a high level of amylase activity.

b) Root and stem extracts

Each extract gave one fast moving band.

2.6 Black eye bean seed extract

At least five distinct electrophoretic bands of amylase activity were detected on the gel. The slowest migrating component (Rf 0.27) appeared to be the most active and gave a very wide band. The fastest migrating component (Rf 0.59) appeared to have the second highest activity, while the component at Rf 0.51 appeared to be less active

Fig. 27

Zymogram pattern changes of amylase in extracts from scarlet runner bean (Phaseolus coccineus) seedlings at different stages of seed germination.

a. Root

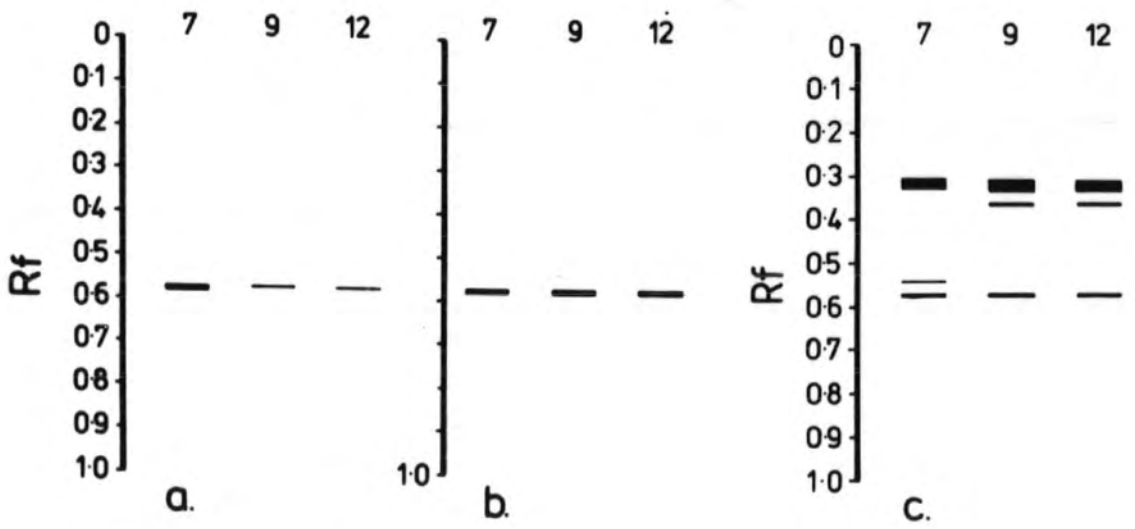
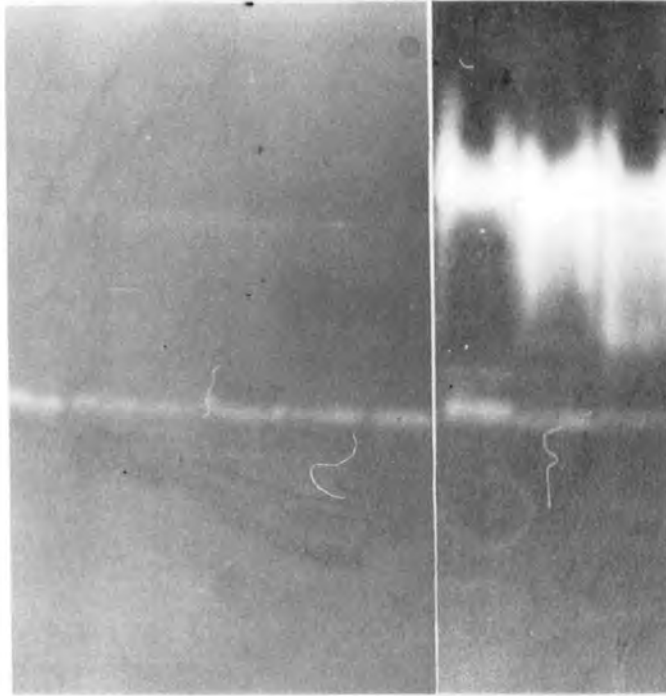
b. Stem

c. Cotyledons

Numbers are days of germination.

Fig. 27

a_{7 9 12} b_{7 9 12} c_{7 9 12}



than the fastest migrating component and more active than the other components at Rf 0.46 and 0.48 which were faint, low in amylase activities and they were resolved more clearly when 50 μ l extract was used.

2.6.1 During seed germination

The activity and zymogram patterns of amylase were studied in seed extracts at five stages of seed germination, up to the ninth day. The zymogram patterns were compared in extracts from cotyledons, leaves, root, hypocotyl and stem. Fig. 28 shows the electrophoretic patterns of the different extracts.

a) Cotyledon extracts

The level of amylase activity was very low in extracts from 1 and 2 day-old seedlings. Three faint, fast moving bands, barely visible on the gel, and one slow moving component, which appeared to have the highest level of amylase activity, were present.

The level of amylase activity appeared to be high in extracts from 4, 6 and 9 day-old seedlings. Two bands in extract from 4 day-old seedlings and three bands in each extracts from 6 and 9 day-old seedlings were detected and the slowest moving component had the highest activity in all extracts.

b) Leaf extracts

Amylase activity was studied in extracts from 6 and 9 day-old seedlings and a total of five faint bands were present in each extract.

Fig. 28

Zymogram pattern changes of amylase in extracts from black eye bean (Vigna unguiculata) seedlings at different stages of seed germination.

a. Cotyledons

b. Leaves

c. Root

d. Hypocotyl

e. Stem

Numbers are days of germination.

Fig. 28

1^a 2 4 6 9^b 6 9 2 4^c 6 9

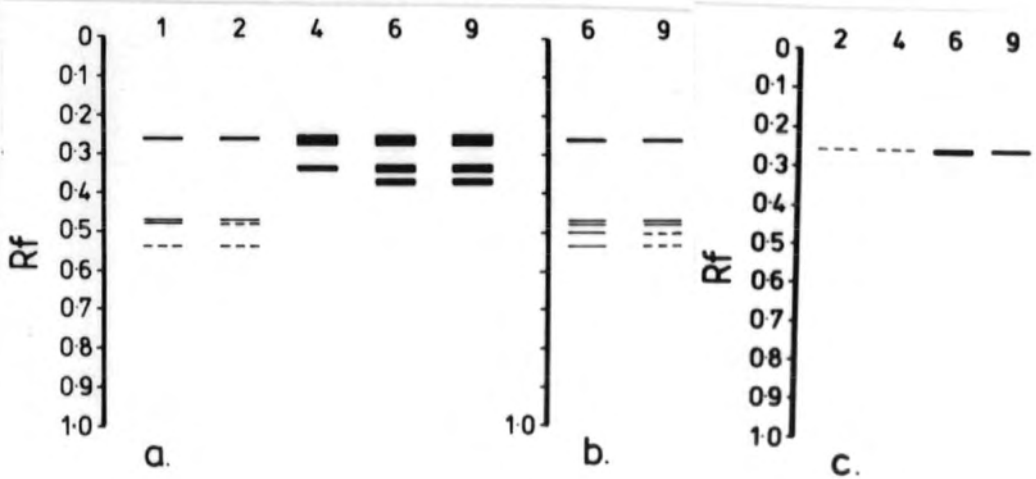
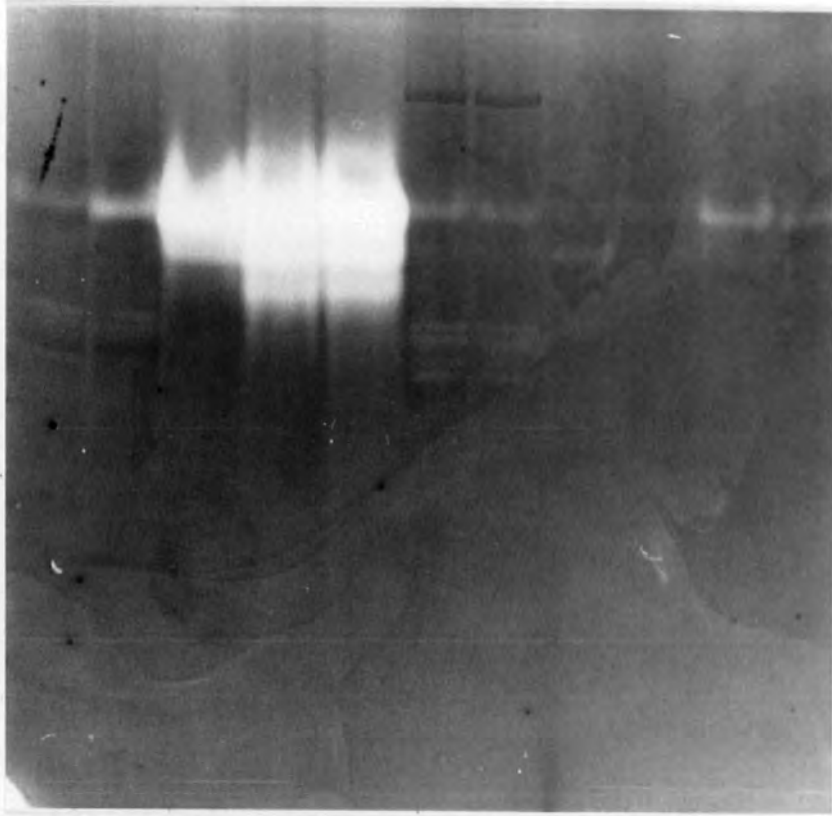
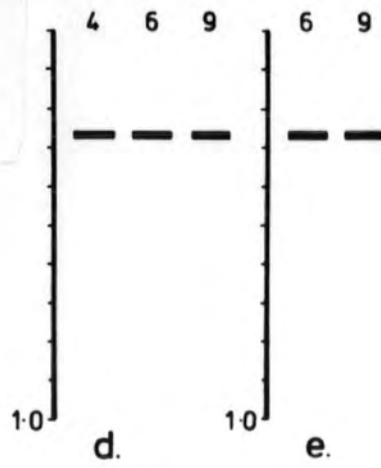


Fig. 28

d₄ 6 9 e₆ 9



c) Root extracts

The zymogram patterns of amylase activity were studied in extracts from 2, 4, 6 and 9 day-old seedlings and one faint band was present in each extract.

d) Hypocotyl extracts

The zymogram patterns of amylase were studied in extracts at three stages of seed germination and only one band was present in each extract.

e) Stem extracts

One band was present in each extract from 6 and 9 day-old seedlings.

2.7 Mung bean seed extract

The zymogram patterns of amylase activity of dry seed extract gave five distinct electrophoretic bands. The fastest migrating component (Rf 0.59) appeared to have the highest activity, while the three slowest migrating components (Rf 0.31, 0.4 and 0.49) were low in amylase activities.

2.4.1 During seed germination

Zymogram patterns of amylase were studied in extracts at four stages of seed germination, up to the fifth day. The activity and the zymogram patterns were compared in extracts from plumule hook, stem, root, hypocotyl and cotyledons. Fig. 29 shows the electrophoretic patterns of amylase in the different extracts.

a) Cotyledon extracts

A total of seven bands were detected. The extracts from 2 and 3 day-old seedlings had similar zymogram patterns and each extract gave five bands.

On the fifth day of seed germination, there was a noticeable change in the zymogram patterns and as shown in fig. 29a, three bands were present. The slowest migrating component (Rf 0.27) was very thick, had the highest activity and was similar to the slowest migrating component in extracts from 1, 2 and 3 day-old seedling, while the other two were additive bands.

b) Plumule hook, stem, hypocotyl and root extracts

Only one band was present in each extract and corresponded to the slowest migrating band in the germinated cotyledon extracts.

2.8 Broad bean (*Vicia faba*) seed extract

Three distinct electrophoretic bands were detected. The fastest migrating component (Rf 0.56) was thick and appeared to have the highest amylase activity. On the other hand, the slowest migrating component (Rf 0.25) was faint and had low amylase activity.

2.8.1 During seed germination

The activity and zymogram patterns of amylase were examined in extracts at different stages of seed germination, up to the eighth day. The electrophoretic patterns of amylase activity were compared in extracts from cotyledons, plumule hook and stem. Fig. 30 shows the changes in zymogram patterns observed in the different extracts.

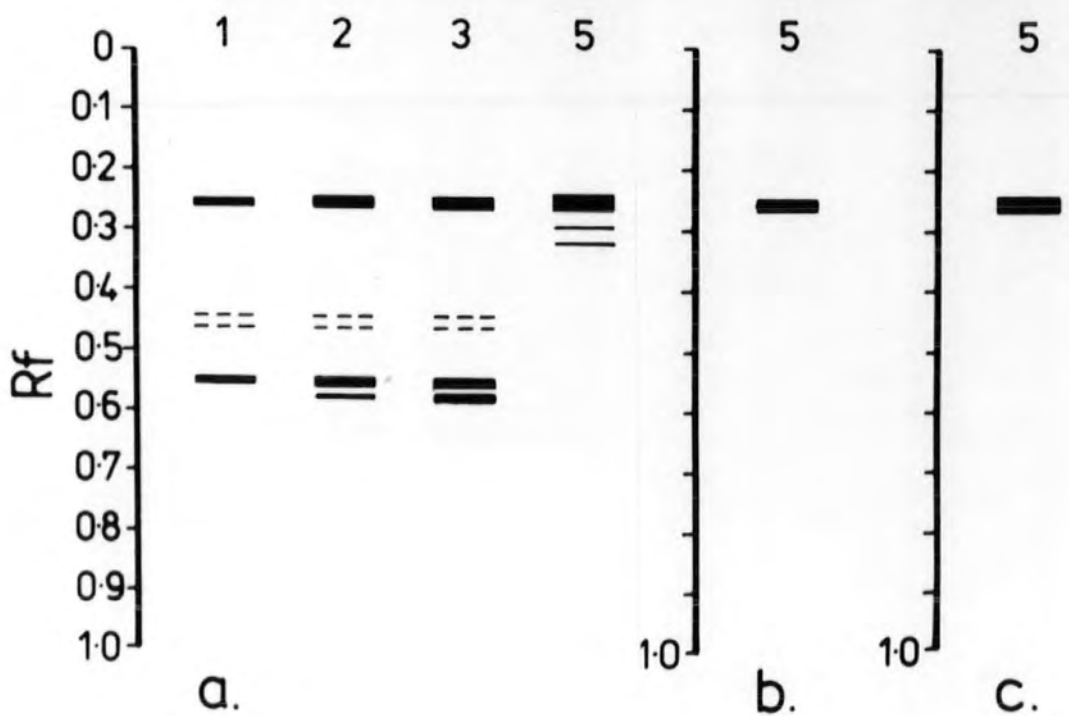
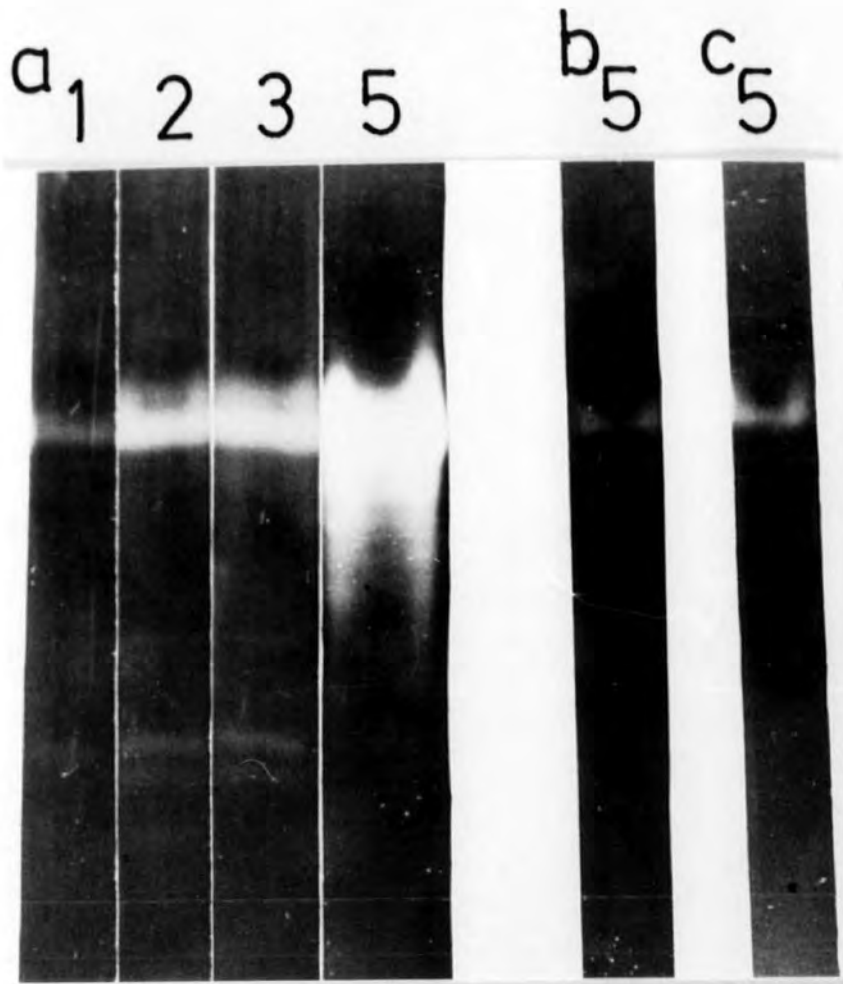
Fig. 29

Zymogram pattern changes of amylase in extracts from mung bean (Vigna radiata) seedlings at different stages of seed germination.

- a. Cotyledons
- b. Plumule hook
- c. Stem
- d. Root
- e. Hypocotyl

Numbers are days of germination.

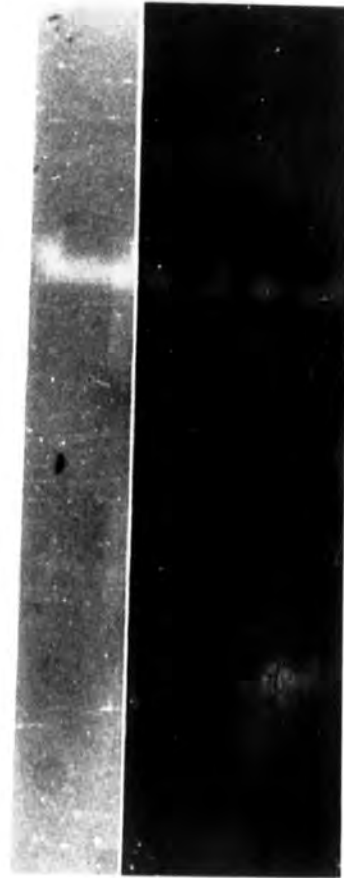
Fig. 29



d₂ 3 5



e₂ 3 5



R_f

0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

d.

10

e.

Germinated cotyledon extracts had the highest amylase activity and the largest number of bands, as compared with that of other parts of the seedling.

a) Cotyledon extracts

Amylase activity was studied in extracts at five stages of seed germination. As shown in fig. 30a the zymogram patterns of amylase activity showed remarkable changes during seed germination and a total of seven bands were observed.

b) Plumule hook extracts

The activity and electrophoretic patterns of amylase were studied in extracts at three stages of seed germination and the zymogram patterns of amylase gave a maximum of four bands (see fig. 20b).

c) Stem extracts

Amylase activity was studied in stem extracts from 5, 7 and 8 day-old seedlings and the results indicated that amylase activity was completely lacking in stem extracts.

2.8.2 During seed development

The activity and zymogram patterns of amylase were studied in seed extracts at 14 stages of seed development and the results in fig. 31 revealed that the fastest moving component had a high level of activity in extracts from young seeds and was not present in extracts at the late stages of developing seeds.

The activities of the two pink components, at position c) in the photograph, increased in extracts at the late stages of developing seeds. The faster moving component, which was more active than the



Fig. 30

Zymogram pattern changes of amylase in extracts from broad bean (Vicia faba) seedlings at different stages of seed germination.

a. Cotyledons

b. Plumule hook

Numbers are days of germination.

FIG. 30

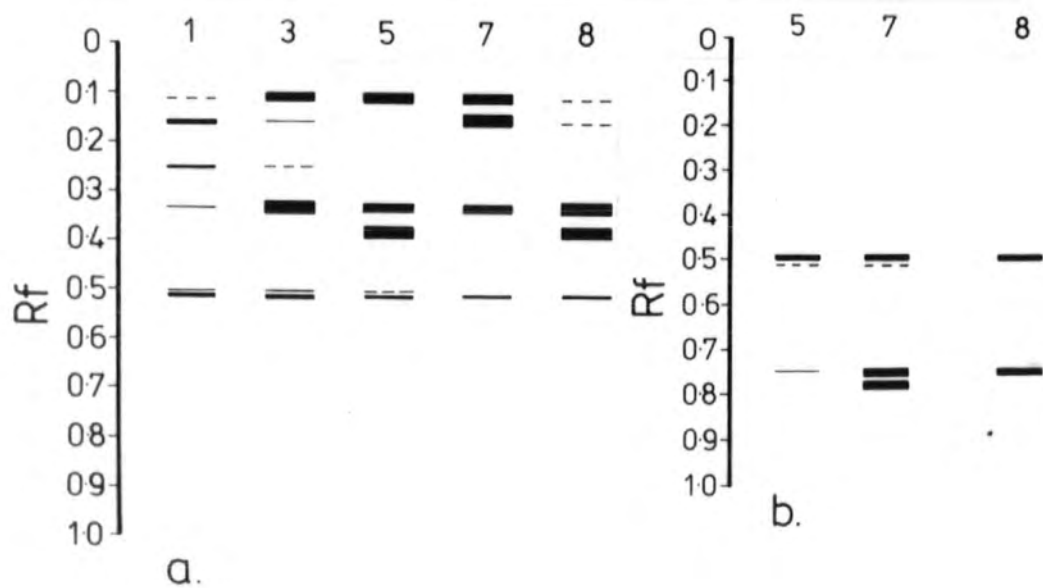
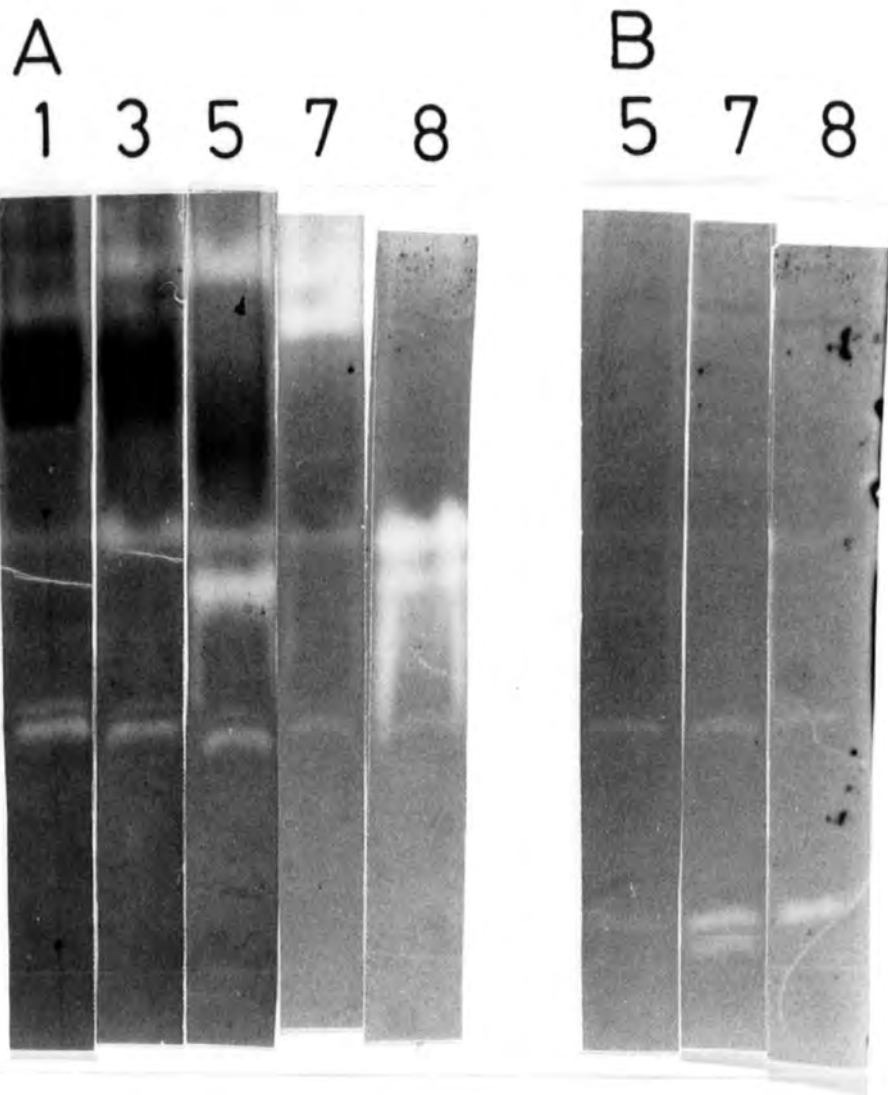


Fig. 31

Zymogram pattern changes of amylase in broad bean (Vicia faba) seed extracts at different stages of seed development.

Track:

- No. 1: 15 day-old seeds
- No. 2: 18 day-old seeds
- No. 3: 23 day-old seeds
- No. 4: 20 day-old seeds
- No. 5: 25 day-old seeds
- No. 6: 28 day-old seeds
- No. 7: 30 day-old seeds
- No. 9: 35 day-old seeds
- No. 10: 40 day-old seeds
- No. 11: 44 day-old seeds
- No. 12: 48 day-old seeds
- No. 13: 52 day-old seeds
- No. 14: 54 day-old seeds

Fig. 31

1 2 3 4 5 6 7 8 9 10 11 12 13 14



← D
← C

other component, appeared in extracts from 23 day-old seeds, while the other component appeared in extracts from 33 day-old seeds.

The component, at position b), had its highest level of amylase activity in extracts from 15 and 18 day-old seeds and was not present in extracts at late stages of developing seeds.

The slowest moving component, at position a) in the photograph, but not clear, had a low level of activity and was present only in extracts at the late stages of developing seeds.

2.9 Bean (*Vicia narbonensis*) seed extract

One active component (pink in colour) was detected (fig. 32 track dr.).

2.9.1 During seed germination

Zymogram pattern changes of amylase were studied in seedling extracts at four stages of seed germination, up to 10th day, and fig. 32 shows these changes.

a) Cotyledon extracts

Each extract from 2, 5, 8 and 10 day-old seedlings gave two bands and the slow moving component increased in activity during seed germination, while the fast moving component decreased slightly in activity over the same period.

b) Root extracts

One fast moving band was present in each extract from 8 and 10 day-old seedlings and an additional faint band was present in extract from 10 day-old seedlings.

Fig. 32.

Zymogram pattern changes of amylase in extracts from broad (Vicia narbonensis) seedlings at different stages of seed germination.

Track dr: dry seed

a. Cotyledons

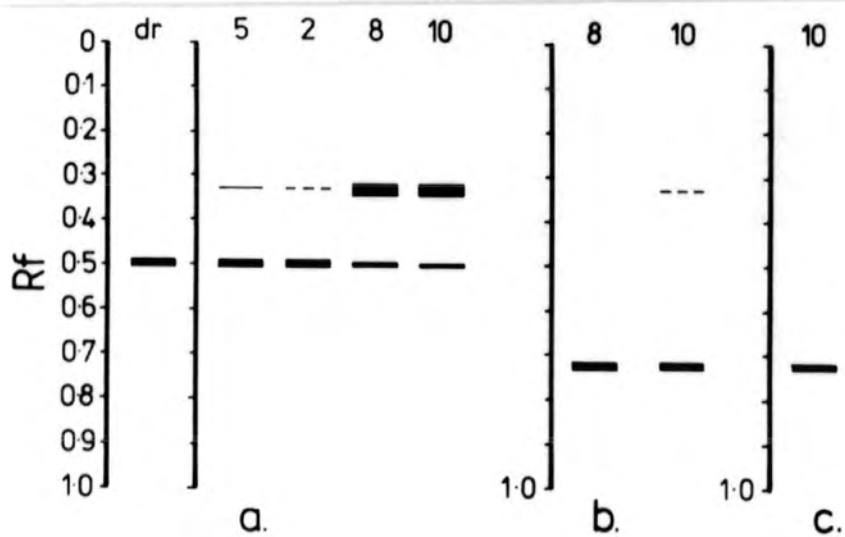
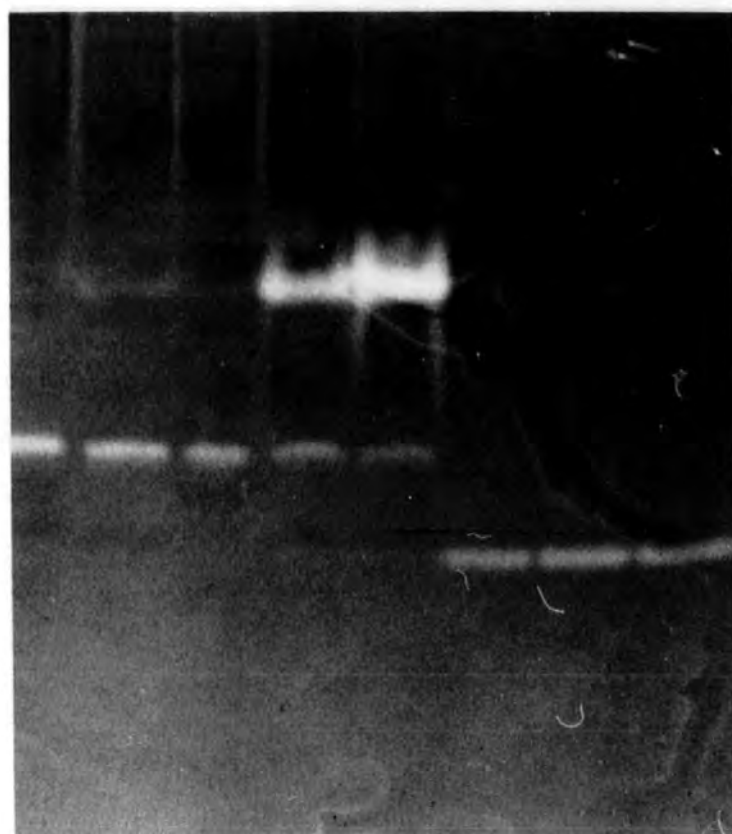
b. Root

c. Stem

Numbers are days of germination.

Fig. 32

dr. ^a5 2 8 10 ^b8 10 ^c10



c) Stem extract

One fast moving band was detected in an extract from 10 day-old seedlings.

3. Non-specific esterase activity

The activity of EST was detected on the gel as a brown-stained band in a yellow background. The bands developed about 20min. after incubation in the staining solution.

3.1 Inbred lines of pea

The zymogram patterns of EST activity in extracts from the dry pea seeds gave three distinct electrophoretic bands for each line of the six inbred lines studied. These bands were polymorphic (see fig. 33).

As shown in fig. 33 the fastest migrating component had the highest EST activity and the other components had low EST activities.

3.1.1 During seed development

The changes in activity and electrophoretic patterns of EST in relation to seed development were investigated in extracts from three varieties of Pisum sativum and the results showed that EST activity exhibited remarkable changes during seed development with decreasing number and intensities of the bands as the seed of the three varieties developed.

The extracts from the var. Feltham First had the most intense stained bands and as shown in figs. 34, 35 and 36 the activity of EST

Fig. 33

Zymogram patterns of EST in pea seed extracts.

Track:

- No. 1: Inbred line no. 379
- No. 2: Inbred line no. 611
- No. 3: Inbred line no. 1293
- No. 4: Inbred line no. 851
- No. 5: Inbred line no. 2162
- No. 6: Inbred line no. 1263

Fig. 34

Zymogram pattern changes of EST in extracts from pea (Pisum sativum var. Feltham First) seeds at different stages of seed development.

Track:

- No. 1: 10 day-old seeds
- No. 2: 11 day-old seeds
- No. 3: 12 day-old seeds
- No. 4: 14 day-old seeds
- No. 5: 15 day-old seeds
- No. 6: 17 day-old seeds
- No. 7: 19 day-old seeds
- No. 8: 21 day-old seeds
- No. 9: 23 day-old seeds
- No. 10: 25 day-old seeds
- No. 11: 27 day-old seeds
- No. 12: 28 day-old seeds
- No. 13: 30 day-old seeds

Fig. 33

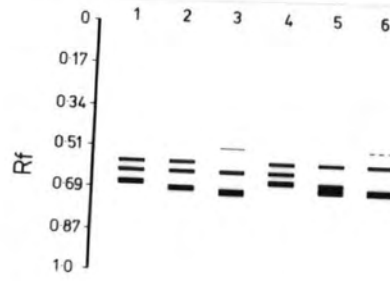
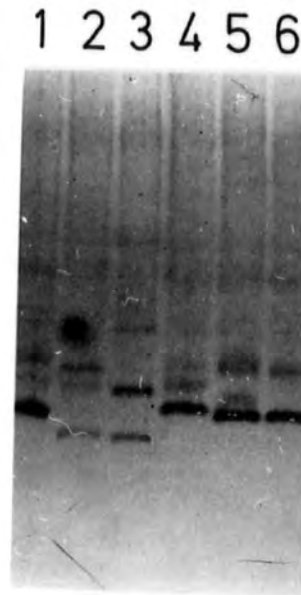


Fig. 34

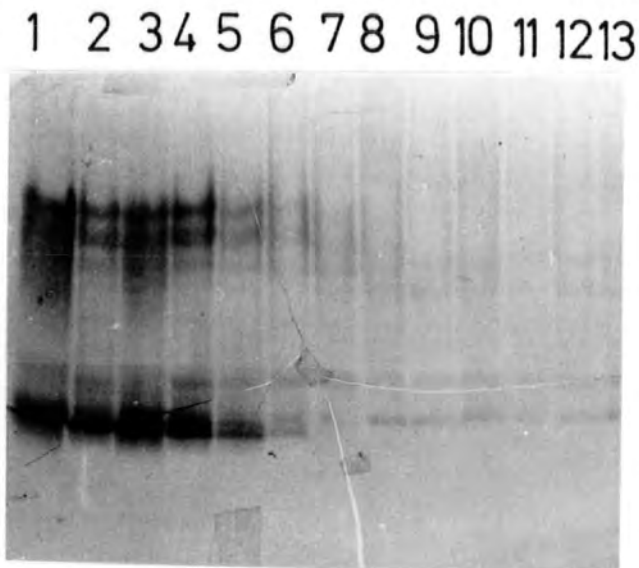


Fig. 35

Zymogram pattern changes of EST in extracts from pea (Pisum sativum var. English Wonder) seeds at different stages of seed development.

Track:

No. 1: 13 day-old seeds

No. 2: 14 day-old seeds

No. 3: 15 day-old seeds

No. 4: 16 day-old seeds

No. 5: 17 day-old seeds

No. 6: 18 day-old seeds

No. 7: 19 day-old seeds

No. 8: 20 day-old seeds

No. 9: 21 day-old seeds

No. 11: 26 day-old seeds

No. 12: 27 day-old seeds

No. 13: 30 day-old seeds

Fig. 36

Zymogram pattern changes of EST in extracts from pea (Pisum sativum var. Mangetout) seeds at different stages of seed development.

Track:

No. 1: 10 day-old seeds

No. 2: 11 day-old seeds

No. 3: 13 day-old seeds

No. 4: 15 day-old seeds

No. 5: 16 day-old seeds

No. 6: 17 day-old seeds

No. 7: 18 day-old seeds

No. 8: Mature seeds

Fig.35

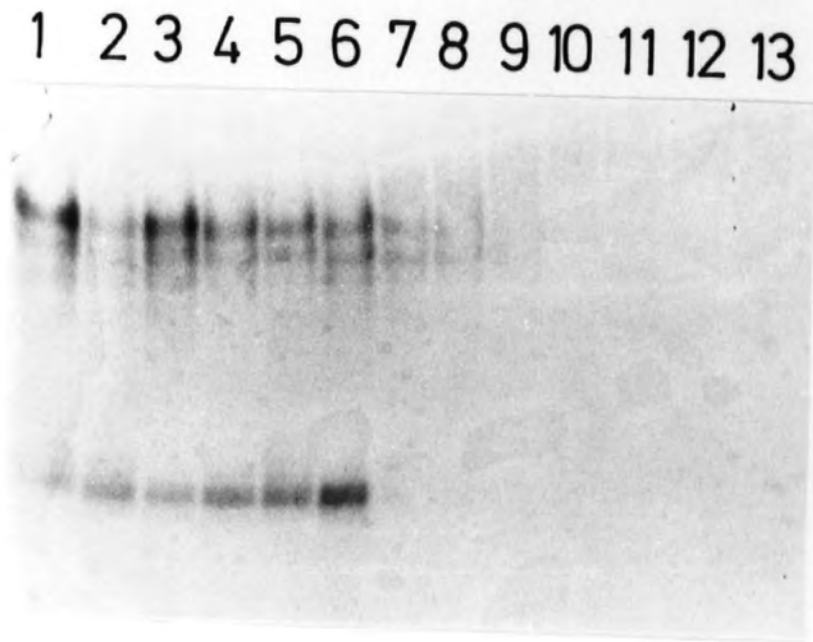
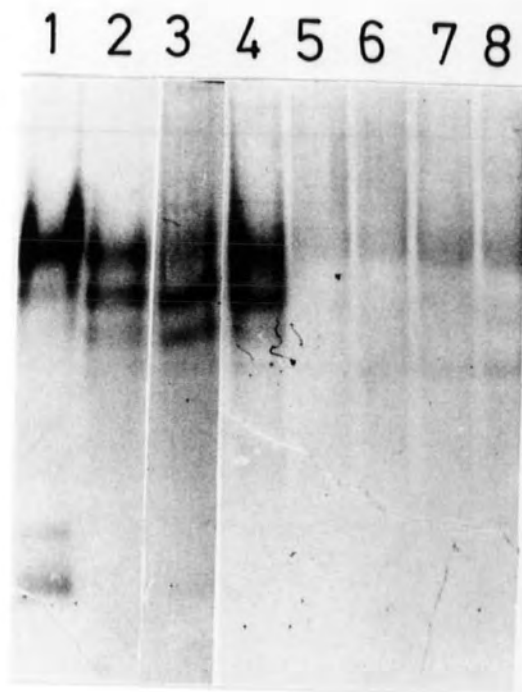


Fig. 36



was lacking in the extracts at the late stages of developing seeds of the varieties English Wonder and Mangetout.

3.2 Soyabean seed extract

No bands of EST activity were detected in mature seed extract.

3.2.1 During seed development

EST activity was studied in extracts at five stages of seed development and the results in fig. 37 showed that the level of EST activity was high in extract from 21 day-old seeds when 3 bands were detected. The two fastest moving components had a high level of EST activities.

The extract from 27 and 35 day-old seeds gave three faint bands.

3.3 Broad bean (*Vicia faba*) seed extract

Three distinct electrophoretic bands were present and the fastest moving component had the lowest activity (see fig. 38 track 14).

3.3.1 During seed development

The results in fig. 38 show that two bands are present in each extract at the different stages. The extracts from some stages gave two additional slow moving bands and the extracts at the early stages of developing seeds had an additional faint, fast moving band.

Fig. 37

Zymogram pattern changes of EST in extracts from soyabean (Glycine max) seeds at different stages of seed development.

Track:

No. 1: 21 day-old seeds

No. 2: 27 day-old seeds

No. 3: 35 day-old seeds

No. 4: 52 day-old seeds

No. 5: Mature seeds

Fig. 38

Zymogram pattern changes of EST in extracts from broad bean (Vicia faba) seeds at different stages of seed development.

Track:

No. 1: 18 day-old seeds

No. 2: 20 day-old seeds

No. 3: 21 day-old seeds

No. 4: 23 day-old seeds

No. 5: 25 day-old seeds

No. 6: 28 day-old seeds

No. 7: 30 day-old seeds

No. 8: 35 day-old seeds

No. 9: 40 day-old seeds

No. 10: 44 day-old seeds

No. 11: 48 day-old seeds

No. 12: 52 day-old seeds

No. 13: 54 day-old seeds

No. 14: Mature seeds

Fig. 37

1 2 3 4 5

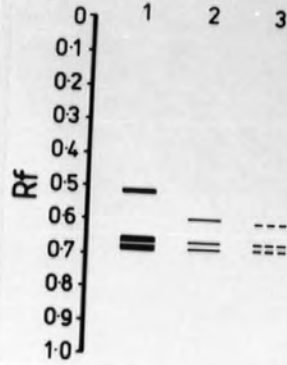
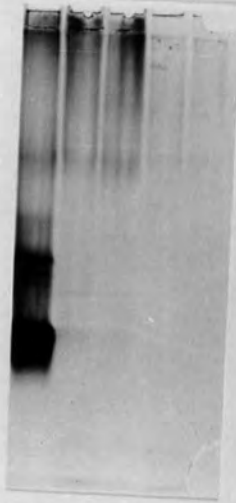
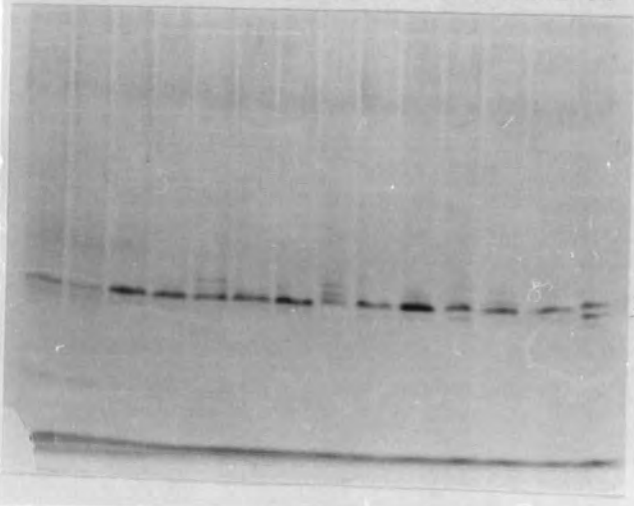


Fig. 38

1 2 3 4 5 6 7 8 9 10 11 12 13 14



3.4 During seed development in dwarf French bean

The extracts from the varieties Processor and Masterpiece had a low level of EST activity at the different stages of seed development and two faint bands were present in each extract (see fig. 39).

4. Glutamate-oxaloacetate transaminase activity

The sites of GOT activity were detected on the acrylamide gel as red-stained bands in a yellow background. The bands developed in about 10min. as the gel incubated in the staining solution. Fig. 40 shows the GOT electrophoretic patterns of the different legume seed extracts.

4.1 Inbred lines of pea

Three distinct electrophoretic bands were detected in seed extracts for each inbred line examined, except seed extract from inbred line 2162 which had an additional faint band at Rf 0.46. The fastest migrating component had the lowest GOT activity (see fig. 41).

a) The variants of GOT

As shown in fig. 41, the three bands were classified as GOT¹ for the slowest migrating band (Rf 0.38), GOT² for the intermediate migrating band (Rf 0.42) and GOT³ for the fastest migrating band. Only the fastest migrating band was present in a polymorphic pattern, while the other two bands were monomorphic. These variants of GOT³ were classified according to their migration toward the anode as F (faster, Rf 0.53) and S (slower Rf 0.47); only one variant occurred in any

Fig. 39.

Zymogram pattern changes of EST in extracts from dwarf French bean (Phaseolus vulgaris) seeds at different stages of seed development.

a. Var. Masterpiece

Track:

No. 1: 20 day-old seeds

No. 2: 24 day-old seeds

No. 3: 38 day-old seeds

No. 4: Mature seeds

b. Var. Processor

Track:

No. 1: 23 day-old seeds

No. 2: 26 day-old seeds

No. 3: 33 day-old seeds

No. 4: Mature seeds

Fig. 39

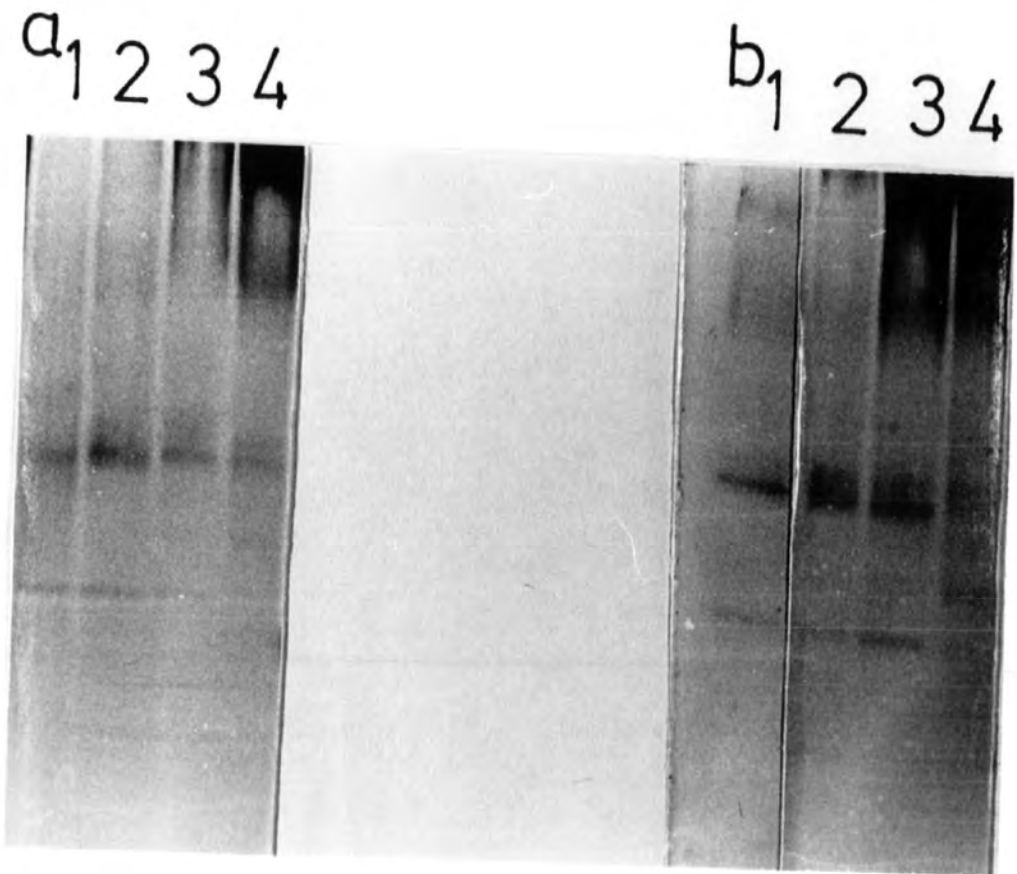


Fig. 40

Diagrammatic zymogram patterns of GOT in different legume seed extracts.

No. 1: Pea (Pisum sativum var. Feltham First)

No. 2: Black eye bean (Vigna unguiculata)

No. 3: Jack bean (Canavalia ensiformis)

No. 4: Dwarf French bean (Phaseolus vulgaris var. Processor)

No. 5: Dwarf French bean (Phaseolus vulgaris var. Masterpiece)

No. 6: Broad bean (Vicia faba)

No. 7: Mung bean (Vigna radiata)

No. 8: Pigeon pea (Cajanus cajan)

fig.40

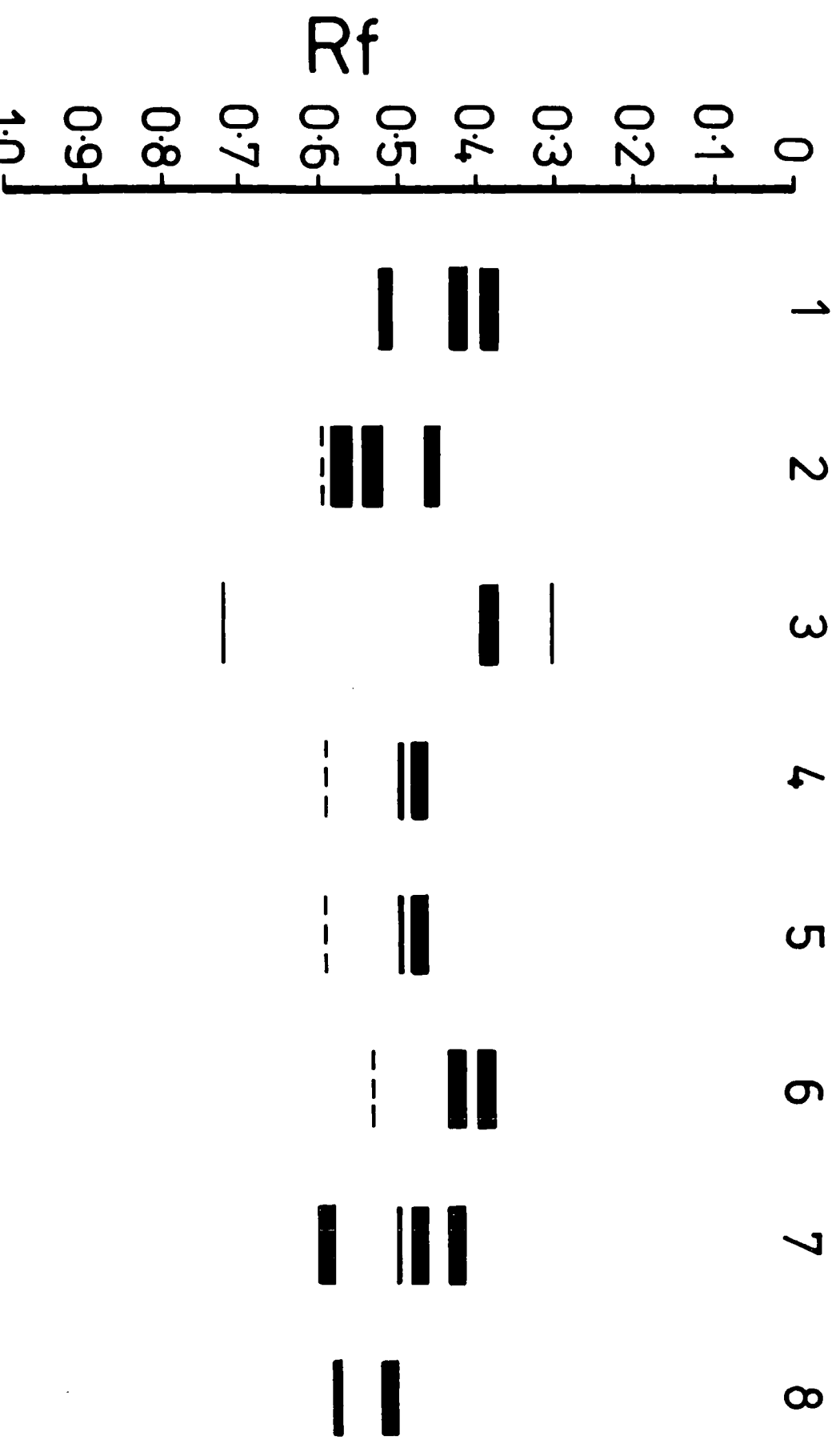


Fig. 41

Zymogram patterns of GOT in pea seed extracts.

Track:

No. 1: Inbred line no. 851

No. 2: Inbred line no. 110

No. 3: Inbred line no. 180

No. 4: Inbred line no. 2162

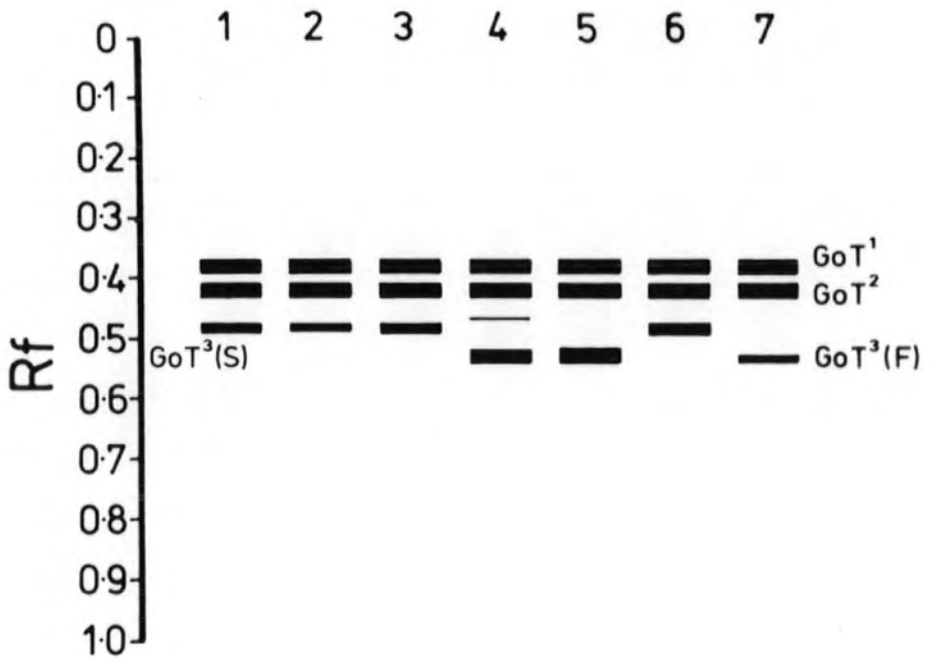
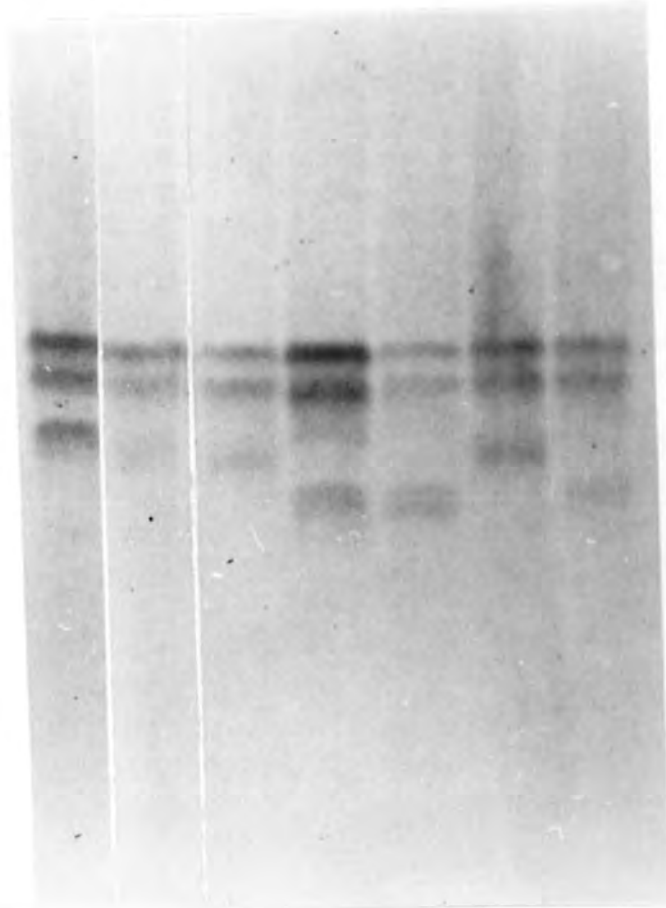
No. 5: Inbred line no. 1293

No. 6: Inbred line no. 1263

No. 7: Inbred line no. 1256

Fig. 41

1 2 3 4 5 6 7



inbred line.

4.1.1 During seed development

The electrophoretic patterns of GOT were studied in seed extracts from three varieties of Pisum sativum at different stages of seed development and the results in figs 42, 43 and 44 indicated that in the three varieties, the dry mature seed extract and the immature seed extract had the same electrophoretic patterns and apparently equal levels of enzyme activity.

The extract of each stage gave three distinct electrophoretic bands and the extracts from the var. Feltham First had the most intensely stained bands. The extracts from the var. Mangetout had the less intensely stained bands especially the fastest migrating band, which was very faint and hardly detectable on the gel.

4.2 Broad bean (*Vicia faba*) seed extract

Three bands were detected and the fastest migrating component (Rf 0.57) was faint and had the lowest activity.

4.2.1 During seed development

The activity and zymogram patterns of GOT were studied in seed extracts at 13 stages of seed development and the results showed that there were no changes in the electrophoretic patterns of GOT as the seed developed and each extract giving three distinct electrophoretic bands, the fastest moving component having the lowest activity (see fig. 45).

Fig. 42

Zymogram pattern changes of GOT in extracts from pea (Pisum sativum var. Feltham First) seeds at different stages of seed development.

Track:

- No. 1: 10 day-old seeds
- No. 2: 11 day-old seeds
- No. 3: 12 day-old seeds
- No. 4: 14 day-old seeds
- No. 5: 15 day-old seeds
- No. 6: 17 day-old seeds
- No. 7: 19 day-old seeds
- No. 8: 21 day-old seeds
- No. 9: 23 day-old seeds
- No. 10: 25 day-old seeds
- No. 11: 30 day-old seeds
- No. 12: 31 day-old seeds

Fig. 43

Zymogram pattern changes of GOT in pea (Pisum sativum var. English Wonder) seeds at different stages of seed development.

Track:

- No. 1: 15 day-old seeds
- No. 2: 16 day-old seeds
- No. 3: 17 day-old seeds
- No. 4: 18 day-old seeds
- No. 5: 19 day-old seeds
- No. 6: 20 day-old seeds
- No. 7: 23 day-old seeds
- No. 8: 25 day-old seeds
- No. 9: 26 day-old seeds
- No. 10: 27 day-old seeds
- No. 11: 28 day-old seeds
- No. 12: 30 day-old seeds

Fig. 42

1 2 3 4 5 6 7 8 9 10 11 12

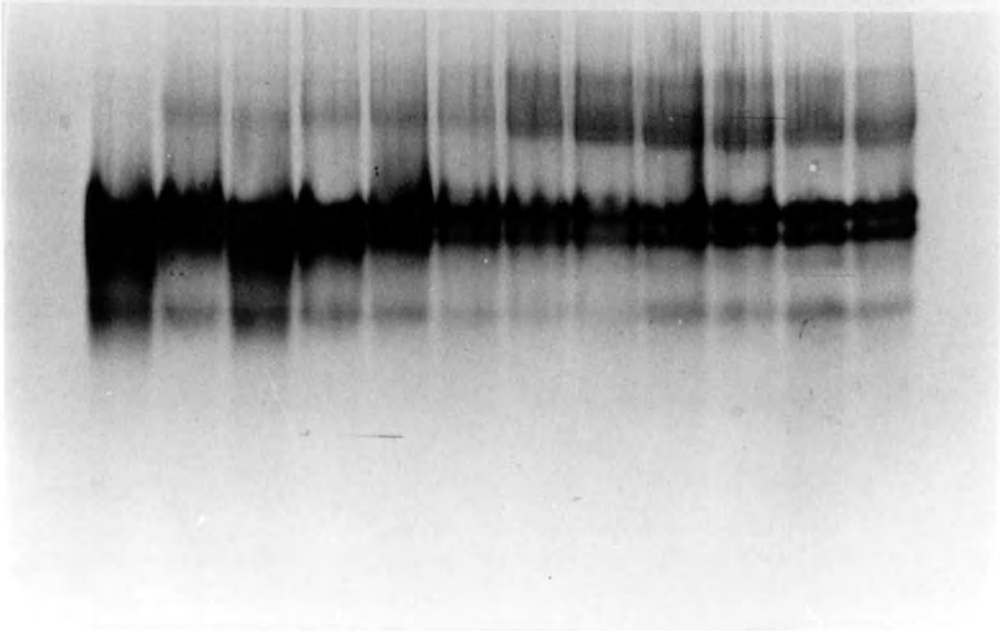


Fig.43

1 2 3 4 5 6 7 8 9 10 11 12 13

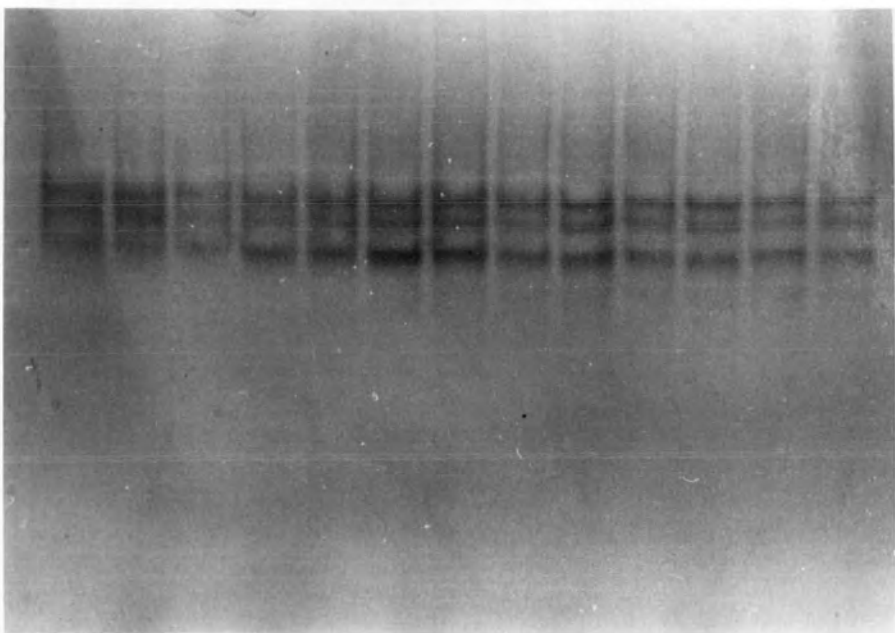


Fig. 44

Zymogram pattern changes of GOT in extracts from pea (Pisum sativum var. Mangetout) seeds at different stages of seed development.

Track:

- No. 1: 10 day-old seeds
- No. 2 and 3: 12 day-old seeds
- No. 4: 13 day-old seeds
- No. 5: 14 day-old seeds
- No. 6: 15 day-old seeds
- No. 7: 16 day-old seeds
- No. 8: 17 day-old seeds
- No. 9: 18 day-old seeds
- Nos 10 and 11: Mature seeds

Fig. 45

Zymogram pattern changes of GOT in extracts from broad bean (Vicia faba) seeds at different stages of seed development.

Track:

- No. 1: 12 day-old seeds
- No. 2: 18 day-old seeds
- No. 3: 20 day-old seeds
- No. 4: 21 day-old seeds
- No. 5: 23 day-old seeds
- No. 5: 23 day-old seeds
- No. 6: 25 day-old seeds
- No. 7: 28 day-old seeds
- No. 8: 30 day-old seeds
- No. 9: 35 day-old seeds
- No. 10: 40 day-old seeds
- No. 11: 44 day-old seeds
- No. 12: 48 day-old seeds
- No. 13: 52 day-old seeds
- No. 14: 54 day-old seeds

Fig. 44

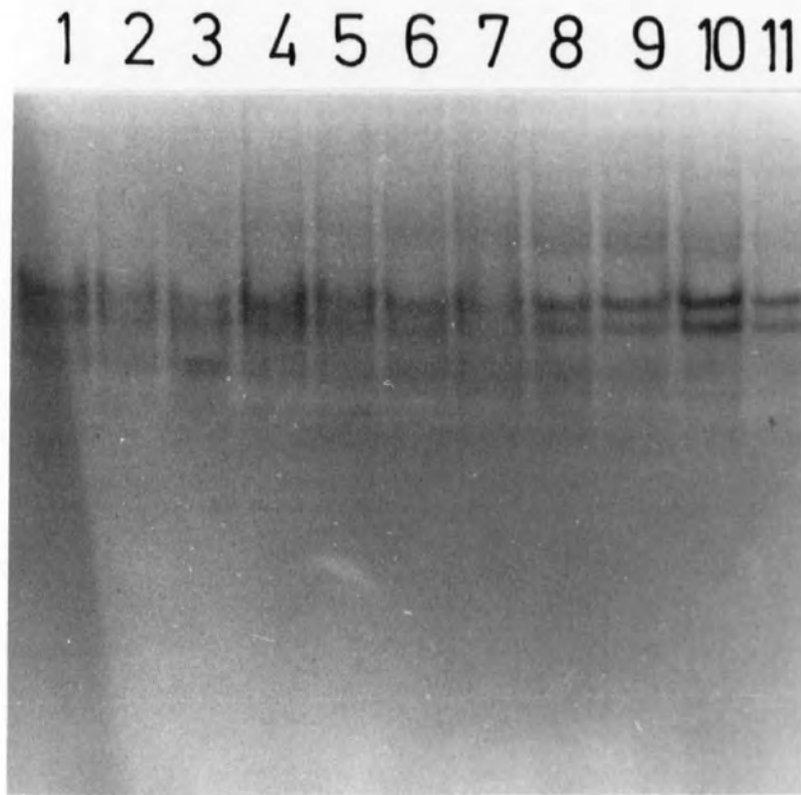
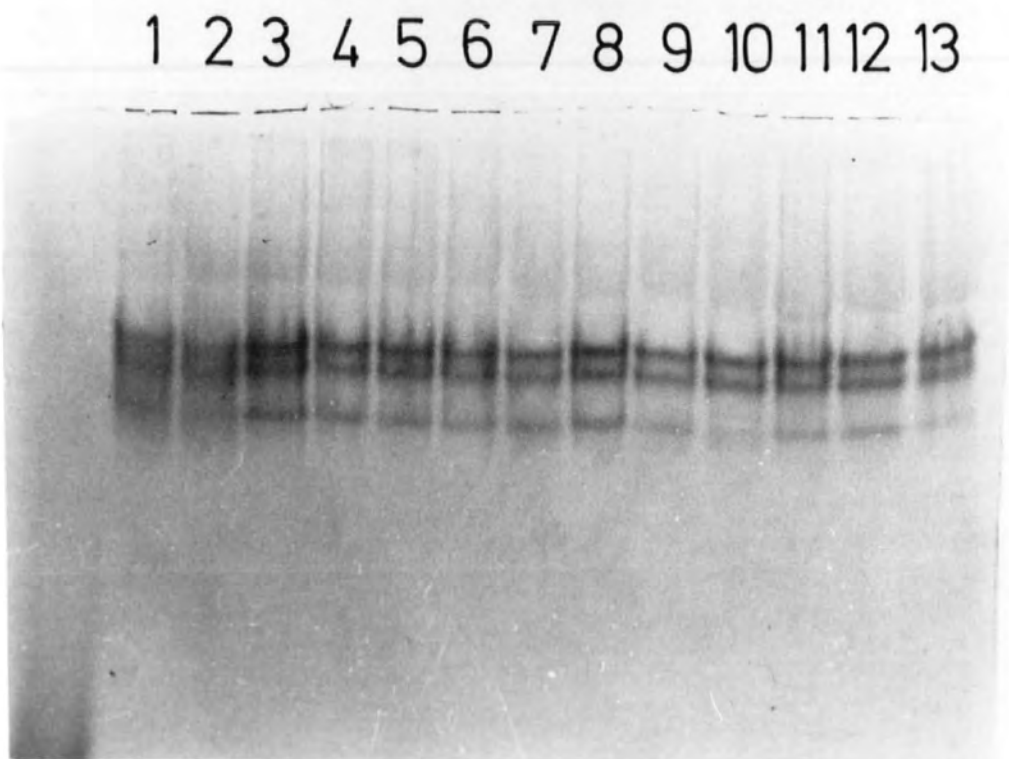


Fig. 45



4.3 Dwarf French bean seed extract

The zymogram patterns of GOT were similar in seed extracts from both varieties (Processor and Masterpiece), and the level of enzyme activity appeared to be the same. Three bands were detected in each seed extract.

4.3.1 During seed development

Each extract at the early stages of seed development of both varieties (Processor and Masterpiece) gave four bands. The fastest moving component decreased in activity as the seed developed and the band, at position a) in the photograph, disappeared in the mature seed extract. See Figs. 46 and 47.

4.4 Black eye bean seed extract

Four distinct electrophoretic bands were observed. The fastest migrating component (Rf 0.60) was faint and appeared to have the lowest GOT activity.

4.5 Jack bean seed extract

The zymogram pattern of GOT gave three distinct electrophoretic bands. The intermediate migrating component had the highest activity.

4.6 Pigeon pea seed extract

Two bands were detected. The slowest moving component (Rf 0.51) was slightly more active than the other component.

Fig. 46

Zymogram pattern changes of GOT in extracts from dwarf French bean (Phaseolus vulgaris var. Masterpiece) seeds at different stages of seed development.

Track:

No. 1: 24 day-old seeds

No. 2: 27 day-old seeds

No. 3: 30 day-old seeds

No. 4: 33 day-old seeds

No. 5: 38 day-old seeds

No. 6: 46 day-old seeds

No. 7: Mature seeds

Fig. 47

Zymogram pattern changes of GOT in extracts from dwarf French bean (Phaseolus vulgaris var. Processor) seeds at different stages of seed development.

Track:

No. 1: 23 day-old seeds

No. 2: 24 day-old seeds

No. 3: 25 day-old seeds

No. 4: 27 day-old seeds

No. 5: 28 day-old seeds

No. 6: 30 day-old seeds

No. 7: 33 day-old seeds

No. 8: 43 day-old seeds

No. 9: 45 day-old seeds

No. 10: Mature seeds

Fig. 46

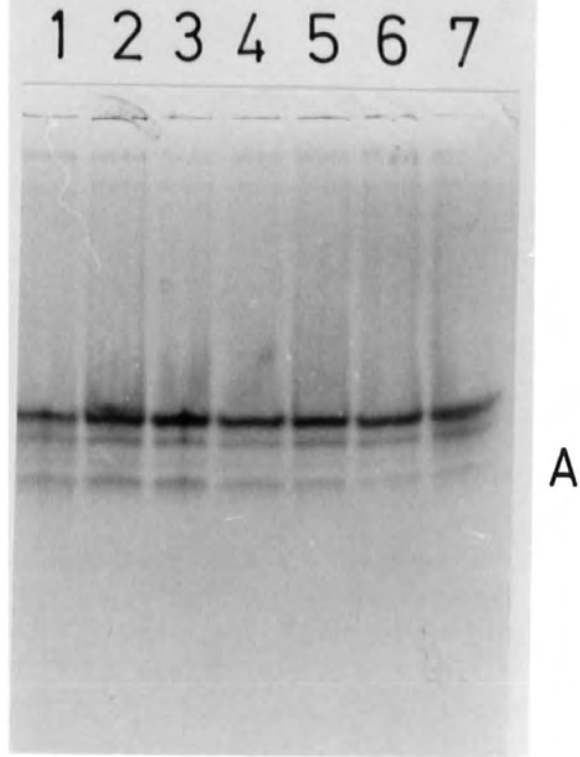
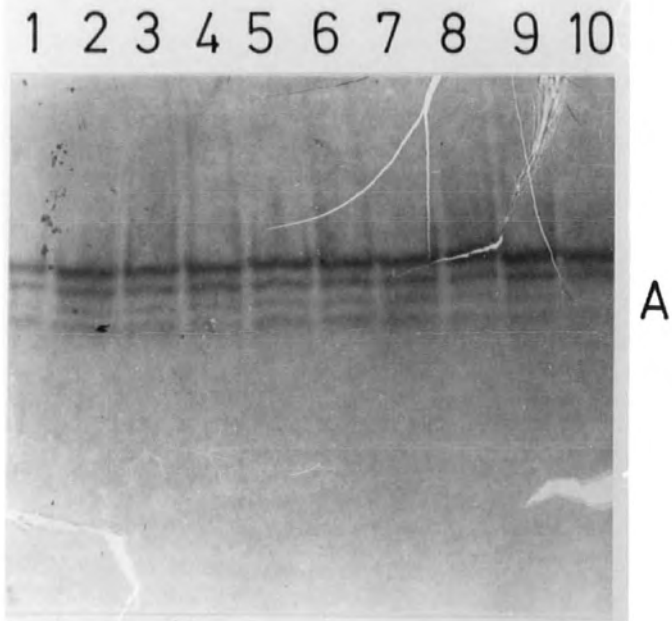


Fig. 47



4.7 Mung bean seed extract

Four bands were observed and the component at Rf 0.45 appeared to have the lowest activity.

4.8 During seed development in soyabean

Two bands were detected in each seed extract at the different stages of seed development (see fig. 48).

5. Leucine-aminopeptidase activity

The activity of LAP was detected on the acrylamide gel as a pink-stained band on a slightly yellow background. The bands developed after about 35min. incubation in the staining solution.

5.1 Pea seed extract

The zymogram of LAP activity of mature pea (var. Feltham First and var. English Wonder) seed extracts showed only one band (Rf 0.66). The level of LAP activity appeared to be low in the seed extract, since the activity was only detected on the gel when no less than 60 μ l extract (1:5 (w/v) seed meal to extraction buffer) was loaded on the gel. See figs. 49 and 50.

5.1.1 During seed development

Zymogram pattern changes of LAP were investigated in relation to seed development in extracts from two varieties, Feltham First and English Wonder and the results in fig. 49 and 50 showed one band was

Fig. 48

Zymogram pattern changes of GOT in extracts from soyabean (Glycine max) seeds at different stages of seed development.

Track:

No. 1: 21 day-old seeds

No. 2: 25 day-old seeds

No. 3: 27 day-old seeds

No. 4: 30 day-old seeds

No. 5: 33 day-old seeds

No. 6: 52 day-old seeds

Fig. 48

1 2 3 4 5 6

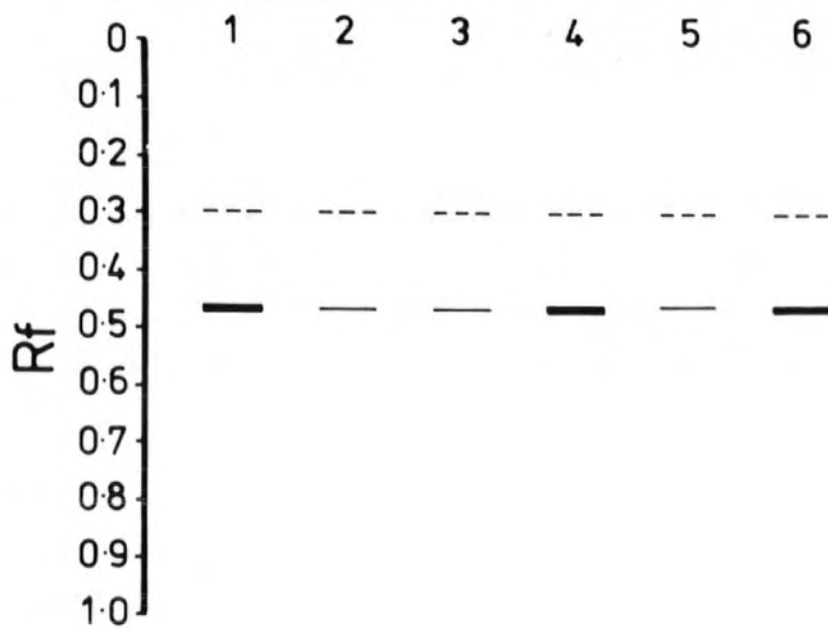
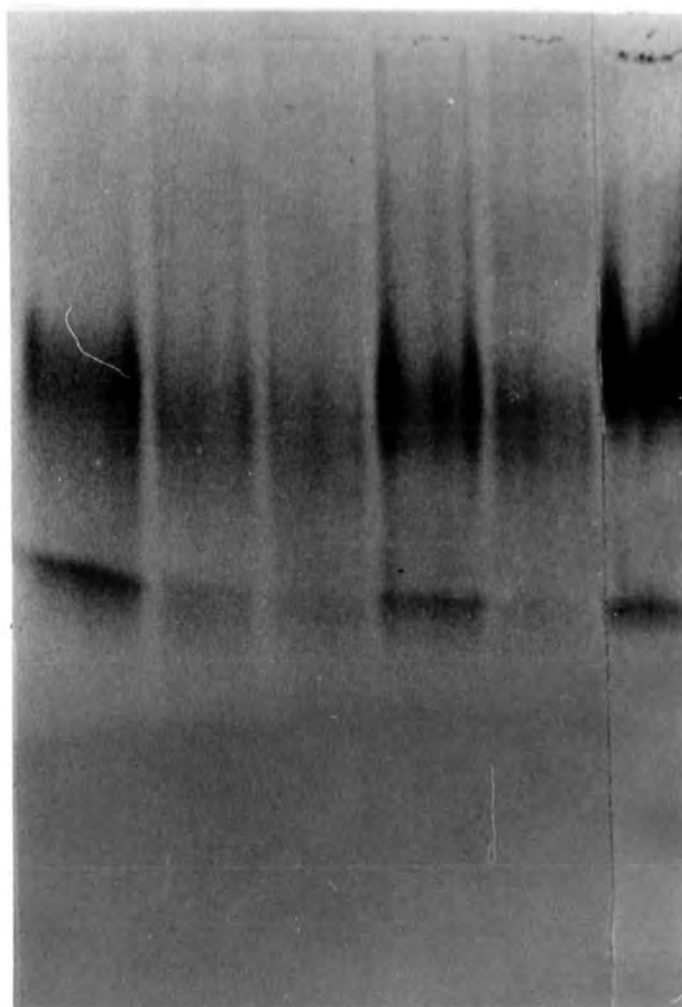


Fig. 49

Diagrammatic zymogram pattern of LAP in pea (Pisum sativum var. Feltham First) seed extracts at different stages of seed development.

No. 1: 10 day-old seeds

No. 2: 11 day-old seeds

No. 3: 15 day-old seeds

No. 4: 17 day-old seeds

No. 5: 19 day-old seeds

No. 6: 21 day-old seeds

No. 7: 23 day-old seeds

No. 8: 30 day-old seeds

Fig. 50

Zymogram pattern changes of LAP in extracts from pea (Pisum sativum var. English Wonder) seeds at different stages of seed development.

Track:

No. 1: 13 day-old seeds

No. 2: 14 day-old seeds

No. 3: 15 day-old seeds

No. 4: 16 day-old seeds

No. 5: 18 day-old seeds

No. 6: 19 day-old seeds

No. 7: 26 day-old seeds

No. 8: 32 day-old seeds

fig. 49

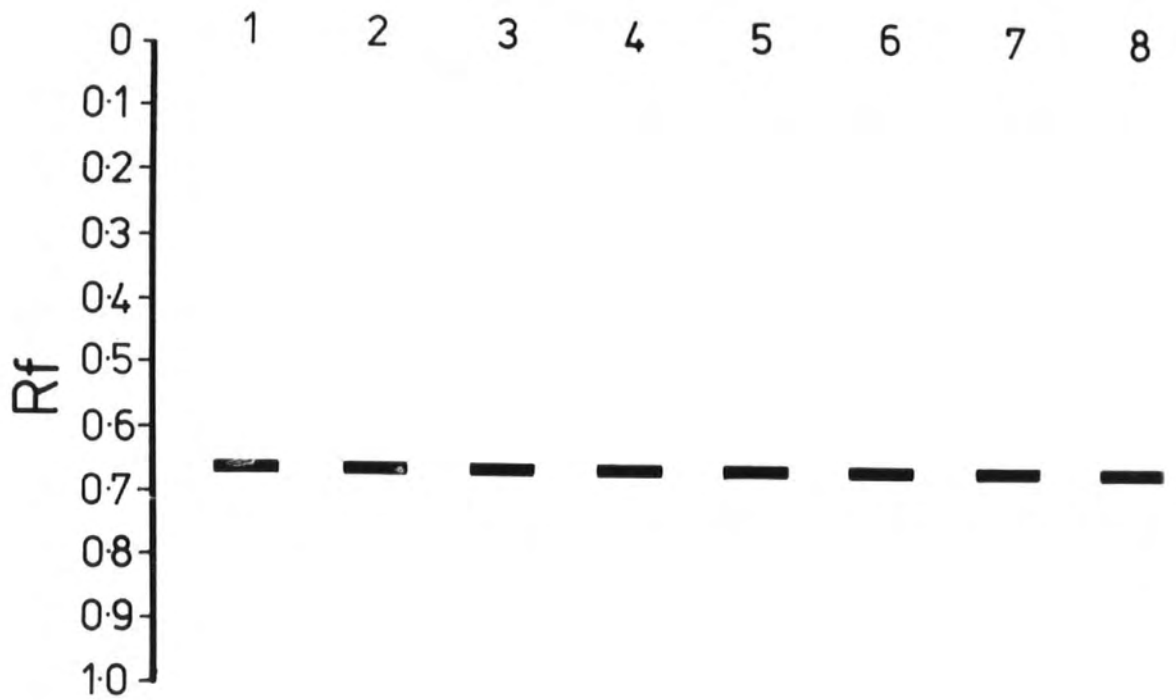
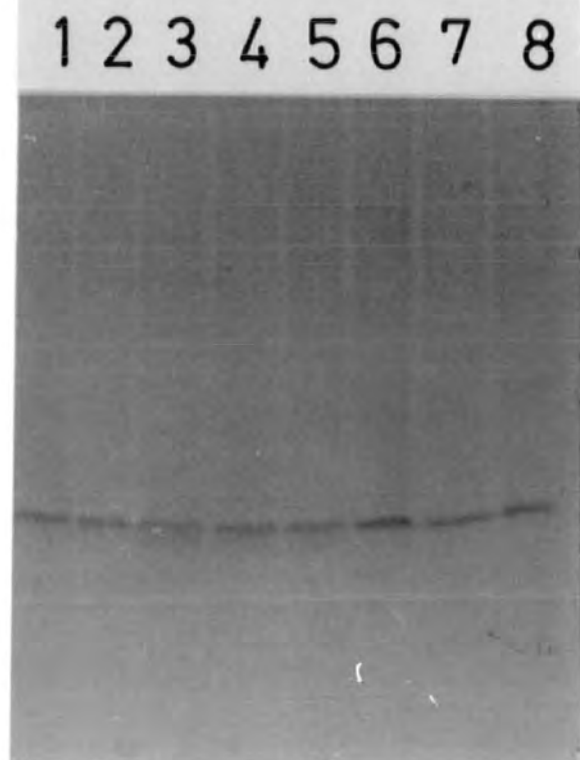


Fig. 50



present in each extract.

5.2 Dwarf French bean seed extract

Each extract from the var. Masterpiece and the var. Processor gave one band. See Fig. 51.

5.2.1 During seed development

One band was present in each extract at the different stages of seed development (see fig. 51) with no apparent change in the level of LAP activity as the seed developed.

6. Malate dehydrogenase activity

The sites of dehydrogenase activities were detected on the acrylamide gel as heavily blue-stained bands in blue background.

The different legume seed extracts appeared to have a high level of MDH activity, since only a small volume of seed extract was needed (15 μ l of 1:20 (w/v) seed meal to extraction buffer).

Fig. 52 shows the zymogram patterns of MDH in the different legume seed extracts.

6.1 Dwarf French bean seed extract

The seed extracts from the varieties Processor and Masterpiece each gave two distinct electrophoretic bands and the fast moving component (Rf 0.51) was more active than the slowest moving component.

Fig. 51

Zymogram pattern changes of LAP in extracts from dwarf French bean (Phaseolus vulgaris) seeds at different stages of seed development.

a. Var. Processor

Track:

No. 1: 24 day-old seeds

No. 2: 25 day-old seeds

No. 3: 28 day-old seeds

No. 4: 30 day-old seeds

No. 5: 43 day-old seeds

No. 6: Mature seeds

b. Var. Masterpiece

Track:

No. 1: 24 day-old seeds

No. 2: 27 day-old seeds

No. 3: 33 day-old seeds

No. 4: 38 day-old seeds

No. 5: 42 day-old seeds

No. 6: 46 day-old seeds

No. 7: Mature seeds (small volume was used)

Fig. 51

^a 1 2 3 4 5 6 1 2 3 4 5 6 7

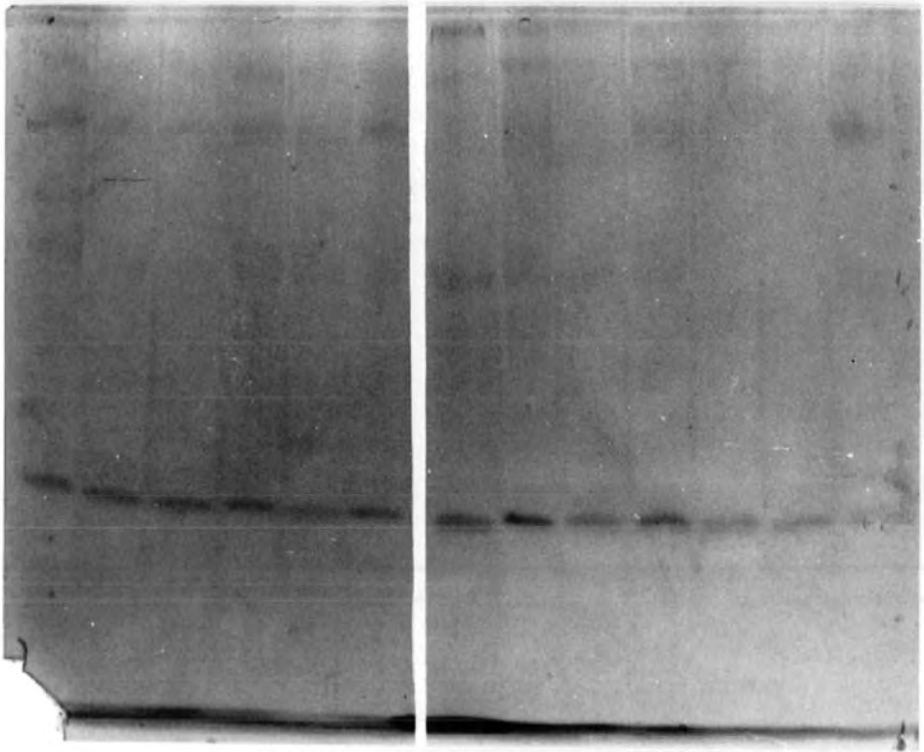


Fig. 52

Zymogram patterns of MDH in different legume seed extracts.

Track:

No. 1: Dwarf French bean (Phaseolus vulgaris var. Masterpiece)

No. 2: Pea (Pisum sativum var. Feltham First)

No. 3: Mung bean (Phaseolus vulgaris var. Processor)

No. 5: Black eye bean (Vigna unguiculata)

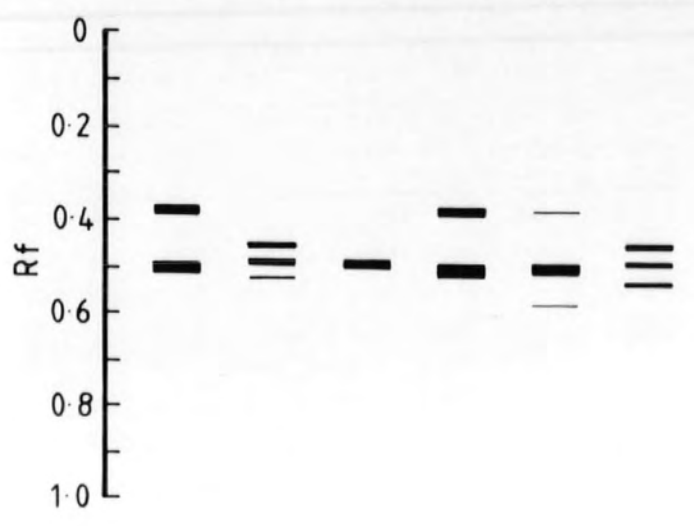
No. 6: Broad bean (Vicia faba)

fig.52

1 2 3 4 5 6



1 2 3 4 5 6



6.2 Pea seed extract

The seed extract from the var. Feltham First gave three bands and the fastest moving component had the lowest level of enzyme activity.

6.2.1 Inbred lines

Each seed extract from the eleven inbred lines studied, gave three monomorphic bands (see fig. 53).

6.2.2 During seed development of var. Feltham First

Each seed extract at the early stages of developing seeds (7 and 12 days), gave two slow moving bands.

The fastest moving band appeared in extracts from 17 day-old seeds onwards (see fig. 54).

6.3 Mung bean seed extract

Only one thick band was detected.

6.4 Black eye bean seed extract

Three bands were detected and the intermediate moving component (Rf 0.51) was thick and had the highest level of MDH activity.

6.5 Broad bean (*Vicia faba*) seed extract

Three distinct electrophoretic bands were present.

Fig. 53

Zymogram patterns of MDH in pea seed extracts.

Track:

- No. 1: Inbred line no. 360
- No. 2: Inbred line no. 1256
- No. 3: Inbred line no. 2163
- No. 4: Inbred line no. 807
- No. 5: Inbred line no 1238
- No. 6: Inbred line no. 1263
- No. 7: Inbred line no. 1478
- No. 8: Inbred line no. 379
- No. 9: Inbred line no. 1673
- No. 10: Inbred line no. 808
- No. 11: Inbred line no. 851
- No. 12: Inbred line no. 611

Fig. 54

Zymogram pattern changes of MDH in extracts from pea (Pisum sativum var. Feltham First) seeds at different stages of seed development.

Track:

- No. 1: 7 day-old seeds
- No. 2: 12 day-old seeds
- No. 3: 17 day-old seeds

Fig.53

1 2 3 4 5 6 7 8 9 10 11 12

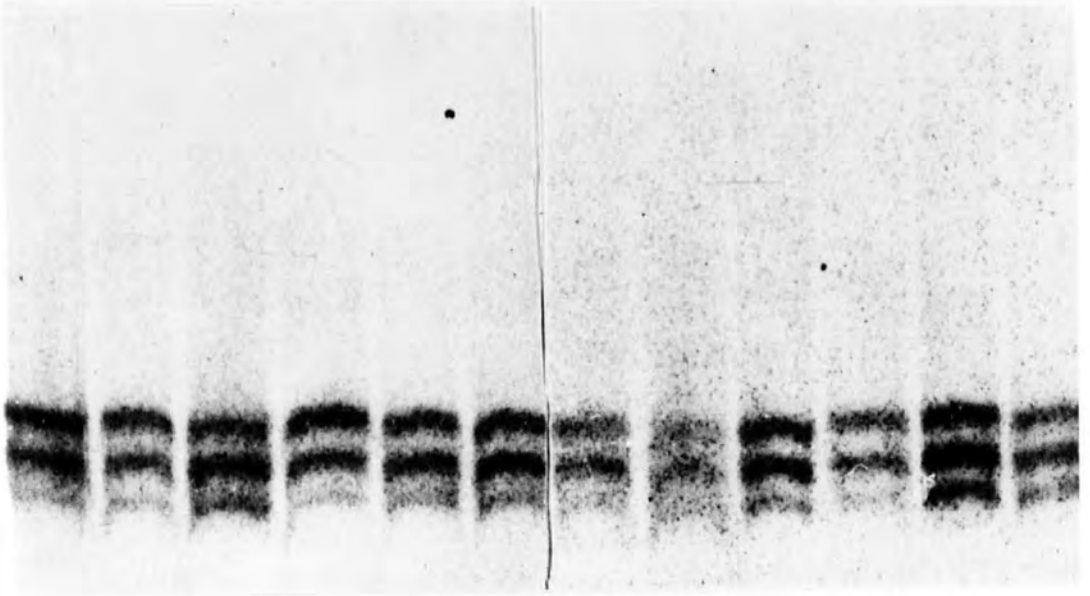
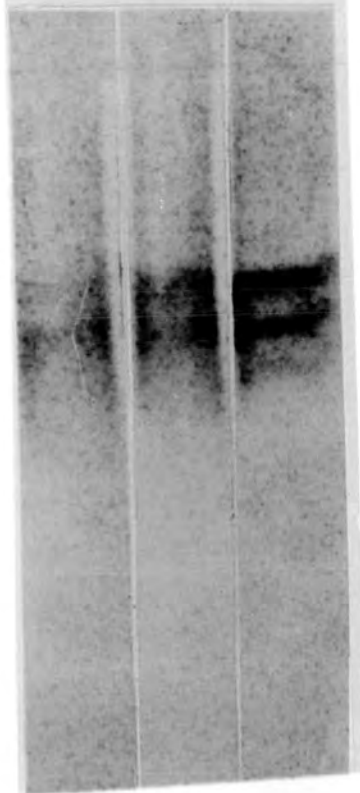


Fig.54

1 2 3



7. Glutamate dehydrogenase activity

Fig. 55 shows the electrophoretic patterns of GDH in extracts from the different legume seeds.

7.1 Pea seed extracts

7.1.1 Inbred lines

The zymogram patterns of GDH consisted of seven monomorphic distinct electrophoretic bands in each extract of the eleven inbred lines studied.

As shown in fig. 56, the two slowest moving components were the most active, while the other bands were faint and needed longer incubation time in the staining solution.

7.1.2 Var. Feltham First

Seven bands were detected and the slowest moving component was thick and had a high level of GDH activity.

7.1.3 Var. English Wonder

Seven bands were detected and the two slowest moving bands were thick and the most intensively stained bands (see fig. 57 track 5).

7.1.4 Var. Mangetout

Seven bands were present (see fig. 58 track 6).

Fig. 55

Zymogram patterns of GDH in different legume seed extracts.

Track:

No. 1: Pea (Pisum sativum var. Feltham First)

No. 2: Broad bean (Vicia faba)

No. 3: Dwarf French bean (Phaseolus vulgaris var. Processor)

No. 4: Black eye bean (Vigna unguiculata)

No. 5: Soyabean (Glycine max)

No. 6: Mung bean (Vigna radiata)

No. 7: Dwarf French bean (Phaseolus vulgaris var. Masterpiece)

Fig. 56

Diagrammatic zymogram patterns of GDH in pea seed extracts.

No. 1: Inbred line no. 807

No. 2: Inbred line no. 808

No. 3: Inbred line no. 2162

No. 4: Inbred line no. 1263

No. 5: Inbred line no. 110

No. 6: Inbred line no. 1293

No. 7: Inbred line no. 58

No. 8: Inbred line no. 1238

No. 9: Inbred line no. 1256

No. 10: Inbred line no. 851

No. 11: Inbred line no. 360

Fig.55

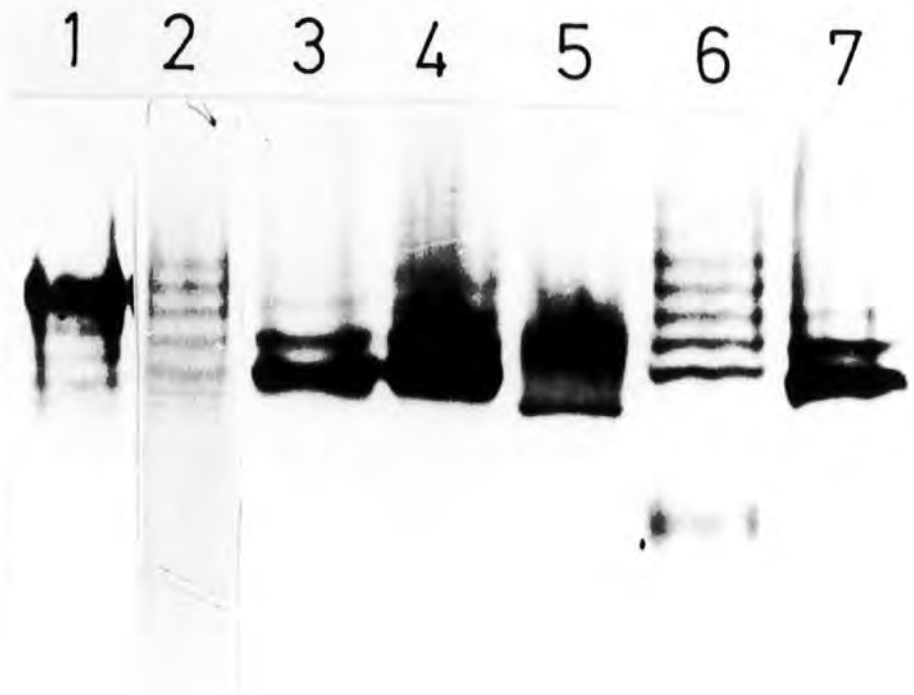
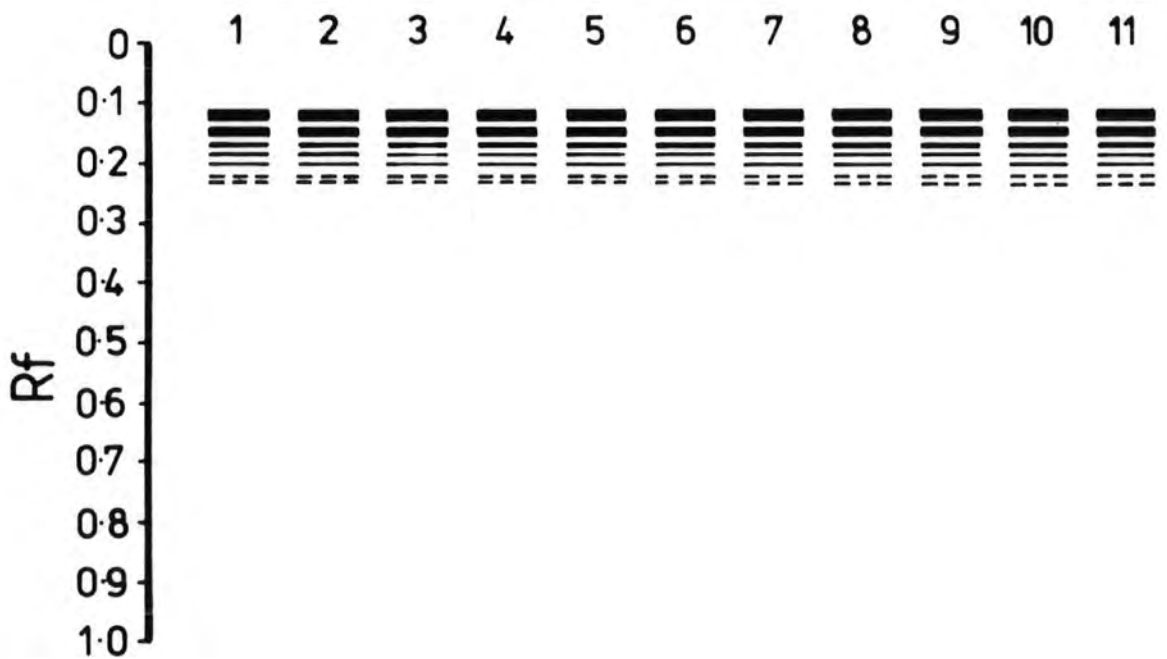


fig. 56



7.1.5 During seed development

7.1.5.1 Var. English Wonder

In the extracts at the early stages of developing seeds, the fastest moving band was the most intensively stained band, while in the seed extracts at the late stages of developing seeds, the slowest migrating two bands were the most intensively stained, although they were faint in the seed extracts at the early stages of developing seeds (see fig. 57).

7.1.5.2 Var. Mangetout

Seven bands were detected in each extract at the different stages at seed development and the obvious change was the apparent increase in the intensity of the slowest moving band as the seed developed (see fig. 58).

7.2 Dwarf French bean seed extract

The seed extract from the varieties Processor and Masterpiece each gave three bands and the fastest moving band was thick, while the slowest moving band was faint.

7.2.1 During seed development

Each seed extract at the early stages of developing seeds gave two bands, while each seed extract at the late stage of developing seeds, gave three bands (see figs 59 and 60).

Fig. 57

Zymogram pattern changes of GDH in extracts from pea (Pisum sativum var. English Wonder) seeds at different stages of seed development.

Track:

No. 1: 13 day-old seeds

No. 2: 14 day-old seeds

No. 3: 28 day-old seeds

No. 4: 32 day-old seeds

Fig. 58

Zymogram pattern changes of GDH in extracts from pea (Pisum sativum var. Mangetout) seeds at different stages of seed development.

Track:

No. 1: 12 day-old seeds

No. 2: 11 day-old seeds

No. 3: 14 day-old seeds

No. 4: 15 day-old seeds

No. 5: 16 day-old seeds

No. 6: Mature seeds

Fig.57

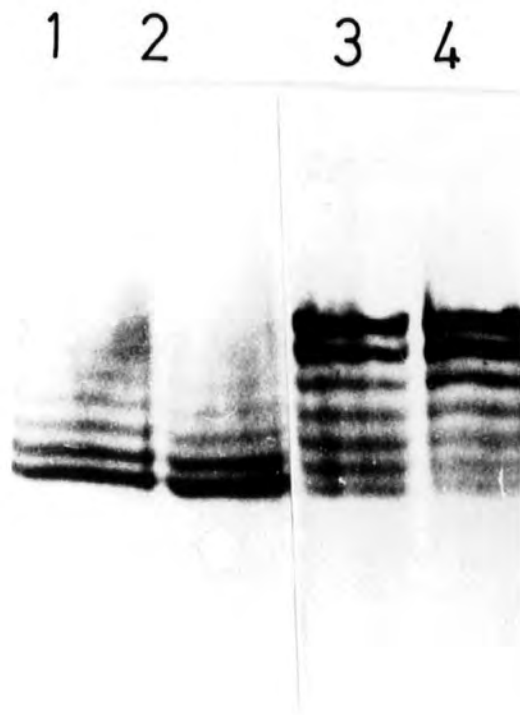


Fig.58

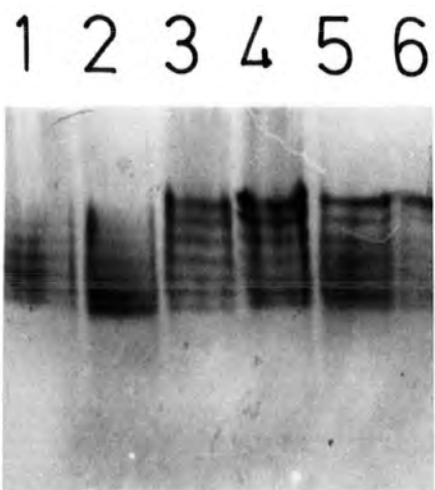


Fig. 59

Zymogram pattern changes of GDH in extracts from dwarf French bean (Phaseolus vulgaris var. Processor) seeds at different stages of seed development.

Track:

No. 1: 24 day-old seeds

No. 2: 26 day-old seeds

No. 3: 27 day-old seeds

No. 4: 28 day-old seeds

No. 5: 30 day-old seeds

No. 6: 33 day-old seeds

No. 7: 45 day-old seeds

Fig. 60

Zymogram pattern changes of GDH in extracts from dwarf French bean (Phaseolus vulgaris var. Masterpiece) seeds at different stages of seed development.

Track:

No. 1: 24 day-old seeds

No. 2: 26 day-old seeds

No. 3: 27 day-old seeds

No. 4: 28 day-old seeds

No. 5: 33 day-old seeds

No. 6: Mature seeds

Fig. 59

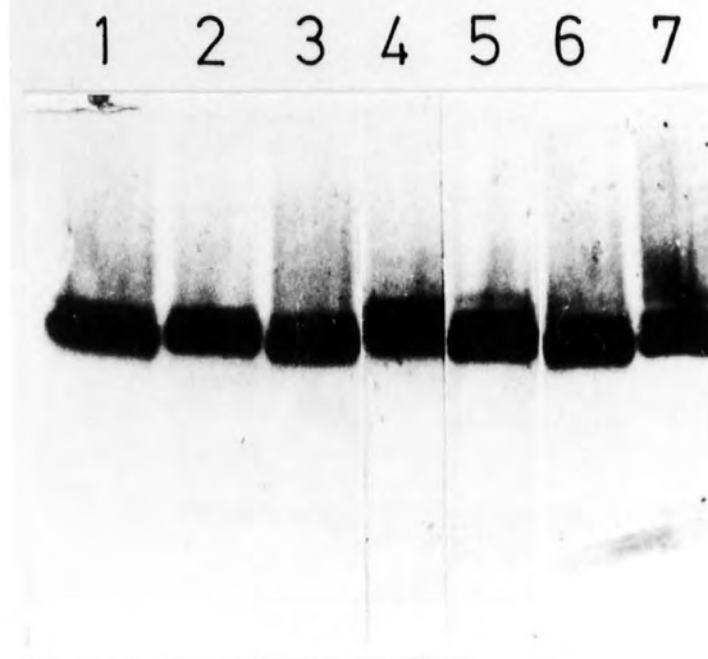
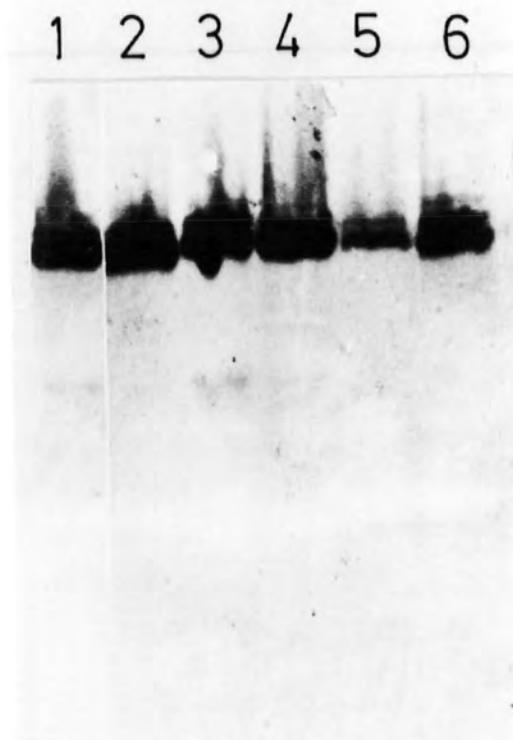


Fig. 60



7.3 Soyabean seed extract

Two bands were present.

7.3.1 During seed development

The extracts at early stages of developing seeds appeared to be low in GDH activity. Two bands were present in each seed extract (see fig. 61).

7.4 Black eye bean seed extract

Four distinct electrophoretic bands were observed and the two fastest moving components had the highest levels of enzyme activities.

7.5 Mung bean seed extract

Eight bands were present.

7.6 Broad bean (*Vicia faba*) seed extract

Seven bands were observed and when the patterns of GDH were studied in extracts at different stages of seed development, no changes were observed.

8. Alcohol dehydrogenase

8.1 Mung bean seed extract

Two distinct electrophoretic bands were detected and the slow moving component appeared to be more active than the other component (see fig. 62 track 1).

Fig. 61

Zymogram pattern changes of GDH in extracts from soyabean (Glycine max) seeds at different stages of seed development.

Track:

No. 1: 25 day-old seeds

No. 2: 35 day-old seeds

No. 3: 37 day-old seeds

No. 4: 40 day-old seeds

No. 5: 45 day-old seeds

No. 6: 52 day-old seeds

Fig. 62

Zymogram patterns of ADH in seed extracts of mung bean and pea.

Track:

1: Mung bean (Vigna radiata)

2: Pea (Pisum sativum var. Feltham First)

Fig. 61

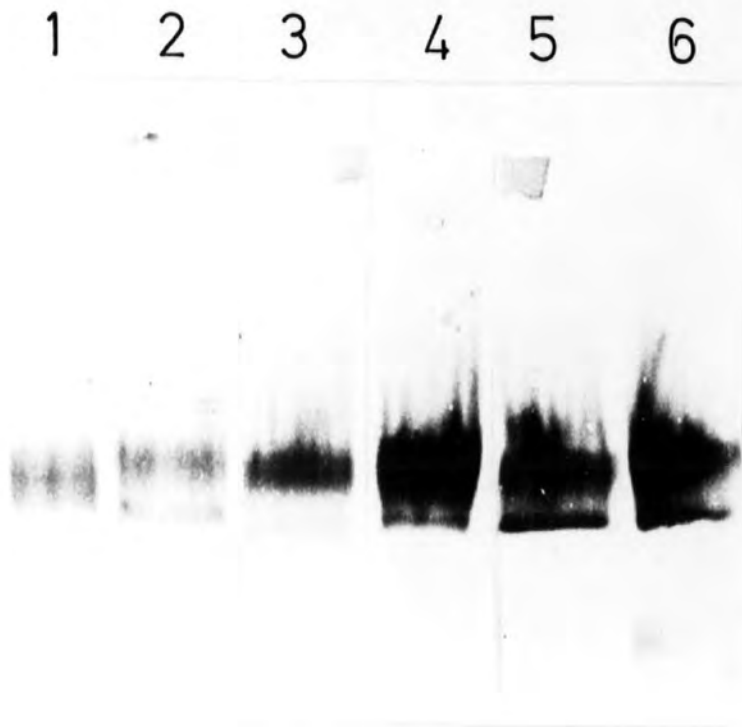


Fig. 62



8.2 Pea seed extract

8.2.1 Var. Feltham First

Two bands were detected and the slow moving band was faint (fig. 62 track 2) and barely visible on the gel.

8.2.2 Var. English Wonder

Two bands were detected in each extract from 16, 19 and 30 (mature) day-old seeds and the fast moving band was faint and barely visible on the gel (see fig. 63).

9. Lactate dehydrogenase

Fig. 64 shows the zymogram patterns of LDH in seed extracts from the different legumes. No bands were detected in extracts from pea (var. Feltham First), different inbred lines of pea and broad bean (Vicia faba) seed extracts.

9.1 During seed development in dwarf French bean

Zymogram pattern changes were studied in extracts from two varieties, Masterpiece and Processor, at different stages of seed development and the results in figs 65 and 66 show that there were no changes in the zymogram patterns of LDH as the seed developed.

Fig. 63

Zymogram pattern changes of ADH and GDH in extracts from pea (Pisum sativum var. English Wonder) seeds at different stages of seed development.

Track:

No. 1: 16 day-old seeds

No. 2: 19 day-old seeds

No. 3: 30 day-old seeds

Note: Arrows point at ADH bands and all the other bands are due to GDH activity.

Fig. 64

Diagrammatic zymogram patterns of LDH in different legume seed extracts.

No. 1: Black eye bean (Vigna unguiculata)

No. 2: Soyabean (Glycine max)

No. 3: Dwarf French bean (Phaseolus vulgaris, var. Processor)

No. 4: Dwarf French bean (Phaseolus vulgaris var. Masterpiece)

No. 5: Mung bean (Vigna radiata)

Fig.63

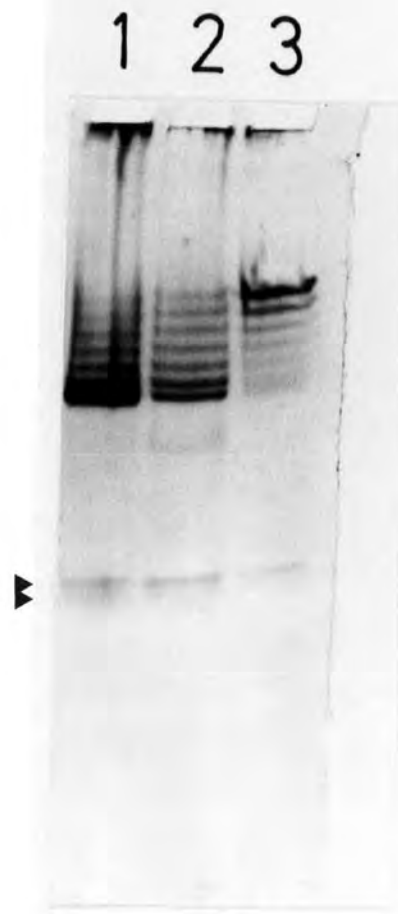


fig.64

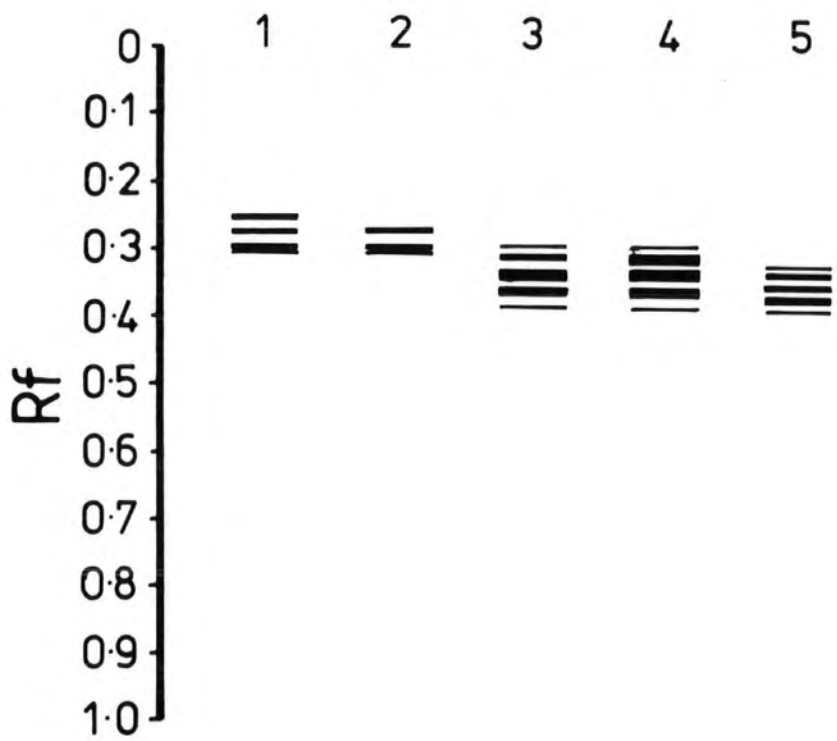


Fig. 65

Zymogram pattern changes of LDH in extracts from dwarf French bean (Phaseolus vulgaris var. Processor) seeds at different stages of seed development.

Track:

No. 1: 24 day-old seeds

No. 2: 25 day-old seeds

No. 3: 28 day-old seeds

No. 4: 35 day-old seeds

No. 5: 38 day-old seeds

No. 6: 45 day-old seeds

No. 7: Mature seeds

Fig. 66

Zymogram pattern changes of LDH in extracts from dwarf French bean (Phaseolus vulgaris var. Masterpiece) seeds at different stages of seed development.

Track:

No. 1: 20 day-old seeds

No. 2: 24 day-old seeds

No. 3: 33 day-old seeds

No. 4: Mature seeds

Fig. 65

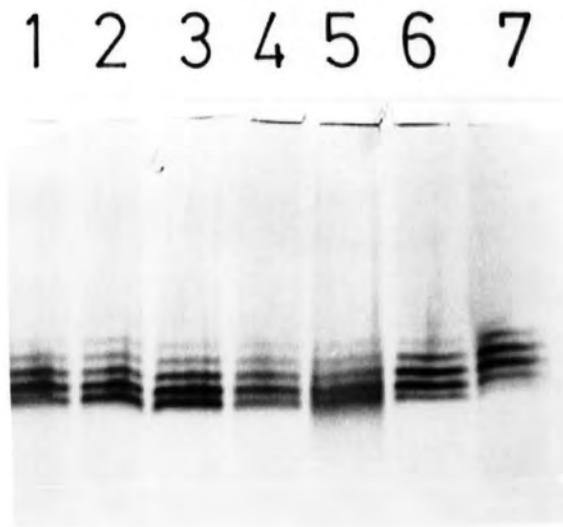
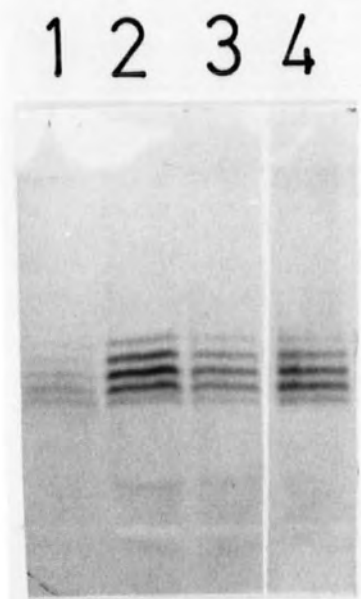


Fig. 66



10. Protease, catalase, peroxidase and glucose-6-phosphate dehydrogenase activities in pea seed extracts

No bands of their activities were detected in the seed extracts of the different inbred lines studied.

Also, the activity of protease was investigated in seed extracts at different stages of seed development and germination in pea (var. Feltham First) and no bands were detected at any stage.

11. Amylase activity of legume plants

11.1 Leaf extracts

The zymogram pattern of amylase activity was studied in leaf extracts of dwarf French bean, pea, soyabean and broad bean, at the time when the plants were flowering. Amylase activity was detected in all extracts studied (see fig. 67).

Soyabean leaf extract had high levels of amylase activity and several bands were observed.

The patterns observed in leaf extracts of pea, var. Feltham First and var. English Wonder, were similar to those observed in the leaf extracts of the seedling of each of them.

The leaf extract of dwarf French bean showed relatively high amylase activity; 4 bands were observed in leaf extract of var. Masterpiece and 3 bands in the leaf extract of the var. Processor.

Only one band of amylase activity was detected in leaf extract of broad bean.

11.2 Leaf, pollen grain and stem of broad bean (inbred line PMS)

The results in fig. 68 showed the zymogram pattern of each extract.

11.3 Pea pollen grains

The zymogram pattern of amylase activity was studied in pollen extract of inbred line no. 1238 and inbred line Meteor and the results in fig. 69 tracks 1 and 2 showed that each extract consisted of one active fast moving component and one slow moving component with very low activity (hardly visible in the photograph).

11.4 Pea pod

The zymogram pattern of amylase activity of extract from pea (inbred line Meteor) pod was similar to that of pollen grains, containing one active fast moving component and another slow moving component with low enzyme activity (fig. 69 track 3).

12. GOT of pea pollen grains

The zymogram pattern of GOT in extract from pea (inbred line no. 1238) pollen grains is very similar to that of the seed extract.

Fig. 67

Diagrammatic zymogram patterns of amylase in extracts from different legume leaves.

- No. 1: Dwarf French bean (Phaseolus vulgaris var. Masterpiece)
- No. 2: Dwarf French bean (Phaseolus vulgaris var. Processor)
- No. 3: Broad bean (Vicia faba)
- No. 4: Pea (Pisum sativum var. English Wonder)
- No. 5: Pea (Pisum sativum var. Feltham First)
- No. 6: Soyabean (Glycine max)

Fig. 68

Zymogram patterns of amylase in extracts from different parts of broad bean (Vicia faba)

Track:

- No. 1: Pollen grains
- Nos 2 and 3: Leaf
- No. 4: Stem

Fig. 69

Zymogram patterns of amylase in extracts from pod and pollen grains of pea.

Track:

- No. 1: Pollen grains of inbred line no. 1238
- No. 2: Pollen grains of inbred line Meteor
- No. 3: Pod of inbred line Meteor

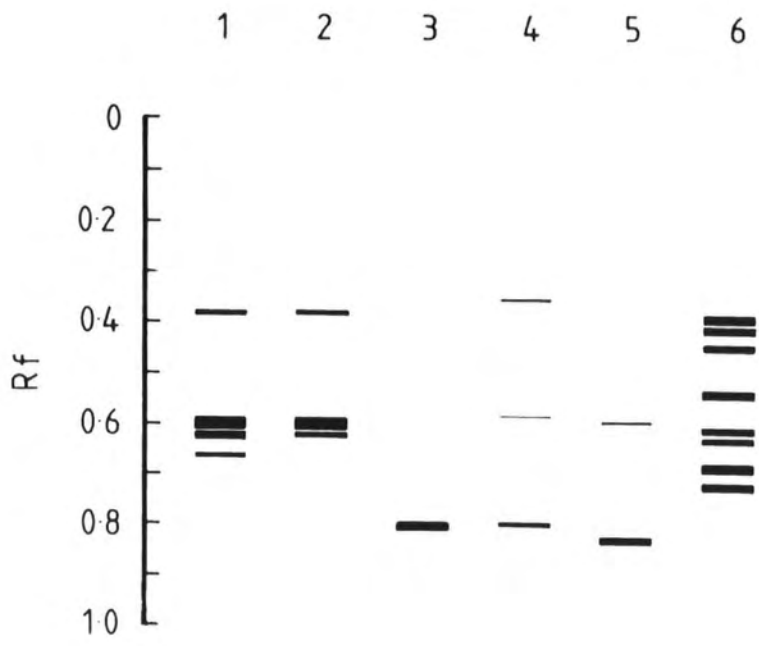


fig. 68

fig. 69



13. Effect of aging and storage on EST and GOT patterns of broad bean
(inbred line no. 22)

13.1 EST

The zymogram patterns and activity of EST were studied in extracts from freshly harvested seeds, seeds that were stored for 1 year at room temperature, seeds that were stored for 2 years at 4°C, seeds that were stored for 5 years at room temperature and seeds that were stored at -20°C for 21 days. The results in fig. 70 showed that the extracts from seeds that were stored at 4°C for 2 years had an additional slow moving component with relatively high enzyme activity as compared to the patterns of the other seed extract. The patterns of extracts from seed stored for 5 years at room temperature and from seed stored for 21 days at -20°C are not present in the photograph, but the patterns are similar to that of freshly harvested seed with slight decrease in the activity of EST component in seeds stored for 5 years at room temperature.

13.2 GOT

The GOT patterns and activity were studied in extracts from seeds that were stored for different periods of time at different temperatures and the results in fig. 70b showed that different storage conditions and the storage period had no effects on GOT patterns and activity. The zymogram patterns of extracts from seeds stored at room temperature for 5 years and from seeds stored at -20°C for 21 days are not present in the photograph but were similar to those of all other extracts.

Fig. 70

Zymogram patterns of EST and GOT of broad bean (Vicia faba) seeds that were stored at different conditions.

a. EST patterns

Track:

No. 1 (80 μ l) and 4(40 μ l) freshly harvested seeds

No. 2 (80 μ l) and 5(40 μ l) seeds were stored for 2 years at 4°C.

No. 3 (80 μ l) and 6(40 μ l) seeds were stored for 1 year at room temperature

b. GOT patterns

Track:

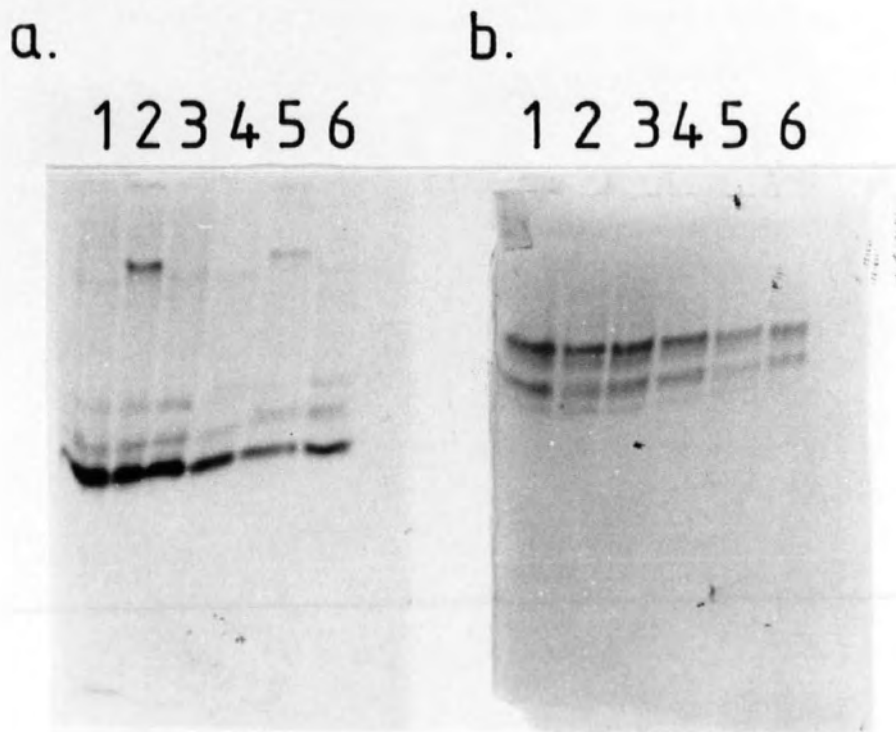
No. 1: (50 μ l) and 4(30 μ l) freshly harvested seeds

No. 2: (50 μ l) and 5(30 μ l) seeds were stored for 2 years at 4°C.

No. 3: (50 μ l) and 6(30 μ l) seeds were stored for 1 year at room temperature

Note:- Numbers in parenthesis indicate the volume of sample loaded. Samples were extracted in 0.4M NaCl (1:3 seed meal to extraction buffer), overnight at 4°C. After centrifugion, the extract was dialyzed against tap water overnight at 4°C and then centrifuged and 20% w/v sucrose was added to the extract.

fig. 70



14. Studies on pea (Pisum sativum var. Feltham First) amylase isoenzymes

14.1 Calibration curves for measuring amylase activity

The semi-quantitative method is not very reliable for accurate measurement of amylase content, since large differences in enzyme concentration gave only small differences in the diameter of the hydrolyzed area (see fig. 71, table 3 and plate 1). However, it is very useful for a comparative study and screening since it utilizes only small volumes.

Table 3: The activity of α -amylase (porcine, Sigma) as measured by the diameter of the hydrolyzed area of starch by enzyme activity for 20h. at room temperature.

Amylase concentration $\mu\text{g/ml}$	10g_{10} $\mu\text{g/ml}$	Diameter (mm) of the hydrolyzed area for 4 replicates	Average area diameter (mm)
20	1.3010	4.0, 4.0, 4.0, 4.0	4.0
25	1.3979	4.5, 4.6, 4.4, 4.5	4.5
50	1.6990	6.4, 6.6, 6.5, 6.3	6.4
100	2.0	7.7, 7.9, 7.8, 7.8	7.8
200	2.3010	9.2, 9.3, 9.1, 9.2	9.2
250	2.3979	9.6, 9.8, 9.8, 9.6	9.7
500	2.6990	11.1, 11.4, 11.1, 11.2	11.2
1000	3.0	12.4, 12.4, 12.4, 12.5	12.4
1250	3.0969	13.0, 12.6, 12.8, 12.9	12.8
2500	3.3979	15.0, 14.9, 14.7, 14.6	14.8
5000	3.6990	16.7, 16.7, 16.7, 16.7	16.7

The alternative procedure is very reliable for quantitative measurement. The incubation time of the enzyme with the substrate was

Fig. 71

Calibration curve for estimating amylase activity. See also table 3 and plate 1.

Plate 1

The activity of α -amylase (porcine, Sigma) as measured by the diameter of the hydrolyzed area of starch by enzyme activity for 20h. at room temperature. Numbers represent amylase concentration in $\mu\text{g/ml}$.

Note:- The plates were produced by photographing the agarose/starch gels. Therefore, the hydrolyzed areas appear as white circles on a dark background.

Fig. 72

Calibration curve for measuring amylase activity. See table 4.

Fig. 71

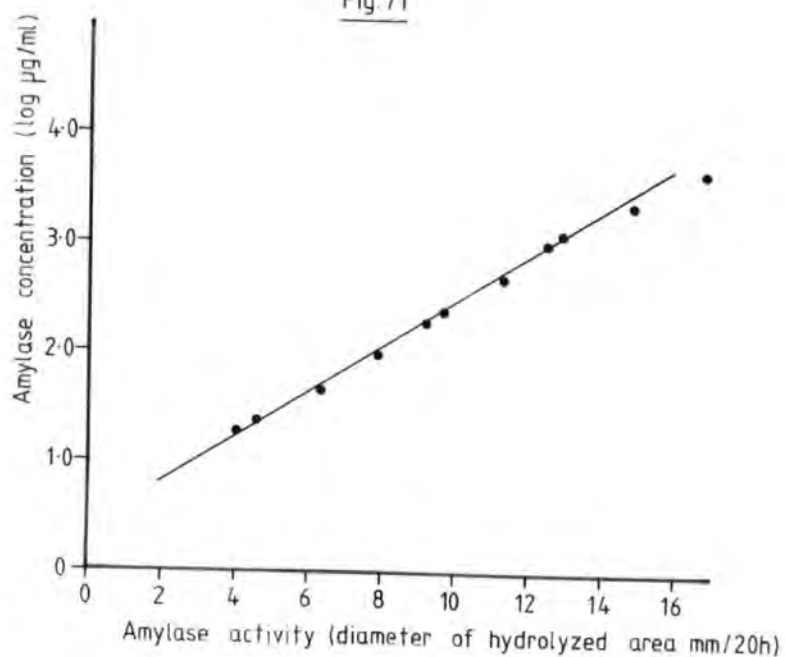


plate 1

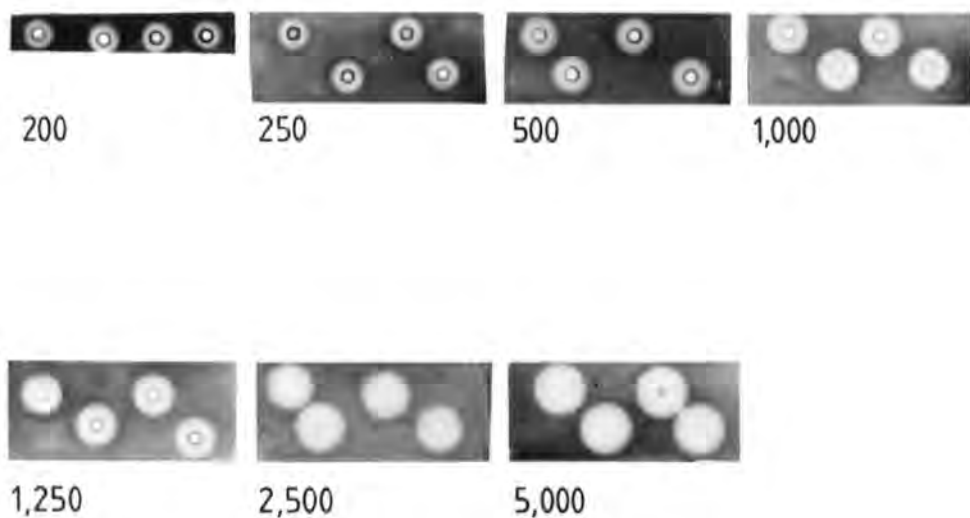
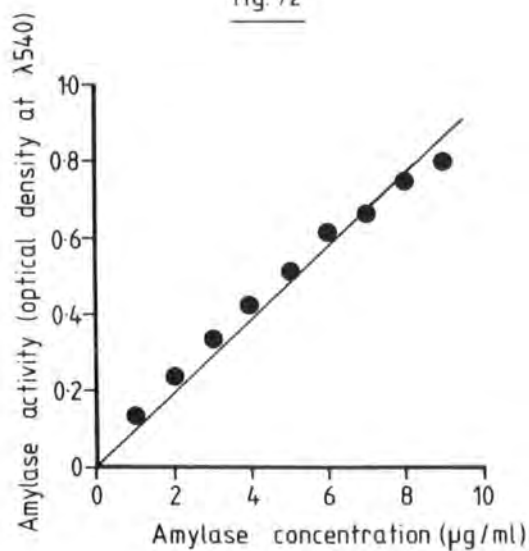


fig. 72



found to be very critical (3min), therefore only a limited number of tubes were used at a time (see fig. 72 and table 4).

Table 4: The activity of α -amylase (porcine, Sigma) as measured spectrophotometrically by the reduction of 3.5 dinitro-salicylic acid, due to the production of reducing groups liberated from starch as the result of enzyme activity for 3min. at room temperature.

Amylase concentration ($\mu\text{g/ml}$)	Amylase activity (optical density (O.D.) at 540nm) for three replicates	Average O.D.
1	0.13, 0.135, 0.13	0.132
2	0.25, 0.24, 0.25	0.247
3	0.38, 0.335, 0.33	0.348
4	0.44, 0.44, 0.41	0.43
5	0.54, 0.50, 0.52	0.52
6	0.58, 0.63, 0.63	0.613
7	0.67, 0.65, 0.64	0.653
8	0.76, 0.745, 0.74	0.748
9	0.80, 0.78, 0.78	0.787

14.2 Amylase activity in pea seedlings

As estimated by the semi-quantitative procedure on a fresh weight basis, the extract from etiolated leaves of 7 day-old seedlings had the highest level of enzyme activity as compared to that from root and stem. On a fresh weight basis, the extracts from stem and root had nearly equal levels of enzyme activity (see table 5 and plate 2).

Plate 2

Amylase activity in extracts from different tissues of 7 day-old seedlings of pea (Pisum sativum var. Feltham First). See table 5 and 7.

Numbers are

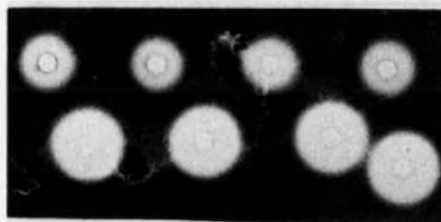
- 1: Root extracted in tris/HCl pH 6.9 buffer
- 2: Leaves extracted in tris/HCl pH 6.9 buffer
- 3: Stem extracted in tris/HCl pH 6.9 buffer
- 4: Stem extracted in acetate pH 6.3 buffer
- 5: Stem extracted in borate pH 7.0 buffer
- 6: Stem extracted in phosphate pH 7.25 buffer
- 7: 10C μ g/ml porcine α -amylase
- 8: Stem extracted in tris/HCl pH 6.9 buffer containing 2-mercaptoethanol

Note:- The plates were produced by photographing the agarose/starch gel. Therefore the hydrolyzed areas appear as white circles on a dark background.

plate 2

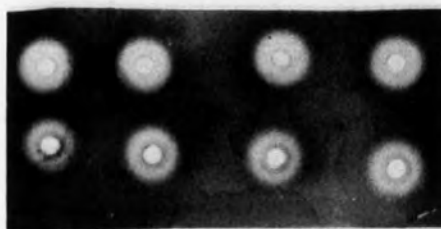
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2



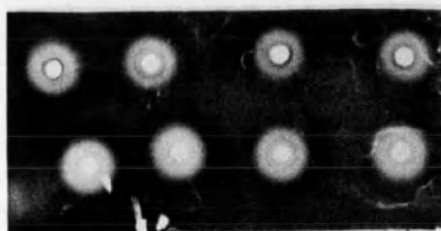
3

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5

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7

8

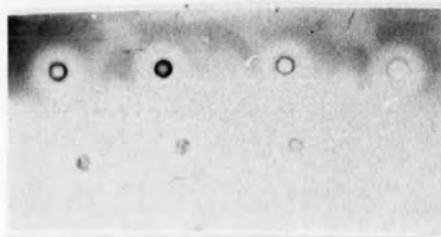


Table 5: β -amylase activity in extracts from different tissues of 7 day-old pea (var. Feltham First) seedlings. Enzyme extracted in 0.2M tris/HCl buffer pH 6.9.

	Diameter(mm) of the hydro- lyzed area after 20h.at room temp.	10g ₁₀ μ g/ml amylase con- centration	μ g/ml amylase con- centration	amylase con- centration μ g/g tissue fresh weight	g dry weight tissue ----- g fresh weight tissue	amylase con- centration mg/g dry weight tissue
Leaves	10	2.500	316.2	948.6	0.165	5.75
Root	7	1.8667	73.57	220.71	0.0861	2.56
Stem	6.8	1.8333	67.97	203.91		

Each value the average of 4 replicates

As shown in table 6, green leaves had higher levels of amylase activity than the etiolated leaves. Enzyme activity seems to decrease in leaf extract between 7 and 11 days of germination.

Table 6: Amylase activity in extracts from 11 day-old pea (var. Feltham First) seedlings.

Tissue	Diameter (mm) of hydrolyzed area after 20h. at room temperature	Average diameter (mm)
Green leaves extracted in 0.023M sodium phosphate buffer pH 7.25	9.2, 9.2, 9.2, 9.2	9.2
Etiolated leaves extracted in 0.025M sodium phosphate buffer pH 7.25	8.3, 8.5, 8.3, 8.3, 7.6, 7.7	8.06
Etiolated leaves extracted in 0.2M tris/Hcl pH 6.9	7.4, 7.4, 7.4, 7.4, 7.4	7.4

14.3 Effect of extraction buffer

In the case of stems, no large differences were found between the four different buffers which were investigated (table 7 and plate 2); tris/HCl buffer and phosphate buffer gave more clearly visualised areas and appeared to be better than borate and acetate buffers.

Table 7: The effect of extraction buffer on amylase activity in extracts from stems of 7 day-old pea (var. Feltham First) seedlings.

Buffer	Diameter (mm) of the hydrolyzed area after 20h. at room temperature (4 replicates)	Average diameter (mm)
0.025M sodium phosphate pH 7.25	6.8, 6.8, 6.8, 6.8	6.8
0.1M sodium borate pH 7.0	6.7, 6.6, 6.0, 6.1	6.35
0.2M tris/HCl pH 6.9	6.7, 6.7, 6.7, 6.7	6.7
0.025M sodium acetate pH 6.3	6.1, 6.2, 6.1, 6.8	6.3

Etiolated leaves extracted in phosphate buffer had slightly higher amylase activity than those extracted in tris/HCl buffer (table 6). This difference might suggest that phosphate buffer is better as an extraction medium for leaves or this slight difference might be due to the activation of phosphorylase known to be active in hydrolyzing starch in the presence of orthophosphate.

14.4 Effect of 2-mercaptoethanol

2-mercaptoethanol had no effect on the α -amylase activity in extract from the stem. However, the results showed that this chemical could not be used since it interfered with the assay procedure, causing the disappearance of the iodine colour (plate 2).

14.5 Effect of lyophilizing the tissue on amylase activity

Amylase activity of germinated cotyledons, leaves and root was not affected by freeze drying the tissues, while that of stem was completely inactivated by this procedure. However activity was retained in frozen tissues in liquid nitrogen.

14.6 Fractionation with ammonium sulphate

14.6.1 β -amylase of shoot

The results in table 8 demonstrated that some amylase activity was present at 40% ammonium sulphate saturation, nearly no amylase activity at 50% and 60% saturation, and that the activity increased at 70% and 90% saturation, the latter having the highest level of enzyme activity. The presence of some activity at 40% saturation is due to another form of β -amylase (the cotyledonary form), which is also present in leaf extract (see section 2.1.5.1d). In this form most of its activity was present at 40% ammonium sulphate saturation (see section 14.6.2).

Table 8: Fractionation of pea protein, in extracts from shoots of 7 day-old seedlings, with ammonium sulphate.

Ammonium sulphate saturation %	Amylase activity (diameter (mm) of hydrolyzed area after 20h. at room temperature
40	4.5, 4.5, 4.5
50	0
60	trace
70	6, 6, 6
90	10, 10, 9.5
supernatant	0

14.6.1.1 Effect of ammonium sulphate on the assay procedure

After dialyzing and freeze drying the fractions that contained enzyme activities, an attempt was made to measure accurately the enzyme level in each fraction using the most sensitive assay procedure, but no enzyme activity could be detected, so it was suspected that the enzyme might have lost activity on either dialysis or freeze-drying. Therefore the procedure of Swain and Dekker (1960a) was carried out to concentrate the enzyme, but no amylase activity was detected at any ammonium sulphate saturation, using the more accurate procedure for assaying the enzyme activity. Nevertheless, amylase activity was detected after lyophilising the materials, by using the semi-quantitative method, suggesting that the problem lay in the assay method of the more accurate procedure.

An investigation was carried out to see if ammonium sulphate had any effect on the assay colour of the more sensitive procedure. Maltose was dissolved in distilled water and divided into two

fractions; one fraction was diluted with distilled water and the other with concentrated ammonium sulphate solution. When both were incubated in boiling water, the fraction which was diluted with ammonium sulphate failed to give any assay colour as compared to the other fraction. Also when concentrated ammonium sulphate was added to the fraction giving the assay colour, the colour disappeared. This indicated that ammonium sulphate interfered with the assay colour of this procedure, and it was concluded that this method cannot be used in the presence of ammonium sulphate.

14.6.1.2 SDS-acrylamide gel

The results of SDS-acrylamide gel electrophoresis of the different fractions are presented in fig. 73. The results showed that the fractions at 70% and 90% $(\text{NH}_4)_2\text{SO}_4$ saturation had large numbers of subunits. Large numbers of protein components are expected to be present in the leaf extract at this stage of seed development, since the leaves have many active enzymes.

14.6.2 Amylase of mature cotyledons

As shown in table 9, most activity was present at 40% ammonium sulphate saturation.

Fig. 73

Zymogram patterns of total protein of extract from pea (Pisum sativum var. Feltham First) shoot. The extract was precipitated with different concentrations of ammonium sulphate.

a. Reducing conditions

Track:

No. 1: 40% $(\text{NH}_4)_2\text{SO}_4$

No. 2: 50% $(\text{NH}_4)_2\text{SO}_4$

No. 3: 60% $(\text{NH}_4)_2\text{SO}_4$

No. 4: 70% $(\text{NH}_4)_2\text{SO}_4$

No. 5: 90% $(\text{NH}_4)_2\text{SO}_4$

No. 6: Supernatant

b. Non-reducing conditions

Track:

No. 1: 90% $(\text{NH}_4)_2\text{SO}_4$

No. 2: 90% $(\text{NH}_4)_2\text{SO}_4$

No. 3: 70% $(\text{NH}_4)_2\text{SO}_4$

No. 4: 60% $(\text{NH}_4)_2\text{SO}_4$

No. 5: 50% $(\text{NH}_4)_2\text{SO}_4$

No. 6: 40% $(\text{NH}_4)_2\text{SO}_4$

Table 9: Fractionation of pea protein, in extracts from mature dry cotyledons, with ammonium sulphate.

Ammonium sulphate saturation %	Amylase activity diameter (mm) of hydrolyzed area after 20h. at room temperature
40	8.3
50	4.2
60	4.2
70	3.5
90	0
supernatant	0

14.6.3 Amylase of germinated cotyledons

High activity was present at all fractions, but slightly higher activity was detected at 70% ammonium sulphate precipitation (see table 10).

Table 10: Fractionation of pea protein, in extracts from germinated cotyledons of 7 day-old seedlings, with ammonium sulphate.

Ammonium sulphate saturation %	Amylase activity (diameter (mm) of hydrolyzed area after 20h. at room temperature
40	10
50	10
60	10
70	12
90	10
supernatant	0

14.7 Purification of shoot β -amylase

The extraction of this enzyme in an active form in media containing EDTA indicates that this amylase is β -amylase.

In the first attempt to purify this isoenzyme, 120g shoot tissues were used and only 5mg protein containing enzyme activity was recovered after ion-exchange chromatography. The profile of ion-exchange chromatography is presented in figs. 74 and 75. The profile of amylase activity is not presented for the first attempt, but is presented for the second attempt in table 11 and plate 3A. In the second attempt 600g shoot tissue were used and after ion-exchange chromatography, 28mg protein was recovered, contains about 4mg protein enzyme (as estimated by semi-quantitative method).

Table 11: Profile of amylase activity on ion-exchange chromatography.

The protein was extracted from shoots of 7 day-old pea seedlings and precipitated at 71%-90% $(\text{NH}_4)_2\text{SO}_4$ saturation. From fig. 75, see plate 3A.

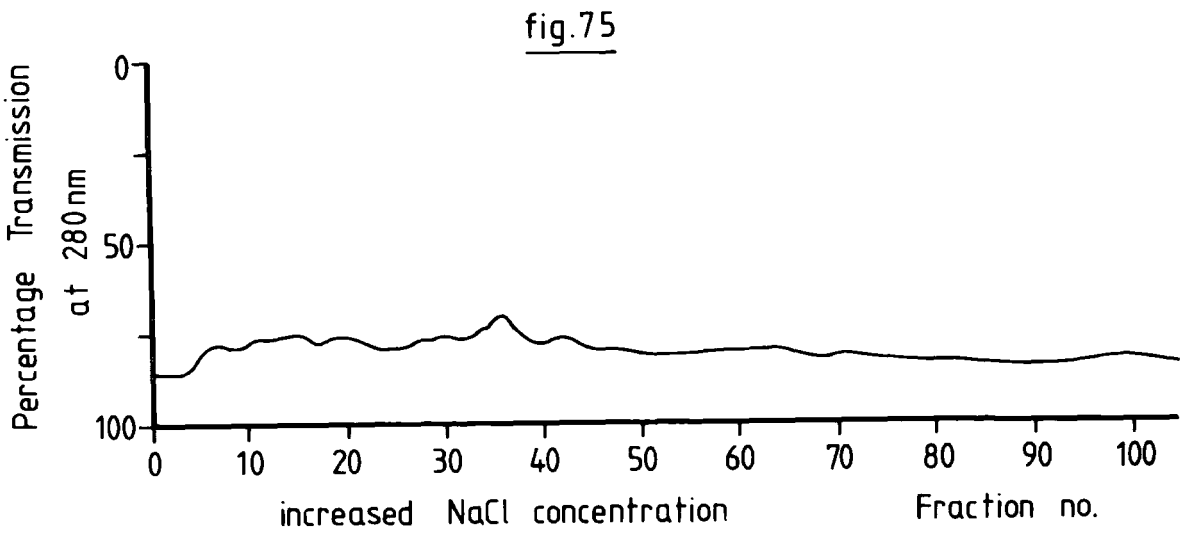
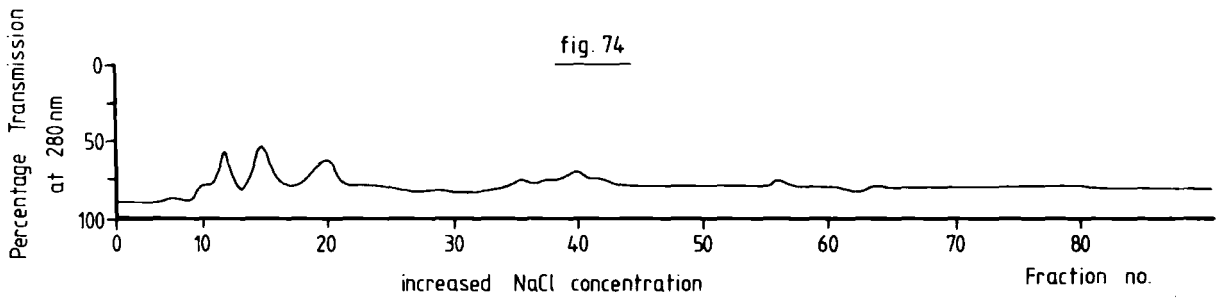
Fraction number	Diameter (mm) of hydrolyzed area after approx. 20h. at room temperature
66	6.0
68	8.0
69	10.0
70	11.0
71	10.0
72	8.0
73	6.0
74	6.0
75	5.5
79	4.5
80	4.0

Fig. 74

Profile of purification of pea (Pisum sativum var. Feltham First) protein, by ion-exchange chromatography. The protein was extracted from the shoot of 7 day-old seedlings and precipitated at 61-90% $(\text{NH}_4)_2\text{SO}_4$ saturation.

Fig. 75

Profile of purification of pea (Pisum sativum var. Feltham First) protein, by ion-exchange chromatography. The protein was extracted from the shoot of 7 day-old seedlings and precipitated at 71-90% $(\text{NH}_4)_2\text{SO}_4$ saturation.



The profile of the first attempt is different from that of the second, because in the first attempt the protein used was precipitated at 61%-90% $(\text{NH}_4)_2\text{SO}_4$ saturation, while in the second attempt, the protein used was precipitated in 71%-90% $(\text{NH}_4)_2\text{SO}_4$ saturation.

The lyophilised protein contained enzyme activity, after ion-exchange chromatography, always associated with yellow pigments.

When the purified protein containing the amylase activity was studied on 7.5% acrylamide gel, the results in fig. 76A showed that this enzyme was the enzyme which was studied during seed germination and development in pea. The non-SDS gel stained for protein had a thick band, plus several thin bands which were not very visible because they probably did not stain well in the non-SDS gel.

The SDS gel (fig. 76B) with a heavily loaded sample showed that the enzyme is far from being pure (fig. 83 tracks 10 and 11). When 9mg purified protein containing enzyme activity were applied to a gel filtration column, about 1mg protein containing enzyme activity was recovered. This 1mg of purified protein which was dissolved in about 0.6ml tris/HCl buffer contained about 200~~ug~~ug protein enzyme (as estimated by the semi-quantitative procedure) (see plate 3C). The 17% SDS-acrylamide gel (fig. 83 track 12) showed two major bands beside many faint bands. The profile of gel filtration and enzyme activity are presented in fig. 77, table 12 and plate 3B.

Plate 3

Amylase activity of pea (Pisum sativum var. Feltham First) shoot.

A. Profile of amylase activity, from fig. 75, see table 11.

Numbers represent fraction number.

B. Profile of amylase activity, from fig. 77, see table 12.

Numbers represent fraction number.

C. Amylase activity in lyophylyzed protein, purified by ion exchange chromatography and gel filtration.

1) The activity in the dissolved purified protein.

2) The activity in 1:5 dilution of 1

D. Amylase activity in purified protein, by ion-exchange chromatography, dissolved in tris/HCl buffer containing 2% (w/v) SDS

Numbers represent fraction number.

Note:- The plates were produced by placing the agarose/starch gel on photographic paper, which was then exposed to light for a short time.

Therefore, the hydrolyzed areas appear darker than the background.

Fig. 76

7.5% non SDS-acrylamide gel of protein, purified by ion-exchange chromatography, of shoots.

A. Gel was stained for amylase activity

B. Gel was stained for protein

Fig. 77

Profile of purification of pea (Pisum sativum var. Feltham First) protein, by gel filtration. The protein was extracted from shoot of 7 day-old seedlings, precipitated at 71-90% $(\text{NH}_4)_2\text{SO}_4$ saturation and purified by ion-exchange chromatography.

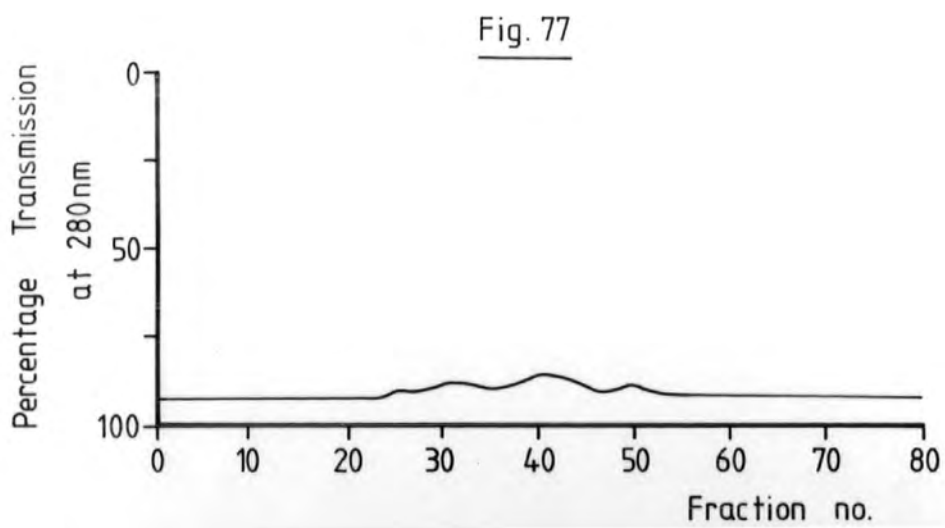
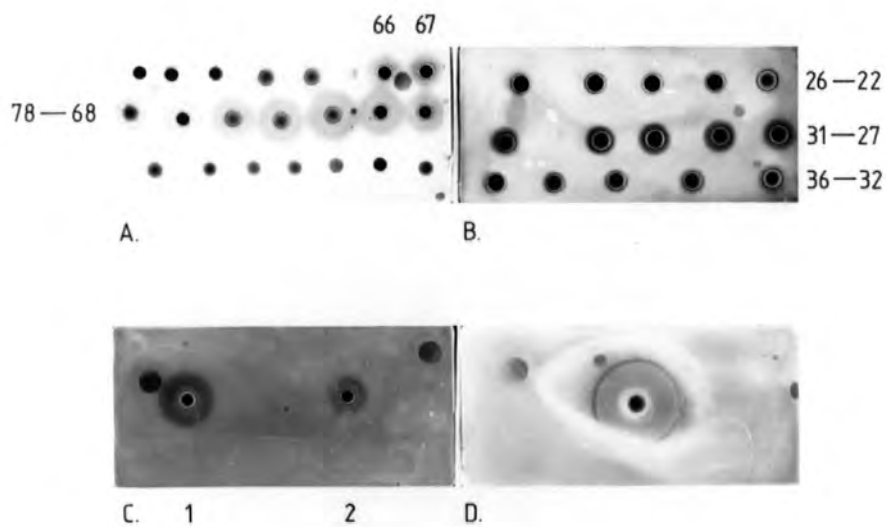


Table 12: Profile of amylase activity on gel filtration. The protein was extracted from shoots of 7 day-old pea seedlings and purified by ion-exchange chromatography. From fig. 77 (see plate 3B).

Fraction number	Diameter (mm) of hydrolyzed area after about 3.5h. at room temperature
26	2.0
27	4.4
28	4.4
29	4.0
30	3.0
31	3.0
32	1.5

The results in plate 3D showed that the enzyme was not inactivated by SDS and when an attempt was made to locate the enzyme protein on the SDS-acrylamide gel, the SDS interfered with the iodine staining reaction colour. Even when the gel was shaken in acetate buffer to remove the SDS, the gel did not stain sufficiently.

14.8 Ion-exchange chromatography of mature cotyledon protein

The profile of ion-exchange chromatography is presented in fig. 78. The profile of enzyme activity, as presented in table 13 and plate 4, showed that the enzyme activity was very low and the 17% SDS gel (fig. 79 tracks 8 and 9) showed that the enzyme protein was highly impure and the enzyme protein was not one of the major bands.

Fig. 78

Profile of purification of pea (Pisum sativum var. Feltham First) protein, by ion-exchange chromatography. The protein was extracted from mature cotyledons and precipitated at 40% $(\text{NH}_4)_2\text{SO}_4$ saturation.

Plate 4

Profile of amylase activity of mature pea (Pisum sativum var. Feltham First) cotyledons, from fig. 78, also see table 11.

Numbers represent fraction number.

Note:- This plate was produced by placing the agarose/starch gel on photographic paper, which was then exposed to light for a short time. Therefore, the hydrolyzed areas appear darker than the background.

Fig. 79

17% SDS-acrylamide gel of the different fractions of pea (Pisum sativum var. Feltham First) mature cotyledons protein which were purified by ion-exchange chromatography. From fig. 78.

Track:

No. 1: vicilin

No. 2: fractions 1-6

No. 3: fractions 7-12

No. 4: fractions 13-18

No. 5: fractions 32-35

No. 6: fractions 40-43

No. 7: fractions 55-67

Nos 8 and 9: fractions contain amylase activity

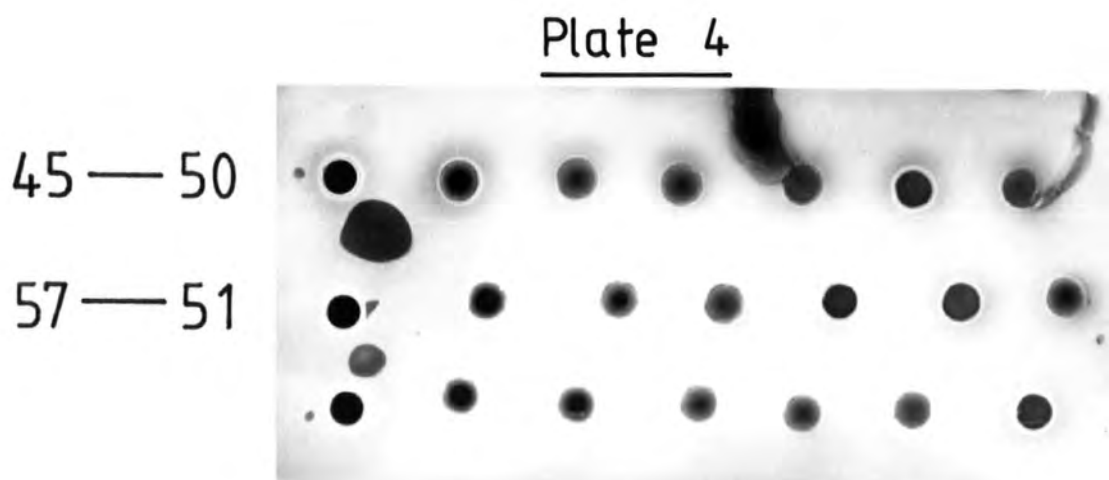
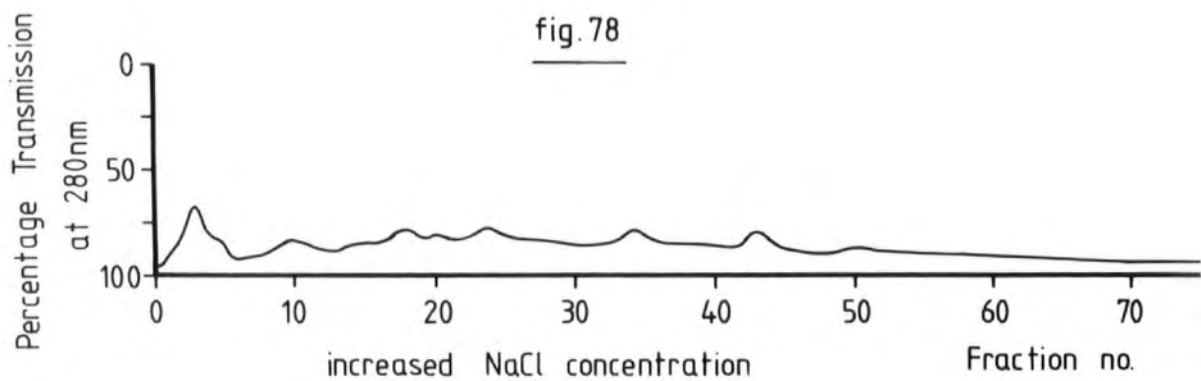


fig. 79

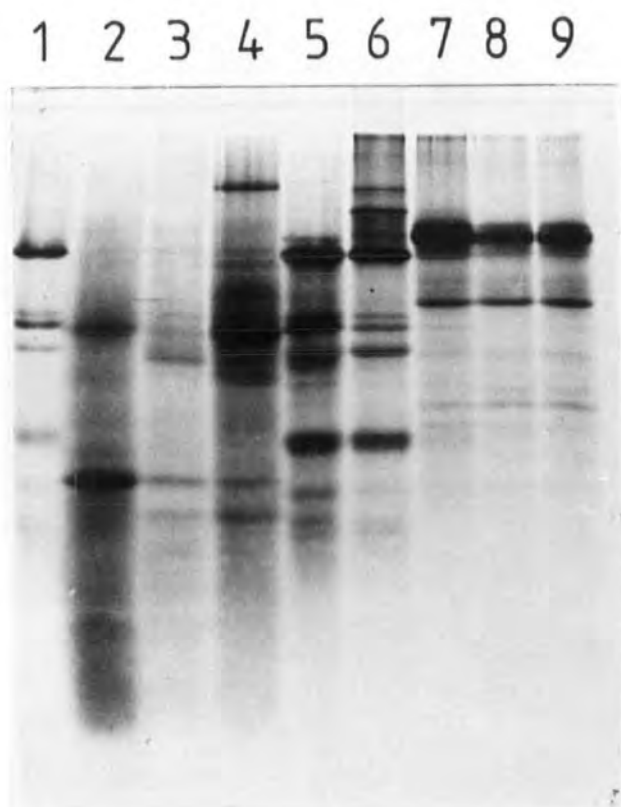


Table 13: Profile of amylase activity after ion-exchange chromatography. The protein was extracted from mature pea cotyledons and precipitated at 40% $(\text{NH}_4)_2\text{SO}_4$ saturation. From fig.78, see plate 4.

Fraction number Diameter (mm) of hydrolyzed area after approx. 20h.
at room temperature

45	5.0
46	5.5
47	5.5
48	4.0
49	4.0
50	4.0
51	4.0
52	3.5
53	3.0
54	2.0

14.9 Purification of α -amylase of germinated cotyledons

The stability of this enzyme under heat treatment in the presence of added CaCl_2 and its ability to form a complex with glycogen, confirmed its identity as α -amylase.

The SDS-acrylamide gel of the enzyme-glycogen complex showed three thick bands in addition to few thin bands (fig. 80).

The profile of the hydroxylapatite column in fig. 81 showed one peak of high enzyme activity (plate 5 and table 14). The SDS-acrylamide gel showed three thick bands beside a few very faint bands (fig. 83 tracks 8 and 9), and no difference between the reduced and non-reduced samples. The 7.5% non-SDS acrylamide gel (fig. 82)

Fig. 80

17% SDS-acrylamide gel of germinated pea (Pisum sativum var. Feltham First) cotyledon protein (7 day-old seedlings) after complexing with glycogen.

Fig. 81

Profile of purification of pea (Pisum sativum var. Feltham First) protein, by hydroxylapatite column chromatography. The protein was extracted from cotyledons of 7 day-old seedling.

Plate 5

Profile of pea (Pisum sativum var. Feltham First) α -amylase activity, from fig. 81, also see table 14.

Numbers represent fraction number.

Note:- These plates were produced by placing the agarose/starch gels on photographic paper which was then exposed to light for a short time. Therefore, the hydrolyzed areas appear darker than the background.

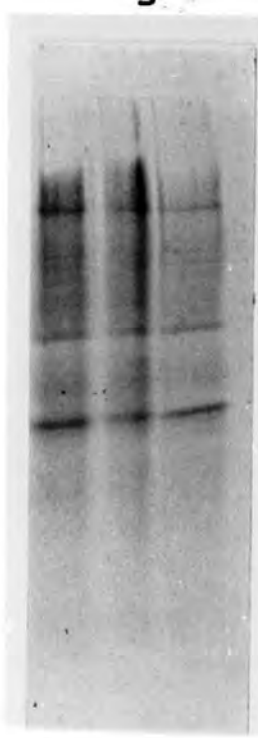


Fig.81

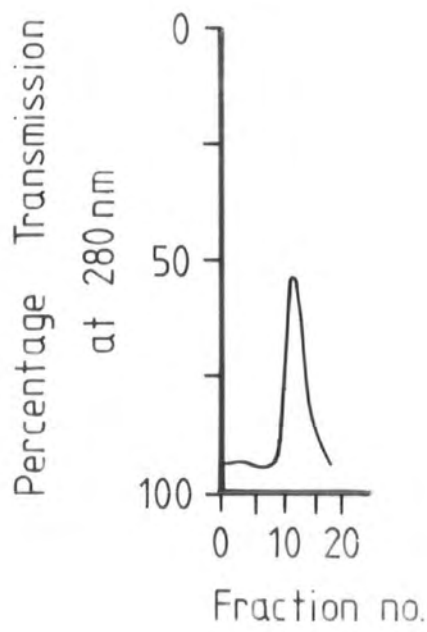


Plate 5

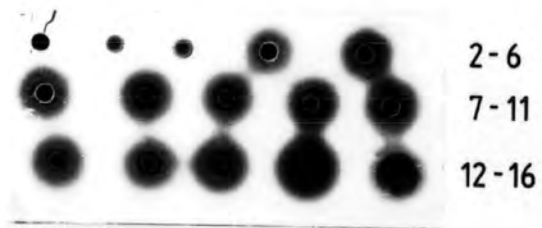


Fig. 82

7.5% non SDS-acrylamide gel of protein of germinated pea (Pisum sativum var. Feltham First) cotyledons. The protein was purified on a hydroxylapatite column.

A. Gel was stained for amylase activity

B. Gel was stained for protein

Fig. 83

17% SDS-acrylamide gel of standard protein and purified protein of pea (Pisum sativum var. Feltham First)

Track:

No. 1: albumin egg

No. 2: albumin bovin

No. 3: α -chymotrypsinogen

No. 4: transferrin

No. 5: myoglobin

No. 6: albumin rind

No. 7: cytochrome C

No. 8: purified pea (Pisum sativum var. Feltham First) protein, from germinated cotyledons, by hydroxylapatite column, under non-reducing conditions. The protein contains α -amylase activity

No. 9: as in 8, but under reducing conditions

No. 10: purified pea (Pisum sativum var. Feltham First) protein, from shoot, by ion exchange chromatography, under non-reducing conditions. The purified protein contains β -amylase activity.

No. 11: as in 10, but under reducing conditions

No. 12: as in 10, but the protein was also purified by gel filtration

fig. 82

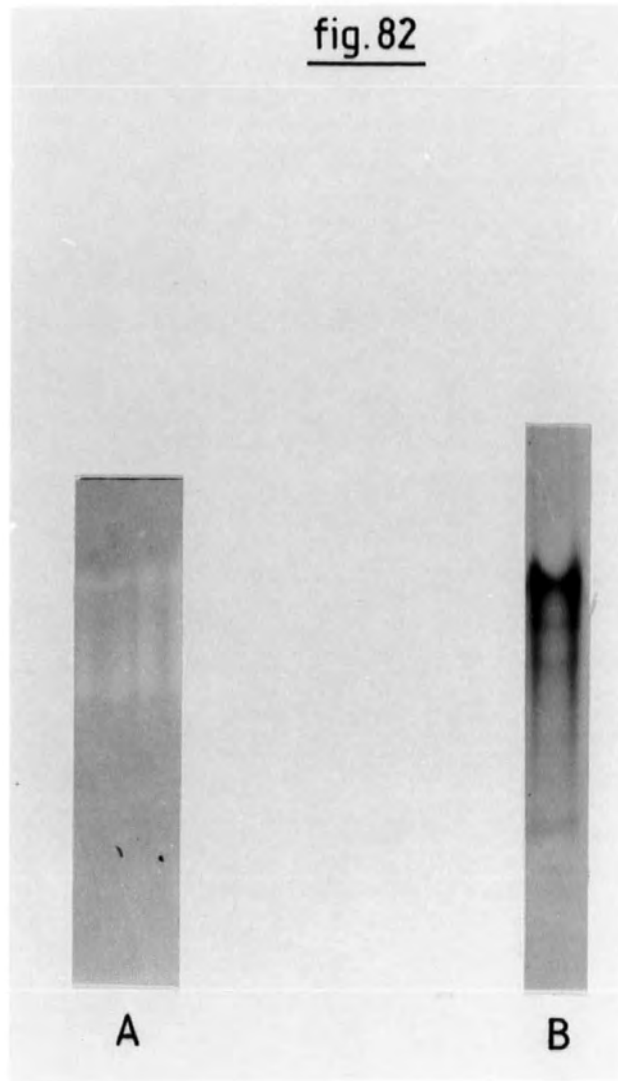


fig. 83



seed germination, over a 15 day period.

The total protein of pea in crude extracts from the germinated cotyledons was analyzed on 17% SDS-polyacrylamide gel under reducing and non-reducing conditions and the results are presented in fig. 85 and 86. Legumin subunits were followed during seed germination in second dimensional gels and the results are present in figs. 87-96.

The major storage protein bands were identified on the SDS-acrylamide gel as follows:-

1. The band of mol. wt. 78,000 is convicilin.
2. The bands of mol. wt. 56,000 and 37,000 are vicilin.
3. The bands of mol. wt. about 65,000 under non-reducing conditions, are legumin.
4. The bands of mol. wt. about 42,000 under reducing conditions, are the acidic subunits of legumin.
5. The bands of mol. wt. about 23,000 under reducing conditions, are the basic subunits of legumin.

15.1 Convicilin

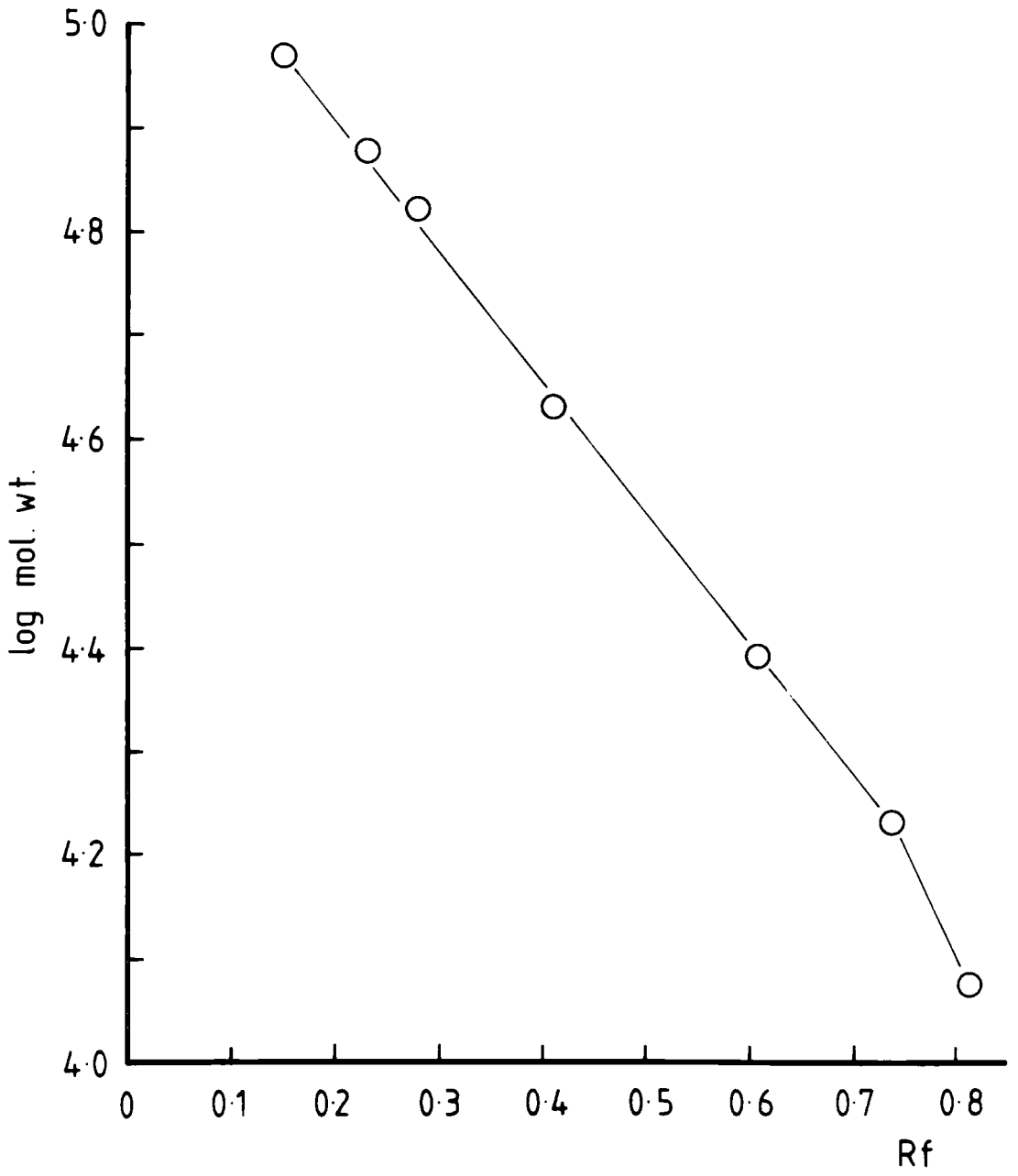
The results in fig. 85 and 86 showed that the level of convicilin decreased gradually during seed germination. As evident from fig.85A , when a low sample volume was used, the convicilin subunit started to decline from the first day of germination and was degraded rapidly between the 3rd and 5th day of seed germination, and could hardly be detected in cotyledon extracts from 5 day-old seedlings, and was not present in extracts from 7, 9, 11, 13 and 15 day-old seedlings.

Under reducing conditions, another band was present in extracts

Fig. 84

Standard curve for molecular weight measurement of protein.

fig.84



from 7, 9, 11, 13 and 15 day-old seedlings, having the same mobility on the gel as the convicilin band, and was not present in the former extracts under non-reducing conditions.

15.2 Vicilin

The subunit of mol. wt. 56,000 had the most obvious decline in its content between the 2nd and 3rd day of seed germination, was hardly detected on the gel in extracts from 7 day-old seedlings and was not present in extracts from 9, 11, 13 and 15 day old seedlings.

The subunit of mol. wt. 37,000 started to be utilized slowly from the 1st day of germination, and disappeared most rapidly from the 3rd day and was not present in extracts from 7, 9, 11, 13 and 15 day-old seedlings.

15.3 Legumin

The bands of mol. wt. about 65,000 under non-reducing conditions were very well resolved in extracts from the germinated cotyledons up to 5 days after seed germination, and were not present in extracts from 7, 9, 11, 13 and 15 day-old seedlings. It appears that they were utilized slowly from the first day of germination and very rapidly from the 5th day.

The acidic subunits of mol. wt. about 42,000, under reducing conditions, were utilized very rapidly from the 3rd day of germination and were not present in extracts from 7, 9, 11, 13 and 15 day-old seedlings. The basic subunits of mol. wt. about 23,000 were very well resolved in extracts up to 9 day-old seedlings and were very faint and

Fig. 85

SDS-acrylamide gels of total protein (under non-reducing conditions) in extracts from pea (Pisum sativum var. Feltham First) cotyledons at different stages of seed germination.

A: Sample used was 10 μ l of 1:100 cotyledon meal: extraction buffer.

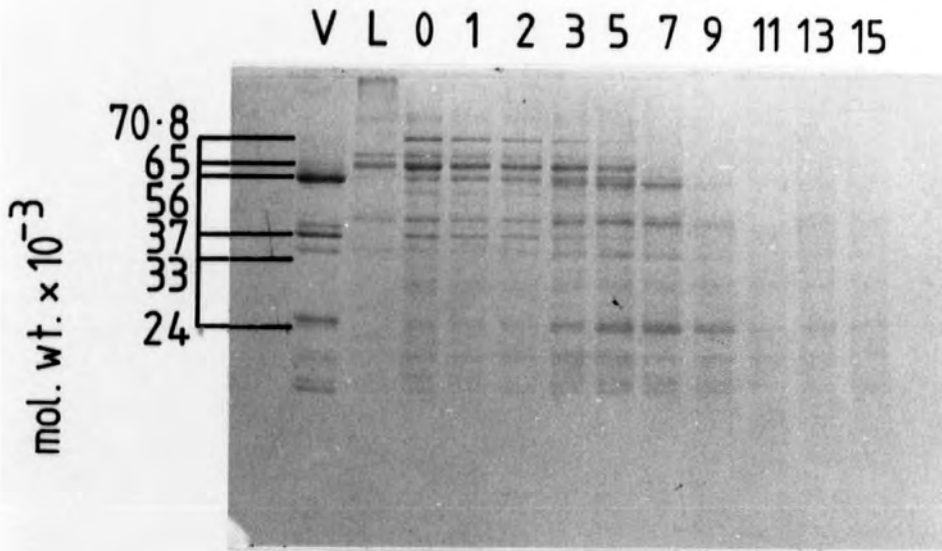
B: Sample used was 30 μ l of 1:100 cotyledon meal: extraction buffer.

Numbers are days of germination.

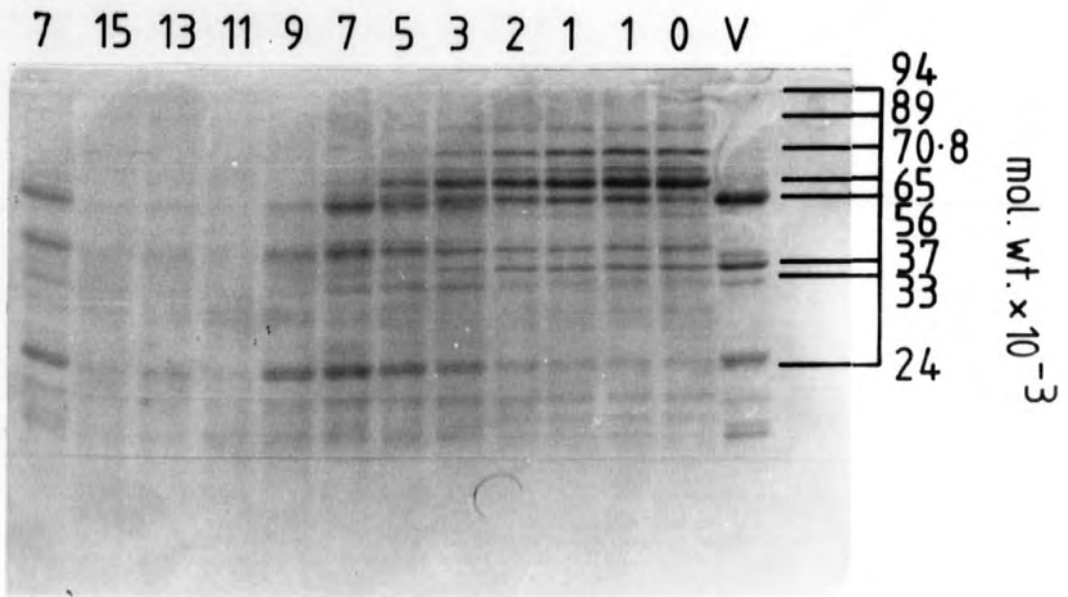
V = standard vicilin

L = standard legumin

fig. 85



A.



B.

hardly detectable on the acrylamide gel in extracts from 11, 13 and 15 day-old seedlings. In another experiment, the gel was incubated only for 7h. in the staining solution (usually the gels were stained overnight, 18h.), and the results in fig. 86c show the absence of these bands in extracts from 11, 13 and 15 day-old seedlings indicating the low levels of these bands in the former extracts.

15.4 Other protein

The subunit of mol. wt. about 54,000, which was very close to the vicilin subunit of mol. wt. 56,000, had its highest level in extracts from 3, 5 and 7 day-old seedlings, and was not present in extracts from 11, 13 and 15 day-old seedlings.

The level of subunit of mol. wt. about 89,000 decreased gradually during seed germination and was not present in extracts from seedlings after 7 days of seed germination.

The subunit of mol. wt. about 32,000 was present in extracts from 3, 5, 7 and 9 day-old seedlings and had its highest level in extracts from 7 day-old seedlings.

The two subunits of mol. wt. about 28,000 under reducing conditions, were very well resolved in cotyledon extract from seedlings up to 9 days old and were faint in extracts from 11, 13 and 15 day-old seedlings.

The subunit of mol. wt. about 24,000, under non-reducing conditions, had its highest level in extracts from 3, 5, 7 and 9 day-old seedlings.

Fig. 86

SDS-acrylamide gels of total protein (under reducing conditions) in extracts from pea (Pisum sativum var. Feltham First) cotyledons at different stages of seed germination. A and B are 17% gels, while C is 12.5% gel and stained only for 7h. See text for details.

Sample used in A was 10 μ l of 1:100 cotyledon meal:extraction buffer, in B was 17 μ l of 1:50 and in C was 16 μ l of 1:20.

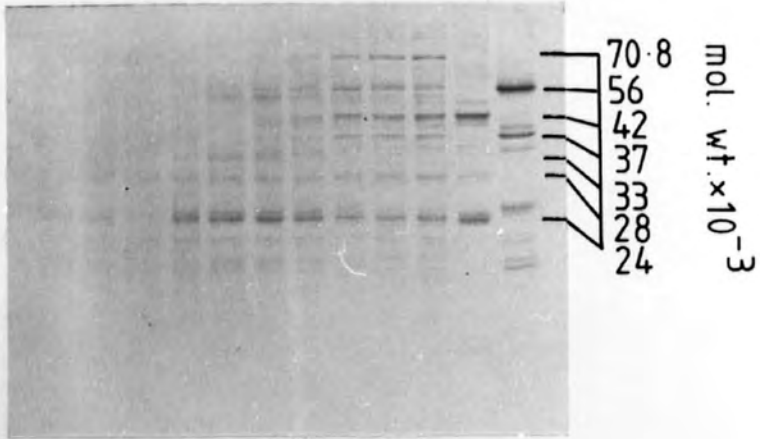
Numbers are days of germination.

V = standard vicilin

L = standard legumin

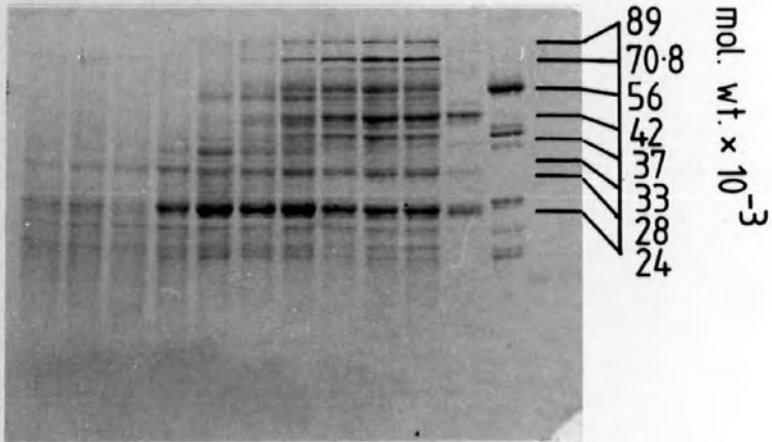
fig. 86

15 13 11 9 7 5 3 2 1 0 L V



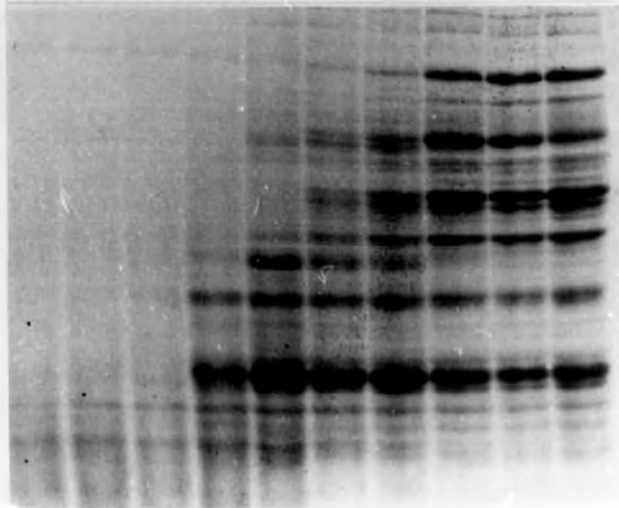
A.

15 13 11 9 7 5 3 2 1 0 L V



B.

15 13 11 9 7 5 3 2 1 0



C.

15.5 Second dimensional gels

The subunits of legumin were followed in the different extracts from germinated cotyledons in second dimensional acrylamide gels. The total protein was first analyzed on 17% SDS-acrylamide gels in the first dimension.

In a preliminary experiment, when a low volume of cotyledon extract (10 μ l of 1:100 cotyledon meal to extraction buffer) was used in the first dimension, the subunits of protein were faint in the second dimensional gel, so larger volumes of cotyledon extract (14 μ l of 1:20 cotyledon meal to extraction buffer) were used in the first dimension. The results are illustrated in fig. 87-96.

In the second dimensional gel, the legumin subunits left the diagonal path, whereas the subunits of other protein remained along the diagonal path.

15.5.1 Control

The second dimensional polyacrylamide gel of extract from cotyledons of mature dry seeds revealed the presence of several subunits of mol. wt. about 42,000 (the acidic subunits of legumin) and several subunits of mol. wt. about 23,000 (the basic subunits of legumin. See fig. 87).

There were about 6 acidic subunits, two of them were large in size, two small and two appeared faint on the polyacrylamide gel.

There were about 6 basic subunits and only three of them appeared to be abundant.

Fig. 87

Two dimensional SDS-acrylamide gel of extract from dry pea (Pisum sativum var. Feltham First) cotyledons.

R: total protein patterns under reducing conditions

U: total protein patterns under non-reducing conditions

Fig. 88

Two dimensional SDS-acrylamide gel of extract from pea (Pisum sativum var. Feltham First) cotyledons of 1 day-old seedlings.

R: total protein patterns under reducing conditions

U: total protein patterns under non-reducing conditions

fig.87

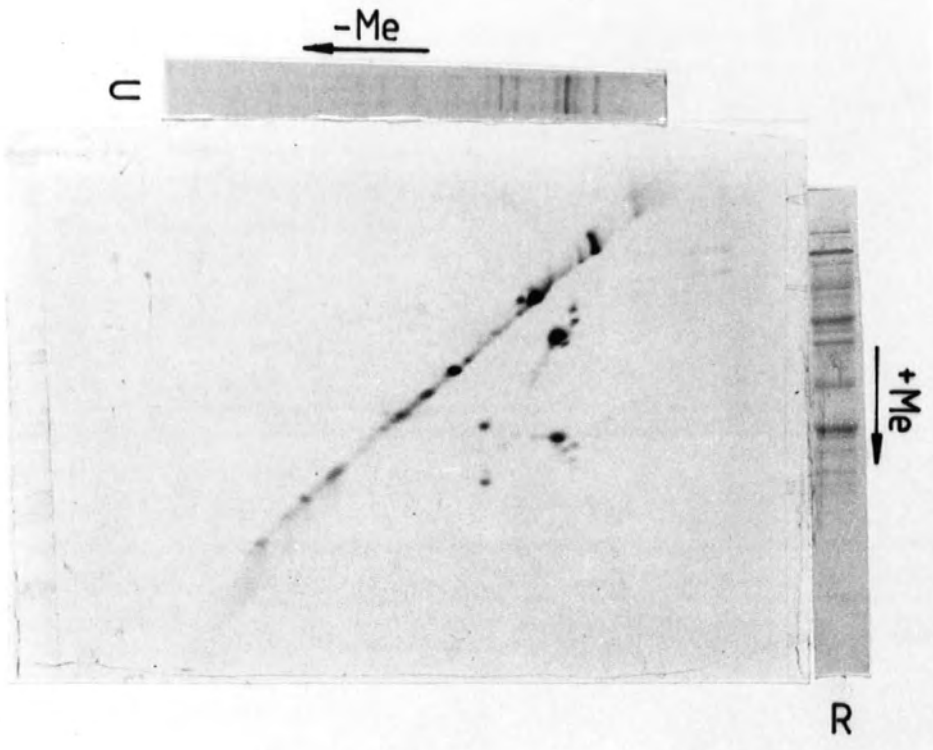
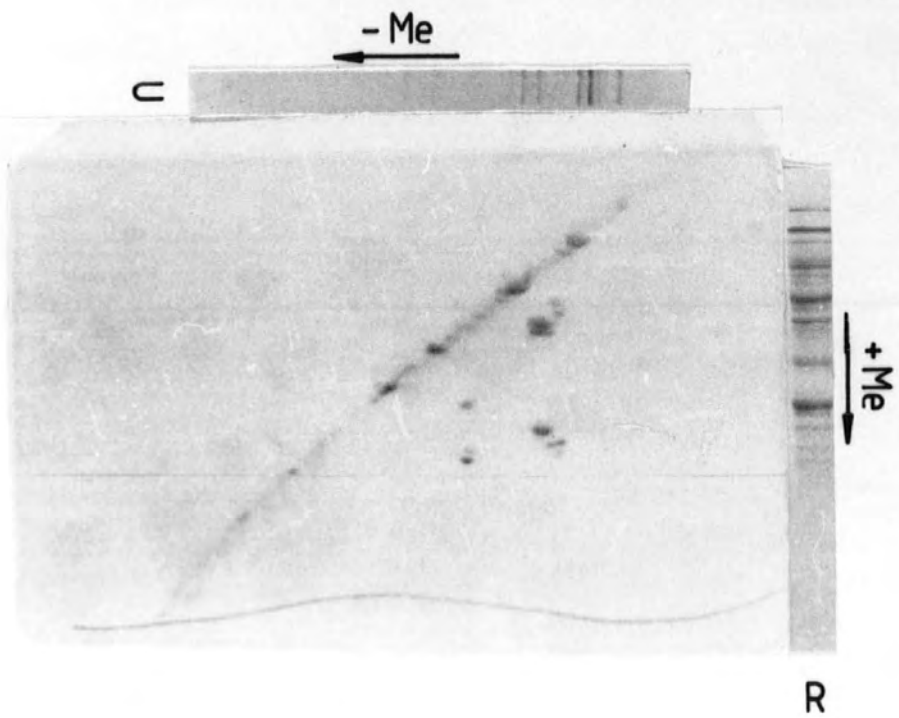


fig. 88.



15.5.2 One day-old seedlings

The second dimensional polyacrylamide gel of cotyledon extracts was similar to that of the control except that two of the basic subunits which were very close to each other in the control, moved slightly apart, with no change in their molecular weight (see fig. 88).

15.5.3 Two day-old seedlings

The second dimension polyacrylamide gel of cotyledon extracts showed some changes, at this stage of germination, in the structure of legumin subunits. The two largest acidic subunits decreased in size, but not in molecular weight, and an additional subunit appeared (see fig. 89).

15.5.4 Three day-old seedlings

The second dimensional polyacrylamide gel of cotyledons revealed that the legumin subunits undergo dramatic changes at this stage of seed germination. The two largest, acidic subunits were broken down and produced several subunits of smaller molecular weight and content (see fig. 90).

15.5.5 Five day-old seedlings

The second dimensional polyacrylamide gel of cotyledons at this stage of seed germination is similar to that of 3 day-old seedlings, except that there is an apparent increase in the content of products of the breakdown of acidic subunits (see fig. 91).

Fig. 89

Two dimensional SDS-acrylamide gel of extract from pea (Pisum sativum var. Feltham First) cotyledons of 2 day-old seedlings.

R: total protein patterns under reducing conditions

U: total protein patterns under non-reducing conditions

Fig. 90

Two dimensional SDS-acrylamide gel of extracts from pea (Pisum sativum var. Feltham First) cotyledons of 3 day-old seedlings.

R: total protein patterns under reducing conditions

U: total protein patterns under non-reducing conditions

fig.89

-Me

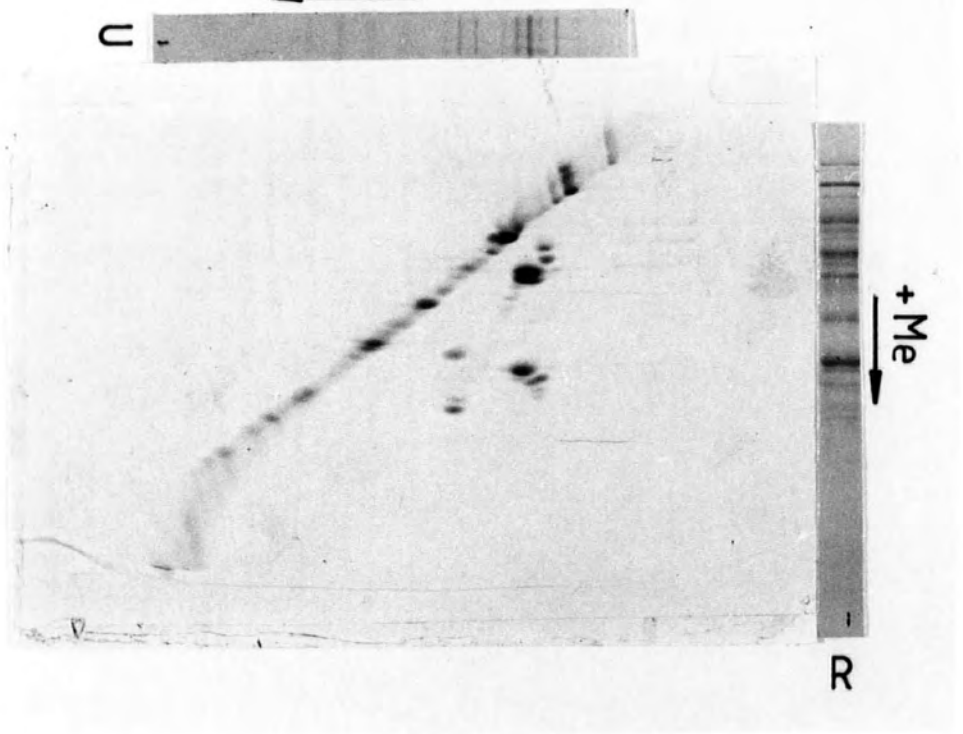


fig.90

-Me

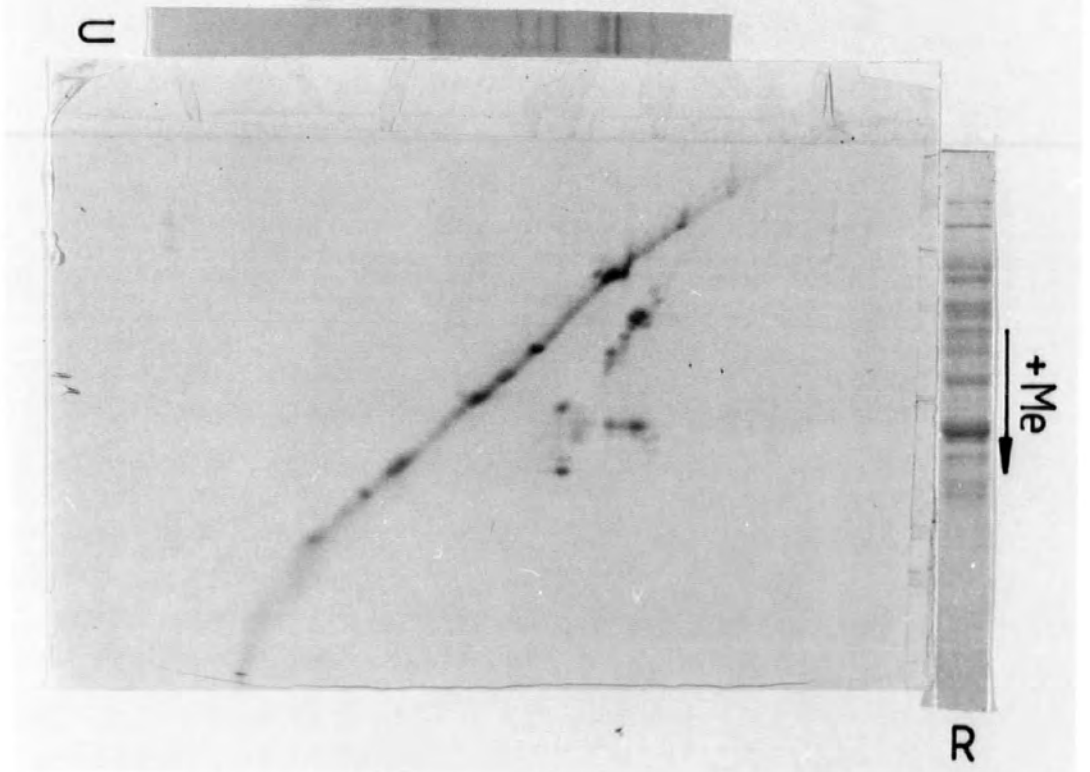


Fig. 91

Two dimensional SDS-acrylamide gel of extract from pea (Pisum sativum var. Feltham First) cotyledons of 5 day-old seedlings.

R: total protein patterns under reducing conditions

U: total protein patterns under non-reducing conditions

Fig. 92

Two dimensional SDS-acrylamide gel of extract from pea (Pisum sativum var. Feltham First) cotyledons of 7 day-old seedlings.

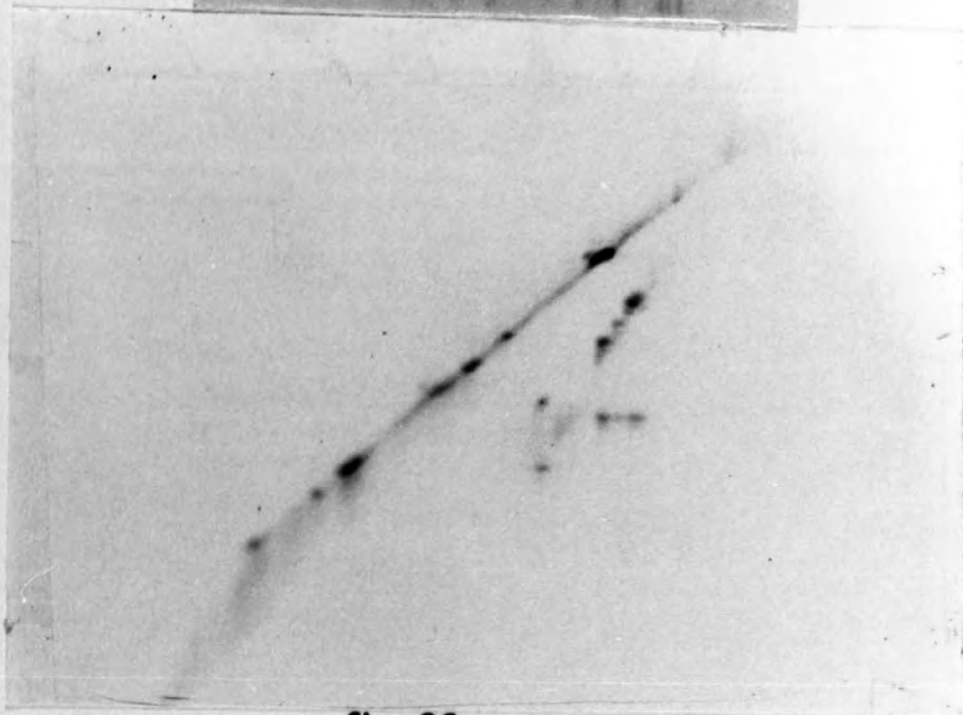
R: total protein patterns under reducing conditions

U: total protein patterns under non-reducing conditions

fig. 91

-Me

C

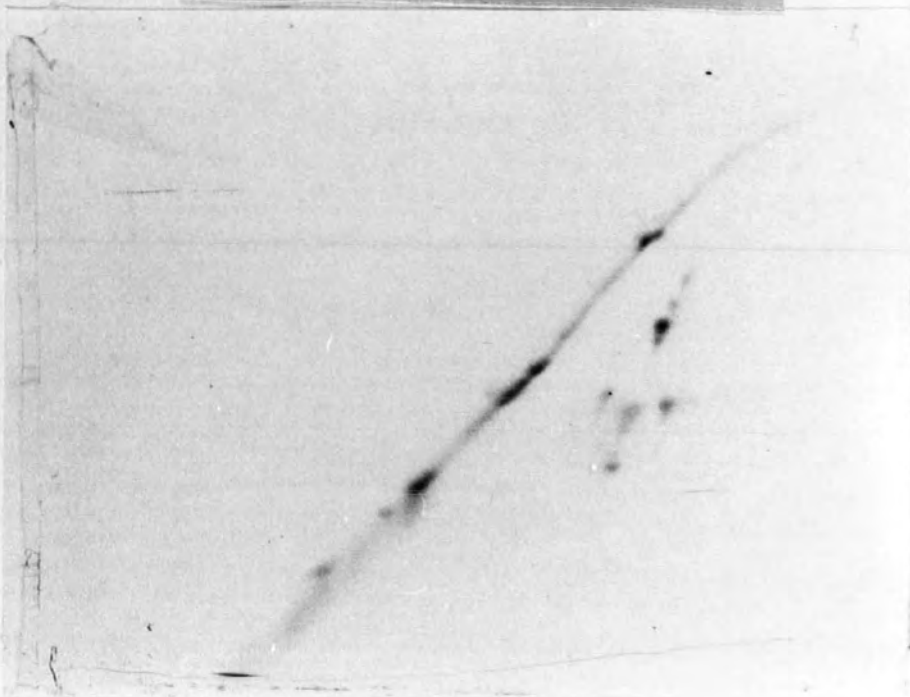


+Me

fig. 92

-Me

C



+Me

R

15.5.6 Seven day-old seedlings

The second dimension polyacrylamide gel of cotyledon extract at this stage of seed germination showed the disappearance of all the remaining acidic subunits and there were four subunits, which were produced by the degradation of the acidic subunits, two relatively large in content and two small (see fig. 92).

15.5.7 Nine day-old seedlings

There was an obvious decline in the content and number of the basic subunits at this stage of seed germination, and only two basic subunits remained. Also, the number and the content of the breakdown of acidic subunits declined and only two breakdown subunits remained at this stage of seed germination (see fig. 93).

There was an apparent increase in the staining of the background between the basic subunits, on the polyacrylamide gel, in cotyledon extracts from the third day of seed germination, increasing in intensity up to the 9th day.

15.5.8 Eleven, thirteen and fifteen day-old seedlings

The legumin subunits and the breakdown of the acidic subunits of legumin were not observed in the second dimensional gels of cotyledon extracts at these stages of seed germination (see figs. 94-96).

Fig. 93

Two dimensional SDS-acrylamide gel of extract from pea (Pisum sativum var. Feltham First) cotyledons of 9 day-old seedlings.

R: total protein patterns under reducing conditions

U: total protein patterns under non-reducing conditions

Fig. 94

Two dimensional SDS-acrylamide gel of extract from pea (Pisum sativum var. Feltham First) cotyledons of 11 day-old seedlings.

R: total protein patterns under reducing conditions

U: total protein patterns under non-reducing conditions

fig.93

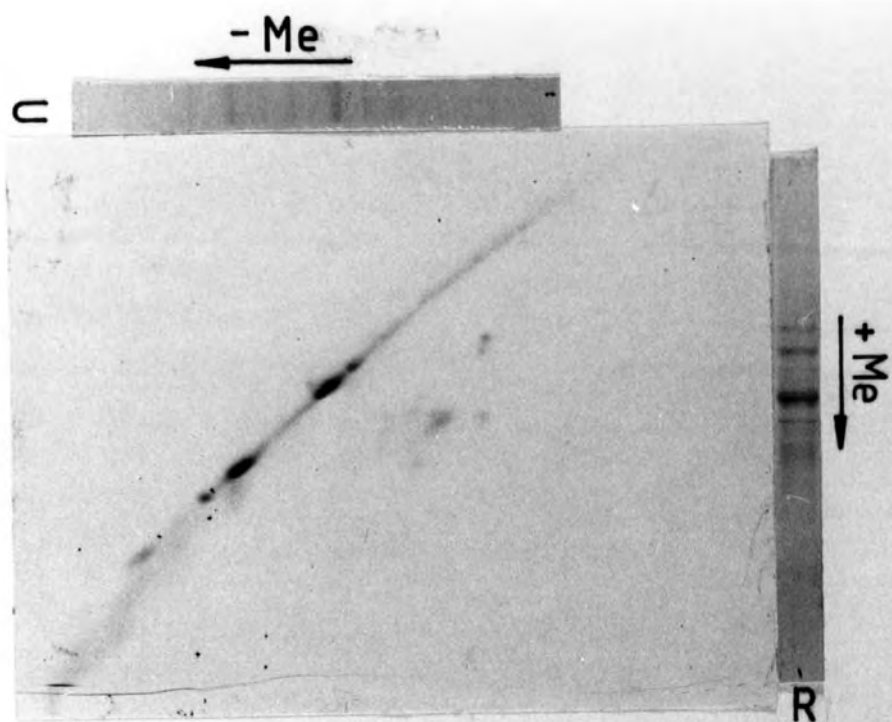


fig.94

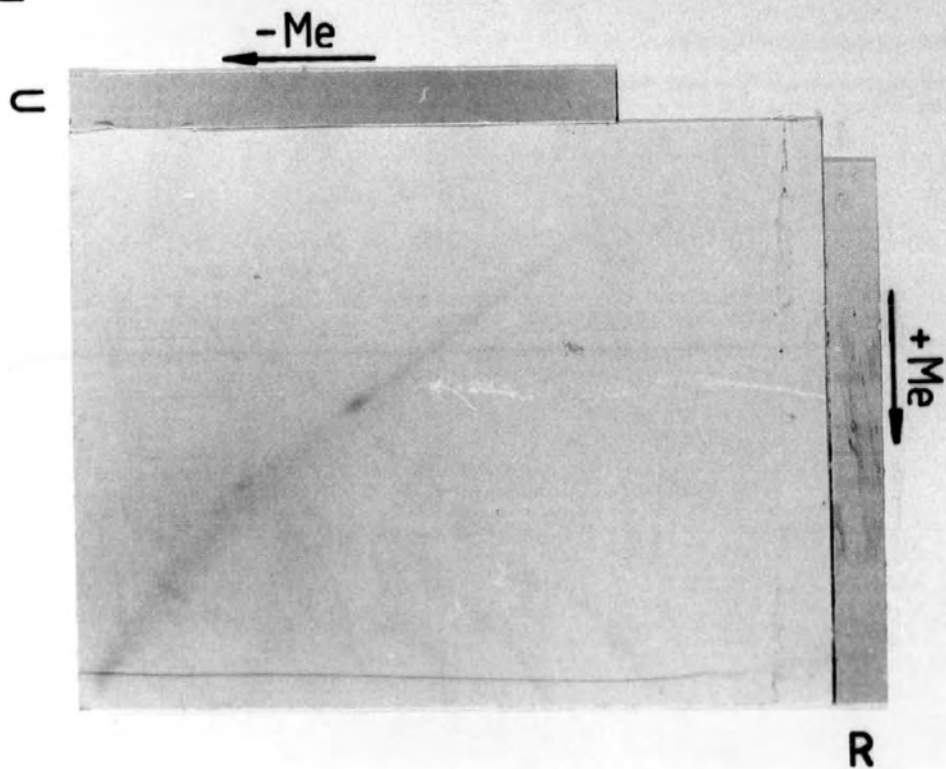


Fig. 95

Two dimensional SDS-acrylamide gel of extract from pea (Pisum sativum var. Feltham First) cotyledons of 13 day-old seedlings.

R: total protein patterns under reducing conditions

U: total protein patterns under non-reducing conditions

Fig. 96

Two dimensional SDS-acrylamide gel of extract from pea (Pisum sativum var. Feltham First) cotyledons of 15 day-old seedlings.

R: total protein patterns under reducing conditions

U: total protein patterns under non-reducing conditions

fig.95

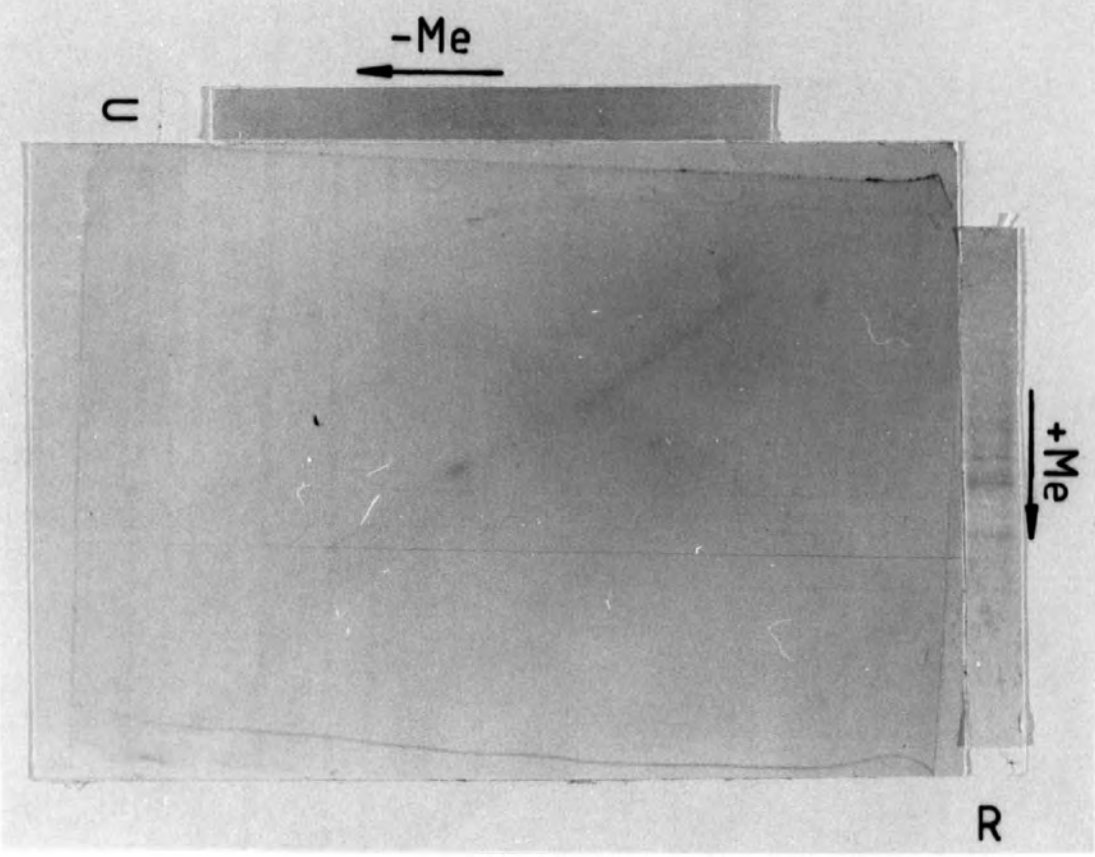
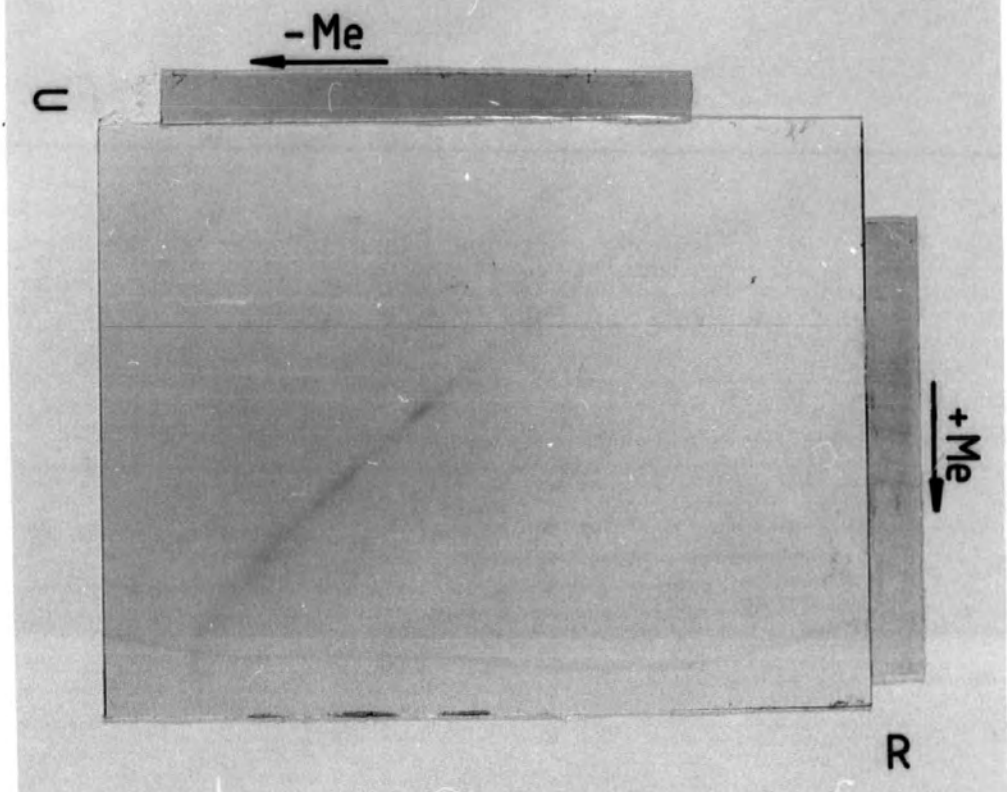


fig.96



Conclusion

It was concluded from this set of results that there was a decrease in the level of total protein in the cotyledon extracts during seed germination, and the extracts from 11, 13 and 15 day-old seedlings were very low in protein content and only few faint bands were present.

Some of the acidic subunits of legumin were broken down into smaller subunits in extracts from 3 day-old seedlings and no similar breakdown was observed for the basic subunits.

Table 15: The days after germination at which a specific band or bands of the major storage protein was (were) completely utilized.

Band or bands	Days after germination at which the band or bands, was (were) not detected
Convicilin (mol. wt. 70,800)	7
Vicilin (mol. wt. 56,000)	9
Vicilin (mol. wt. 37,000)	7
Legumin (mol. wt. 65,000)	5
Legumin (acidic subunits, mol. wt. 42,000)	7
Legumin (basic subunits, mol. wt. 23,000)	very faint hardly detected after 9

16. The application of isoenzyme techniques to a plant breeding experiment

16.1 **The use of seed isoenzyme patterns to assess to relative competitive ability of pea pollen grains of different genotypes**

Two Pisum sativum inbred lines, 1238 and Meteor, were selected as parents for this study on the basis of their differing seed GOT isoenzyme patterns. Reciprocal pollinations with pollen mixtures were made as described in methods section 5.1.

The patterns of GOT isoenzymes were used to identify the genotype of seeds resulting from the pollinations.

16.1.1 **GOT patterns of the hybrid seed**

GOT patterns obtained from extracts of hybrid seed contained five bands, comprising four parental bands and an additional hybrid band. The latter was intermediate in mobility between the fast-moving, parental polymorphic bands. See figs. 97b and 98.

There was an apparent decrease in activity of the fast-moving component in the hybrid seed extract, as compared to that of the parental seeds and seeds derived from self-pollination of parent plants, suggesting that the formation of new bands did not result in an overall increase in GOT activity, and perhaps indicating the presence of a mechanism regulating enzyme activity between the different components.

Fig. 97

Zymogram patterns of GOT and amylase in pea (Pisum sativum) seed extracts.

a. GOT patterns

Track:

No. 1: Inbred line no. 1238

No. 2: Inbred line Meteor.

No. 3: Mixed extracts from both inbred lines

b. Amylase patterns

Track:

No. 1: Inbred line no. 1238

No. 2: Inbred line Meteor

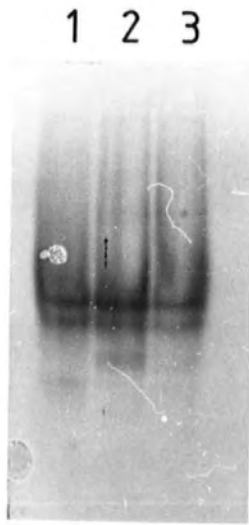
No. 3: Mixed extracts from both inbred lines

Fig. 98

Zymogram patterns of GOT in extracts from pea (Pisum sativum inbred line Meteor) seeds. Flowers were pollinated with pollen of two genotypes (line 1238 and Meteor).

fig. 97

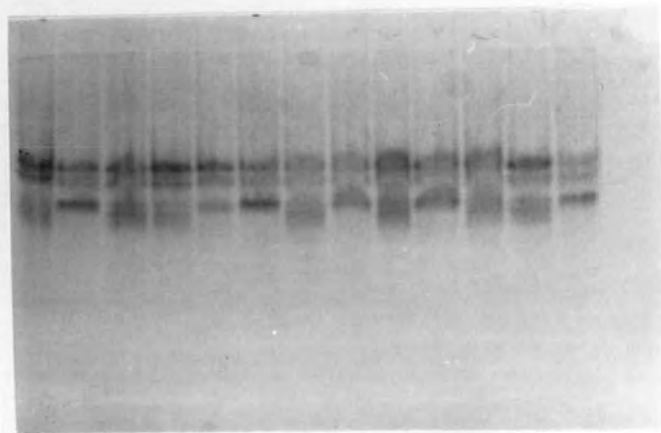
a.



b.



fig. 98



16.1.2 The fertilisation ability of competing pea pollen genotypes

The results in table 16 indicate that of the 112 seeds harvested from both female parents pollinated with 1:1 mixtures of pollen of both genotypes, 60.7% of those were the result of fertilisation by pollen of line 1238, whilst only 39.3% were fertilised by pollen of the inbred line Meteor. This result shows a significant deviation, at the 5% probability level, from the expected 1:1 ratio.

When the results presented in table 17 are examined, representing data from pollination with mixtures of pollen genotypes in varying ratios, analysing both female parents separately, the ratios of parentage of seeds differ significantly from the expected result in several instances. When the inbred line Meteor was pollinated with 1:1 and 2:1 mixtures of Meteor (self): 1238 (cross) pollen a greater-than-expected level of cross fertilisation occurred, and when line 1238 was pollinated with 1:2 and 1:3 mixtures of 1238 (self): Meteor (cross) pollen an excess of self fertilisation resulted.

16.1.3 Stigma size and style length

Figs 99 and 100 show that there are no large differences in stigma size and style length between the two genotypes.

16.1.4 Pollen germination

Fig. 101 illustrates the high levels of self pollen germination observed on inbred line Meteor stigmas.

Fig. 99

Photomicrographs of pea (Pisum sativum) gynecia

a. Inbred line Meteor

b. Inbred line no. 1238

Magnification is 20.

fig.99



A.

B.



Fig. 100

Photomicrographs of pea (Pisum sativum) stigmas

a. Inbred line Meteor

b. Inbred line no. 1238

Magnification is 100



Fig. 101

Fluorescence micrographs of in vivo germination of pea (Pisum sativum inbred line Meteor) pollen grains.

a. After 7h. and 25min. of pollination

b. After 4h. of pollination

Magnification is 100.

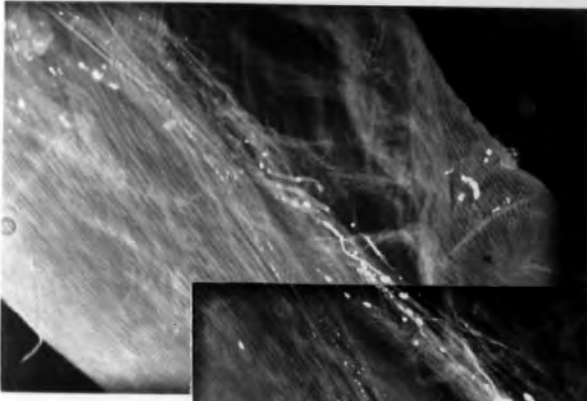


fig. 101 A.

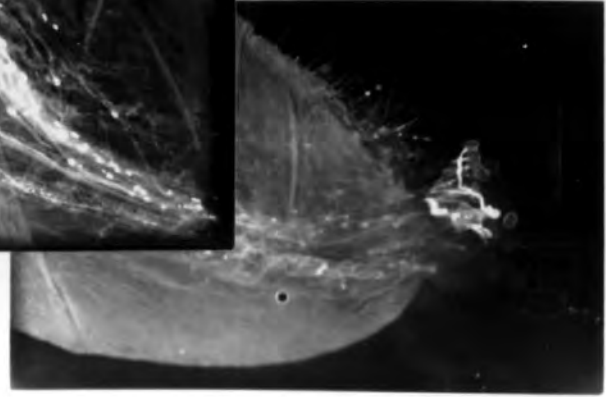
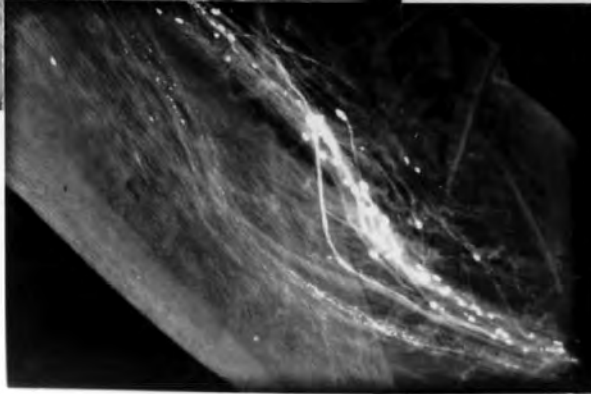


fig. 101 B.

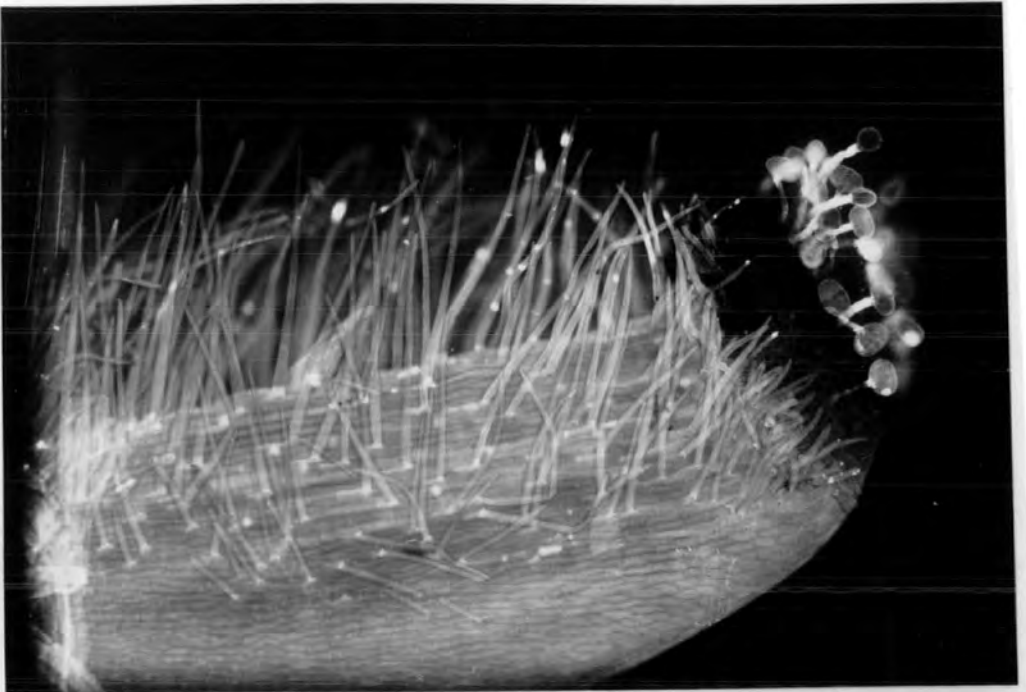


Table 16: Competitive success of 1:1 pollen mixtures of genotypes 1238 and Meteor

	Pollen genotype effecting successful fertilisation	
	<u>1238</u>	<u>Meteor</u>
Expected	56	56
Observed	68	44

$\chi^2 = 5.14$

$p = 0.05$

Table 17: Numbers of cross and self seed obtained after pollination with varying ratios of cross and self pollen genotypes

cv. Meteor as female parent	seed offspring genotype		χ^2	
Ratio self: cross pollen	self	cross		
3:1	9	7	3.00	N.S.
2:1	17	22	9.34	**
1:1	20	36	4.58	*
1:2	3	11	0.88	N.S.
1:3	1	12	2.08	N.S.

cv. 1238 as female parent

3:1	4	0	1.33	N.S.
2:1	28	7	2.79	N.S.
1:1	32	24	1.04	N.S.
1:2	17	7	15.18	***
1:3	13	11	10.89	***

* Significant at $p \leq 0.05$

** Significant at $p \leq 0.01$

*** Significant at $p \leq 0.001$

Discussion

The identification of phosphorylase activity on the acrylamide gel was firstly based on the ability of this enzyme to hydrolyze starch in the presence of a high concentration of orthophosphate in the assay medium, producing colourless bands (white bands) on the acrylamide gel stained with KI/I₂ solution, and secondly on its ability to synthesize starch from glucose-1-phosphate in the presence of starch as a primer, producing deeply blue-stained bands on the acrylamide gel stained with KI/I₂ solution. These characteristics were used by Juliano and Varner (1969) and Yomo and Varner (1973) to identify phosphorylase activity on the acrylamide gel.

The presence of only one band of phosphorylase activity in pea seed extracts is in a good agreement with the results of Juliano and Varner (1969) and Yomo and Varner (1973), while Matheson and Richardson (1976) and Richardson and Matheson (1977) reported that they purified two types of phosphorylase from pea seeds. However, their results showed that one form, as the enzyme was fractionated on a DEAE-cellulose column chromatography and the activity was measured spectrophotometrically, had extremely low activity of only about 1/10th of the other form in the mature seeds and 1/26th to 1/36th in the immature seeds and the cotyledons of germinated seeds. Their results also showed that the phosphorylase activity in pea is cultivar dependent.

It has been reported that sucrose favours the dissociation of the

phosphorylase forms that associate with the plastids (Müntz , 1977) and since in this work the general procedure used for extracting phosphorylase was a sucrose-free buffer, it might be argued that more forms of phosphorylase might be present in association with the plastids and that they require sucrose for dissociation. This possibility is ruled out because no additional bands of phosphorylase activity were detected in the pea seed extract when sucrose was included in the extraction buffer.

Also, Frydman and Slanbink (1973) reported that they were unable to detect the activity of the unprimed phosphorylase in some potato extracts and that this was due to the presence of a thermolabile inhibitor for this form of phosphorylase which could be removed during the process of phosphorylase purification, so that subsequently the unprimed phosphorylase regained its activity. If this is the case for pea phosphorylase, then an additional form(s) might be present in the crude extract but in an inactive form, probably due to the binding of an inhibitor to it; during the course of purification the inhibitor could be removed from the isoenzyme which subsequently become active.

The presence of inactive forms of phosphorylase that may complex with a non-dialyzable, heat labile inhibitor has been described for maize (Tsai and Nelson, 1969) and this complex can be dissociated by protamine sulphate fractionation, but not by DEAE-cellulose column chromatography.

The present results showed that no electrophoretic variants were present for this form of phosphorylase in the 36 inbred pea lines studied. So, on the basis of these results it can be assumed that no

allelic variation exists, for the gene or genes coding for this isoenzyme, in pea. No allelic variation for phosphorylase in legumes has been reported elsewhere.

The results suggested that there was no apparent changes in phosphorylase activity during the development of pea seeds, as judged by band intensity on the acrylamide gel. However, the relative intensities of the bands on the gel cannot be expected to provide a reliable quantitative estimation, especially if the differences are small. It is probable that quantitative changes can be detected spectrophotometrically, as reported for pea by Matheson and Richardson (1976) and Richardson and Matheson (1977) and for maize (Tsai and Nelson, 1968).

The detection of one band of R-enzyme (debranching enzyme) activity, identified as a blue band on acrylamide gels incubated in acetate buffer, containing amylopectin and stained with KI/I₂ solution, in extract from pea (var. Feltham First) seeds confirms the results of Shain and Mayer (1968), who reported the presence of one band of R-enzyme activity in extracts from pea seedlings but not in dry seeds extract. Also, in this work one band of R-enzyme activity was detected in the cotyledon extracts at the various stages of seed germination. The results of Voldawsky et al (1971) showed the presence of very high activity of R-enzyme in 3h. incubated pea seeds.

An investigation was carried out to purify and study amylase of pea in various cultivars with respect to germination, development and genetic control. The results showed that amylase activity is low in extracts from mature seeds and a limited number of bands were detected

(2-3 bands). These components were identified as β -amylase in the var. Feltham First and the different inbred lines, because they retained full activities in the presence of EDTA and lost their activities if heated at 70°C in the presence of added Ca^{2+} . Therefore, it was concluded that only β -amylase is present in the mature pea seeds and α -amylase activity is completely lacking. Similar results were reported for pea seeds (Swain and Dekker, 1966; Juliano and Varner, 1969; Shain and Mayer, 1968; Yomo and Varner, 1973), wheat (Abbot and Matheson, 1972) and for rice (Tanaka et al, 1970). However, Davis (1977) claimed the presence of α -amylase in the embryonic axes extracts of different varieties of the ungerminated pea seeds. Also, Baun et al (1970) and Adams et al (1981a) reported the presence of low α -amylase activity in mature rice grains and soyabean seeds.

As evident from the results, the processes of seed development and germination in pea (var. Feltham First) are accompanied by interesting changes in activity and zymogram patterns of amylase, which showed a time and tissue specificity, and there was an apparent shift in activity from one electrophoretic form to another. Although the physiological significance of these changes is unknown, the results revealed that amylase is a convenient, useful system for studying gene expression and the coordination of the different parts of the seed during the life cycle of pea. The results suggested that the patterns of amylase in pea may be used to reflect the physiological age of the seeds. Similar observations have been reported by Duffus and Rosie (1973), who showed that the activity of β -amylase in barley may reflect the age and the degree of senescence of different tissues.

Moreover, from the results which showed that the different organs had distinct patterns and content of amylase activity, it can be speculated that this variation in the expression of the genes which are responsible for amylase activity in the different parts of the seed might be due to the possibility that the amylase genes active in the cotyledons are controlled by different regulators than the amylase genes active in the embryonic axes tissue. The presence of separate systems controlling the synthesis or release of β -amylases have been suggested for the different parts of barley grain (Duffus and Rosie, 1973).

One of the interesting features observed for amylase patterns during seed germination and development of pea (var. Feltham First) was the presence of α -amylase activity at the early stages of pea development, which disappeared at the late stages and reappeared in the cotyledon extracts during seed germination. The later observation is very well known and in a good agreement with the results reported by several workers (Swain and Dekker, 1966b; Yomo and Varner, 1973; Juliano and Varner, 1969), who all demonstrated the formation of α -amylase during seed germination of pea and showed that its activity is restricted to cotyledons, and Davis (1977 and 1979) reported the presence of α -amylase in the axis of germinated pea. However, the detection of α -amylase in the seed extracts at the early stages of seed development is an original observation. The physiological significance of its presence at these stages of seed development is obscure, but its presence might be essential for some specific biochemical processes that plays an important part in the process of

seed development, since it has been demonstrated that it is the only form of amylase which can attack starch grains directly (Dunn, 1974). So, it might be assumed that the presence of α -amylase is to provide soluble oligo-saccharides for the other starch-hydrolyzing enzymes.

Another feature of amylase pattern changes during the life cycle of pea seed, was the presence of one fast-moving component whose activity was restricted to the embryonic axes extracts, with high levels of activity in extracts at the early stages of development, declining in activity thereafter, and disappearing in the mature seeds, and reappeared during seed germination in root, stem and leaf extracts. The identification of this component as β -amylase was based on its ability to retain full activity in the presence of EDTA, and it was not heat liable in the presence of added Ca^{2+} . Although some investigators reported that β -amylase produces pink bands on the acrylamide gel because of the incomplete breakdown of starch, this component produced a white band on the acrylamide gels and it has β -amylase properties. However, the colour of the bands on the acrylamide gel (α -amylase produces white bands and β -amylase produces pink bands) is not a reliable major criterion for distinguishing between the two types of amylases, since Frydenberg and Nielson (1965) reported that some forms of α -amylase from barley produced bands with a bluish tinge on the acrylamide gel, and many investigators do not report the colour of the bands produced by amylase activity. However, the identification of the amylase in extracts from pea (var. Feltham First) axes during seed germination,

as a β -type is in good agreement with the results of Swain and Dekker (1966b) who reported the presence of relatively high activity of β -amylase in the axis extracts of germinated pea seeds and who reported that α -amylase activity is restricted to cotyledons extracts.

One of the qualitative changes observed for amylase patterns in pea (Var. Feltham First) was the appearance of another band of β -amylase activity only found in extract from mature seeds. Since this form of β -amylase had an extremely low amylase activity and required large volumes of seed extract in order to detect it on the acrylamide gel, its presence in extracts from seeds at another stage of seed development can not be ruled out. This result is parallel to the result of Scandalios (1974), who reported the presence of one band of catalase activity in extracts from maize seeds at the early stages of seed development and another band first detected in extracts from the mature seeds.

Although the results suggested the presence of two different kinds of β -amylase in pea (var. Feltham First), one type produced white bands on the acrylamide gel (its activity was restricted to the embryonic axes extract) and the other produced pink bands. The chemical differences in their catalytic activities and their precise biochemical role in vivo are not known. Also the results showed that their relative abundance differs in the different organs during seed germination and development, but it is premature at the present time to draw any reliable conclusion to the physiological and genetic processes that control their expression and their respective sites of

synthesis. So, the precise function and the regulation of the synthesis of the different forms of pea β -amylase remain to be elucidated.

It was very unexpected to find that embryonic axis β -amylase retained its activity in extracts from the freeze-dried immature seeds of pea and lost its activity in lyophilized stems of seedlings. This either indicates that this enzyme is protected in the immature seeds during the process of freeze drying or this might suggest that the observed activity of this enzyme in the extracts from the immature seeds only represented the activity that was present in the plumule and the radical, but not that which was present in the epicotyl, since both leaves and root retained enzyme activity when lyophilized.

As shown in the results, the α -amylase band of pea cotyledon extract was slower migrating than all other α -amylase bands, and these results are in contrast to the results of Juliano and Varner (1969), who showed that the migration of α -amylase bands in extracts from germinated pea cotyledons was faster than the migration of all β -amylase bands that were present in the extract. Also, Yomo and Varner (1973) reported different patterns of amylase in extracts from pea germinated cotyledons. The faster moving band of the two α -amylase bands which were detected occupied the same position on the acrylamide gel as the fastest moving β -amylase band (three bands of β -amylase activity were detected), and the second α -amylase band had an intermediate migration rate as compared to the other two bands of β -amylase activity. Therefore, the different varieties of pea seem to have different zymogram patterns with respect to the

migration of α -amylase and β -amylase bands on the acrylamide gel.

The appearance of an additional band of amylase activity in extracts from mature pea seeds when developed on acrylamide gel containing 1% (w/v) soluble starch in the separating gel, as compared to the zymogram pattern that were present in the acrylamide gel, seems to be a strange phenomenon, but a similar observation has been reported for phosphorylase in extract from potato (Gerbrandy and Verleur, 1971). The authors reported that one band of phosphorylase activity developed in acrylamide gel containing glycogen as a primer separated into two bands when developed in acrylamide gel containing starch as a primer, but they did not speculate on this phenomenon. Also, it has been demonstrated that human acid α -glucosidase (maltase) polymorphism could be detected by using affinity electrophoresis in a starch gel matrix only (Swallow, 1977). The two isoenzymes had the same isoelectric point and the same molecular weight, but they differed in their affinity toward starch, and this is the reason why they could be only separated on starch media. In pea it is probable that two amylase isoenzymes have the same electrophoretic mobility but differ in their affinity toward starch, such that one of them might have a high affinity to starch, therefore retarding its mobility in the acrylamide gel containing starch, whilst the other form has no affinity for starch and migrated freely in the acrylamide gel that contained starch. However, if this assumption is true, then both isoenzymes should produce a white band (due to complete starch breakdown), but not a pink band (due to incomplete starch breakdown), since the additional band that was observed on the acrylamide gel

containing starch, was white in colour. However, in this study this band has not been included in the total number of amylase bands that are present in pea seed extract because it needs further investigation.

In order to see whether or not the changes that were observed for amylase zymogram patterns during the seed development and germination of pea (var. Feltham First) are common for the various cultivars of pea and among legumes, the zymogram patterns of amylase were investigated during seed development and germination in another two varieties of the species Pisum sativum and one variety of the species Pisum elatius and in several varieties of various legume species. Since amylase of pea was intensively studied by several investigators and a reasonable body of information is available on the mechanisms that regulate its activity and development during seed germination, therefore it will be used as an example to illustrate the possible mechanisms that regulate enzyme synthesis and activity during the processes of seed development and germination.

The regulation of enzyme activity is a very complicated phenomenon, which involves the spatial and temporal regulation of gene expression and includes the rate of degradation of the messenger RNA, the activation of stored mRNA, and the activation, inactivation and interconversion of an existing enzyme (Smith et al, 1977). Since the mechanisms which regulate the development of α -amylase activity in pea cotyledons during seed germination are unknown, the observed activity may have resulted from induced catalytic activity of enzyme proteins that were already present previously in an inactive state or from de novo synthesis of enzyme protein. Although the detection of an

active α -amylase in seed extracts at early stages of seed development might support the former possibility, work on the formation of α -amylase in pea cotyledons, reported by Shain and Mayer (1968) support the latter possibility. However, there has been no conclusive demonstration of the de novo synthesis of α -amylase in the germinated legume seeds and, evidence from the results of Juliano and Varner (1969) showed that the treatment of pea seeds, prior to germination, with protein synthesis inhibitors does not prevent the appearance of α -amylase activity in the cotyledon extract, but retards the full development of amylase activity. The de novo synthesis of α -amylase activity has been demonstrated for cereals during seed germination (see introduction).

Another possibility that might account for the regulation of α -amylase activity is that α -amylase might become bound to starch granules at the later stages of seed development and be released during seed germination. This possibility is not supported by the results of Swain and Dekker (1969), who were unable to demonstrate the presence of a bound α -amylase in pea.

Alternatively, α -amylase might become bound tightly to an amylase inhibitor at the later stages of seed development and this complex might dissociate during seed germination. Again this possibility is not supported by the results of Swain and Dekker (1969), since they did not detect such complexes. Koshiba and Minamikawa (1983) were unable to detect either active or inactive α -amylase in Vigna mungo seeds using immunological techniques specific for this enzyme.

The results demonstrated that the gene coding for α -amylase is expressed during the early stages of developing seeds, but did not indicate whether α -amylase is produced by de novo synthesis during seed germination or arises from an activation or release of an already present form. They might indicate the activation of a possible presence of an inactive α -amylase in the mature seeds, but this can not account for all α -amylase activity present in the extract of germinated cotyledons, since much higher activity is present in the latter. Therefore, most, if not all, α -amylase activity should be de novo synthesized during seed germination, assuming that all α -amylase protein present in the seeds at the early stages of developing seeds is active.

The disappearance of the fast moving band of β -amylase activity from seed extracts at the late stages of seed development of pea, might be due to 1) Faster rate of inactivation than formation of an active enzyme protein; 2) Faster rate of degradation than synthesis of an active enzyme protein; or 3) A fast rate of degradation or inactivation of the already present active forms with no new active enzyme protein being synthesized. The presence of β -amylase in an inactive form in higher plants have been reported by Duffus and Rosie (1973) and Rowsell and Goad (1962), who showed that β -amylase of the ungerminated wheat seed was present in an inactive latent form, apparently bound chemically to the wheat glutenin by disulphide linkages, and they suggested that the appearance of β -amylase activity during seed germination might be due to the secretion of a substance capable of releasing this bound enzyme. No similar investigation has been carried out on pea.

There are possibly many factors that regulate amylase synthesis and activity in pea, including the level of maltose. It has been shown that maltose, which is one of the end products of the amylolytic breakdown of starch, has an inhibitory effect on amylase activity in barley (Schwimmer, 1950), and maize leaves (de Fekete and Vieweg, 1973), and in germinated pea seeds it has been demonstrated that the amylolytic activity of the cotyledons and embryonic axes was regulated by the level of reducing sugars (Parys et al, 1983). Also, Briggs and Clutterbuck (1973) demonstrated that the level of sugars in barley grains restricted the initial production of α -amylase by repressing gibberellin production. Moreover, it has been shown that the rate of pea amylase synthesis was regulated by the level of free sugars in the germinated cotyledons and the accumulation of the latter reduced amylase synthesis and activity (Garcia-Luis and Guardiola, 1978). So, during seed development and germination amylase synthesis and activity might be regulated by the end products of starch breakdown. During seed development the end products might accumulate to a level that inhibits enzyme synthesis and activity, while during seed germination, the end products of starch hydrolysis might be transferred to the growing embryonic axes so that they do not accumulate to a level that inhibits amylase synthesis and activity. The removal of the end products as a regulatory mechanism for enzyme activity has been suggested by Garcia-Luis and Guardiola (1978). However, the results of Juliano and Varner (1969) showed that the level of free sugars in the germinated cotyledons of pea seed, at the stage of highest level of amylase activity is only slightly less than that present in the mature seeds, where amylase activity was very low.

The presence of relatively high levels of β -amylase activity in pea embryonic axes at the early stages of seed development is in contrast to that of barley embryo, which had no amylase activity at any stages of seed development (Duffus and Rosie, 1973). Also, these authors showed that the total amylase activity in the seeds increased during seed development and β -amylase activity was always higher than α -amylase activity. The former observation is in contrast with the results obtained for pea in this investigation, but the latter is in a good agreement with results obtained for pea. However, it has been shown that the embryonic axes of pea had an effect on the seed development (Hedley and Ambrose, 1980) and therefore the presence of a relatively high level of β -amylase activity in the embryonic axes at the early stages of seed development might play an important role in starch breakdown and the process of seed development.

It is surprising to find comparatively high levels of amylase activity in pea seeds at the early stages of seed development, characterized by rapid starch synthesis (Biliaderis, 1982). However, high level of amylolytic activity is not always associated with rapid starch hydrolysis, as demonstrated by the results of Koshiha and Minamikawa (1980) who showed that Vigna mungo seeds grown at 15°C had high levels of amylolytic and proteolytic activities with no change in the level of the storage reserves of the cotyledons. Baun et al (1970) also found high levels of α -amylase and β -amylase activities at the time of rapid starch synthesis during the development of rice grains and the authors suggested that the function of amylases is to provide primer molecules, by hydrolyzing starch, for the synthesis of

amylose by starch synthetase.

The observed pattern changes of amylase in pea is in good agreement with the pattern changes of peroxidase during seed development of maize, as reported by Scandalios (1969), who showed that some existing forms disappeared and new forms appeared, that there were apparent shifts in the activity from one form to another, and that the different tissues had distinct variations in enzyme content.

The presence of a nearly equal level of amylase activity in stem and root extracts of pea seedlings (as measured by the semi-quantitative method) is in contrast to the results of Davis (1977), who reported the presence of a higher amylase activity in the stem extract than in the root extract.

The absence of amylase activity in extract from the seed coat of pea seed is in contrast to the results that were obtained for acid phosphatase (Murray and Collier, 1977) where most of its activity was associated with the seed coat at the early stages of seed development, and the enzyme remained active in the seed coat of the mature seeds. Although, amylase activity was detected in the testa and pericarp of immature seeds of barley, no activity was present in the testa and pericarp of mature seeds (Duffus and Rosie, 1973). Also, no esterase activity was detected in the seed coat of broad bean (Gates, 1978).

Although glycogen reagent has been used to purify α -amylase of germinated pea cotyledons (Swain and Dekker, 1966b), neither the percentage of the enzyme protein in the purified protein, nor the purity of the enzyme are known. In animal tissue the purity of

α -amylase in glycogen precipitation ranged from 75% in human saliva to 95% in rat parotis (Loyter and Schramm, 1962). The results suggested that α -amylase of germinated pea cotyledons can not be purified to homogeneity following the procedure of Swain and Dekker (1966). Also Rodaway (1978) followed a similar method for barley α -amylase and this author obtained only a partially pure enzyme, and after fractionation of the purified protein with $(\text{NH}_4)_2\text{SO}_4$, only 80% pure enzyme was obtained.

An attempt was made to allocate the gene or genes coding for β -amyl² (see results), but the number of replicates available was not sufficient. However, the results indicated the presence of allelic variation for both bands and these results are in good agreement with the results reported for amylase of maize (Scandalios, 1965) and barley (Frydenberg and Nielsen, 1965). The F_2 data showed that in some extracts the electrophoretic mobility of the two bands of β -amyl² were altered from that of the parental enzymes and the reason for this is obscure, but might be attributable to an interaction between the products of the two alleles which resulted in the formation of hybrid molecules. Scandalios (1966) studied the inheritance of amylase in maize and he showed that in some crosses the F_1 hybrids contained a band having an intermediate mobility with respect to the parental enzymes.

The presence of high level of amylase activity in soyabean seeds (mature and immature) is in a good agreement with the results of Laufter et al (1944), Newton and Naylor (1939) and Adams et al (1980), who all showed the presence of high levels of amylase activity in

soyabean seed extract. The latter authors also demonstrated that some varieties had low levels.

Comparing the profile of starch content and amylase activity in an oil-seeded legume, such as soyabean, and a starch-seeded legume, such as pea, during the processes of seed development and germination, the literature showed that the percentage content of starch (on a dry weight basis) in pea seeds, increased rapidly between 17 and 27 days after anthesis (from 20% - 46%), due mainly to the increase in amylose content (Biliaderis, 1982), and that round seeds contained more starch than smooth seeds at all the stages of seed development (Stickland and Wilson, 1983), while during seed germination starch content decreased (Juliano and Varner, 1969). In contrast to pea, the different varieties of soyabean accumulated starch at the early stages of seed development and mobilized it at the later stages to reach a very low level in the mature seeds, while during seed germination, starch content increased in the first few days then declined (Adams et al, 1980 and 1981b; Hildebrand and Hymowitz, 1981). The results showed that amylase activity in soyabean was high throughout the different stages of seed development and germination; in contrast, the results of Hildebrand and Hymowitz (1981) showed that some varieties of soyabean had low levels of amylase activities, increasing slightly in the first few days of seed development, coincident with increased in starch content and high level of soluble sugars, then declining in the later stages of seed development and throughout the process of seed germination. However, the results of Adams et al (1981b) showed that some varieties of soyabean had high levels of β -amylase activity

throughout the processes of seed development and germination, while other varieties had low levels of amylase throughout these processes (low level of α -amylase activity increased at the early stages of seed development and declined thereafter, and low levels of β -amylase activities, increased during seed development). The authors were unable to detect any phosphorylase activity. From all this and the results obtained for amylase activity during pea seed development and germination, it can be concluded that the oil-seeded legumes appear to be radically different from starch-seeded legumes in respect to starch metabolism and amylase activity during seed development and germination.

In contrast to pea amylase, which has been intensively studied in the past two decades, there is a lack of information on the patterns of amylase of broad bean, mung bean, black eye bean and jack bean, therefore the results will be discussed in general terms.

The presence of low amylase activity in the mature legume seeds, except soyabean, is in agreement with the results reported for pea (Sprent, 1968; Yomo and Varner, 1973; Shain and Mayer, 1968), rice (Baun et al, 1970), and Phaseolus vidissimus Ten (Yamasaki and Suzuki, 1979). Some investigators reported the lack of amylolytic activity in dry seeds and one-day old seedlings of dwarf French bean (Gepstein and Ilan, 1970; Onkelen et al, 1977), maize (Scandalios, 1966) and Vigna mungo (Koshiha and Minamikawa, 1980).

The increase in the amylolytic activity during seed germination in the various varieties of legume, from a very low level in the dry seeds to a remarkably high level during seed germination, is

consistent with the results reported for pea (Varner et al, 1963; Sprent, 1968a and b; Locker and Ilan, 1975; Abbot and Matheson, 1972; Swain and Dekker, 1966 and 1969; Yomo and Varner, 1973; Shain and Mayer, 1968; Juliano and Varner, 1969), wheat (Abbot and Matheson, 1972; Marchylo et al, 1981), dwarf French bean (Gepstein and Ilan 1970 and 1981; Onckelen et al, 1977), maize (Scandalios, 1966), rice (Miyota et al, 1981; Tanaka et al, 1970) Vigna mungo (Koshihira and Minamikawa, 1980, 1981 and 1983) and for Phaseolus vidissimus Ten (Yamasaki and Suzuki, 1979). The increase in amylolytic activity during seed germination appears to be universal among higher plants, since it is required for the breakdown of the storage starch in the seeds, to be used as nutrient source for the growing embryo axes. Several investigators showed that the increase in the amylolytic activity during seed germination was accompanied by a decline in starch content, e.g. pea (Sprent, 1968; Juliano and Varner, 1969) and Vigna mungo (Koshihira and Minamikawa, 1980). However, the mechanisms that regulate the increase and the appearance of new forms during seed germination of legumes are unknown and the literature on this subject is confusing and contradictory (see introduction). On the other hand, in cereals, such as barley, it is very well known that gibberellin which is produced in the embryo moves to the aleurone layers and promotes the formation of α -amylase during seed germination (Bernal-Lugo et al, 1981).

The detection of amylase activity in the various parts of the seedling axes during seed germination is supported by the results of Swain and Dekker (1966) and Clum (1967). The latter showed that the

level of α -amylase activity in dwarf French bean was under hormonal control. The amylase of the seedling axes has been shown to function in the degradation of the oligosaccharides that are transported from the cotyledons during seedling growth (Swain and Dekker, 1966). However, the mechanisms which regulate amylase development in the seedling axes are not known.

The roles and the involvement of the different forms of amylase in the process of seed maturation in legumes, are obscure and the present investigation may provide an insight into the possibility of their involvement in this process, since their patterns undergo changes during seed development. The involvement of α -amylase in the development of field bean pod has been demonstrated by Müntz (1969) who showed the presence of an additional band of α -amylase activity in pod extracts at the middle stages of pod development. The appearance of this additional band was accompanied by the rapid breakdown of starch. Hildebrand and Hymowitz (1981) and Adams et al (1981b) concluded that β -amylase probably had no essential role in the carbohydrate metabolism during seed development and germination of soyabean seeds. Their conclusion was based on the large differences in β -amylase content of the different phenotypes with no significant differences between them in starch and soluble sugar levels during seed development and germination. Therefore, the authors questioned the role of β -amylase in higher plants./

The observed patterns of amylase of broad bean seedlings contrast with the results of Gerbrandly and Verleur (1971), who observed only one fast moving band of amylase activity in extracts from young seeds

and the various parts of 14 day-old seedlings (root, cotyledons and leaves). In this investigation several bands were observed with tissue specificity. However, EDTA was used in their extraction buffer, which is known to inactivate α -amylase activity (see introduction).

The detection of seven bands of NAD-GDH in each extract from mature seeds of pea and broad bean, with the two fastest moving components having very low enzymatic activity, is consistent with the results reported by Thurman et al (1965) and Fawole (1977).

In pea (var. English Wonder), the noticeable shift in the relative staining intensity (the amount of formazan dye precipitate at the site of a particular isoenzyme on the acrylamide gel) of GDH isoenzymes from the two fastest moving components to the two slowest moving components, indicated that there was an actual shift in GDH activity between forms as the seeds developed. This independent expression of the individual isoenzymes of GDH might reflect the possibility of a different specific metabolic role of the different forms, and they may play an important role in the process of seed maturation in this variety. However, the mechanism which regulates the relative distribution of GDH isoenzymes is obscure, but it is known that GDH of pea is a single protein (Pahlich, 1972) and that the isoenzymes were the result of the variation in the net charge of the protein molecule. So, the change in the activity of the different isoenzymes should be due to the mechanism that regulates the abundance of each isoenzyme, rather than the changes in the protein content. Moreover, it has been demonstrated that the fastest moving component of GDH is specific for shoot and the radical extracts in pea seeds and in the second fastest

moving component was more active in radical extract than in cotyledon extract (Thurman et al, 1965). It is interesting to note that in this variety, the amylase isoenzyme and GDH isoenzymes which are specific for embryonic axes extract decreased in activity as the seed developed. These observed changes in GDH isoenzymes activities were restricted for the variety English Wonder, and did not appear in the variety Mangetout. Variety-specific changes for isoenzymes have been reported for LAP in pea (Collier and Murray, 1977).

Furthermore, this shift in GDH might be related to metabolic change in pea seeds during seed development. It has been shown that GDH activity is induced by ammonia (e.g. Shepard and Thurman, 1973) and by amino acids (Bayley et al, 1972), and Stewart and Rhodes (1977) suggested that perhaps there are different functional GDH isoenzymes; assimilatory isoenzymes induced by ammonia and catabolic isoenzymes induced by amino acids. Barash et al (1975) showed that ammonia promotes the de novo synthesis of a specific GDH isoenzyme in oat leaves. Therefore, this shift in enzyme activity between forms might be related to changes in the endogenous levels of ammonia and amino acids during pea (var. English Wonder) seed development, and in vitro examination of the effect of ammonia and amino acids on the activity of the individual isoenzymes might verify this possibility and demonstrate a metabolic role for isoenzymes in seed development.

The detection of two bands of GDH activity in extracts from mature soyabean seeds is consistent with the results of Yue (1969) but in contrast with the results of McKenzie and Lees (1981), who reported the presence of three bands.

The detection of four bands of GDH activity in extracts from mature seeds of black eye bean is in contrast to the results reported by Fawole and Boulter (1977), who reported the homogeneity of GDH in extracts from seedlings.

The detection of eight bands of GDH activity in extract, from mature seeds of mung bean is in contrast to the results of Yue (1969) who showed the presence of only seven bands.

The detection of multiple forms of ADH in extracts from pea and mung bean seeds is parallel to the results of Scandalios (1966) who reported the presence of ADH in multiple forms in higher plants.

The results demonstrated that the inbred lines of pea studied were homogeneous with respect to the isoenzymatic zymogram patterns of GDH. Similar results have been reported for the different varieties of Vicia faba (Fawole, 1977).

The apparent increase in MDH activity as pea seed developed, as judged by the increase in the staining intensity of the bands and the appearance of the third band at the later stages of seed development, coincide with the results of Duffus (1970), who showed that the activities of several dehydrogenases, including MDH, increased during the early stages of barley grain development then declined. Also, Choniski and Trelease (1978) showed that MDH activity was barely detected in the extracts from cotton seeds at the early stages of seed development and the activity increased dramatically 22 days after anthesis.

The detection of only one band of LAP activity in seed extracts of pea and dwarf French bean confirms the results reported for broad bean

(Gates, 1978), but is in contrast to the results reported for pea by Scandalios (1969) and Collier and Murray (1977), who all reported the presence of two LAP isoenzymes.

The results showed that the seed extracts from soyabean and the three varieties of pea at the early stages of seed development, were characterized by a noticeably high level of the activity of EST, as compared to extracts from mature seeds. This many-fold decrease in the EST activity has not hitherto been reported to occur in legume seeds. It is difficult to assess the metabolic significance of these changes in activity, since neither the metabolic roles nor the natural substrates for this enzyme are known *in vivo*. But, the results might suggest its possible involvement in the process of seed maturation in pea and soyabean.

Nevertheless, the relative abundance of EST seems to be a convenient parameter for estimating the developmental state of pea and soyabean seeds as pointed out by several investigators (e.g. Markert and Møller, 1959; Yue, 1969), who demonstrated that the electrophoretic patterns of isoenzymes can be used as a parameter for estimating the state of development and cell differentiation.

The rapid decline in the activity of the fast moving component of EST in extracts from broad bean as the seed developed is in a good agreement with the results of Gates and Boulter (1979), but their results did not show similar fluctuations in EST patterns during seed development, as presented in the results of this work. However, the results of Cherry and Ory (1973) showed some fluctuations in EST patterns in peanut seeds during seed development and germination.

The results showed that the GOT zymogram patterns of pea pollen grains and seeds are identical. This observation is parallel to the results reported by Tanksley et al (1981) who showed that 60% of the structural genes coding for nine enzymes, which are normally present in the sporophyte, are expressed in the gametophyte (pollen). This observation suggests that GOT patterns of pea pollen can be used for breeding studies. Also the results showed that GOT patterns of pea seeds were stable throughout the different stages of seed development, so that GOT patterns of immature seed can also be used in breeding studies, unlike amylase and EST patterns.

The detection of amylase activity in pea pod confirms the results reported for broad bean (Müntz, 1977), where the author demonstrated that amylase plays an important role in starch breakdown in pods.

The appearance of an additional band of GOT in hybrid seeds suggests that GOT of pea is a dimeric protein. Similar results have been reported for GOT of Pinus sylvestris (Rudin, 1975). Also, from the results it can be concluded that the two slowest moving bands of GOT in pea are monomorphic, since no variation in their mobility was detected in the various inbred lines studied, and the fastest moving band is coded by two alleles at the same locus. In contrast β -amyl² of pea is coded by three alleles at the same locus and β -amyl¹ is coded by two alleles at the same locus.

The detection of amylase activity in pea and broad bean pollen is in a good agreement with results reported for several plant species (Johri and Vasil, 1961 and the references within).

In conclusion, the following observations are made from the results:

1. A unique feature has been observed for pea amylase only, in that all the forms present in the different extracts of the seedlings were also expressed at some stages of seed development, while all the other legumes studied in this investigation had additional bands of amylase activity in the various seedling extracts which were not present in seed extract at any stage of seed development.
2. Nearly all the different varieties of legumes studied showed decreases in the activity levels of most of the components present in the mature seed extracts, during seed germination, except soyabean, where all amylase components of the mature seeds remain active during seed germination.
3. Only extracts from broad bean (Vicia faba) seeds showed similarity to the major changes of amylase zymogram patterns which were observed in pea during seed development.
4. Apart from dwarf French bean, all the other species of legume studied during seed germination showed that the level of amylase activity in the different parts of the embryonic axes was nearly the same, while the former showed a considerably higher level of amylase activity in the leaf extracts as compared to that of the other parts of the growing embryo axes.
5. In the different varieties of legume species studied, all the amylase components present in the extracts from the different parts of the growing embryo axes of the seedlings were also present in the extracts from germinated cotyledons, except the

extracts from broad bean leaves and dwarf French bean leaves and the different parts of pea embryonic axes, which had bands not present in extracts from germinated cotyledons.

6. The amylase patterns of extracts from the different parts of seedlings of Vicia narbonensis were similar to the amylase patterns of extracts from the various parts of pea (var. Feltham First) seedlings, but different from these of Vicia faba seedlings.
7. The presence of additional bands of amylase activity in freeze-dried extracts from germinated pea (var. Feltham First) cotyledons, as compared to that from fresh materials, may have resulted from a modification or degradation of enzyme molecules, during the process of lyophylyzing. Artifacts of amylase have been reported to occur in barley (Tanaka and Akazawa, 1970).
8. All the genes responsible for GOT in pea, broad bean and soyabean, were expressed at all the stages of seed development, since no change in GOT patterns was observed as the seed developed. However, GOT of dwarf French bean had slight changes shown by the disappearance of a faint band as the seed developed.
9. The process of soyabean seed development was characterized by a progressive increase in GDH activity.
10. Among the isoenzymes studied in pea seeds only amylase, GOT and EST are suitable for genetic studies.

The detection of an additional band of EST activity in broad bean seeds stored for two years at 4°C appears to be a strange phenomenon and it is difficult at the present time to speculate on the cause of this, and there is no available information on the effect of storage

conditions on isoenzyme patterns. However, there are probably two possibilities; the seeds that were stored at 4°C might have been hybrid seeds (although there was no morphological differences between them and the other seeds of the same inbred line and the seeds were self pollinated for at least seven generations) or this additional band might have arisen by physical and biochemical mechanisms (see introduction, Section 1.2.2). Gates (1978) suggested that the presence of additional bands of EST in F₁ hybrids of broad bean might arise from recombination of subunits of dimeric enzyme. In conclusion, the storage condition of the seeds might affect the isoenzyme patterns and a further study is required to verify this possibility.

Globulin proteins of pea (Var. Feltham First) during seed germination

The storage proteins of pea, which are a very useful source of human dietary protein, have been intensively investigated in the last decade and all the major globulin storage proteins of pea have been purified and characterized by several investigators (Gatehouse et al, 1980 and 1981; Croy et al, 1979; Casey, 1979; Croy et al, 1980a and b; Thomson et al, 1978). Therefore, no attempt was made to characterize the major globulin proteins, but standard purified samples of pea (Var. Feltham First) legumin and vicilin were run beside the crude extracts from the cotyledons at the various stages of seed germination, with the aim of recognizing the legumin and vicilin subunits on the SDS polyacrylamide gels.

The results of the SDS polyacrylamide gels and the two dimensional gels of crude extract from cotyledons of mature dry pea seeds, showed

that legumin consists of several acidic subunits (mol.wt. of about 42,000) and of several basic subunits (mol.wt. of about 23,000), linked covalently by disulphide bonds. These observations are in good agreement with the results of Gatehouse et al (1980) and with the accepted model for the structure of legumin (Wright and Boulter, 1974).

The results showed that staining intensity of protein bands on the SDS polyacrylamide gel is a very reliable method for detection of the large quantitative changes in protein content, but not for detection of the very small quantitative changes, and also the results revealed that the resolution of protein bands in the SDS polyacrylamide gel electrophoresis depends on sample loading. Some of the broad bands resolved very poorly when the amount of sample loaded was high and some of the weak bands of protein fail to be detected if the amount of sample loaded was very low. Consequently, it is important that as appropriate various concentrations of the extracts be used before an accurate quantitative estimation of the protein content can be obtained.

As was evident from the number of the bands and their staining intensity on the SDS polyacrylamide gel, the results suggested that the most rapid decline in globulin protein of cotyledons occurred after a few days of seed germination. These observations coincide with those reported for pea (Basha and Beevers, 1975) and for mung bean (Kern and Chrispeels, 1978), while Chrispeels and Boulter (1975) showed that the protein content of mung bean cotyledons declined at a linear rate from the second day of seed germination.

The SDS-polyacrylamide gel of cotyledon extracts at the different stages of seed germination, as developed under non-reducing conditions, suggested that the storage legumin protein was completely utilized by the seventh day of seed germination, since the subunits of the mol.wt. 65,000 disappeared from the cotyledon extracts at this stage. However, the SDS-polyacrylamide gel, as developed under reducing conditions, showed that the basic legumin subunits were still a major component of the protein content up to the ninth day of seed germination. The disappearance of legumin subunits of mol.wt. 65,000 before the disappearance of the basic legumin subunits is due to the complete degradation of the acidic subunits to smaller subunits of mol.wt. 32,000 by the seventh day of seed germination, as evident from the results of the second dimensional gels. Therefore legumin subunits of mol.wt. 65,000 no longer existed in the cotyledon extracts after seven days of seed germination.

The early feature of legumin utilization appears to be the degradation of the acidic subunits to subunits of smaller molecular weight. It appears that the subunits of mol.wt. of about 32,000 on the SDS-polyacrylamide gel are the breakdown products of acidic subunits because

- 1) they appeared on the SDS polyacrylamide gel from the third day of germination, at the time when the acidic subunits started to be degraded, as evident from the second dimensional gel;
- 2) the disappearance of one subunit of them coincided with the disappearance of two breakdown subunits, as evident from the second dimensional gel, by the ninth day of seed germination; and

3) they disappeared from the gel at the time of the disappearance of the breakdown subunits from the second dimensional gel.

The question arises - are the breakdown subunits covalently linked to the basic subunits? There are three possibilities to be considered:

- 1) All the breakdown subunits are covalently linked to the basic subunits;
- 2) Some of the breakdown subunits are covalently linked to the basic subunits and the others are not; and
- 3) None of them are covalently linked to the basic subunits.

If the breakdown subunits are covalently linked to the basic subunits then subunits of mol.wt. about 55,000 (i.e. the added mol.wt. of basic subunits, 23,000 and the mol.wt. of the breakdown, 32,000) should be present on the SDS polyacrylamide gel under the non-reducing conditions only. A protein subunit of mol.wt. about 55,000 is present on the SDS-polyacrylamide gel in the cotyledon extracts from the third day of seed germination, at the time of the degradation of the acidic subunits to smaller ones, but on SDS polyacrylamide gels developed under both reducing and non-reducing conditions. Therefore both the first and the second possibilities are ruled out.

If the breakdown acidic subunits are not covalently linked to the basic subunits, then subunits of mol.wt. 23,000 (the basic subunits) and subunits of mol.wt 32,000 (the breakdown subunits) would be present on the SDS polyacrylamide gels under both reducing and unreducing conditions, in cotyledon extracts from the third day of seed germination. Such subunits were detected. Therefore the third possibility seems to be the most likely.

The mechanisms that initiate and regulate the degradation of pea cotyledonary storage protein during seed germinations are not well understood. Although it has been reported that the protease activities increase in pea cotyledons during seed germination (Basha and Beevers, 1979; Yomo and Varner, 1973; Beevers and Splittstoessor, 1968; Beevers, 1968; Guardiola and Sutcliffe, 1971) their respective roles in protein degradation are not understood since increase in activities occur after the initiation and the bulk mobilization of storage protein (Beevers, 1968; Guardiola and Sutcliffe, 1971; Beevers and Splittstoesser, 1968). Also, Basha and Beevers (1975) concluded that an acid sulphhydryl protease plays an important role in protein depletion in pea cotyledons, but does not initiate the modification of protein before it is degraded.

Peptidases seems more likely to be involved in the mobilization of cotyledonary reserve protein in pea (Beevers, 1968), since their activity is higher than the proteolytic activity at the beginning of seed germination. Peptidases has been reported to be present in pea seeds and seedlings (Beevers and Splittstoesser, 1968; Caldwell and Sparrow, 1976 and 1980; Alleman, 1974). Also, in this investigation LAP and BAPA activities were detected in pea seeds. Detailed data for the latter are not presented, but activity increased during seed development then declined.

Assessment of the competitive fertilisation ability of pollen genotypes using seed isoenzyme markers

It has been widely assumed that the fertilisation ability of the male germate is generally independent of its genetic constitution, but in recent years evidence has steadily accumulated that the genotype of the male parent may play a decisive role in determining its competitive ability (Barnes and Cleveland 1963; Pfahler 1965 and the literature cited therein).

The data presented here resulted from an investigation of the fertilisation ability of pea pollen mixtures of different genotypes, using isoenzyme markers to identify the genotypes of resulting seeds.

The results clearly indicated that when 1:1 mixtures of pollen of genotypes 1238 and Meteor were used to pollinate both parents, overall pollen of line 1238 was significantly more successful in effecting fertilisations (table 16). When the data from each female parent line was considered independently, then line 1238 pollen was only more competitive in 1:1 mixtures on stigmas of inbred line Meteor. A gametophytic self-incompatibility system is common amongst members of the Leguminosae, and although no such system is known to operate in Pisum sativum, this latter result might be interpreted to indicate the persistence of a low level of self incompatibility in this line, which was not manifest in line 1238. Constant selection for self fertility in crops with a long history of domestication is known to reduce the expression of self incompatibility.

However, when the results in table 17 are considered, it is

evident that pollen of line 1238 is more competitive when applied in mixtures to stigmas of both female parents. This observation would indicate that the greater competitive ability of 1238 pollen itself may be the consequence of its own genetic constitution and not that of the female parent. Falconer (1966) termed this class of phenomenon assortative mating, as opposed to random mating. Pollen of inbred line 1238 exhibits positive assortative mating, since the uniting gametes tend to be of the same genotype more often than would occur by chance, whereas inbred line Meteor is negatively assortative. Similar results have been reported for maize by Pfahler (1967).

Table 17 illustrates that the most significant deviations generally occurred from the expected frequencies of fertilisations when the proportion of line 1238 pollen in the pollen mixtures was lowest. There is no obvious explanation for this curious result, which requires further investigation.

There are several possible contributing factors which may account for the competitive success of pollen of inbred line 1238. These are:

- a) 1238 pollen may germinate more rapidly on the stigma than that of Meteor;
- b) 1238 pollen may inhibit Meteor pollen germination;
- c) 1238 pollen viability levels may be higher than those of Meteor;
- d) 1238 pollen tube growth may be faster than that of Meteor.

Although detailed examination of these possibilities was not pursued in the present study, the high levels of self seed set observed throughout this study indicate that pollen inviability is not

an important factor. Self pollination of Meteor stigmas resulted in vigorous pollen germination and pollen tube growth (fig. 101). Inter-specific pollen allelopathy has recently been described by Suknada and Jayachandra (1980), but no evidence for intraspecific pollen allelopathy (b, above) exists. Differential fertilisation ability due to differences in pollen tube growth rate would be exaggerated by differences in style length, so that in longer styles the competitive ability of faster growing pollen would be enhanced. Style lengths of the two parental lines were similar (see fig. 99), so this hypothesis could not be tested in this study, but should be exhaustively tested, along with the other possibilities outlined above, in any future investigation of pollen competitive ability.

The effect of the genetic constitution of pollen and stigma has been studied in maize by Pfahler (1967) using pollen mixtures. This author showed that in maize the deviation from random mating may occur as a result of differential pollen transmission, which was associated not only with the pollen genotype, but also with the genetic constitution of the female sporophyte. The author concluded that the nuclear elements contained in the pollen grain itself probably influence its fertilization ability. This author also found that the genetic constitution of the female sporophyte altered the fertilization ability of the pollen grains. The effect of female sporophyte was attributed to the production of a physiologically suitable surface medium to initiate the germination and pollen tube growth and to the possession of morphologically and physiologically suitable conditions in the styla tissue, so that pollen tube penetration was permitted and pollen tube growth was maintained.

The rate of pollen tube growth in various inbred lines has been studied in maize. Mulcahy (1971) found that if a mixture of genotypes of corn pollen, identified by genetic markers, was applied to the silks of other inbred lines, the rate of pollen tube growth often varied with the genotype of pollen. The author showed that the rate of growth of pollen tubes was correlated with the weight of the resulting seeds, so that heavier seed in seed mixtures result from fertilization by gametes from faster growing tubes and the author attributed the increased in seed weight to the greater competitive ability of the zygotes thus formed. Also, Mulcahy (1974) found variation in the relative speed of pollen tube penetration of the style in maize. The variation was statistically significant and correlated with the relative weight of the resultant seedlings. Pfahler (1968) studied the in vitro germination of maize pollen from six single cross hybrids on media containing various combination of B and Ca and the results showed that the germination percentage was dependent on the genetic source of pollen, and the rate of pollen tube growth was affected by pollen source. Also, Pfahler (1970) showed that in the in vitro germination of maize pollen grains from three inbred lines and two of their single cross hybrids, the germination characteristics of pollen were influenced by its genotype.

The phenomenon of gametophytic competition has extremely important implications for plant breeders. The correlation between vigour of pollen germination and pollen tube growth rate in vitro, and the superior vigour of seedlings produced from fertilisations with these genotypes raises the possibility of selecting parental genotypes in

vitro for F_1 hybrid breeding programmes. In addition, the possibility of assortative mating should be considered and investigated when synthetic varieties are constructed, since their level of hybridity and genetic structure depends on the operation of random mating.

In conclusion, the investigation discussed above illustrates the value of using isoenzyme patterns as a marker to detect hybridity. The system can be used where morphological markers are absent, or when morphological markers are available but are only expressed late in plant development. In the latter instance the ability to identify genotypes of seeds formed after fertilisation has the advantage of saving time and obviates the need to grow large numbers of plants to maturity.

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Appendix

Peptidase activity of pea

Ap.1. Method

Ap.1.1 Enzyme extraction

The enzyme was extracted by homogenizing 20mg of seed meal in 500 μ l 0.05M sodium phosphate buffer pH 8.0 containing 10 μ l 2-mercapto-ethanol/1ml buffer, overnight at 4°C. The crude extract was centrifuged for 5min. at 500g and the clear supernatant was used for enzyme assay.

Ap.1.2 Enzyme assay

The activity of the enzyme in the extracts from the seeds at the various stages of seed development was determined by using N-benzoyl-arginine p-nitroanilide (BAPNA).

20mg of the substrate was dissolved in 1ml dimethyl sulphoxide, then diluted to 50ml with 0.05M sodium phosphate buffer pH 8.0.

Assay protocol

The assay mixture consisted of:

2.5ml substrate solution

100 μ l crude extract

The hydrolysis of the substrate was followed on the spectrophotometer at 410nm.

Results

As evident from the table and the figure, the enzyme activity increased rapidly in seed extract from the 9th to the 10th days of seed development, then started to decline, followed by a slow increase from day 21.

Table appendix: BAPNAan activity in extracts from pea seeds at the different stages of seed development.

Age of seeds in days	Rate of substrate hydrolysis $\times 10^{-5}$
9	5.2
10	9.6
13	9.5
15	5.5
17	2.5
19	2.0
21	4.0
32	4.3

Fig. Appendix

PABNAase activity in pea (Pisum sativum var. Feltham First) seed
extracts at the various stages of seed development



