Studies on the purification and characterisation of the major albumin seed proteins from pea (pisum sativum L.)

Hoque, Mohammed Shamsul

ABSTRACT

The seeds of pea (Pisum sativum L.) contain several proteins in the albumin solubility fraction which are significant components (5-14%) of the total cotyledonary protein, and are accumulated in developing seeds concurrently with storage protein synthesis. Two related schemes are described for the fractionation of the predominant pea albumin proteins.

Using the first scheme two closely related major albumin proteins have been isolated and purified to homogeneity for the first time. The larger protein, designated PMA-L, has an Mr ∿ 53,000 and consists of two 25,000 Mr subunits, whereas the smaller, PMA-S has Mr ∿ 48,000 and contains two 24,000 Mr subunits. There was no evidence of mixed dimers of the two subunit sizes, despite their close homology as judged by immunological cross reaction, amino acid compositions, N-terminal amino acids, tryptic peptide mapping, cyanogen bromide cleavage products, and lack of detectable functional properties. PMA-L and PMA-S contain significant amounts of both sulphur amino acids, cysteine and methionine, and have a normal distribution of amino acids.

Under the second scheme, a low molecular weight albumin protein, designated PLA has been purified, characterized and sequenced. PLA has an Mr of ∿ 11,000 and contains polypeptides of Mr 6,000, suggesting that the protein molecules are dimeric. The amino acid sequence of this protein contains 54 residues, and has a high content (10/54) of asparagine/aspartate residues. Its N-terminal amino acid (aspartate) is different from that of both PMA-L and PMA-S (threonine) and PMA and PLA do not appear to be in any way homologous. Like PMA, PLA has no detectable functional properties, and is distinct from the trypsin/chymotrypsin inhibitors found in pea seeds.

PLA does not contain methionine, but contains a relatively high level of cysteine (4 residues per polypeptide) suggesting a possible role as a sulphur storage protein. However, its sequence is not homologous to low Mr (2S) storage proteins from castor bean (Ricinus communis) or rape (Brassica napus); PLA therefore represents a new type of low Mr seed protein.

The putative storage role of PMA and PLA is not supported by studies on germinating seeds which show that PMA-L, PMA-S and PLA are not significantly degraded on or after germination during the period in which the major globulin fraction storage proteins are utilised. It is possible that these proteins have no signficant role in seed physiology, since a few different pea lines are shown to lack either PMA-L, PMA (L + S), or other proteins of the albumin solubility fractions, although none lack PLA. Further evidence for the non-functional role of PMA is provided by its species distribution; preliminary screening indicates the presence of homologous major albumin proteins only in closely related legume species.

STUDIES ON THE PURIFICATION AND CHARACTERISATION OF THE MAJOR ALBUMIN SEED PROTEINS FROM PEA (*PISUM SATIVUM* L.)

A

THESIS

SUBMITTED IN ACCORDANCE WITH

THE REQUIREMENTS OF THE UNIVERSITY

OF DURHAM FOR THE DEGREE OF

MASTER OF SCIENCE

BY

MOHAMMED SHAMSUL HOQUE, M.SC. (DHAKA)

SEPTEMBER, 1984

DEPARTMENT OF BOTANY

UNIVERSITY OF DURHAM

UNITED KINGDOM

ABSTRACT

The seeds of pea (Pisum sativum L.) contain several proteins in the albumin solubility fraction which are significant components (5-14%) of the total cotyledonary protein, and are accumulated in developing seeds concurrently with storage protein synthesis. Two related schemes are described for the fractionation of the predominant pea albumin proteins.

Using the first scheme two closely related major albumin proteins have been isolated and purified to homogeneity for the first time. The larger protein, designated PMA-L, has an Mr ∿ 53,000 and consists of two 25,000 Mr subunits, whereas the smaller, PMA-S has Mr ∿ 48,000 and contains two 24,000 Mr subunits. There was no evidence of mixed dimers of the two subunit sizes, despite their close homology as judged by immunological cross reaction, amino acid compositions, N-terminal amino acids, tryptic peptide mapping, cyanogen bromide cleavage products, and lack of detectable functional properties. PMA-L and PMA-S contain significant amounts of both sulphur amino acids, cysteine and methionine, and have a normal distribution of amino acids.

Under the second scheme, a low molecular weight albumin protein, designated PLA has been purified, characterized and sequenced. PLA has an Mr of ∿ 11,000 and contains polypeptides of Mr 6,000, suggesting that the protein molecules are dimeric. The amino acid sequence of this protein contains 54 residues, and has a high content (10/54) of asparagine/aspartate residues. Its N-terminal amino acid (aspartate) is different from that of both PMA-L and PMA-S (threonine) and PMA and PLA do not appear to be in any way homologous. Like PMA, PLA has no detectable functional properties, and is distinct from the trypsin/chymotrypsin inhibitors found in pea seeds.

PLA does not contain methionine, but contains a relatively high level of cysteine (4 residues per polypeptide) suggesting a possible role as a sulphur storage protein. However, its sequence is not homologous to low Mr (2S) storage proteins from castor bean (Ricinus communis) or rape (Brassica napus); PLA therefore represents a new type of low Mr seed protein.

The putative storage role of PMA and PLA is not supported by studies on germinating seeds which show that PMA-L, PMA-S and PLA are not significantly degraded on or after germination during the period in which the major globulin fraction storage proteins are utilised. It is possible that these proteins have no signficant role in seed physiology, since a few different pea lines are shown to lack either PMA-L, PMA (L + S), or other proteins of the albumin solubility fractions, although none lack PLA. Further evidence for the non-functional role of PMA is provided by its species distribution; preliminary screening indicates the presence of homologous major albumin proteins only in closely related legume species.

ACKNOWLEDGEMENTS

I wish to express my heart-felt gratitude to Professor Donald Boulter, my supervisor and the Head of the Department of Botany, for his guidance, valuable advice and encouragement throughout, and for providing the facilities to do the present work. I am indebted to Dr. John A. Gatehouse for his continued support and sustained interest in this research. I thank him for allowing me to include his work on hydrophobicity profile and secondary structure prediction. I am grateful to Dr. R.R.D. Croy for providing the anti-PMA antibodies and anti-lectin antibodies. I thank him for the kind help, useful suggestions and unfailing interest he rendered whenever sought during the progress of this work.

I wish to express my thanks to John Gilroy, who carried out the amino acid sequence analyses, to Dr. A.M.R. Gatehouse for carrying out the insect bioassays, to David Bown for carrying out the amino acid analysis, and to Paul Preston who carried out α-amylase assays. I also acknowledge the cordial co-operation of all my friends and colleagues in the Botany Department, who have helped in various ways.

A grant of a British Technical Co-operation Training Award by the British Council, United Kingdom, is thankfully acknowledged. I also thank the Secretary, Ministry of Agriculture and Forestry, Government of the People's Republic of Bangladesh, for granting me study leave to undertake this course of study.

I am thankful to Mrs. M. Raine for typing this thesis.

Finally, I would like to thank members of my family and friends from Bangladesh, especially my brother, Advocate Abdul Jalil and my wife, Mrs. Monowara Hoque.

ABBREVIATIONS

The following abbreviations have been used in this thesis :

BAPNA	:	α -N-Benzoyl-DL-arginine-p-nitroanilide HCl
Bisacrylamide	:	N,N'-methylene bisacrylamide
BTEE	:	N-Benzoyl-L-tyrosine ethyl ester
CNBr	:	Cyanogen bromide
Dansyl	:	5-dimethylaminonaphthalene-1-sulphonyl
DABITC	:	4-NN-dimethylaminoazobenzene-4'-isothiocyanate
DABTH	:	4-NN-dimethylaminoazobenzene-4'-thiohydantoin
DEAE-cellulose	:	diethylamino ethyl cellulose
DTT	:	dithiothreitol
DMSO	:	dimethyl sulphoxide
EDTA	:	ethylene diamine tetra acetic acid
HMW-albumin	:	high molecular weight albumin
h.p.l.c.	:	high pressure liquid chromatography
IgG	:	immunoglobulin fraction from serum
Mr/mol.wt.	:	molecular weight
PAGE	:	polyacrylamide gel electrophoresis
PBS	:	phosphate buffered saline
PITC	:	phenyl isothiocyanate
PLA	:	Pisum low molecular weight albumin
PMA	:	Pisum major albumin
PMA-L	:	Pisum major albumin, large
PMA-S	:	Pisum major albumin, small
rel.satn.	:	relative saturation
SDS	:	sodium dodecyl sulphate

TCA	:	tri-chloro acetic acid
TEMED	:	N,N,N'N' - tetra methyl ethylene diamine
TFA	:	tri-fluro acetic acid
TLC	:	thin-layer chromatography
Tris	:	tris (hydroxymethyl) amino methane
2ME	:	2-mercaptoethanol

CONTENTS

SECTION 1

1. Legumes as crops

Food is the primary necessity of life. It serves three main functions - physiological, social and psychological. Physiological functions are related to (i) supply of energy, (ii) building and maintaining the cells and tissues and (iii) regulating the body processes. These needs are satisfied by the nutrient present in the food. The selection of a particular food helps in developing social relationships among people to whom it is served, depending upon their taste. Food also has a psychological effect when it satisfies certain emotional needs; an example of these effects is the reactions of persons who adjust themselves to unfamiliar food in a new place.

This thesis is concerned with one important component of food namely the amino acids required for adequate nutrition.

The major source of dietary amino acids for non-ruminant animals is the protein present in the harvested grain of crop plants. Of this, about 50% is composed of a fraction termed 'storage proteins' (Miflin and Shewry, 1980). The chief contribution of seed proteins and calories for both human and animal consumption is made by the cereal crops, such as rice, wheat and maize, etc., with legumes the second most important source - other crops, such as potatoes, make a relatively small contribution.

However legumes are high protein crops (> 20% dry weight protein in seeds). There are about a dozen major legume crops (Table 1), each of which is used in a variety of different diets (Boulter and Crocomo, 1979). These legume foods are being cultivated in virtually every temperate region, including high altitude locations

in tropical countries.

On average the world production of legumes is about one-tenth (1/10th) that of cereal (Burr, 1975) and in 1973 the Protein Advisory Group (P.A.G.) of the United Nations recommended urgent research attention to be given to "eight major legume foods" (*Phaseolus vulgaris*, *Cajanus indicus*, *Vigna unguiculata*, *Cicer arietinum*, *Vicia faba*, *Glycine max*, *Arachis hypogaea* and *Pisum sativum*) which account for over 90% of total world legume production.

Legume seeds are of particular importance as a supply of dietary proteins in those parts of the world where protein deficiency is a serious problem, such as in Bangladesh and other countries of the third world. They are also important sources of energy and contain significant amounts of both oil and carbohydrate in varying proportions (Boulter, 1983). In western developed countries grain legumes may be primarily used as animal feed or as a source of oil. More recently they have been utilized to a small extent for the production of protein concentrates e.g. textured plant protein for human consumption (Boulter, 1982).

On the other hand, legumes play an essential role in crop rotation and improving soil facility by fixing atmospheric N_2. In western developed countries they play another important role as a 'break-crop' that improve soil fertility, since by virtue of their association with Rhizobia they can fix N_2. In view of the energetically costly industrial process of manufacturing nitrogen fertiliser, legumes offer interesting possibilities both from the ecological and economic standpoints (Boulter and Crocomo, 1979). Worldwide, most legumes are used directly in human nutrition, but some, notably soya bean (*Glycine max*) and peanuts (*Arachis hypogaea*), are often grown

as a 'cash crop' for their oil and/or as a source of animal feed and it is important to distinguish whether their ultimate role is direct human consumption or animal feed. Many legumes, of course, are important forage crops (Boulter, 1982). The world production figures for major legume crops are given in Table 2. (Boulter and Crocomo, 1979).

TABLE 1

LEGUMES OF MAJOR IMPORTANCE

Botanical name	Common name	Areas of Consumption
Cajanus cajan	Pigeon pea	India, Pakistan, Middle East, East Africa
Cicer arietimum	Chickpea	India, Pakistan
Lens esculenta	Lentil	Near East, North Africa, India Central and South America
Vigna radiata (Phaseolus aureus)	Mung bean	South, Southeast, and East Asia, East Africa, India
Phaseolus lunatus	Lima bean	Tropical America, West Indies, Madagascar
Vigna mungo (Phaseolus mungo	Black gram	India, Iran, East Africa, West Indies
Phaseolus vulgaris	Kidney bean	North, Central and South America, Mexico, East Africa
Pisum sativum	Green pea	Mainly temperate zones, parts of India and Africa
Vicia faba	Broad bean	Temperate zones, Near East, North Africa, Central and South America
Vigna unguiculata (Vigna sinensis)	Cowpea	Asia, Tropical Africa, West Indies, China
Oil Seeds, etc.		
Arachis hypogaea	Ground nuts	Asia, Africa, North, Central and South America
Glycine max	Soya bean	North and Central America, Asia, Europe

SOURCE: Modified from Siegel and Fawcett, 1976 by Boulter and Crocomo, 1979.

TABLE 2

WORLD PRODUCTION OF MAJOR LEGUMES, 1972

Botanical name	World Production (1000 metric tons)	World Legume Production (%)
Glycine max	53,024	49.4
Arachis hypogaea	16,887	15.7
Phaseolus vulgaris	10,899	10.2
Pisum sativum	10,218	9.5
Cicer arietinum	6,718	6.3
Vicia faba	5,326	5.0
Cajanus cajan	1,720	1.6
Vigna unguiculata (Vigna sinensis)	1,260	1.2
Lens esculenta	1,182	1.1
World Total	107,234	100

SOURCE: FAO 1973; modified from Siegel and Fawcett, 1976

2. Nutritional properties of legume seed meals and the role of legumes in the diet

Legumes are of special significance as a plant food source since they are high protein crops. On average, cereal grains contain 10-15% of the dry weight as protein, whereas legume seeds contain 20-30% and up to 50% of the dry weight as protein in some varieties of soya bean (Glycine max). In contrast, a typical vegetative organ, such as the leaf, has only 3-5% of its dry matter as protein (Derbyshire et al, 1976; Boulter, 1982). Some legume seeds are also rich in oil; concentrations vary from 1% to more than 40% (Boulter, 1982). Although relatively poor in some vitamins, such as retinol, riboflavin and ascorbic acid, legumes have reasonable quantities of thiamin and nicotinic acid. The nutritionally important minerals, calcium and iron are also present as well as fibre. Legume fibre consists of polysaccharides and lignins that resist hydrolysis by human digestive enzymes and form viscous solutions or gels with water. This fibre is of particular importance medically due to its component seed storage galactomannans, important in the treatment of diabetes (Boulter, 1983). In the final analysis the nutritional importance of a seed constituent must be related to the purpose for which the seed meal is being used, e.g. animal feed, human food etc., either directly or, more recently, as 'textured vegetable protein foods'. The nutritional significance of legume components must also be considered in relation to other feed components in the diet.

Amino acid analyses of a variety of legume seed meals, indicate that the sulphur-amino acids, cysteine and methionine, are usually the 'first-limiting' amino acids for animal and human nutrition. This suggestion has been supported by experiments on rat-feeding (Aykroyd and Doughty, 1964; Boulter et al., 1973; Boulter, 1982).

The amino acid composition of the whole seed meal is a reflection of the fact that the seed 'storage globulins , the major proteins of the meal, are low in sulphur-amino acids (Jackson et al., 1969; Shewry et al., 1984; Boulter et al., 1973; Spencer, 1984). Analogously storage proteins of major cereal crops, such as rice, wheat, maize, barley and rye etc. are deficient in certain amino acids, i.e. lysine, tryptophan and threonine, which are the 'limiting factor nutritionally (Shewry et al., 1984; Spencer, 1984). Another important consideration is that cereals and legumes nutritionally complement one another; the low levels of sulphur-amino acid in legumes are largely offset by their higher concentration in many cereals, and the legumes compensate for the low levels of lysine in cereals (Boulter and Crocomo, 1979). Thus a deficiency in the sulphur-amino acid content of legume seeds, in a diet which has sufficient of these amino acids supplied from other sources is relatively unimportant. Most of the sulphur-amino acids are in the protein, and levels of free sulphur-amino acids are low (Boulter, 1982); increasing the free sulphur amino acid content would appear to be theoretically possible, since legume seeds lose little of their sulphur-amino acids during normal cooking procedure (Boulter, 1983).

Legumes play a key role in developing countries, specifically increasing the low protein content of the diet whether the main staples are cereals, roots crops, e.g. cassava or yams or starchy fruits, e.g. plantain (Boulter and Crocomo, 1979).

With present population trends, the importance of these crops in the diet will increase. However, world cereal production is increasing much more rapidly than that of legumes, and this imbalance should be corrected. Thus, in the developing countries between 1952 and 1972,

population increased 53%, food production 62%, but legumes only 40%. In Asia and the Far East, population increased 51%, food production 55% and legumes 21% (FAO, 1971, 1973).

Legumes are less important in developed countries, where the main protein sources are of animal origin; but even so, many of these countries are looking for a legume that can replace soya as a source of textured protein products. Furthermore, although it is relatively less efficient to produce protein from animals than plants, the increasing demand for meat and animal products means that the need for high protein feed supplements (e.g. legume seed meals) will continue to rise concurrently (Boulter and Crocomo, 1979). It is generally accepted that animal proteins are far more expensive commodities than the plant-proteins, as the animal is a poor converter of food. The people of the developing countries of the third world in comparison to the developed countries cannot afford to buy animal proteins as they are costly, or do not use them because of religious beliefs. As a result, the third world countries may have a serious deficiency of protein in the diets and people may suffer from poor health including protein-energy malnutrition (PEM), 'kwashiorkor'and 'marasmus'; the extreme forms of PEM, are caused primarily by caloric inadequacy (e.g. inadequate food) and secondarily to protein deficiency in diets (Srikantia, 1982). There is a clear case to improve supplies of legumes for consumption by third world countries.

In this regard it is generally conceded that legume yields are well below optimum levels, especially in comparison with cereals, due to (i) lack of breeding efforts so far (soya beans are the exception); (ii) lack of basic understanding of the underlying physiological constraints; (iii) low pest and disease resistance of many semi-tropical

and tropical legume varieties in cultivation.

The best strategy for improving protein supplies lies in stabilising and increasing overall grain yields. Breeders such as Jain (1971) have suggested that this is best accomplished by the development of a new legume ideotype. Chapman et al., (1979) and Gates (1981) have also suggested that flower and pod abortion could be reduced by the breeding of improved floral architectures. An alternative to altering the relative synthesis of carbohydrate and protein in the seed in order to obtain a higher protein yield, might be increasing the input of N_2 and photosynthate to the seeds. In addition, protein quality is also of prime importance. The quality of a protein (either high or low) is generally known to be affected by (a) essential amino acid composition, (b) amino acid imbalance, (c) digestibility, (d) interference in protein utilization by anti-nutritional factors, and (e) biological availability of the essential amino acids Lysine, Threonine, Valine, Leucine, Isoleucine, Methionine, Tryptophan, Phenylalanine, Histidine and Arginine (Gupta, 1983), which cannot be adequately synthesized by humans.

As described earlier, the limiting amino acid for legumes generally is methionine, but it is useful to consider both the sulphur amino acids, methionine and cysteine together, since evidence from animal feeding trials suggests that cysteine can 'spare' methionine i.e. if excess cysteine is present, methionine need not be used in the animal to produce this amino acid (Boulter et al., 1973; Boulter, 1982). Legume breeding programmes aimed at improving seed protein quality therefore usually involve screening for both sulphur amino acids. The anti-nutritional factors in legume seeds are described in the next section.

3. Drawbacks to the use of legume seeds

Anti-nutritional factors in plant food stuffs are of great importance since they can limit the nutritional potential of these materials both for human and animal consumption. In the raw state, most legume seeds contain a variety of anti-metabolites, these are usually classified as 'heat-stable' or 'heat-labile' substances (Liener, 1962, 1974a; Boulter, 1983). The heat-stable compounds include (i) phytate, which interferes with the availability of minerals, e.g. zinc, manganese, copper and iron in a soy protein containing diet (Odell and Savage, 1960) and zinc in a pea meal for chicks (Kienholz et al., 1962); (ii) tannins, which may affect digestion processes. For example, the true digestibility of the protein of a typical legume, e.g. Phaseolus vulgaris, is of the order of 80% and can be increased to about 90% by removal of the testa, which contains most of the condensed tannins (Boulter, 1983); (iii) Lathyric factor causing Lathyrism (Gupta, 1983; Mirsa et al., 1980); (iv) compounds such as vicine and convicine found in faba beans, which probably cause 'favism' and (v) other compounds such as Saponins or modified nucleotides and amino acids (which give rise to several clinical syndromes) and a variety of ill-defined antivitamin factors etc. (Liener, 1974b; Boulter, 1983). The heat-labile compounds include: (a) Non-protein antimetabolites: (i) Goitrogenic factors, e.g. 'goitrogens' found in soya bean and peanuts (Van Etten, 1969; Liener, 1974a) and (ii) cyanogenic factors, e.g. glycosides from which HCN may be released by hdrolysis and cause cyanide poisoning if improperly cooked (Gupta, 1983; Liener, 1974b). For example, 'Lima-beans' in particular contain high levels of cyano-glycosides (Montgomary, 1969). The biological functions of these nutritionally unwholesome compounds in seeds are still unknown. There is evidence that some

of these anti-metabolites and toxic substances play a defensive (or protective) role against pests and disease (insect, fungi and bacteria) (Gatehouse et al., 1979; Bell, 1981). If this is the case their removal might not be advantageous.

(b) 'Anti-nutritional' proteins

Most of these anti-nutritional proteins can be classified into three groups, namely (i) the proteinase inhibitors; (ii) the lectins (phytohaemagglutinins) and (iii) the antigens (or food allergens).

(i) Proteinase inhibitors: By definition proteinase inhibitors are substances that inhibit proteolytic enzyme activity and are specific in their interactions with proteinases. In plants the important specific proteinase inhibitors are polypeptides and proteins. Protein proteinase inhibitors of plant origin are known to inhibit the proteinases found in animal, bacteria and fungal fluids and secretions but only occasionally do they inhibit plant proteinases (Ryan, 1973; Ryan et al., 1981). Plant proteinase inhibitors have been essentially divided into four major groups (Hartley, 1960), namely those that specifically inhibit the Serine proteinases, those that inhibit the Sulphydryl proteinases, those that inhibit metallo-carboxy peptidases and finally those that inhibit the acid proteinases: most plant proteinase inhibitors fall into the first group, such as trypsin and chymotrypsin inhibitors.

Of these protease inhibitors, most work has been done on trypsin inhibitors, because of their abundance in many legume seeds including soya beans (Glycine max) from which inhibitors were isolated at a relatively early date (Kunitz, 1945). Trypsin inhibitors of many legumes are high in sulphur amino acids, but there is no nutritional advantage in this since at least in the case of the Phaseolus vulgaris, the inhibitors are not readily digested in the rat gut (Phillips et al.,

1981). Ingestion of trypsin inhibitors in the form of raw beans, such as Haricot, Kidney, Navy, Pinto Bean (Phaseolus vulgaris) and Lima bean (Phaseolus lunatus), causes a growth inhibitory effect accompanied by pancreatic hypertrophy in experimental animals e.g. rats and chicks (Evans and Brandemer, 1967: Jaffe and Vega Lette, 1968; KaKade and Evans, 1965; Wagh et al., 1963; Rackis, 1974; Tauber et al., 1949). Heat treatment is beneficial to the nutritive value of these raw beans (Jaffe and Vega Lette, 1968; Richardson, 1948). It is thought that pancreatic hypertrophy leads to an excessive loss of endogenous protein secreted by the pancreas (Booth et al., 1960). Since this protein consists largely of pancreatic enzymes, it is quite rich in cysteine and the resulting effect is a net loss of sulphur-containing amino acids from the body. This would explain why the need for methionine, which is inherently limiting in soya bean protein is more acute in diets containing raw soya beans. There is also evidence that trypsin and chymotrypsin in the intestine suppresses pancreatic enzyme secretion by 'feed-back' inhibition and that trypsin inhibitors evoke increased enzyme secretion by counteracting the suppression produced by trypsin (Green and Lyman, 1972; Niess et al., 1972). A lot of investigations into the adverse effect of trypsin inhibitors have mainly been carried out on animals and it is therefore most important to note that a major part of human trypsin fails to be inhibited by soya bean trypsin inhibitors (Travis and Roberts, 1969; Figarella, Negri and Guy, 1975). So it is essential to establish the correct processing procedure depending on whether soya bean products are for human or animal consumption. Trypsin inhibitors have also been found in a large number of other legumes including Arachis hypogaea, Vicia faba, cow pea, chick pea and Pisum sativum etc., in fact all legumes appear

to contain trypsin inhibitors to varying degrees (Liener and KaKade, 1969; Gatehouse and Boulter, 1980).

(ii) Lectins: The poor nutritive value of many raw legumes (beans and peas) have not only been ascribed to the low sulphur amino acid content of their storage protein (Liener, 1962) and the presence of proteolytic enzyme inhibitors, but also to the presence of significant amounts of another class of proteins, the lectins (Lis and Sharon, 1973; Liener, 1974b, 1980). Lectins are carbohydrate-binding proteins, and because of their property of being able to agglutinate red blood cells (erythrocyte) (Gatehouse et al., 1980), they are also known as (Phyto) haemagluttinins (Stillmark, 1889). From work on rats fed Phaseolus vulgaris haemagglutinin, Jaffe (1960, 1969) demonstrated that haemagglutinin combined with cells lining the intestinal wall causing a non-specific decrease in absorption of nutrients leading to growth inhibition. Pusztai et al. (1975, 1979) using gnotobiotic rates showed that lectin toxicity in Phaseolus vulgaris (kidney bean) is due to the leakage of bacterial toxins through the damaged intestinal wall. In a preliminary study lectins isolated from Phasoelus vulgaris were shown to be toxic to larvae of the bruchid beetle (Callosobruchus maculatus), a major storage pest of many legumes (Janzen et al., 1976). Recently Gatehouse et al. (1984) have demonstrated that the mechanism of this lectin toxicity in the developing larvae of C. maculatus is analogous to that known to occur in the rat.

On the other hand, many physiological roles have been attributed to lectins; and it has been suggested that they play a role as "antibodies" to counteract soil bacteria (Bohlool et al., 1976; Calvert et al., 1978; Dazzo, 1978); are involved in plant protection against insect

(Janzen et al., 1976); pathogenic bacterial attack (Sequeira, 1978) and fungal attack also during seed germination and early seedling growth (Mirelman et al., 1975).

The toxic effects of the lectins present in plant foodstuffs can generally be eliminated by adequate heat treatment, although in practice their complete destruction may not always be achieved, especially if ground seeds are used or industrial processes for quick cooking products are applied (Stein, 1976). Since many seed lectins appear to be resistant to inactivation by dry heat (du Muelenaera, 1964), soaking prior to cooking is often required for complete elimination of toxicity of Kidney beans (Jaffe and Vega Lette, 1968) and field beans (Phadke and Sohonie, 1962).

(iii) Food allergens (antigens):

A third type of 'anti-nutritional' protein present in plants is the allergens (antigens). The term "allergic" was first introduced in 1906 by Von Pirquet to describe the altered capacity of a human to react to a second infection of horse-serum (Von Pirquet, 1906). Subsequently all types of hypersensitivity in humans to any allergen-antibody reaction that causes release of chemical mediators of hypersensitivity have been called 'allergies' (Austin, 1965), and there is now increasing awareness that allergy to food substances may play a major role in either causing or exacerbating a variety of diseases. It is important to note here that unlike true toxins, which exhibit their effects on any individual who consumes them, the severity of toxicity being roughly proportional to the quantity consumed (Austin, 1965), allergens are usually normal food constituents, and the abnormality rests in the individual who has an altered reactivity to such otherwise innocuous substances. The

intensity of the reaction depends on the degree of hypersensitivity in the consumer rather than on the quantity consumed (Gatehouse, 1984). There are three types of allergy (Spies, 1974). They are: (a) 'atopic allergies' which are caused by the specific reaction of the allergen with either skin-sensitising antibodies, homocytotropic antibodies or immunoglobulin-E (IgE); in such cases the symptoms appear within a few minutes and continue for up to an hour after exposure. (b) 'Delayed allergies' are triggered by a specific reaction between the allergen and small lymphocytes and the symptoms may not appear until several hours later. (c) 'The anaphylactic allergies', are the most severe and whilst they are triggered by reaction of allergen with most cell bound IgE, the symptoms appear within a few seconds or minutes after exposure, can be violent and sometimes fatal.

Allergens are generally large molecular weight compounds, non-dialysable, and are most often detected with the protein moiety of the material (Gatehouse, 1984). Almost any plant food can act as an allergen, but the most common offenders among them are the cereal grains (rice, wheat, corn, barley, oat and rye), legumes and nuts, i.e. oil seeds (castor bean and cotton seed). Of the cereal grains the most common offending allergen is wheat and it is thought that the allergenic fraction is probably an "albumin protein" (Goldstein, 1969), since other fractions including gliadin and gluten (major protein fractions) failed to give positive skin test reactions in wheat-sensitive patients. This wheat allergen is reduced in activity by temperatures above 120°C. Although the grains of rice, rye, oat and others do cause allergenic responses in some people, they are not such potent allergens and are often used as substitutes for wheat. There is wide variation both qualitatively and quantitatively regarding allergenic responses of legume seeds (Perlman, 1966). The peanut is the most highly allergenic

member. The various stages of ripening exhibit changes in allergenicity, and thermostability to these allergens is more evident in the ripened stages. Age of the consumer may also be another important factor in allergenicity since soya bean, which has been successfully used as a milk substitute, occasionally proves to be highly allergenic in older children than adults (Fries, 1966). Among the most highly potent allergens are those oil seeds such as Castor bean, and cotton seed: they are not generally consumed by man (Ory and Sekul, 1983).

Areas of the body affected by allergens include the skin which may result in eczema or urticaria, and the respiratory tracts, which may produce symptoms such as allergic rhinitis (hay fever), asthma, or allergic pneumonitis. Vomiting and diarrhoea are common symptoms of allergens affecting the gastrointestinal tract. Allergy to foods may be manifested in skeletal structures where it results in acute or chronic swelling of the joints (Zussman, 1966). These allergies to foods are destroyed by cooking. For example, vegetables such as carrots or potatoes when eaten raw may produce allergenic responses but when cooked are relatively innocuous (Gatehouse, 1984).

Conclusion

Fortunately most anti-nutritional proteins are heat-labile and on proper cooking conditions become non-toxic. Although most legumes are eaten cooked, it should be borne in mind that in developing countries of the third world the amount of fuel available for cooking is often limited and furthermore, since animals are fed raw seeds, large amounts of these 'anti-nutritional' factors may influence animal nutrient utilization.

On the other hand, cooking should not be too prolonged as there may be a decrease in nutritional value. This decrease may be due

either to destruction of vitamins (ascorbic acid and thiamin are particularly susceptible, for example) or to heat denaturation or modification of nutrients, in particular lysine and methionine . This is especially important in child nutrition since, unlike adults, children are often unable to renature partially denatured nutrients (Boulter, 1983).

Nevertheless, taking account of all these anti-nutritional factors, legumes still provide a valuable source of dietary protein.

4. Albumin fraction proteins in legume seeds

Having outlined the major nutritionally important legume proteins, specific attention is now given to the fractions which contain albumin type proteins. The proteins of seeds were studied as long ago as the 18th century with a considerable amount of work being done throughout the 19th century by different groups of people using different techniques. The modern study of seed proteins is founded on the pioneering work of T.B. Osborne as summarised in his book 'The Vegetable Proteins' (Osborne, 1924). He classified proteins into four groups (often called Osborne groups or fractions) on the basis of their extraction and solubility characteristics in series of solvents. These groups are (a) Albumins: These proteins are soluble in water and the group is mainly made up of enzymes i.e. metabolic proteins (Gatehouse et al., 1984). (b) Globulins: These are insoluble in water but soluble in dilute salt-solution, most storage proteins of legumes belong to this solubility class. (c) Prolamins: These are proteins which are soluble in 70-80% ethanol and insoluble in both water and salt-solutions. In addition, these proteins are also soluble in propan-1-ol (50% by volume) or propan-2-ol (55% by volume), which give more complete extraction (Shewry et al., 1984). The major cereal endosperm storage proteins, e.g. wheat, (gliadin), barley (hordein) and rye (secalin) belong to this solubility class. (Shewry et al., 1978a, 1982, 1983a, 1983b). (d) Glutelins: These are insoluble in all the above solvents but soluble in dilute acids or dilute alkalies. Cereals like rice are especially rich in glutelins, but they also occur in the other cereals although to a lesser extent than prolamins. Many storage proteins of cereals were originally thought to belong to the glutelin solubility fractions. However, more recent studies carried out by Byers et al., (1983); Wilson et al., (1981); Shewry et al., (1978b, 1983b), have shown

that these proteins are also extractable by the prolamin solvents under reducing conditions, and thus it is now believed that few, if any, cereal storage proteins belong to the glutelin solubility fractions. The true glutelins are probably a mixture of structural and metabolic proteins (Shewry et al., 1984).

Plant proteins may be alternatively classified in terms of their function i.e. enzymatic, structural, storage and regulatory etc. or in terms of their location within the plant, e.g. membrane, chloroplast stroma, peroxisomal, protein bodies etc. (Boulter and Derbyshire, 1971; Miflin and Shewry, 1980). Thus, with these possible approaches in mind, some of the basic properties of seed proteins are that :-

a) Enzymes : These are proteins characterised by their specific catalytic functions. Most proteins in legume seeds are probably enzymes, but the number present in a 'typical' legume cell is still unknown; and may be of the order of several thousand. As well as the enzymes necessary for the basic metabolic processes found in all cells, legume cells contain the enzymes responsible for the production of so-called secondary plant constituents.

b) Structural proteins : Proteins which are constituents of cell walls or membranes of the cells, and which are without catalytic activity, have been isolated from mitochondria and chloroplasts of plants (Criddle and Willemot, 1967). Similar proteins may be present in legumes, since legume cells contain these organelles (Boulter and Derbyshire, 1971).

c) Storage proteins :

(i) Their predominant function is to act as a store of nitrogen and sulphur (and in some cases carbon) that is laid down during seed development and utilised during germination.

(ii) They accumulate relatively late in seed development.

(iii) Their synthesis is preferentially stimulated by increasing the nitrogen supply to the plant.

(iv) Generally they consist of proteins chiefly of one solubility class for a given species and consist of a polymorphic series of related polypeptides.

(v) They are specified by a relatively small number of genetic loci, probably complex, each consisting of a number of duplicated sequences.

(vi) They are organ specific, i.e. only found in the seed storage organs.

(vii) They are usually deposited in protein bodies (Miflin and Shewry, 1980).

Globulins and secondarily albumins are always predominant fractions over the other protein fractions in legume seeds (Blagoveshchenskii, 1967). For example, in Vicia faba, 80% of the total protein consisted of albumins and globulins, and glutelins formed 15% and prolamin less than 5% of the total (Fox, 1964; Schroeder, 1982). Vicia faba appears typical and similar protein ratios have also been found in a large number of legumes (Klydzhev and Pleshkov, 1965); Blagoveshchenskii, 1967; Pant et al., 1968). Although globulin/albumin ratio varies in the legume seeds of different species, the globulin fraction is always the major one (Brohult and Sandegren, 1954). The ratio of globulin to albumin in pea seeds is found to be 4:1 (Basha, 1974). However, the proportions represented by the different solubility fractions are very different in the vegetative organs (Boulter and Derbyshire, 1971). Studies carried out by Ripa and Geidans (1964), showed that vegetative organs of several legumes contained mainly alkali-soluble protein and that albumins accounted for less than 10% and globulins for less than 3% of the total

protein nitrogen. For many years, since the work of Danielson (1951, 1956), the albumin fraction from legume seeds has been considered as enzymes, because most of the enzymes are extracted in the albumin fraction, although it is known that not all enzymes are strictly water-soluble, e.g. Urease is soluble in very dilute salt-solution (Sumner, 1926). The globulin fraction has been considered to constitute the storage proteins (McKee, 1962; Fox et al.,1964;Boulter,Thurman and Derbyshire 1967; Morris et al., 1970; Millerd, 1975; Johnston et al., 1977; Thomson et al., 1978; Millerd et al., 1978). The major globulin proteins, legumin and vicilin, are located in membrane bounded protein bodies, in cotyledons of broad bean, Vicia faba L. (Graham and Gunning, 1970) and pea, Pisum sativum L. (Varner and Schidlovsky, 1963; Thomson et al., 1978). Albumins are predominately cytoplasmic (Murray, 1979) and were found to be located in the soluble (cytosol) fraction of pea cotyledon cells (Croy et al., 1984), which has been taken as support for the distinction between globulins and albumins.

From a nutritional point of view most legume seeds are deficient in sulphur amino acids - methionine and cysteine (½ cystine) but contain high levels of acidic and amide amino acids reflecting the amino acid compoition of the storage proteins (Jackson et al., 1969; Spencer, 1984). The albumin fraction of legume seeds contains higher levels of sulphur amino acids, and a more normal distribution of amino acids, than the globulin fraction (Davies, 1976; Hurich et al., 1977; Jakubek and Przybylska, 1978,1979; Gatehouse et al., 1984), and has been variously reported by different groups to constitute - 14% (Grant et al., 1976); 13-38% (Davies, 1976); 40% (Beevers and Poulson, 1972); 42% (Murray, 1979); 20-35% (Schroeder, 1982), of the total seed protein, depending on the genetic line and method of albumin extraction.

5. Albumin proteins in pea seeds

Studies on the seed proteins of pea (Pisum sativum L.) have largely concentrated on the predominant proteins - the globulins or major storage protein fraction (Gatehouse et al., 1984).

Mature pea seeds contain 18-32% (average 26%) of dry weight as protein (Schroeder, 1982) and about 95% of the protein is found in the cotyledons (Müller and Gottschalk, 1973), of which 16-24% is albumin proteins and 50-55% is globulin proteins (neither the glutelin fraction nor the prolamin fraction of legumes (pea) is well characterized, although the prolamin fraction from Glycine max has been separated by DEAE-cellulose and thin-layer chromatography into various components (Nash et al., 1967). The globulin fraction of pea (Pisum sativum L.) is composed of principally three major storage proteins called legumin, vicilin and convicilin (Matta and Gatehouse, 1982) with only small amounts of other proteins present.

The pea albumin fraction has recently been reported to contain a few proteins present in amounts large enough to be conveniently isolated and to be considered important nutritionally. According to Grant et al., (1976), three components, from the albumin fraction, of both 'Century' and 'Trapper' varieties of peas, (extracted by deionised water at pH 5.0) were resolved on G-150 Sephadex, into components corresponding to molecular weights of 78,000, 47,700 and 26,000. Six other major components with molecular weight of 200,000, 110,000, 48,000, 32,000, 21,500 and 18,000; and a few minor components were also identified by gel-electrophoresis. By using SDS-polyacrylamide gel electrophoresis under reducing conditions, only two major albumin bands with subunit molecular weights of 25,000 and 15,000 were detected.

Observations made by Guldager (1978), suggested that three to five

albumin proteins, were present in albumin preparations, from standard pea lines of K-42 (Dark Skin Perfection), obtained by extraction in glass distilled water (Danielson, 1949). The extracts were contaminated with globulin protein vicilin. Subsequent gel filtration chromatography on Ultrogel AcA-34, showed that the molecular weights of these albumin proteins ranged between 15,000 and 68,000. A further 14 different albumin proteins from the same albumin fraction were detected by a crossed-immunoelectrophoresis. Albumins free of globulins were also extracted, using 100 mM Na-acetate buffer at pH 5.0, and 24 different albumin proteins were detected in this extract by immunoelectrophoresis.

Murray (1979) identified more than 10 components, in an albumin fraction, extracted by 5% (w/v) K_2SO4 in 0.1 M Na-phosphate buffer at pH 7.0, from 'Green Feast' variety of peas (Pisum sativum L.). Molecular weights of the polypeptides were shown to lie in the range 8000 to 90,000 by using SDS-polyacrylamide gel electrophoresis. However, these extracts were found to be contaminated with legumin. An albumin polypeptide with molecular weight of 23,000 was a major component in the albumin fraction compared with the standard pea globulin fraction as judged by SDS-polyacrylamide gel electrophoresis under reducing conditions.

Jakubek and Przybylska (1979) reported four major albumin proteins present in albumin fraction extracted according to Grant et al., (1976). Fractionation on sephadex G-100 column and subsequent analysis of peak fractions by SDS polyacrylamide gel electrophoresis showed major subunits of molecular weights : 80,000, 40,000, 18,000 and 7,000 from seed meals of five Pisum 'ecotypes' but not separated from each other.

Other authors have reported that there are two major subunit bands of similar subunit molecular weights of about 25,000 in the albumin

fraction of pea seed meal analysed by SDS-polyacrylamide gel electrophoresis (Tyler, 1981). According to Matta (1981), the albumin fraction prepared according to the methods of Croy (1977) from pea seed meals, followed by hydroxylapatite column chromatography, contained major polypeptides of molecular weights of 80,000, 50,000, 30,000, 25,000, 22,000 and 18,000.

Among the other proteins accumulated by pea seeds (Guldager, 1978) in significant amounts although less than those of the storage proteins, are the lectins (Lis and Sharon, 1981) and a number of enzymes such as carbohydrate hydrolases (Neely and Beevers, 1980) and enzyme inhibitors (Ryan, 1973; Ryan et al., 1981; Kunitz, 1945).

(i) Storage and anti-metabolic roles of pea albumins

In general, storage protein is metabolically inert and often insoluble in aqueous media to allow deposition. It is synthesized only in the developing seed and is subsequently hydrolysed on seed germination to provide a source of amino acids and nitrogen for the growing seedling (as mentioned before). The constraints on solubility and packing of storage proteins result in them often being large, multimeric molecules, whereas their role as a nutrient source is usually reflected in high levels of nitrogen rich amino acids, i.e. amides, lysine and arginine (Boulter and Derbyshire, 1971; Gatehouse et al., 1984). The developing and germinating seed must also carry out normal metabolism and seed proteins may be classified into those that are necessary for the metabolism of the organism, and the accumulated storage material that has no apparent metabolic role. However, the distinction between these two classes is not always clear-cut, since enzymatic or other functional properties have occasionally been assigned to some seed storage proteins.

It is widely held that the albumins of pea seeds are mainly enzymic proteins e.g. metabolic proteins. In agreement with this concept, the albumin fraction from pea cotyledons contains different polypeptides from those of the storage proteins, the globulin fraction (Boulter and Derbyshire, 1971; Murray, 1979). Substantial differences exist between the amino acid composition of the whole fraction and the composition of the storage proteins and the sizes of individual polypeptides also differ between these two fractions (Jackson et al., 1969; Murray, 1979). Studies carried out by Malley et al., (1975) showed that all of the allergenic activity and the major part of the antigenic activity of pea seed proteins reside in the albumin fraction.

Murray (1979) has reported that major polypeptides with molecular weights: 84,000, 78,000, 61,000, 44,000, 42,000, 32,000 and 18,000 of the albumin fraction from the cotyledons of peas appear to be degraded during germination, like storage proteins. However, the predominant major albumin components, molecular weight of 23,000, does not behave as a storage protein, as judged by its continued presence in seeds germinated for 14 days by which time storage proteins have more or less disappeared. It was suggested that these albumins may have some key function as a structural component of nucleus or other organelles. Guldager (1978), who studied 12 day germinating seeds reported that certain predominant major albumin components and the lectin had not been degraded significantly when analysed by SDS-polyacrylamide gel electrophoresis. The pea globulins, legumin, vicilin and convicilin are rapidly degraded during the first 3-5 days of germination, but pea major albumin protein with molecular weight of 25,000 and the lectin, are all maintained longer than 7 days after germination when analysed by SDS-polyacrylamide gels as well as two dimensional gel electrophoresis (Tyler, 1981).

Furthermore, Jakubek and Przybylska (1982), reported the continued presence of a certain major albumin protein with subunit molecular weight of about 23,000 in pea cotyledons of five different Pisum lines: WL 110 (Pisum sativum); WL 936 (Pisum humile); WL 1490 (Pisum cinereum); WL 808((Pisum abyssinicum); and WL 1256 (Pisum fulvum), up to three weeks after germination as judged by SDS-polyacrylamide gel electrophoresis. They suggested that these albumin proteins could function as structural components; although further studies are required to prove this hypothesis.

These observations raise the question as to what the primary function(s) of these major albumin proteins might be. In order to try to answer the past and present questions raised about pea albumin proteins it is necessary to isolate the proteins and describe their detailed properties and functions individually. Although the pea albumin fraction has been described in some detail, few reports exist of comprehensive fractionation schemes for the albumins, or the isolation and study of individual albumin proteins, except in the case of lectins (Trowbridge, 1974). Grant et al., (1976) and Jakubek and Przybylska (1979), used only gel filtration as a crude fractionation technique for the albumin proteins (as mentioned before) but such one step methods are essentially inadequate for purification of proteins to homogeneity and fail to separate the pea major albumins.

The object of this study was to establish methods for isolation, purification, separation and characterization of the pea major albumin proteins. Most of this work has been done on the garden pea or Pisum sativum L. variety Feltham First, a species in the genera Pisum, one of five genera in the tribe Vicieae of the family Leguminosae (Heywood, 1971). It is a typical legume which contains significant amounts of albumin proteins as well as storage proteins. A well-established body

of knolwedge exists about pea genetics and physiology and moreover it is a crop of economic importance (Gatehouse et al., 1984; Boulter, 1984).

(ii) Summary of work

Initial studies involved isolation and purification of pea albumin proteins using ammonium sulphate fractionation and a variety of chromatographic techniques. This was followed by detailed characterization of separated major pea albumin proteins using several types of electrophoresis and chromatography, as well as both N-terminal and C-terminal analysis, amino acid analysis, sequence analysis, sugar analysis, isoelectric focusing; CNBr cleavage, haemagglutination assay, tryptic peptide map analysis, enzymatic assays, inhibitor activity, immunological experiments, quantitative estimation and bio-assays etc. By using a combination of these techniques, the changes in the structure and number of the pea major albumin proteins and lectins, taking place during the processes of seed development and seed germination were followed; the situation in different parts of the pea plant as well as in the different pea lines was also investigated.

MATERIALS AND METHODS

SECTION 2

Materials

1. Biological Materials

Pea (Pisum sativum L.) seeds, variety Feltham First, were obtained from Suttons Ltd., Torquay, Devon, TQ27 6QJ., U.K. Seeds of different pea lines were supplied by Dr. S. Blixt, Weibullsholm Plant Breeding Institute, Landskrona, Sweden. Seeds of other legumes (unspecified varieties) were obtained locally.

2. Chemicals and Reagents

Chemicals and reagents, apart from those listed below, were obtained from BDH Ltd., Poole, Dorset, DH12 4NN, U.K. and were of "Analar" grade, or the best available.

(a) Medicell International Ltd., 239 Liverpool Road, London, N1 1LX, U.K.
Visking dialysis tubing (size 9-35/32" and 2-18/32").

(b) Whatman Chemical separation Ltd., Springfield Mill, Maidstone, Kent, ME14 2LE, U.K.
Diethyl amino ethyl (DEAE) cellulose (preswollen ion exchange cellulose, DE-52).

(c) Koch-light Laboratories Ltd., Colnbrook, Bucks, U.K.
Coomassie brilliant Blue R-250; Bromophenol blue, Dimethyl sulphoxide.

(d) Pharmacia Fine Chemicals, Uppsala, Sweden.
Dextran Blue 2000, Sephadex G-150, Sephadex G-75, Sephadex G-50, Sephacryl S-200, Ampholines for isoelectric focusing (pH3.0-10.0).

(e) Schleicher and Schüll, Dassel, FRG.
Nitro-cellulose (Type BA85).

(f) Sera Laboratories Ltd., Sussex, England, U.K.

Horse serum

(g) Sigma London Chemical Company Ltd., Poole, Dorset, U.K.

Transferrin, bovine serum albumin, catalase, ovalbumin, bovine α-chymotrypsin, lactate dehydrogenase, β-lactoglobulin, soya-bean trypsin inhibitor, bovine trypsin, porcine pancreatic amylase, myoglobin, lysozyme, ferritin, cytochrome c, insulin, BAPNA, BTEE, para-nitrophenyl α-,β-D-glucopyanosides, para-nitrophenyl α-,β-D-galactopyranosides, para-nitrophenyl α-D-mannopyranoside and carboxypeptidase A.

(h) Orion Diagnostica, Helsinki, Finland

Peroxidase-coupled (Goat anti-rabbit lgG)

(i) Miles Laboratories, Stoke Poges, Bucks, U.K.

Staphylococcus aureus V8 protease

(j) Pisum sativum lectin, total major albumin, legumin basic subunit, vicilin, convicilin, concanavalin A, were kindly supplied by Dr. John A. Gatehouse, University of Durham, U.K.

(k) Pisum sativum globulin, anti-lectin lgG and anti-PMA serum preparations were kindly supplied by Dr. Ronald R.D. Croy, University of Durham, U.K.

(l) Un-coated cellophane for drying gels was obtained from W.E. Cannings, Avonmouthway, Avonmouth, Bristol.

(m) Buffers used for column chromatography were prepared using Analar reagents wherever possible. They were adjusted to the required pH by pH meter and were filtered to remove any insoluble materials. 0.1% sodium azide was routinely added as an anti-bacterial agent.

Methods

1. Growth of Biological materials

a) Germinating seeds

Seeds of Pisum sativum L. variety Feltham First, were grown in a dark spray room as described by Evans et al. (1979) for 11 days. The addition of water to the dry seeds was taken as zero time germination. Seedlings were supported by a 4 cm layer of alkathene polyethylene granules in bowls containing distilled water and harvested at intervals of 1, 3, 5, 7, 9 and 11 days.

b) Developing seeds

Seeds were germinated as mentioned above. Seedlings were transferred to water culture bottles of nutrient solution after 4-5 days in the spray room, to be grown under the controlled environmental conditions as follows :- lighting : 16h total (including morning and evening phases), temperature : day 28°C, night 23°C, humidity : 75-80% relative humidity. Harvesting of the pods was carried out at intervals of 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 and 24 days after flowering. The cotyledons were separated aseptically from the testas, radicles and hypocotyls prior to extraction were frozen in liquid N_2 and stored at -80°C.

2. Fractionation of the pea albumins and isolation of the major protein components

(i) Preparation of meals from Pisum sativum L.:

The testas of dried mature pea seeds were removed and the cotyledons ground for 30 seconds to a fine flour using a Janke and Kunkel water-cooled mill. The flour was passed through a 365 µm mesh sieve and stored at 4°C.

Defatting was carried out for half an hour by two hexane extractions (10 ml hexane/g meal) with continuous stirring at 4°C. After filtering off the hexane, the meal was dried under vacuum.

(ii) Preparation of extract from meals:

The total albumin protein fraction of pea seeds was prepared by extracting 50 gm defatted meal twice with 20 mM Na-acetate buffer pH 5.0 at a ratio of 1 gm meal in 5 ml buffer with constant agitation or continuous stirring, for 1-2 h or overnight at 4°C. The suspension was centrifuged in a 6 x 250 ml MSE 18 rotor (250 ml tubes) at 10,000 r.p.m. for 30 minutes at 4°C. The clear supernatant was carefully removed and kept at 4°C; the pellet was re-extracted and centrifuged as above, the supernatant pooled with the first and the pellet discarded.

(iii) Ammonium sulphate precipitation:

Total albumin extract (ii) was fractionated by ammonium sulphate precipitation at 0-50% and 50-90% relative saturation at 4°C.

(a) The volume of the total albumin extract was adjusted to 200 ml and solid ammonium sulphate (29.1 gm per 100 ml i.e. 58.2 gm) was added slowly to 50% saturation with continuous stirring; and was stirred for 2-3 hours or overnight at 4°C. The precipitate was collected by centrifugation at 20,000 r.p.m. for 30 minutes. Both supernatant and pellet, designated as AS-50 were recovered carefully and kept at 4°C.

(b) The supernatant of AS-50 (iii.a) was fractionated by ammonium sulphate precipitation at 90% relative saturation. Solid ammonium sulphate (23.5g per 100 ml) was added as before and stirred for 2-3 h at 4°C. The precipitate was collected by centrifugation as above (iii.a) and the pellet, designated AS-90, was recovered, and kept at 4°C; the supernatant (AS-90) was discarded.

(iv) Sephadex G-150 column chromatography:

The major albumin proteins were purified from the ammonium sulphate fractions by Sephadex G-150 column chromatography. The column was packed with Sephadex G-150 according to the instructions given in the Pharmacia Booklet (Ref. list).

The albumin proteins precipitating in the range 50-90% relative saturation with ammonium sulphate (AS-90) were redissolved in 10 ml of buffer A (50 mM Tris-HCl, 0.2 M NaCl buffer, pH 7.5 containing 0.1% Na-azide), and the solution was centrifuged at 10,000 r.p.m. for 10 minutes at 4°C to remove the insoluble materials. The cleared supernatant (AS-90) was loaded on to the top of a column (3.2 cm diam x 56 cm, flow rate 20 ml/h) of Sephadex G-150 which was eluted with buffer A, and 10 ml fractions collected. The column eluant was continuously monitored at 280 nm using an LKB 8300 Uvicord II detector and control unit, with an LKB chart recorder. Fractions were collected using an LKB ultrorac 7000 fraction collector.

Peak fractions according to the recorder trace were pooled together, dialysed against distilled water for 36 hours at 4°C with several changes of water, and freeze dried. Aliquots of these freeze-dried samples were dissolved in SDS-sample buffer containing 2-mercaptoethanol, at 2 mg/ml (unless otherwise stated) and analysed for protein content by SDS-17% polyacrylamide gel electrophoresis.

(v) Sephadex G-75 column chromatography:

The low molecular weight albumin protein was also purified from the AS-90 $(NH_4)_2SO_4$ fractions from the total albumin protein extract as described previously by Sephadex G-75 column chromatography.

The 90% ammonium sulphate precipitate (AS-90) was redissolved in 5 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.1% NaN_3, the solution was clarified by centrifugation for 10 min as

before. The cleared supernatant (AS-90) was loaded on top of the column (1.6 cm diam x 150 ml vol.) Sephadex G-75, after equilibration with the same buffer. The column was eluted by downward flow (flow rate 6 ml/h) and 2 ml fractions were collected using an LKB fraction collector and pooled by peaks of absorbance at 280 nm, dialysed against distilled water for 24 h at 4°C with several changes of water, lyophilized and analysed by SDS-polyacrylamide gel electrophoresis.

(vi) DEAE-Ion exchange chromatography:

Fractions of the AS-90 from the Sephadex column which contained high concentration of major albumin proteins were further purified by ion exchange chromatography on DEAE-cellulose (DE-52). The DE-52 column was prepared as described in the Whatman Booklet (Ref. list).

Freeze-dried samples of the appropriate fraction (fraction no.2, Fig. 3) of AS-90 after the Sephadex G-150 chromatography were redissolved in 5 ml of buffer B (50 mM Tris-Hcl, pH 7.5, containing 0.1% NaN_3) and applied directly to a column of DE-52 cellulose (2.5 cm diam x 26 cm, flow rate 40 ml/h) equilibrated and washed in with 150 ml of buffer B. Elution was effected with 500 ml of a linear concentration gradient of NaCl, from zero to 0.5 M, in buffer B at a flow rate of 40 ml/h. 10 ml fractions were collected and pooled together according to the uv (280 nm) absorbance trace, dialysed against distilled water and then freeze-dried for subsequent analysis by various electrophoretic techniques.

The molar concentration at which a particular protein was eluted was obtained from the gradient profile as measured by refractive index and determined from a standard curve of refractive index against sodium chloride concentrations. The refractive index measurements were made on an 'Abbe' type refractometer at 20°C.

(vii) Ion exchange chromatography

The AS-90 fractions from the peak which contained high concentrations

of low molecular weight albumin protein were also further purified by ion exchange chromatography on DEAE-cellulose (DE-52).

Freeze-dried samples of fractions no.3 and 4 from the Sephadex G-75 chromatography of AS-90 were redissolved in 3-5 ml column buffer B and were separately applied to a column (1.6 cm diam x 70 ml vol.) of DEAE-cellulose (DE-52), equilibrated with the same buffer B at a flow rate of 15 ml/h. The samples were washed on with 1 column volume of starting buffer, and the column was then eluted with a linear gradient (250 ml + 250 ml) of NaCl (0-0.5 M) in the same buffer. 5 ml fractions were collected as before and pooled together by peaks of absorbance at 280 nm, dialysed against distilled water for 24 h at 4°C with several changes of water and then freeze-dried for subsequent analysis by various electrophoretic techniques. NaCl concentrations in eluted fractions were also determined by refractive index as before.

(viii) Affinity chromatography:

Lectin protein was isolated and purified from albumin extracts by affinity chromatography on Sephadex G-150 by the method of Trowbridge (1974).

The Sephadex G-150 column used to purify the major albumin proteins (2.iv) was washed with the buffer A until all unbound materials had eluted. The column was then eluted with 0.1 M glucose in buffer A. A single peak of seed-lectin was eluted. The peak fractions were pooled together, dialysed against distilled water at 4°C and freeze-dried.

(ix) Sephacryl S-200 column chromatography:

A plot of $^{Ve}/Vo$ against $\log_{10}$ mol.wt. was obtained by chromatographing the following standard proteins (molecular weights in parentheses):- Catalase (240,000 Mr), Vicilin (150,000 Mr), Bovine Serum albumin

(68,000 Mr), Ovalbumin (43,000 Mr) and Myoglobin (17,200 Mr) on a column of Sephacryl S-200 (1.6 cm diam, 400 ml vol.). The column was equilibrated (flow rate 7.0 ml/h) using 0.1 M Tris-HCl buffer pH 8.0, containing 0.25 M NaCl and 0.1% NaN_3 for at least 20 h. Purified albumin protein samples were subsequently chromatographed on the column under identical conditions and the elution volumes of standard proteins and the samples measured. Molecular weights of the albumin proteins were calculated from their $^{Ve}/Vo$ values and the standard graph of $^{Ve}/Vo$ against Log_{10} mol. wt.

(x) Preparation of total protein extract:

Total protein extract of pea-seeds (and different legume species) was prepared by extracting 20 mg of defatted meal in 1.0 ml of SDS-sample buffer (0.2 M Tris-HCl pH 6.8, 2% SDS, 10% sucrose and ± 2% (V/V) 2-mercaptoethanol), stirred overnight at 4°C and then centrifuged at full speed for about 6-10 minutes in a bench centrifuge at room temperature. 15-20 µl of supernatant was analysed by SDS-17% PAGE along with purified pea albumin proteins and other protein fractions with and without 2-mercaptoethanol, on the same gel.

(xi) Sephadex G-50 column chromatography:

A plot of $^{Ve}/Vo$ against log_{10} mol. wt. was obtained by chromatographing the following standard proteins (molecular weights in parentheses) : β-lactoglobulin (35,000), soya-bean trypsin inhibitor (20,100), myoglobin (17,200) and cytochrome c (12,700) on a column of Sephadex G-50 superfine (1.6 cm. dia. x 115 ml. vol.). The column was equilibrated in 50 mM Tris-HCl, 0.1% NaCl, pH 7.5, containing 0.1% NaN_3 overnight at a flow rate of 5 ml/h. It was then loaded with a sample 3-4 mg of low molecular weight albumin, PLA, dissolved in 2 ml column buffer and eluted with the same buffer at the same flow rate.

2.5 ml fractions were collected and the elution volumes of standard proteins and the sample calculated from the UV (280 nm) absorbance profile of the elute. Like before, molecular weight of the albumin protein PLA was also calculated from its $^{Ve}/Vo$ value and the standard graph of $^{Ve}/Vo$ against $\log_{10}$ mol. wt.

(xii) Gel electrophoresis:

The subunit composition of protein preparations were analysed by SDS-polyacrylamide gel electrophoresis in slab gels according to the methods of Laemmli (1970) and as modified by Matta et al. (1981) for various one-dimensional and two-dimensional techniques.

(A) 17% and 12.5% SDS-slab polyacrylamide gel electrophoresis:

(a) Reagents

(i) 1M Tris-HCl pH = 8.8

(ii) 0.2 M Tris-HCl pH = 6.8

(iii) Main gel acrylamide stock solution:

30g acrylamide
0.135 g bisacrylamide
} 100 ml of distilled H_2O

(iv) Stacking gel acrylamide stock solution :

30 g acrylamide
0.433 g bisacrylamide
} 100 ml of distilled H_2O

(v) 10% SDS solution

(vi) ammonium persulphate solution (15 mg/ml)

(vii) ammonium persulphate solution (20 mg/ml)

(viii) TEMED

Gel concentrations refer to total monomer (i.e. acrylamide and bisacrylamide).

(b) Gel preparation

Glass plate assembly and procedure were as described by Payne (1976). Main gels of 17% acrylamide concentration were prepared by

mixing 22.5 ml(i) and 34.5 ml(iii), then degassing the solution. To this 0.6 ml (v), 1.5 ml (vi) and 20 µl (viii) were added, this gave sufficient gel mixture (60 ml) for one good slab gel. The 12.5% main gels were prepared as before after 25.8 ml of (iii) had been made up to 34.5 ml with distilled water.

Stacking gels (3% acrylamide) were prepared by adding 2.5 ml (ii) and 13.8 ml of distilled water to 3.0 ml (iv), then degassing the solution. Following this 0.2 ml (v), 0.5 ml (vii) and 10 µl of (viii) were added.

c) Sample preparation

Protein samples were dissolved at a concentration of about 2mg/ml (unless otherwise stated) in sample buffer .

d) Electrophoresis

Electrophoresis buffer consisted of 141.1g glycine, Tris 30g and SDS 10g made up to 1000 ml with distilled water (pH ∿ 8.3) and was diluted 1:10 before use.

Samples 10-15 µl (unless otherwise stated) containing 2% (v/v) 2-mercaptoethanol were loaded on to each track of the gels and marker dye (0.1% bromophenol blue) added to the upper reservoir. Electrophoresis was carried out using a Raven slab gel apparatus with a Shandon VoKam SAE 2761 power supply, at 25 mA per gel (constant current), for approximately 5-6 hours per gel.

e) Subunits Mr of pea major albumin:

Molecular weights of albumin protein subunits were obtained from a standard graph prepared for the following standard subunits analysed by SDS-17% PAGE and SDS-12.5% PAGE (subunit Mr in parentheses):

(a) transferrin (76,600), (b) pea vicilin (71,000, 50,000, 33,000)

(c) bovine serum albumin (68,000), (d) catalase (60,000),

(e) ovalbumin (43,000), (f) lactic dehydrogenase (36,000), (g) soyabean trypsin inhibitor (20,100), (h) ferritin (18,500) and (i) β-lactoglobulin (18,400).

(B) Non SDS-polyacrylamide slab gel electrophoresis

Non-dissociating polyacrylamide gel electrophoresis was carried out as described above for gel electrophoresis in xii.(A) but SDS was omitted from gel preparations and replaced by the same volume of distilled water. Running buffer was identical except SDS was not added. Sample buffer was prepared without SDS and in dilution of the stock solution, 2-mercaptoethanol was replaced by the same volume of distilled water. Main gels with a polyacrylamide concentration of 8.5% (w/v) were obtained by diluting 17.25 ml of (A.iii) to 34.5 ml with distilled water.

After electrophoresis, all gels were stained for protein bands with 0.025% Coomassie Brilliant Blue R in 50% methanol, 7% acetic acid (Reid and Bielski, 1968) and then destained with 50% methanol and 7.5% acetic acid solutions.

(C) Urea SDS-15% polyacrylamide slab gel electrophoresis

SDS-polyacrylamide (15%) slab gels containing 0.75% bisacrylamide and 7M urea were prepared according to the method of Hashimoto et al., (1983) but without the use of sucrose gradient.

(i) Preparation of gel mixture (50 ml):

a) 7.5 g acrylamide

b) 0.375 g bisacrylamide

c) 21 g urea

d) 22.5 ml of 1M Tris-HCl, pH 8.8

total volume was made 49 ml with distilled water, then degassing the solution. To this 0.5 ml (v), 1.5 ml (vi) and 20 µl (viii) of xii.(A)

were added.

(ii) Preparation of stacking gel:

Stacking gel preparation was same as before (xii. A.b).

(iii) Electrophoresis:

Electrophoresis buffer as well as sample application were the same as (xii. A.d) but gels were run at 60V per gel (constant voltage), for approximately 15-16 h per gel, and then the gel was incubated in a fixative wash solution of 50% methanol and 12% acetic acid with constant agitation at room temperature for 1-2 h to clear up the background of the gel facilitating the detection of the low molecular weight protein bands, according to Merril et al. (1981), prior to staining and destaining as before (xii.B).

(iv) Subunit Mr of PLA:

Subunit mol. wt. of carboxymethylated PLA was determined from a standard graph prepared for the following standard subunits analysed by SDS-PAGE in 7M urea containing gels (15% polyacrylamide)(subunit Mr in parentheses): soyabean trypsin inhibitors (20,100 Mr), β-lactoglobulin (18,400 Mr), pea lectin (17,700 and 5,800 Mr for α- and β-chains),lysozyme (13,200 Mr), cytochrome c (12,700 Mr) and insulin (3,400 Mr, α-chain).

(D) Isoelectric focusing:

One-dimensional isoelectric focusing of albumin proteins PMA-L, PMA-S and PLA was carried out in 7.5% (w/v) polyacrylamide slab gels containing 2.5% (w/v) - bisacrylamide according to the method of Vesterberg (1975). β-lactoglobulin, myoglobin and cytochrome c were also applied to the same gel as markers. Focusing, washing and staining etc. were performed as described by Gatehouse et al (1980).

(xiii) Amino acid analysis:

Protein samples were dissolved in 6M guanidine hydrochloride (in

0.5M Tris-HCl buffer pH 8.0, containing 2 mM EDTA), reduced in excess DTT (300 moles/mole of protein) and carboxymethylated with iodoacetamide in excess as described by Glazer et al. (1975) followed by acid hydrolysis.

Acid hydrolysis of total albumin fractions, PMA-L, PMA-S and PLA w. performed as described by Croy et al. (1980). Samples were subsequently analysed on a Varian 5060 h.p.l.c. system using a standardised Micropak A.A. column (varian) (15 cm x 4 mm diam.). The amino acid peaks were detected with a Varian PCR-1 post-column derivatization and Fluorichrom detection systems using o-phthaldehyde. Cysteine was estimated as its carboxymethyl derivative.

(xiv) N-terminal amino acid determination:

The N-terminal amino acids of PMA-L, PMA-S and PLA were determined by the 'dansyl' technique of Gray (1972) using two dimensional t.l.c. on polyamide sheets to identify the products (Woods and Wang, 1967).

(xv) C-terminal amino acid determination:

The C-terminal amino acids of PMA-L, PMA-S and PLA were determined by digestion with carboxypeptidase A followed the 'dansyl' technique of Gray (1972).

(xvi) Determination of N-terminal amino acid sequence:

(a) The N-terminal amino acids sequences for PMA-L, PMA-S and PLA were determined by manual micro sequence analysis using the DABITC/PITC double coupling method of Chang et al. (1978). The N-terminal DABTH-amino acid was identified by two dimensional chromatography on polyamide thin-layer sheets.

(b) The amino acid sequence determination:

Peptides from PLA were obtained by digestion of 2 mg portions of the protein with bovine trypsin (digestion conditions enzyme:protein 1:50, buffer 0.1M NH_4Co_3, pH 8.0, 18 hours at 25°C), bovine α-chymotrypsin

(digestion conditions enzyme: protein 1:50, buffer 0.2M N-ethylmorpholine-acetate, pH 8.5, 2 hours at 37°C) or Staphylococcus aureus V8 protease (digestion conditions enzyme: protein 1:30, buffer 50 mM ammonium bicarbonate, pH 8.0, 18 hours at 37°C). The resulting peptides were lyophilised and re-dissolved in 100 µl of 0.1% TFA; they were subsequently separated by h.p.l.c. on a reverse-phase column (c_{18}, 5 µm; Micropak MCH-5) as previously described by Gatehouse et al. (1982a). The chromatography conditions were as follows : starting buffer, 0.1% TFA in water, 5 min; gradient, 0-50% acetonitrile containing 0.1% TFA over 100 min, 50-70% over 20 min; flow rate 1.0 ml/min. and column temperature 30°C.

The amino acid sequences of the peptides were determined by manual microsequence analysis using DABITC/PITC double coupling method of Chang et al. (1978) as mentioned before.

(xvii) Tryptic-peptide analysis:

Protein samples dissolved in modification buffer, i.e. in 6M guanidinium chloride (in 0.5M Tris-HCl buffer pH 8.1, containing 0.25M EDTA and 8mM 2-mercaptoethanol) were carboxymethylated with iodoacetamide and then digested with bovine trypsin according to Gray (1972) prior to lyophilisation.

Lyophilised PMA-L and PMA-S protein samples were redissolved in 0.1% TFA and analysed on a Varian 5060 h.p.l.c. system using a Micropak MCH-10 (c_{18}, 10 µm) column as described by Gatehouse et al. (1982a). The peak elution profiles were compared as peptide maps.

(xviii) Cyanogen bromide cleavage:

Native albumins PMA-L and PMA-S; and carboxymethylated PLA (as described previously) were dissolved in 70% (v/v) formic acid and digested for different times (0, 3, 6, 24 and 48 h) with a 100-fold molar excess of cyanogen bromide (2gm/ml solution in acetonitrile)

followed by lyophilization as described by Croy et al. (1980). The resulting fragments were analysed on SDS-PAGE or 7M urea containing SDS-PAGE as described (Section xii. A & C).

Standard proteins used for estimating the Mr values for the fragments were (subunit Mr in parentheses): soyabean trypsin inhibitor (20,100 Mr), β-lactoglobulin (18,400 Mr), myoglobin (17,200 Mr), lysozyme (13,200 Mr) cytochrome c (12,700 Mr) and insulin (5,600).

(xix) Carbohydrate analysis:

Total sugars present in samples of PMA-L and PMA-S, precipitated and washed extensively with 10% (w/v) TCA; and the native form of PLA were measured in solutions by the phenol/H_2SO_4 acid method of Dubois et al. (1956) using standard glucose solution to calibrate the analyses.

(xx) Glycoprotein analysis after gel electrophoresis:

Protein samples containing gels were stained for glycoproteins by the fluorescence method of Segrest et al. (1972) using dansyl-hydrazine as described by Eckhardt et al. (1976).

(xxi) Reaction with concanavalin A:

Precipitation reactions of major albumins with concanavalin A were tested by diffusion in 1% (w/v) agarose gels using a system analogous to immunodiffusion, using concanavalin A in place of antibodies, as described by Croy et al. (1980).

(xxii) Two-dimensional electrophoresis:

This analytical system involved the combined use of polyacrylamide gel electrophoresis (PAGE) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under the conditions already described in sections xii. A & B. These were performed in combination as follows :

(a) Polyacrylamide gel electrophoresis→ SDS-polyacrylamide gel electrophoresis:

Albumin protein samples were electrophoresed in 8.5% (w/v) non-SDS-polyacrylamide slab gels in the first dimension under non-reducing conditions. After staining, destaining and drying the gel, sample tracks were excised in strips which were equilibrated in 0.2M Tris-HCl pH 6.8, containing 50% methanol (unless otherwise stated) overnight with constant agitation at room temperature. Then incubated in SDS-sample buffer containing 2% (v/v) 2-mercaptoethanol for 1 h with one change of buffer after half an hour at room temperature in the dark without agitation. The equilibration step was omitted for wet slab gels. The gel strip was then inserted between the glass plates, with the help of a thin spatula, onto a 17% SDS-polyacrylamide main slab gel with 1 cm, 3% SDS-polyacrylamide stacking gel. No well-former was used and the stacking gel was overlaid with water prior to polymerization, to obtain a flat surface. The SDS-PAGE was then carried out in the second-dimension under the same conditions as described above in xii.A.

(xxiii) Immunodiffusion:

Antibodies against purified total major albumin proteins were raised in New Zealand White rabbits and IgG fractions isolated from the antisera as described previously by Evans et al. (1979). Immunodiffusion of purified albumin proteins against anti-albumin antibodies was carried out on 1% agarose gels by standard methods of Ouchterlony and Nilsson (1978) as described by Croy et al. (1979). Precipitation arcs formed after 48 hours diffusion at 4°C. Gels were pressed, washed twice in 20 mM borate buffer pH = 8.0 containing 0.9% NaCl for 15 minutes each, then pressed again for 15 minutes and dried

under a hair drier for 5 minutes. Precipitation arcs were stained for 25-30 minutes with staining solution (0.25% Coomassie Brilliant Blue, 7.5% glacial acetic acid and 50% methanol in distilled water), then destained for 2 minutes in destaining solution (50% methanol and 7% glacial acetic acid in distilled water) and finally dried as before. The identity of precipitin lines were confirmed by excision from the gel and analysis by SDS-17% PAGE described by Croy et al. (1980).

(xxiv) "Laurell" rocket immunoelectrophoresis:

This immunoelectrophoresis was performed in 1% agarose gels (10 x 20 x 0.15 cm) using a Shandon flat-bed electrophoresis apparatus according to the method of Laurell(1966) as modified by Weeke (1973). 0.3 gm of agarose was dissolved in 15 ml of distilled water by boiling for 2-3 min and then was kept in a water bath at 55-60°C. 15 ml of 0.5M Tris-EDTA borate buffer (0.5M Tris, 1.62 mM EDTA and 0.75M borate pH 8.6) equilibrated to 55-60°C was mixed with the agarose solution and the appropriate antiserum (60 µl of anti-albumin antibodies). The mixture was poured into the gel plate assembly at 55-60°C and then left for 20-30 min at 4°C to set. Samples of total protein extracts were prepared by extracting seed meal(12.5 mg/ml)in 0.5M Tris-EDTA borate buffer, pH 8.6 and total albumin extract(5 mg/ml) in 20mM Na-acetate buffer pH 5.0 for 2 h at 4°C. Samples were loaded along with standard amounts of the appropriate protein (pea major albumin) into wells in the gel. Samples were electrophoresed overnight into the antibody containing gel at a constant voltage of 100 v. Gels were then pressed and washed extensively in 20 mM borate buffer, pH 8.0 containing 0.1% NaCl followed by washing in distilled water. The gels were then pressed, dried, stained and destained as before (xxiii). Calibration curves were prepared by plotting the rocket heights against amounts of the standard proteins.

The amounts of pea major albumin proteins present in the samples were determined from the 'rocket' heights by comparison with this calibration curve.

(xxv) Densitometric scanning after gel electrophoresis:

The amounts of pea major albumin (PMA) and low molecular weight albumin (PLA) in pea seed meals were estimated by comparative SDS-PAGE and Urea/SDS-PAGE respectively. Gel systems were carried out as usual using different concentrations of freshly prepared total ablumin protein extract (100 mg/ml of 20 mM Na-acetate buffer, pH 5.0); lyophilised pea total albumin extract along with different amounts of purified PMA and PLA as standard.. After electrophoresis, staining and destaining as usual, tracks on the gel were cut out and scanned at 605 nm for 10 min using a Gilford 2000 spectrophotometer and densitometer attachment. The areas of peak corresponding to PMA and PLA were estimated by cutting out and weighing the appropriate areas of the trace. Semi-quantitative estimates of both PMA and PLA in seed extracts and albumin proteins were obtained by comparison of peak areas of these tracks with those produced by known amounts of PMA and PLA.

(xxvi) Immunoassay by antibody blot:

Albumin protein subunits from different legume species; from different variant pea lines and from different parts of pea plants were analysed by electro or 'Western blotting'.

Proteins were transferred from SDS-17% polyacrylamide gels (Laemmli, 1970) onto nitrocellulose filter paper (Schleicher and Schüll) by electro-blotting (Bio-Red Trans Blot Cell).

The filters were then reacted with affinity purified anti-PMA-antibodies and then with peroxidase-coupled goat anti-rabbit lgG, before staining with 4-chloro-1-naphthol according to the standard 'Western blotting' protocols of Towbin et al. (1979).

(xxvii) Haemagglutination assays:

Haemagglutination assays of purified albumin and pea lectin proteins were carried out by a serial dilution method in microtitration plates (Lis and Sharon, 1973) using a 2% (v/v) suspension of untreated rabbit erythrocytes in PBS. Concanavalin A was used as a standard lectin. The lectin dilutions at which haemagglutination ceased were estimated visually. Sugar inhibition of haemagglutination was measured at fixed lectin concentration by a serial dilution of 0.1M glucose (prepared serial dilution to X12).

(a) Treatment of blood for agglutination assays:

Reagents:

Buffers: (i) Phosphate buffered saline (PBS)

0.006M phosphate, pH7.5 containing 0.9% NaCl.

(ii) Alserver's solution

2.05 g glucose

0.80 Na-citrate (tri-sodium citrate)

0.42 g NaCl, total volume was made up to 100 ml with distilled water, pH 6.1 (adjusted by adding solid citrate).

(iii) Anti-coagulant solution

8.0 g Na-citrate

54 ml 37% formaldehyde

100 ml saline

(iv) PBS containing 0.1M glucose

36 mg glucose

2 ml PBS

Fresh blood samples were collected into an equal volume of (ii) containing $^{I}/_{30}$ the volume of (iii) equivalent to 0.4% and stored in heparin treated tubes. Before use the erythrocytes were washed three

times in PBS, centrifugation between washes was performed in a bench centrifuge (MSE) at full g force. Erythrocytes were made up to a final concentration of 2% (v/v) with PBS.

(b) Agglutination test:

HMW-albumin, PMA-L, PMA-S and pea lectin were tested for haemagglution from stock solutions of each sample (Img/ml in PBS); in which 100 µl (100 µg) aliquots of the individual samples were mixed with 100 µl of 2% (v/v) rabbit erythrocytes in microtitration plates. Haemagglutination was assessed after 1½-2 h at room temperature. Agglutination of the samples was judged by a failure of the erythrocytes to settle out.

(xxviii) Enzyme activities:

Purified albumin proteins PMA-L, PMA-S, PLA and fractions of total albumin extract obtained after ion exchange chromatography on DEAE-cellulose (DE-52), were tested for potential enzyme and enzyme inhibition activities in the following ways :-

(a) Potential enzyme activities:

(i) α- and β-glucosidases, α- and β-galactosidases and α-D-mannosidase were assayed using the p-nitrophenyl derivaties of the corresponding sugars according to the standard methods of Anstee et al. (1977).

Reagents:

(i) Substrates: p-nitrophenyl-α- or β-D-glucopyranoside
∿ 51.3 mM ≡ 15.5 mg/ml H_2O
: p-nitrophenyl-α- or β-D-galactopyranoside
∿ 35 mM ≡ 10.55 mg/ml H_2O
: p-nitrophenyl-α-D-mannopyranoside
∿ 50 mM ≡ 15.0 mg/ml H_2O.

(ii) Sodiumhydroxide solution:

50 mM NaoH = 0.2 g/100 ml H_2O

(iii) McIlvaine's buffer

0.1M citrate-phosphate buffer, pH5.0

Assay:

0.2 ml (iii) and 50 µl (i) were taken in a 1.5 ml Eppendorf tube, mixed well and 10-20 µl of PMA-L or PMA-S from stock solutions 2 mg/ml in H_2O (unless otherwise stated) was added and incubated in 30-35°C water bath for 30 min. Then the reaction was terminated by adding 1.0 ml (ii) and again incubated for 10 min as before. Optical density readings at 410 nm of uv-spectrophotometer in a reduced volume plastic cuvette against a cuvette containing distilled water only were taken.

(ii) Similarly, α-amylase was assayed using the substrate starch according to the standard method of Mestechy et al. (1969).

(b) Inhibitory activities:

(i) α-amylase inhibitory activity was estimated using porcine pancreatic α-amylase in 1% (w/v) starch-agarose gels by radial diffusion assays on starch-agarose plates according to the method of Mestechy et al. (1969).

(ii) Albumin extract on DE-cellulose experiment:

50 ml extraction of total albumin proteins from meal of mature pea seeds (1 g meal/10 ml 20 mM Na-acetate buffer, pH 5.0) was prepared and centrifuged as before (2.ii). The pH of the clear extract (supernatant) was adjusted to pH 8.0 with 1M Tris base and applied directly to a column of DE-52 cellulose (1.6 cm Diam x 70 ml vol., flow rate 16 ml/h), equilibrated and washed in with the 20 mM Tris-HCl pH 8.0 (containing 0.1% NaN_3) until all unbound material was eluted. A linear gradient

(400 ml + 400 ml) of NaCl (0-0.3M) in the same buffer was then applied at a flow rate of 16 ml/h. 8 ml fractions were collected and the required fractions (7 ml out of 8 ml) were pooled according to the uv (280 nm) absorbance trace, dialysed against distilled water and then lyophilised for subsequent analysis by SDS-polyacrylamide gel electrophoresis.

The remaining 1 ml aliquots of the fractions were assayed for both trypsin and chymotrypsin inhibitory activities.

(a) Trypsin inhibitor assay

Trypsin inhibitors were estimated using BAPNA as substrate, according to the method of Erlanger et al. (1961).

Reagents:

(i) BAPNA = 63 mg in 4 ml DMSO

(ii) Buffer = 0.05 M Tris, 0.02 M $CaCl_2$ pH 8.2

(iii) Bovine trypsin = 100 µg/ml 10^{-3}M HCl pH 2.65

Assay:

1.0 ml (ii), 10 µl (iii) and 50 µl fraction after DE-52, were taken in a reduced volume plastic cuvette, mixed well and preincubated for 5 min at 30-35°C (heater block). Then 100 µl (i) was added, mixed well and the rate of reaction measured at 410 nm with uv-absorbance 0-0.2 and reaction speed 100 sec/cm against a cuvette containing 1.0 ml (ii) and 100 µl (i). For purified PMA-L, PMA-S and PLA, 10-50 µl (unless otherwise stated) from a stock solution of 1 mg/ml in H_2O were used in place of 50 ml fraction (DE-52) and for control, previous mixture was used omitting preincubation step as well as protein solution or fractions.

The compound p-nitroaniline is produced on hydrolysis of the substrate by trypsin which absorbs at 410 nm. The optical density of the solution at 410 nm is followed with time. 1 unit of enzyme activity is defined as causing an increase in O.D. of 0.1 per minute (corrected to a total

of 1.15 ml), under these conditions; trypsin inhibitor activity is expressed in terms of trypsin units inhibited, when a albumin fraction or purified albumin was used as the inhibitor source, all values were corrected for endogenous 'BAPNA-ase activity'.

(b) Chymotrypsin inhibitor assay

α-chymotrypsin inhibitory activity was determined by measuring degree of inhibition of the bovine chymotrypsin catalysed hydrolysis of the synthetic substrate N-benzoyl-L-tyrosine ethyl ester (BTEE) according to the method of Walsh and Wilcox (1970).

Reagents:

(i) BTEE = 3.13 mg/10 ml (10^{-3}M BTEE in 50% (v/v) aqueous methanol)

(ii) Buffer = 0.05 M Tris, 0.02 M $CaCl_2$ pH 8.2

(iii) Bovine α-chymotrypsin = 100 µg/ml 10^{-3}M HCl pH 2.65

Assay:

1.0 ml (ii), 20 µl (iii) and 50 µl albumin fraction after DE-52 or enzyme solution (1 mg/ml H_2O) were mixed together in a 1 cm pathlength cuvette, preincubated for 5 min as before and then 300 µl (i) was added. The optical density of the solution at 256 nm, with uv-absorbance and reaction speed as before, was followed with time vs blank containing 1.0 ml (ii) and 300 µl (i). For control, previous conditions were also maintained. Activity (corrected to a volume of 1.36 ml) was calculated from the slope of the linear portion of the reaction curve. 1 unit is equal to the hydrolysis of 1 micromole of substrate per minute.

(xxix) Insect Bio-assays:

Toxic effect of total pea albumin extracts, pea major albumin, PLA and pea lectin on the development of larvae of Callosobruchus maculatus, 'a major storage pest of many legumes', was carried out over a period of 41 days according to the method of Gatehouse and Boulter (1983).

(xxx) Preparation of total albumin extracts:

(a) Extracts from seed meals of different species:

Albumin protein extracts were prepared from small samples of meal (100-200 mg) from seeds of different legume species, including: Lathyrus odoratus (sweet pea), Vicia faba (broad bean), Lens culinaris (lentil), Cicer arietinum (Chick pea), Glycine max (soya-bean) Vigna unguiculata (cow pea), Vigna mungo (mung bean), Dolichos lablab (horse gram), Cajanus cajan (pigeon pea), Phaseolus vulgaris (dry bean) and Phaseolus coccineus (runner bean), by extraction with 20 mM NH_4- acetate buffer pH 5.0 (1.0 ml) for 1-2 h at 4°C with constant stirring. After centrifugation at 1300 g for 10-15 min the supernatants were used directly for immunodiffusion or were freeze-dried before SDS-polyacrylamide gel electrophoresis.

(b) Extracts from different parts of pea plants and seeds:

Total albumin extracts were also prepared from small samples (100-200 mg) of different freeze-dried tissues of pea plants such as, testa, cotyledons (whole), outer-cotyledons, inner-cotyledons, radicles, hypocotyls/epicotyls, leaves, stems and roots, by extracting with 5.0 mM NH_4- acetate buffer pH 5.0 (1.0 ml) for 2 h with constant stirring at 4°C. After centrifugation as before (a) the supernatants were freeze-dried for subsequent analysis by SDS-polyacrylamide gel electrophoresis followed by "Western blotting".

(c) Extracts from germinating, developing and different lines of pea seeds:

Preparation of total albumin extracts from germinating, developing and different lines of pea seeds meal were the same (unless otherwise stated). 100-200 mg meal of each sample was extracted with 20 mM Na-acetate buffer pH 5.0 (1.0 ml) at 4°C for 2 h with continuous stirring

and then centrifuged for 10 min as before (a). The supernatants were recovered carefully from the pellets (discarded) and kept at -20°C for subsequent analysis by various electrophoretic techniques. For SDS-PAGE and Urea/SDS-PAGE, equal volumes of supernatant (extract) and 2 x SDS-sample buffer were mixed together, from which 20-25 µl (unless otherwise stated) was used. For non SDS-polyacrylamide gel electrophoresis, 2 x non SDS-sample buffer was added in place of 2 x SDS-sample buffer, from which 40-45 µl of each sample was applied to the gels followed by two dimensional electrophoresis as previously described.

RESULTS

SECTION 3

1. Purification of major and low Mr albumin proteins

The schemes outlined in Fig. 1(a) and Fig. 1(b) have been used successfully to purify two major pea albumin proteins to homogeneity and a low Mr albumin protein to homogeneity, as judged by SDS-PAGE. Each consists of single-Mr subunits. The scheme No.1(a) also indicates the initial isolation of a HMW-albumin (subunit Mr about 100,000) which is present in the 40-60% rel.satn.ammonium sulphate fraction.

A. Preliminary experiments:

i) Ammonium sulphate fractionation

Freeze-dried ammonium sulphate fractions from a total albumin protein extract from Pisum sativum L. obtained at 0-40%, 40-50%, 50-60%, 60-70%, 70-80% and 80-90% rel. satn.with ammonium sulphate were analysed by SDS-PAGE under reducing conditions to identify those ammonium sulphate fractions in which the major pea albumin proteins were precipitated. Ammonium sulphate fractionation indicated the following separation of albumin proteins :-

Ammonium sulphate fractionation - Polypeptide (subunits Mr)

0-40% rel. satn. - no detectable significant polypeptide bands

40-60% rel. satn.- HMW-albumin (100,000)

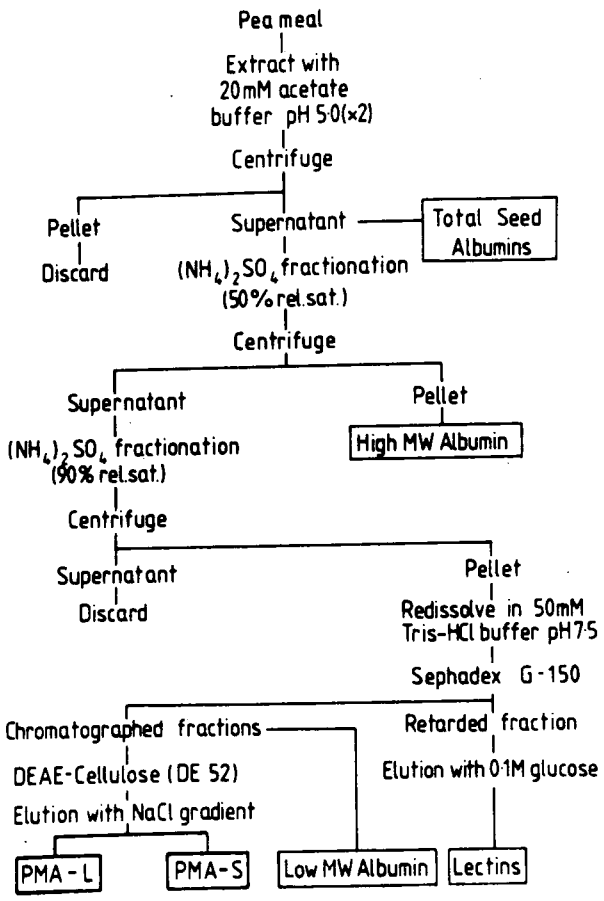
50-90% rel. satn.- PMA(L+S) (25,000, 24,000)

40-60% rel. satn.- Lectin (α+β) (17,700, 5,800)

50-90% rel. satn.- Low Mr albumin (PLA) (6,000 ± 300)

The protein fraction precipitating from total seed albumins between 50-90% rel. satn. with ammonium sulphate contained all or most of the major and low Mr albumin proteins. Pea seed lectins also precipitated in this ammonium sulphate fraction (see Fig. 2).

Fig.1(a)


Pea meal
Extract with 20mM acetate buffer pH 5·0(×2)
Centrifuge
Pellet
Discard
Supernatant
Total Seed Albumins
$(NH_4)_2SO_4$ fractionation (50% rel.sat.)
Centrifuge
Supernatant
$(NH_4)_2SO_4$ fractionation (90% rel.sat.)
Centrifuge
Pellet
High MW Albumin
Supernatant
Discard
Pellet
Redissolve in 50mM Tris-HCl buffer pH7·5
Sephadex G-150
Chromatographed fractions
DEAE-Cellulose (DE 52)
Elution with NaCl gradient
PMA-L
PMA-S
Retarded fraction
Elution with 0·1M glucose
Low MW Albumin
Lectins

(b)

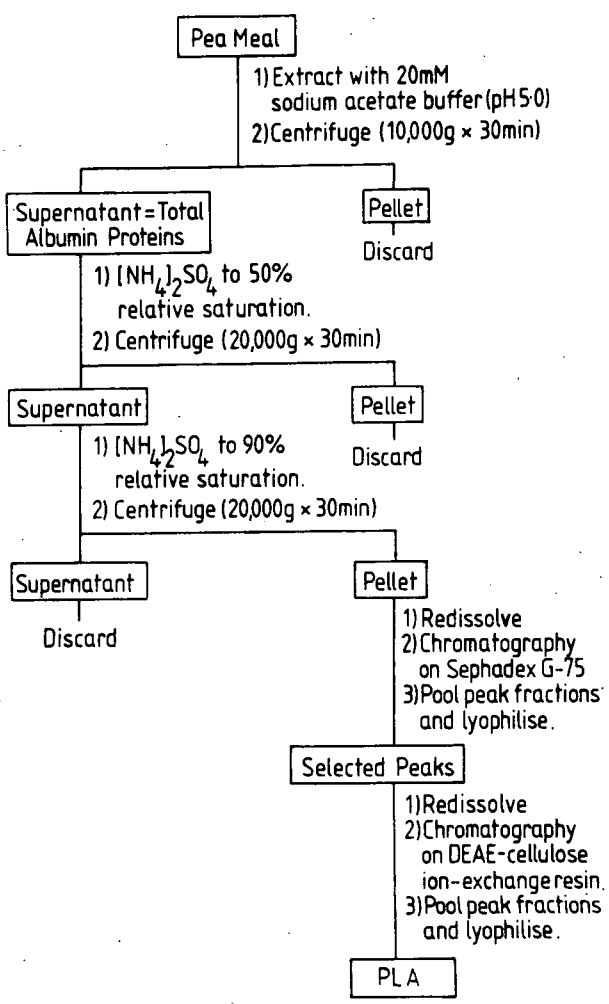
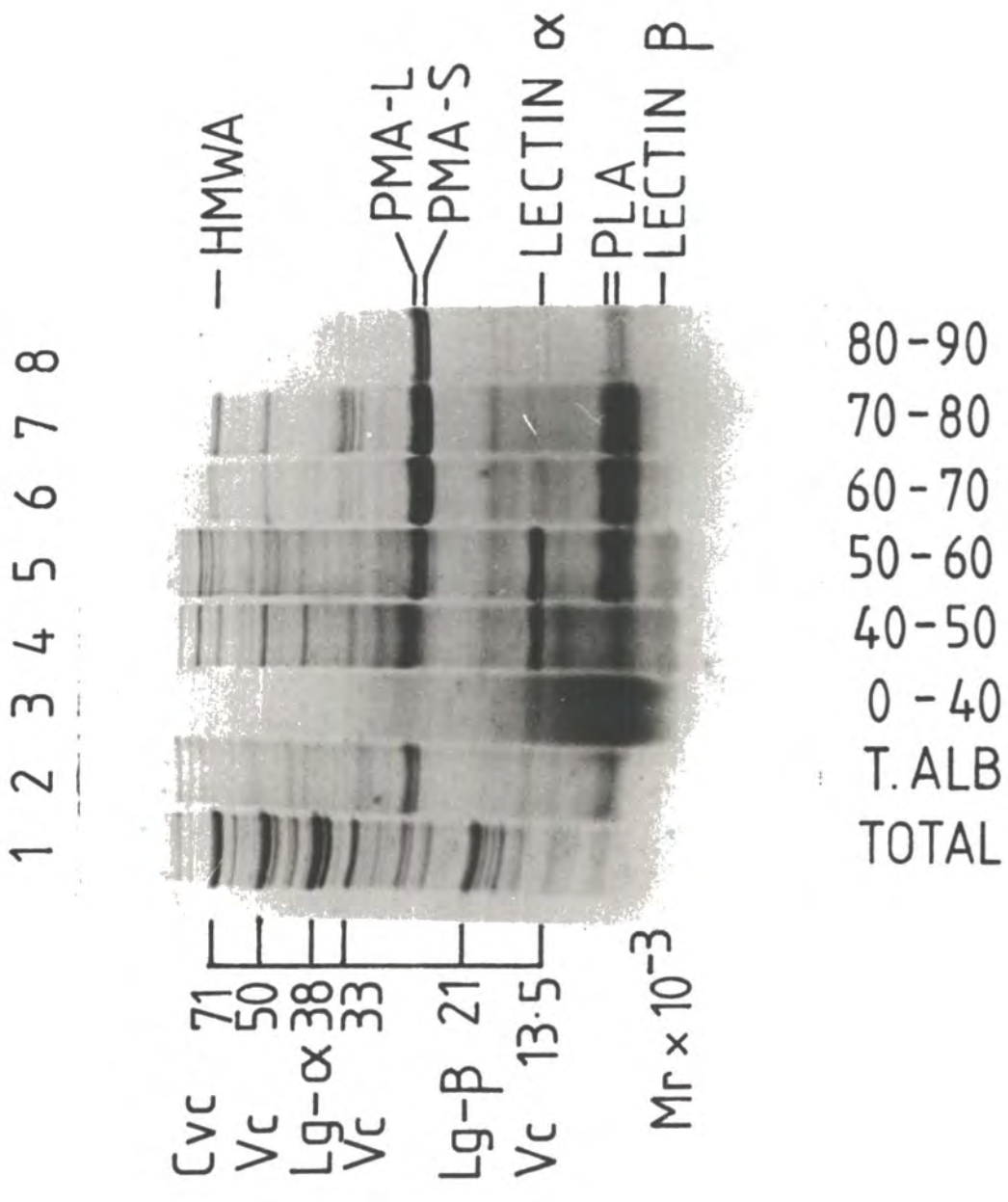

Pea Meal
1) Extract with 20mM sodium acetate buffer (pH 5·0)
2) Centrifuge (10,000g × 30min)
Supernatant = Total Albumin Proteins
Pellet
Discard
1) $[NH_4]_2SO_4$ to 50% relative saturation.
2) Centrifuge (20,000g × 30min)
Supernatant
Pellet
Discard
1) $[NH_4]_2SO_4$ to 90% relative saturation.
2) Centrifuge (20,000g × 30min)
Supernatant
Discard
Pellet
1) Redissolve
2) Chromatography on Sephadex G-75
3) Pool peak fractions and lyophilise.
Selected Peaks
1) Redissolve
2) Chromatography on DEAE-cellulose ion-exchange resin.
3) Pool peak fractions and lyophilise.
PLA

FIG. 2.

Analysis of different ammonium sulphate fractions from total albumin proteins on SDS-17% polyacrylamide gels.

Track no.	Sample
1	total protein extract of mature peas
2	total albumin extract of mature peas
3-8	proteins precipitating from total albumin extract as in track 2 in the indicated concentration ranges of ammonium sulphate
3	0-40% relative saturation
4	40-50% relative saturation
5	50-60% relative saturation
6	60-70% relative saturation
7	70-80% relative saturation
8	80-90% relative saturation

Fig. 2


1 2 3 4 5 6 7 8
HMWA
PMA-L
PMA-S
LECTIN α
PLA
LECTIN β
Cvc 71
Vc 50
Lg-α 38
Vc 33
Lg-β 21
Vc 13·5
Mr x 10⁻³
80-90
70-80
60-70
50-60
40-50
0-40
T. ALB
TOTAL

ii) Purification of major albumin proteins

a) Sephadex G-150 column chromatography:

The elution profile of the AS 50-90% fraction (AS 90) containing the major pea albumin proteins from Sephadex G-150 featured 4 peaks (Fig. 3). Analysis of all the fractions by SDS-PAGE (Fig. 4) showed that the main peak (fraction no. 2, Fig. 3) consisted largely of the major albumin proteins [PMA (L+S), subunit Mr about 25,000 and a minor amount of PLA subunits Mr 6000 ± 300]. Whereas fraction no. 3 (peak no. 3) contained mainly PLA (subunit Mr 6000 ± 300), collected sample was not enough for subsequent analysis. Materials present in other fractions (fractions no.1 and 4) were shown to contain variable amounts of other proteins and were not considered for further purification. Pea lectins, which precipitate in this AS 50-90% fraction, were bound to the Sephadex, and the purified major albumins were completely free from lectins as judged by SDS-polyacrylamide gel electrophoresis (Fig. 4).

b) Ion exchange chromatography

Ion exchange chromatography on a column of DEAE-cellulose was employed in order to further purify the fractions containing major albumins (fraction no. 2) obtained after Sephadex G-150 column. A feeze-dried sample of fraction no. 2 was applied to the column and eluted with a linear salt gradient. The elution profile obtained is shown in Fig. 5 and featurd 8 peaks. Analysis of the fractions by SDS-PAGE (Fig. 6) showed that the proteins eluted in the leading edge of the peak eluting at 0.10M NaCl (fraction 2) contained major albumin protein consisting only of the small subunits (subunit Mr 24,000), whereas the trailing edge of the peak eluting 0.13M NaCl (fraction 5) contained major albumin consisting only of the larger subunit (subunit Mr 25,000). Intermediate fractions (fractions 3 and 4) contained

FIG. 3.

Elution profile obtained after chromatography of Pisum sativum L. Albumin proteins precipitating in the range 50-90% rel.satn. of ammonium sulphate on a column of Sephadex G-150. Hatched areas show the fractions containing major albumin proteins.

FIG. 4.

SDS-17% polyacrylamide gel electrophoresis of fractions indicated in Fig. 3.

Track No.	Sample
1	standard total albumin extract of mature peas
2-3	standard major albumin proteins PMA (L + S).
4	G-150 fraction no. 1
5-6	G-150 fraction no. 2
7	G-150 fraction no. 3
8	G-150 fraction no. 4

Fig. 3

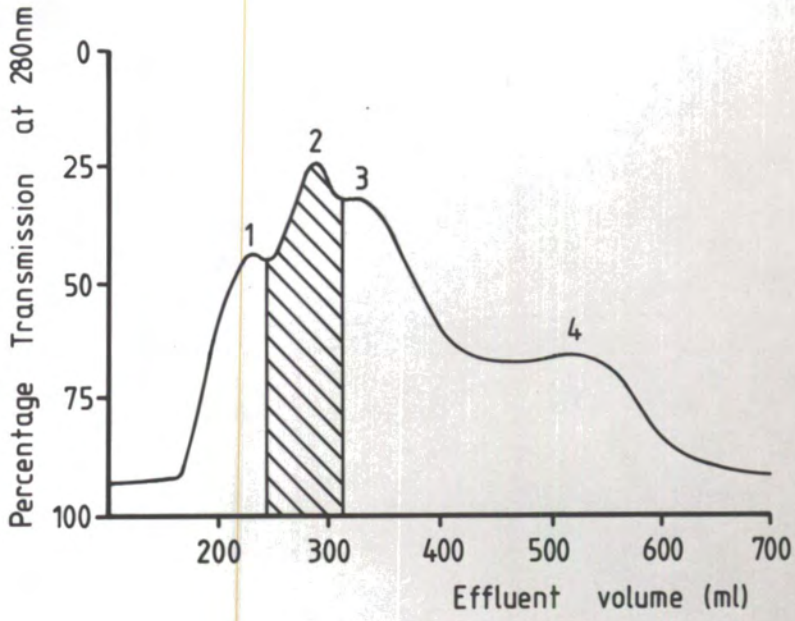
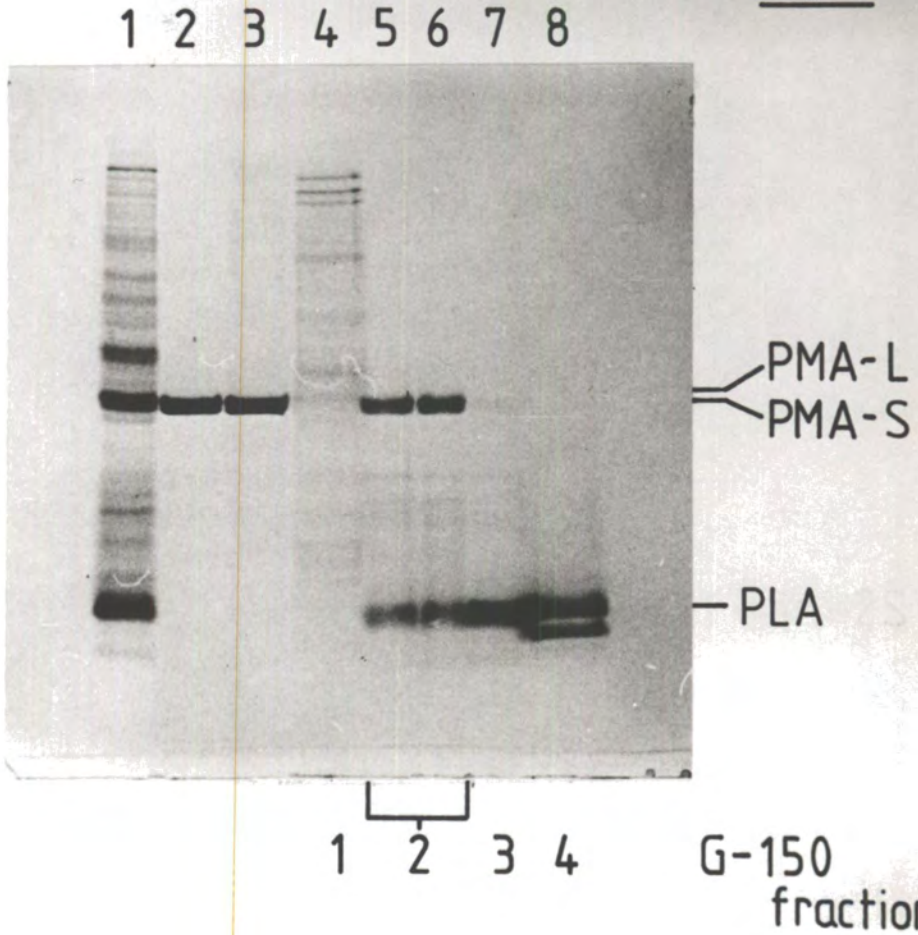

Percentage Transmission at 280nm
0
25
50
75
100
1
2
3
4
200
300
400
500
600
700
Effluent volume (ml)

Fig. 4


1 2 3 4 5 6 7 8
PMA-L
PMA-S
PLA
1 2 3 4
G-150 fractions

variable amounts of small and large subunits contaminated with various other proteins. Fractions eluted up to approx. 0.2M NaCl (fractions 6 and 7) also contained major albumin of the larger subunits (subunit Mr 25,000) but were contaminated with other proteins. The protein eluted in the last peak (0.25M NaCl)(fraction 8) contained low Mr albumin subunits (subunit Mr 6,000 ± 300) contaminated with traces of other low Mr proteins. This material was not further purified. Pea major albumin, small (PMA-S) was eluted at a salt concentration of 0.085M, and pea major albumin, large (PMA-L) was eluted at 0.14M NaCl (Fig. 5) respectively.

PMA-S and PMA-L (total pooled fractions from peaks 2 and 5 respectively; Fig. 5) were used for subsequent analysis by SDS-PAGE, see Fig. 7.

iii) Isolation of pea seed lectin

The elution profile from the column of Sephadex G-150 used to purify major albumin proteins is shown in Fig.8, which featured only a single peak of seed lectin, which was analysed by SDS-PAGE under both reducing and non-reducing conditions on the same gel, see Fig. 9. Lectin was free from contaminants and there was no change in band patterns of α- and β-chains of lectins under non-reducing conditions.

iv) Purification of PLA

a) Sephadex G-75 column chromatography

The elution profile from Sephadex G-75 chromatography of the albumin protein fraction featured 6 peaks (Fig. 10). Analysis of all the fractions by SDS-polyacrylamide gel electrophoresis, (Fig. 11),

FIG. 5.

Elution profile obtained after ion-exchange chromatography of fraction 2 from Sephadex G-150 column (Fig. 3) on a column of DEAE-cellulose (DE-52). Hatched areas show the fractions pooled as PMA-S and PMA-L respectively.

FIG. 6.

SDS-polyacrylamide gel electrophoresis of purified fractions indicated in Fig. 5.

Track no.	Sample
1	standard preparation of total albumin extract of *Pisum sativum* L.
2	standard total major albumins of *Pisum sativum* L.
3	total major albumins containing fraction (G-150).
4	fraction no. 1
5	fraction no. 2 (PMA-S)
6	fractions no. (3 + 4)
7	fraction no. 5 (PMA-L)
8	fraction no. 6
9	fraction no. 7
10	fraction no. 8

Fig. 5

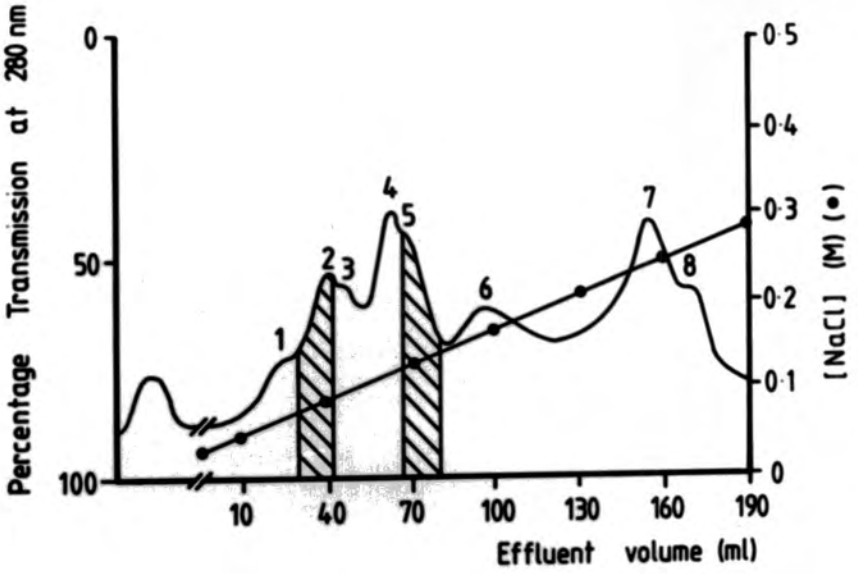

Percentage Transmission at 280 nm
0
50
100
0·5
0·4
0·3
0·2
0·1
0
[NaCl] (M) (•)
1
2
3
4
5
6
7
8
10
40
70
100
130
160
190
Effluent volume (ml)

Fig. 6

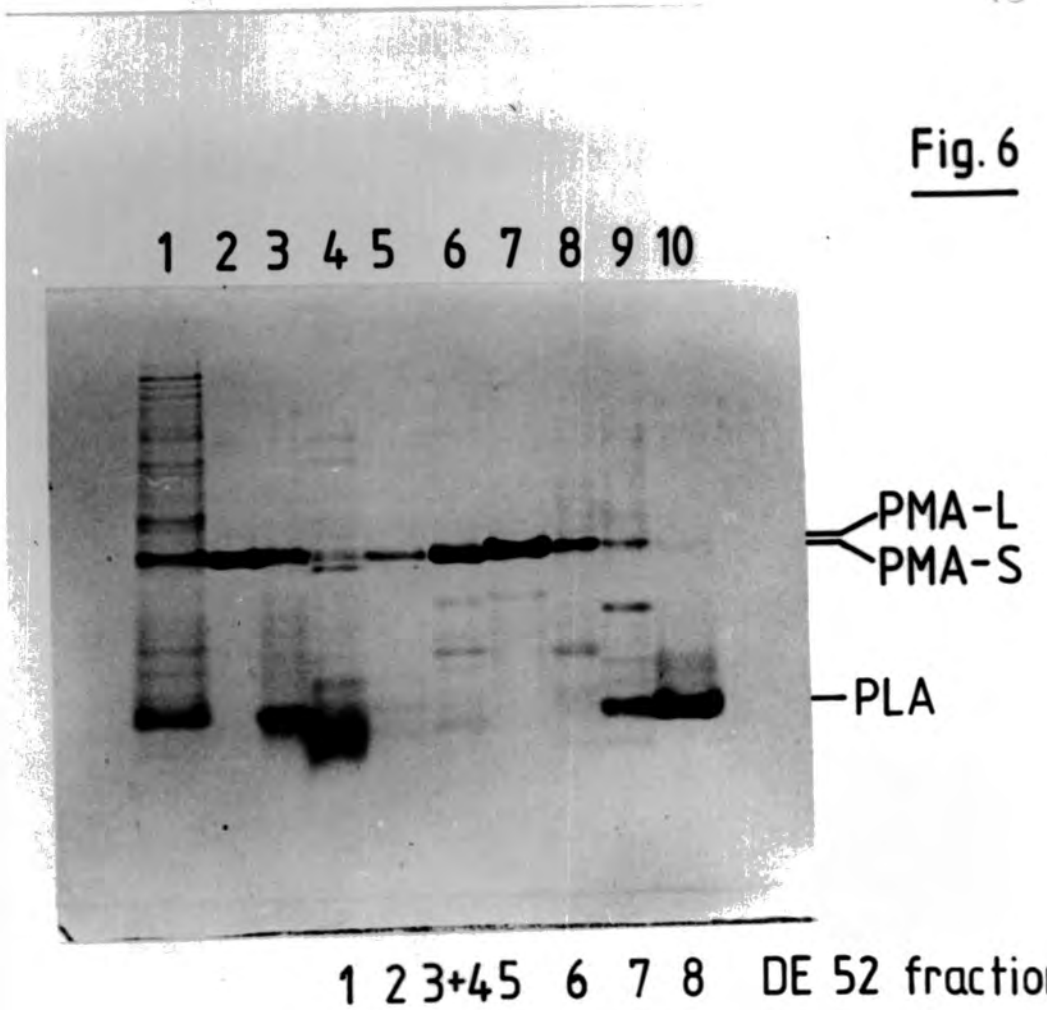

1 2 3 4 5 6 7 8 9 10
PMA-L
PMA-S
PLA
1 2 3+4 5 6 7 8 DE 52 fractions

FIG. 7.

SDS-Polyacrylamide gel electrophoresis of proteins and protein fractions isolated according to the scheme outlined in Fig. 1(a).

Track no.	Sample
1	total seed globulin proteins
2	total seed protein extract
3	total seed albumin extract
4	total major albumin fraction (G-150)
5	purified PMA-S
6	purified PMA-L
7	equal amounts of PMA (L + S) mixed
8	purified pea seed lectin

Fig. 7

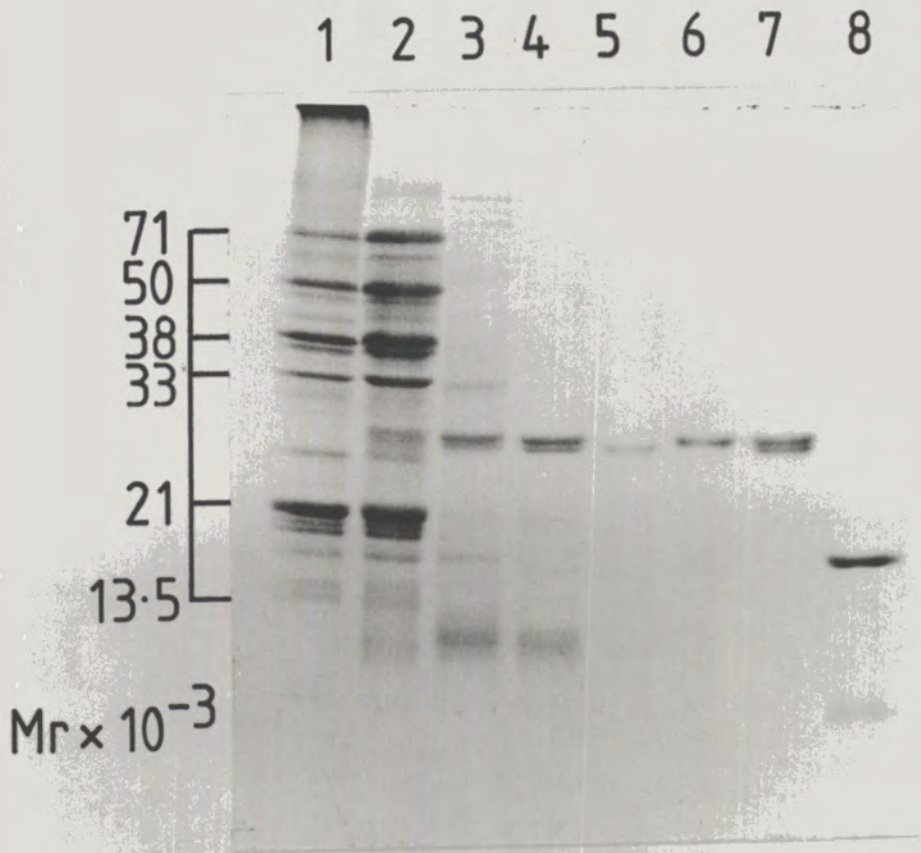


FIG. 8.

Affinity purification of seed lectin by elution of Sephadex G-150 column used to purify albumin proteins (Fig. 3) with buffer containing 0.1M glucose.

FIG. 9.

SDS-17% polyacrylamide gel electrophoresis of isolated seed lectin protein fraction indicated in Fig. 8.

Track no.	Sample
1	total protein extract of mature peas (+ 2 ME)
2	total albumin extract of mature peas (+ 2 ME)
3-4	standard seed lectin of mature peas (+ 2 ME)
5-6	standard major albumins of mature peas (+ 2 ME)
7-8	isolated seed lectin (+ 2 ME)
9	isolated seed lectin (- 2 ME)
10	standard seed lectin (- 2 ME)

Fig. 8

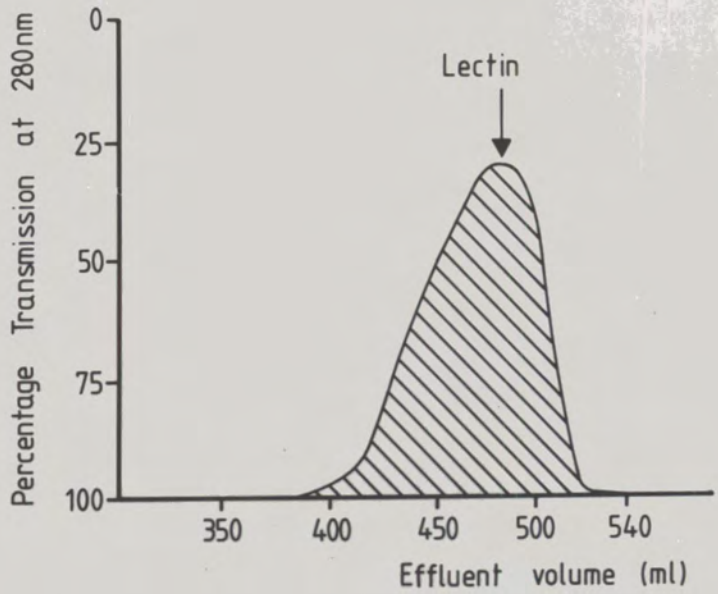
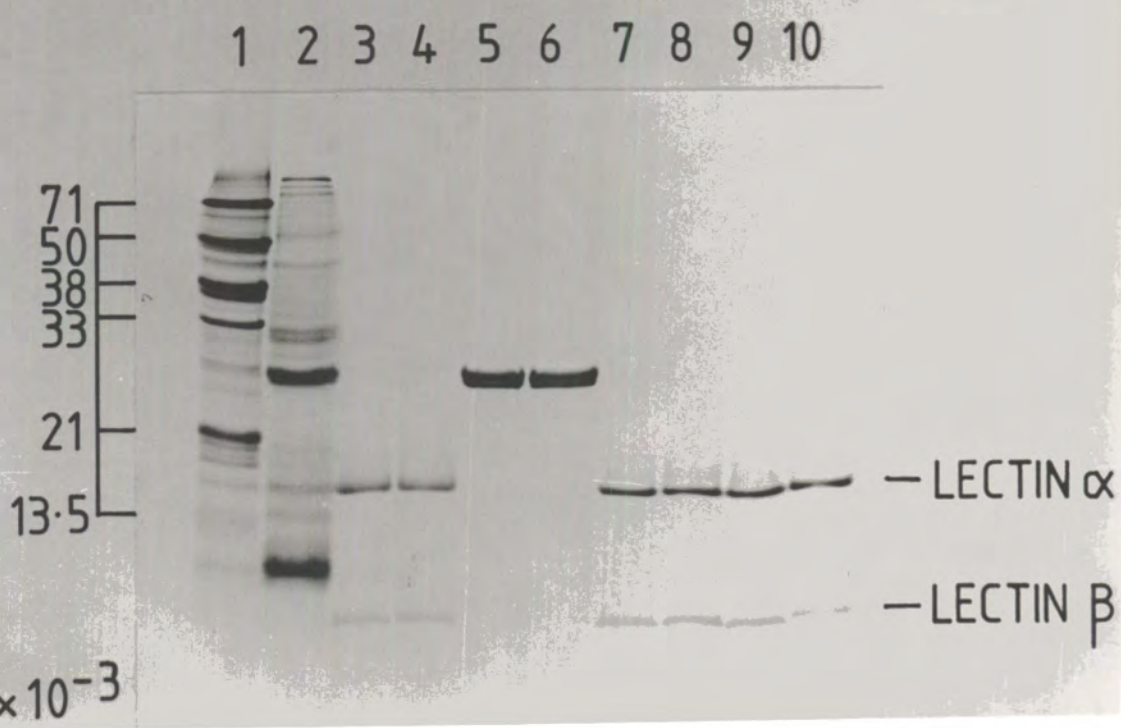

Percentage Transmission at 280nm
0
25
50
75
100
Lectin
350
400
450
500
540
Effluent volume (ml)

Fig. 9


1 2 3 4 5 6 7 8 9 10
71
50
38
33
21
13·5
Mr x 10⁻³
— LECTIN α
— LECTIN β

showed that fractions no.3 and 4 consisted mainly of PLA protein (subunit mol. wt. about 6,000 ± 300), though fraction no. 3 was contaminated with small amounts of major albumin proteins; fraction no. 5 was found to contain insignificant amounts of various low-molecular-weight subunits; after freeze-dried insufficient sample was collected from fraction no. 6 for analysis. Materials present in other fractions were not considered here and discarded.

b) Ion exchange chromatography

Ion exchange chromatography on a column of DEAE-cellulose was successfully employed to further purify the fractions containing PLA protein obtained from the Sephadex G-75 column. Freeze-dried samples of fractions 3 and 4 were applied separately to the column and eluted with a linear concentration gradient of NaCl from 0-0.5M in 50mM Tris-HCl buffer, pH 7.5. The elution profiles are shown in Fig. 12 and Fig. 14, which featured 10 and 11 peaks respectively. On analysis of the fractions by SDS-polyacrylamide gel electrophoresis, see Fig. 13 and Fig. 15, the proteins eluted in peaks 9 (Fig. 12) and 10 (Fig. 14) contained only PLA subunits (subunit mol. wt. 6,000 ± 300). The proteins eluted in peaks 3 and 4 (Fig. 12) were mainly PMA-S (subunit mol. wt. 24,000) and PMA-L (subunit mol. wt. 25,000) repsectively (Fig. 13, tracks no. 4 and 5). Materials present in other fractions contained other proteins and were discarded. PLA was eluted as a single peak at a salt concentration of 0.16 - 0.18M (Fig. 12 and Fig. 14), the last major peak to be eluted from the column.

v) Quantitative determination of pea major albumin and PLA

Estimations of the amount of PMA and PLA proteins in a total seed

FIG. 10.

Elution profile obtained after chromatography of albumin proteins precipitating in the range 50-90% rel. satn. of ammonium sulphate on a column of Sephadex G-75. Hatched areas show the fractions containing low molecular weight albumin (PLA) protein.

FIG. 11.

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig. 10.

Track no.	Sample
1	fraction no.1
2	fraction no. 2
3-4	fraction no. 3
5-6	fraction no. 4
7	fraction no. 5
8	fraction no. 6

Fig. 10

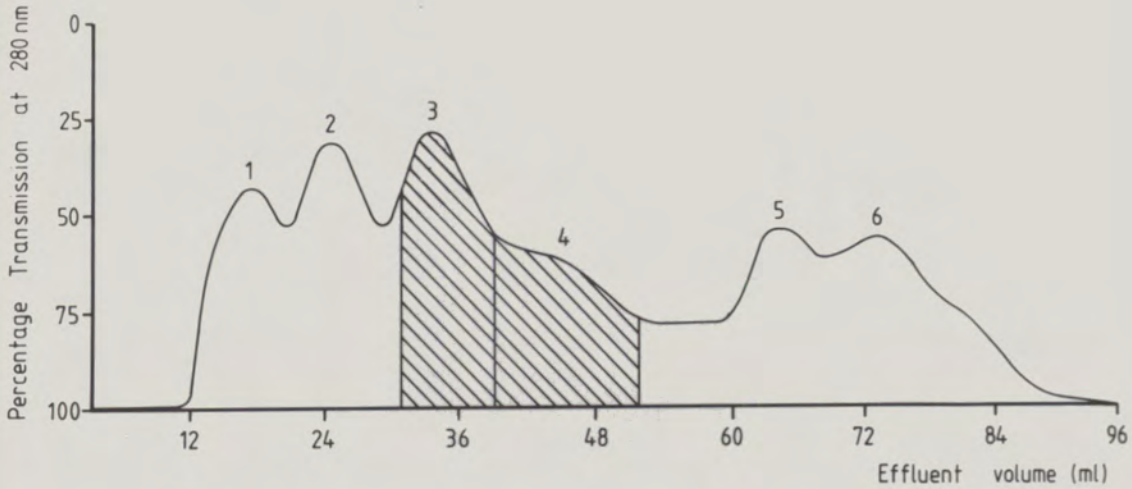

Percentage Transmission at 280 nm
0
25
50
75
100
1
2
3
4
5
6
12
24
36
48
60
72
84
96
Effluent volume (ml)

Fig. 11

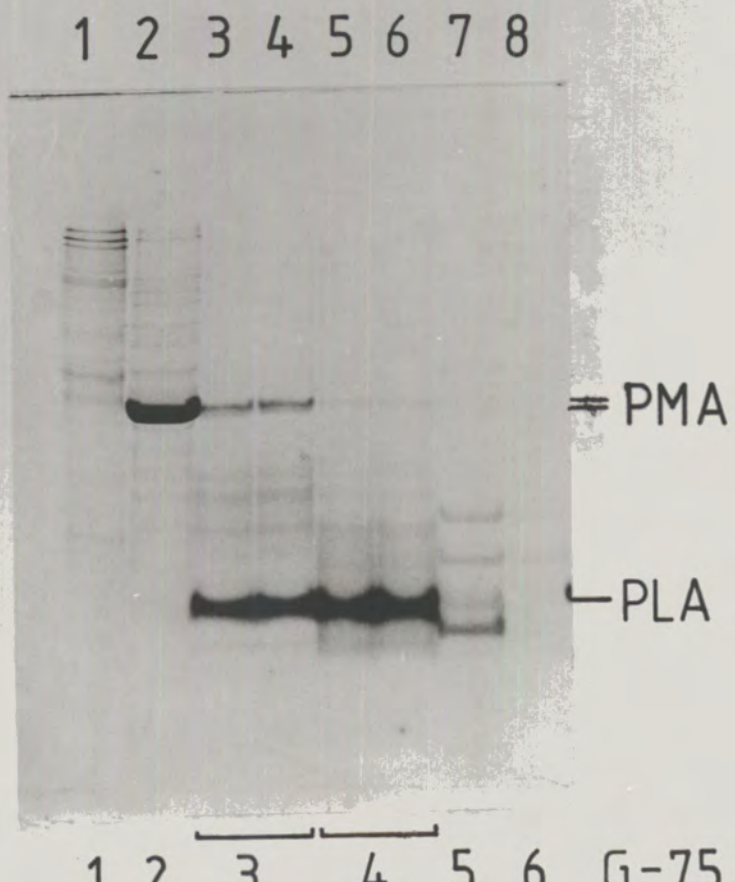

1 2 3 4 5 6 7 8
PMA
PLA
1 2 3 4 5 6 G-75 fractions

FIG. 12.

Elution profile obtained after ion-exchange chromatography of fraction 3 from the G-75 column (Fig. 10) on a column of DEAE-cellulose (DE-52). Hatched areas show the fractions containing PLA proteins.

FIG. 13.

SDS-Polyacrylamide gel electrophoresis of fractions indicated in Fig. 12.

Track no.	Sample
1	fraction no. 3 after G-75
2	fraction no. 1
3	fraction no. 2
4	fraction no. 3 (PMA-S)
5	fraction no. 4 (PMA-L)
6	fraction no. 5
7	fraction no. 6
8	fraction no. 7
9	fraction no. 8
10	fraction no. 9 (PLA)
11	fraction no. 10

Fig. 12

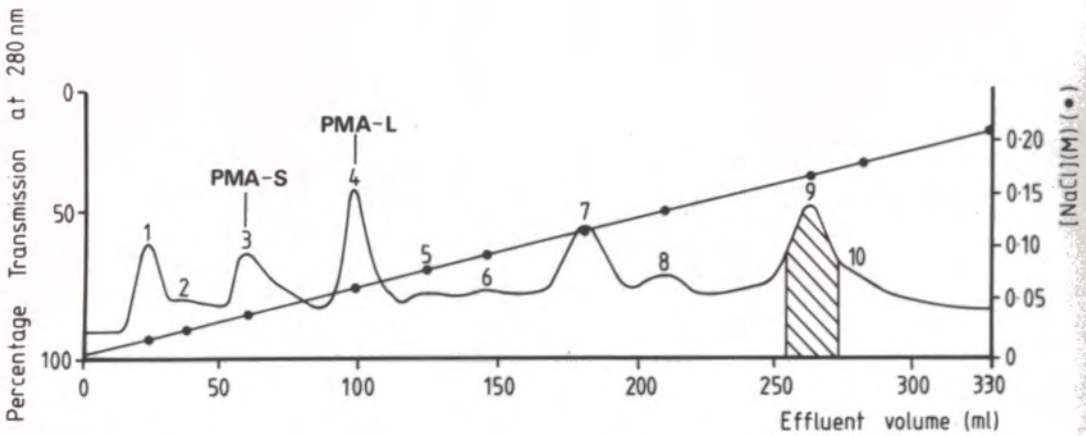

Percentage Transmission at 280 nm
0
50
100
PMA-S
PMA-L
1
2
3
4
5
6
7
8
9
10
0
50
100
150
200
250
300
330
Effluent volume (ml)
0·20
0·15
0·10
0·05
0
[NaCl](M)·(•)

Fig. 13

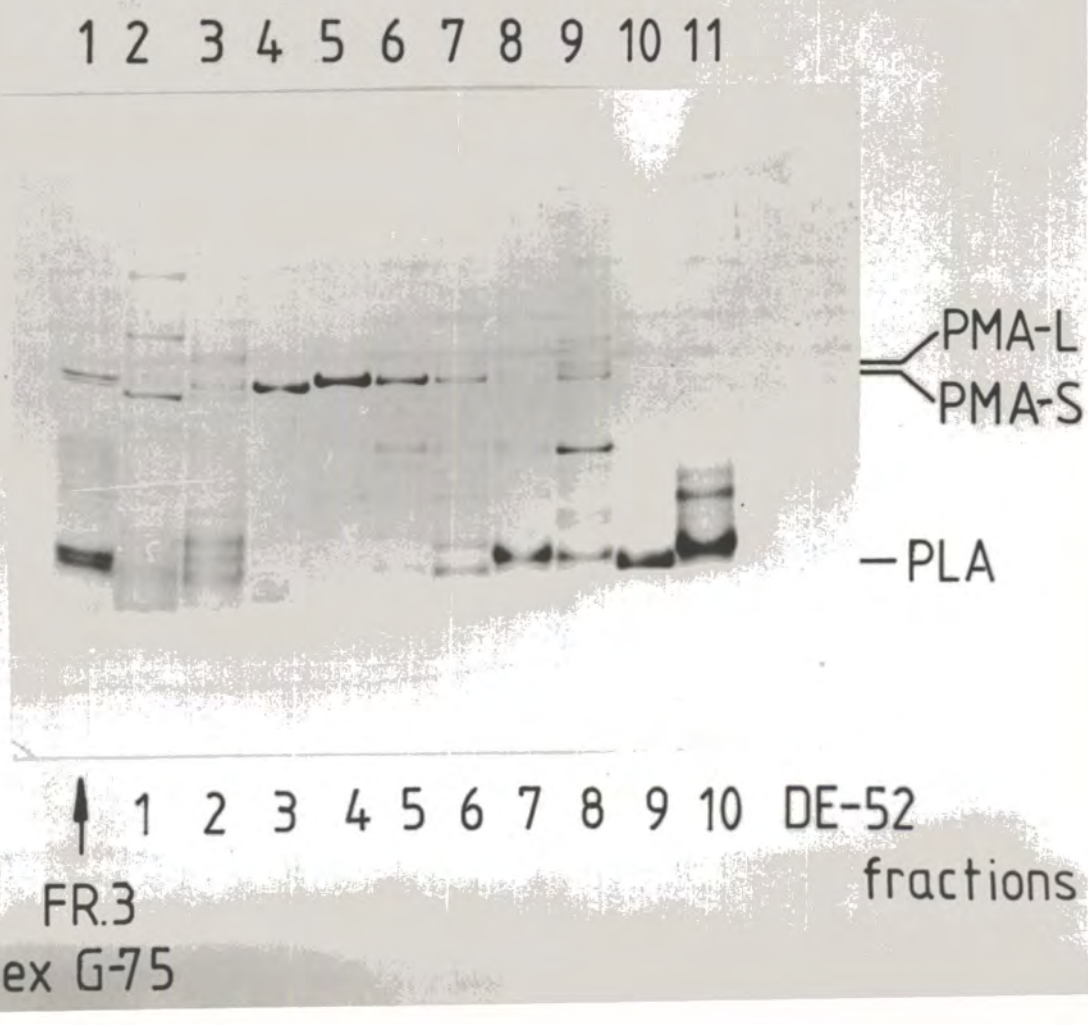

1 2 3 4 5 6 7 8 9 10 11
PMA-L
PMA-S
PLA
1 2 3 4 5 6 7 8 9 10 DE-52
fractions
FR.3
ex G-75

FIG. 14.

Elution profile obtained after ion-exchange chromatography of fraction no. 4 from G-75 column (Fig. 10) on a column of DEAE-cellulose (DE-52). Hatched areas show the fractions containing PLA proteins.

FIG. 15.

SDS-Polyacrylamide gel electrophoresis of fractions indicated in Fig. 14.

Track no.	Sample
1	fraction no. 4 after G-75
2	fraction no. 1
3	fraction no. 2
4	fraction no. 3
5	fraction no. 4
6	fraction no. 5
7	fraction no. 6
8	fraction no. 7
9	fraction no. 8
10	fraction no. 9
11	fraction no. 10 (PLA)
12	fraction no. 11

Fig. 14

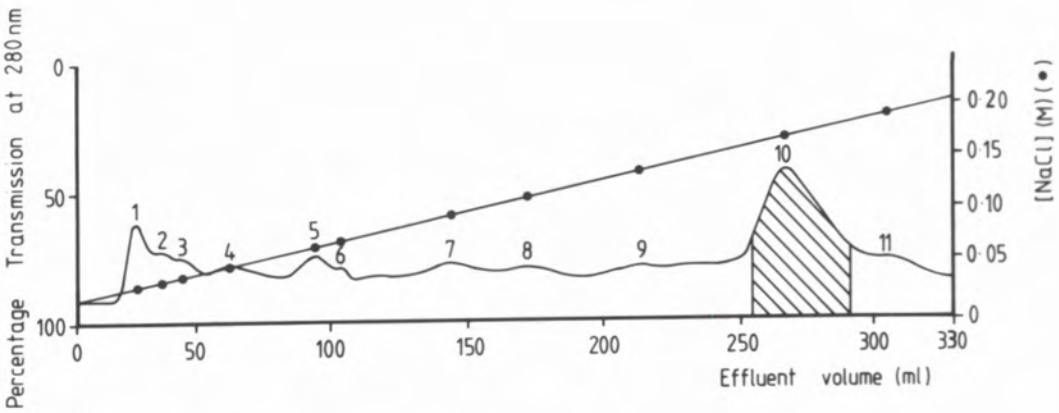
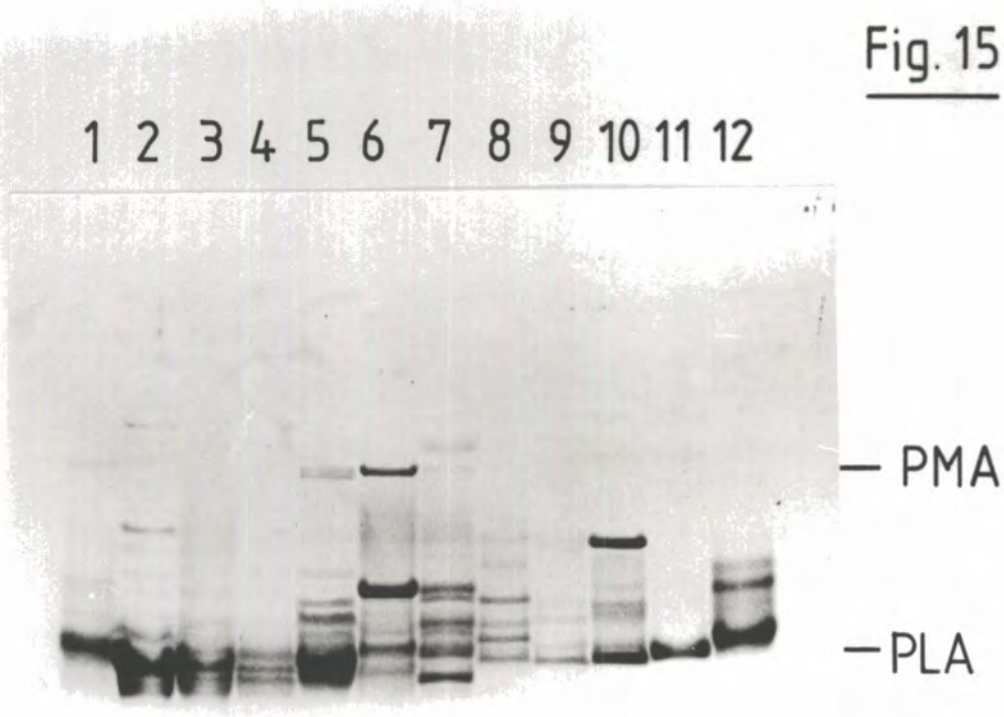

Percentage Transmission at 280nm
0
50
100
0
50
100
150
200
250
300
330
Effluent volume (ml)
0·20
0·15
0·10
0·05
0
[NaCl] (M) (•)
1
2
3
4
5
6
7
8
9
10
11

Fig. 15


1 2 3 4 5 6 7 8 9 10 11 12
PMA
PLA
1 2 3 4 5 6 7 8 9 10 11 DE-52
fractions
FR.4
ex. G-75

protein extract and in a total seed albumin extract were carried out by various electrophoretic techniques in the following ways as described earlier :

(a) Gel electrophoresis and densitometric scanning :

Semiquantitative estimations of PMA and PLA present in seed meal were made by comparison of the intensities of bands on an SDS-PAGE and on a Urea/SDS-PAGE gel respectively by densitometric scanning. Known amounts of PMA and PLA were run on the same gels to obtain calibration curves (see Fig.18 and Fig.19).

It was estimated from the levels of PMA in total seed albumin extracts that PMA represented 2.4% (± 0.6%) i.e. 2-3% of the dry weight of seed meal, corresponding to ∿ 10% of total cotyledonary proteins and ∿ 50% of the total albumin proteins (based on average total protein and total albumin protein contents of 25% and 5% of the dry weight of seed meal respectively). Similarly, PLA represented 1.0% (± 0.3%) of the dry weight of seed meal corresponding to ∿ 4% of the total cotyledonary proteins and ∿ 20% of total albumin proteins respectively.

(b) Rocket immunoelectrophoresis :

Quantitative estimation of PMA was performed by using monospecific anti-PMA IgG in the 'rocket-immunoelectrophoretic' technique described earlier by Laurell, (1966). PMA proteins in a total protein extract and in a total albumin extract of pea seeds were quantitatively determined from standard calibration curve(s) prepared by plotting the rocket height(s) against concentration of standard PMA proteins, see Fig. 20. It was found that total protein extract and total albumin extract from mature pea seeds (Pisum sativum L.) contained about 2.8% (± 0.4%) and 4.4% (± 0.7%) respectively of total major albumin proteins,

FIG. 16.

SDS-17% polyacrylamide gel used for densitometric scanning in semi-quantitative estimation of PMA proteins present in seed meal albumin extracts of Pisum sativum L.

Track no.	Sample
1	500 µg seed meal (freshly prepared extract)
2	1 mg seed meal (freshly prepared extract)
3	2 mg seed meal (freshly prepared extract)
4	100 µg seed meal (freeze-dried extract)
5	200 µg seed meal (freeze-dried extract)
6	400 µg seed meal (freeze-dried extract)
7	10 µg standard PMA
8	20 µg standard PMA
9	30 µg standard PMA
10	40 µg standard PMA

FIG. 17.

Urea/SDS-15% polyacrylamide gel used for densitometric scanning in semi-quantitative estimation of PLA present in seed-meal albumin extracts of Pisum sativum L.

Track no.	Sample
1	500 µg seed meal (freshly prepared extract)
2	1 mg seed meal (freshly prepared extract)
3	2 mg seed meal (freshly prepared extract)
4	100 µg seed meal (freeze-dried extract)
5	200 µg seed meal (freeze-dried extract)
6	400 µg seed meal (freeze-dried extract)
7	10 µg standard PLA protein
8	20 µg standard PLA protein
9	30 µg standard PLA protein
10	40 µg standard PLA protein

Fig. 16

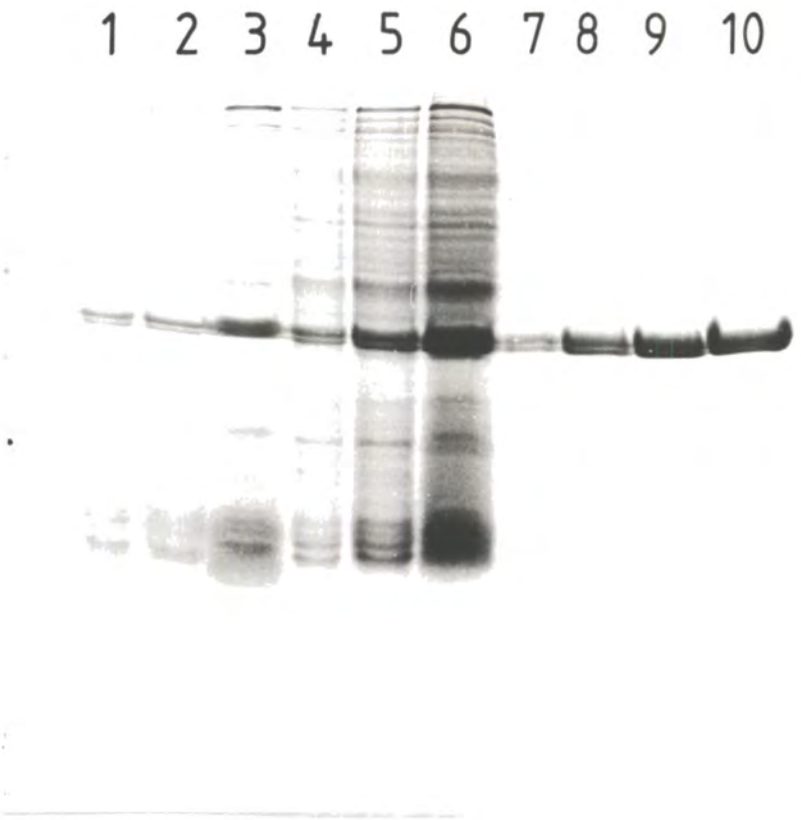


Fig. 17

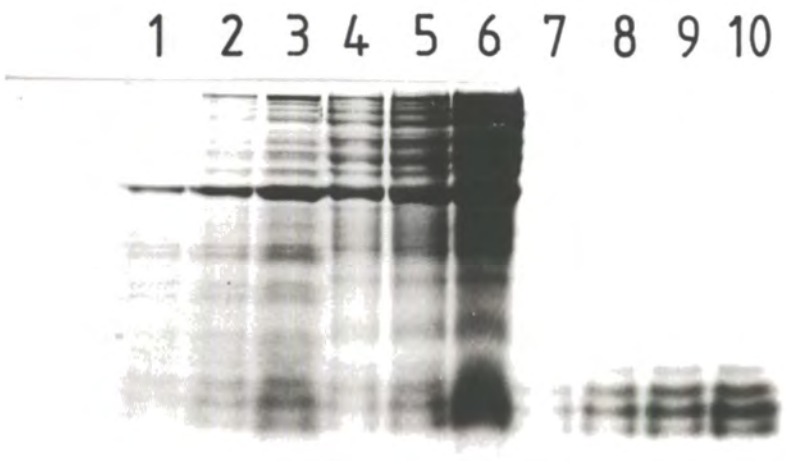


FIG. 18.

Calibration curve prepared by plotting area weight of peak (mg) against amounts of the standard PMA proteins (µg).

Total albumin extract (fresh) = ⟶

Total albumin extract (freeze-dried) = ●

FIG. 19.

Calibration curve prepared by plotting area weight of peak (mg) against amounts of the standard PLA protein (µg).

Total albumin extract (fresh) = ⟶

Total albumin extract (freeze-dried) = ●

FIG. 20.

Calibration curve prepared by plotting 'rocket-heights' against standard amounts of purified total major albumin protein (PMA).

Fig 18

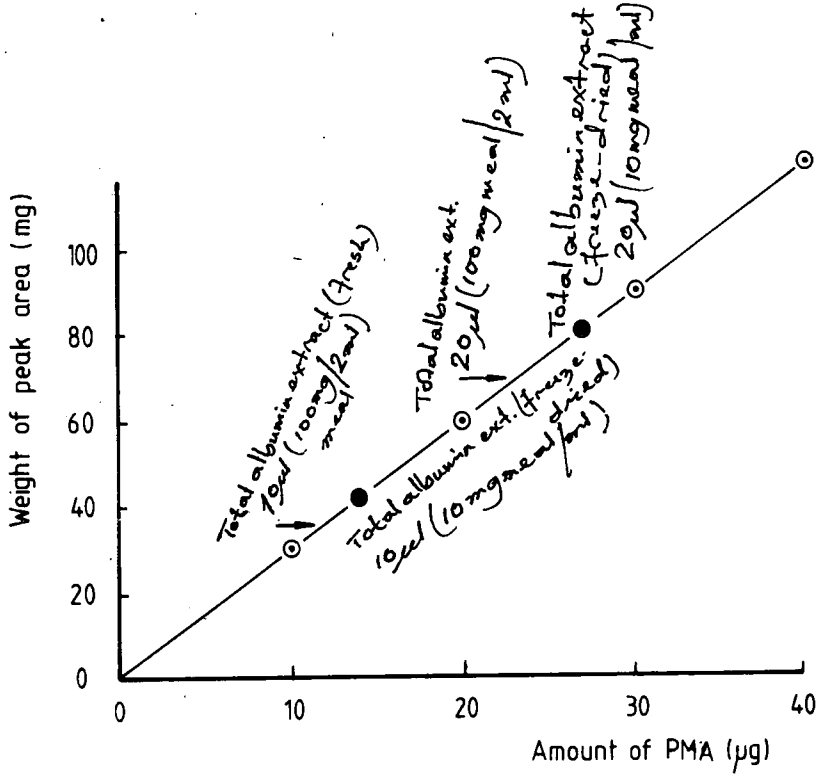


Fig 19

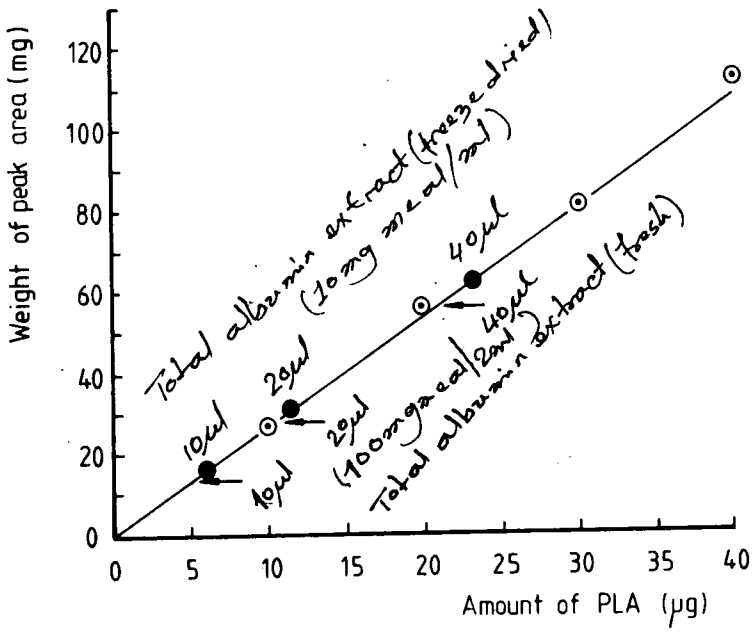
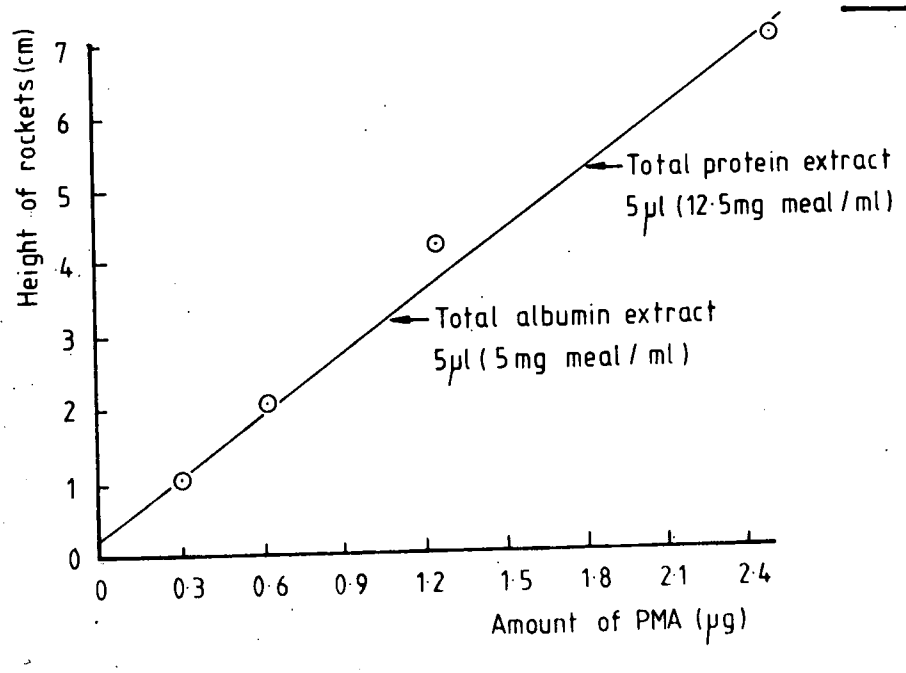


Fig 20



based on weightsof meal extracted, i.e. ∿ 3-4% of the dry weight of seed meal, corresponding to ∿ 14% of total cotyledonary protein (based on an average protein content of 25%). The variation between the estimates from the two different extracts is within the experimental error, and although these values are slightly higher than that from the densitometric method, are in reasonable agreement.

B. Characterization of PMA-L, PMA-S and PLA:

1. Molecular weights and subunit compositions :

(a) Molecular weights of PMA-L and PMA-S

Chromatography of the major albumin proteins on Sephacryl S-200 and comparison of their Ve/Vo (elution volume/void volume) values with those for standard proteins gave molecular weight values of 53000 ± 10% for PMA-L and 48000 ± 10% for PMA-S respectively, see Fig. 21.

(b) Molecular weight of PLA:

The molecular weight of PLA was determined by gel filtration on a column of Sephadex G-50 (superfine) calibrated with standard proteins. A graph of Ve/Vo (elution volume/void volume) against Log (Mr) was plotted for standard proteins and used to predict the Mr of PLA. A value of 11000(± 1,000) was estimated by extrapolation, see Fig.22.

(c) Subunit molecular weights of PMA-L and PMA-S:

Subunits of PMA-L and PMA-S proteins were found to have molecular weights of 25,400 ± 10% and 24,300 ± 10% on 17% SDS-polyacrylamide gels and values of 24,600 ± 10% and 23,700 ± 10% were obtained with 12.5% SDS-polyacrylamide gels when compared to standard proteins, see Fig. 23, Fig. 24, Fig. 25 and Fig. 26. Mean values of 25,000 and 24,000 were adopted as the subunit molecular weights of PMA-L and PMA-S respectively. Such molecular weight estimates are consistent with dimeric molecules for the major albumin proteins.

FIG. 21.

Calibration curve relating $^{Ve}/Vo$ values of standard proteins, chromatographed on Sephacryl S-200, with their molecular weights. Protein standards in order of decreasing size: catalase, Pisum vicilin, bovine serum albumin, ovalbumin and myoglobin.

FIG. 22.

Calibration curve relating $^{Ve}/Vo$ values of standard proteins, chromatographed on Sephadex G-50, with their molecular weights. Standard proteins in order of decreasing size are: β-lactoglobulin, soyabean trypsin inhibitor, myoglobin and cytochrome c.

Fig. 21

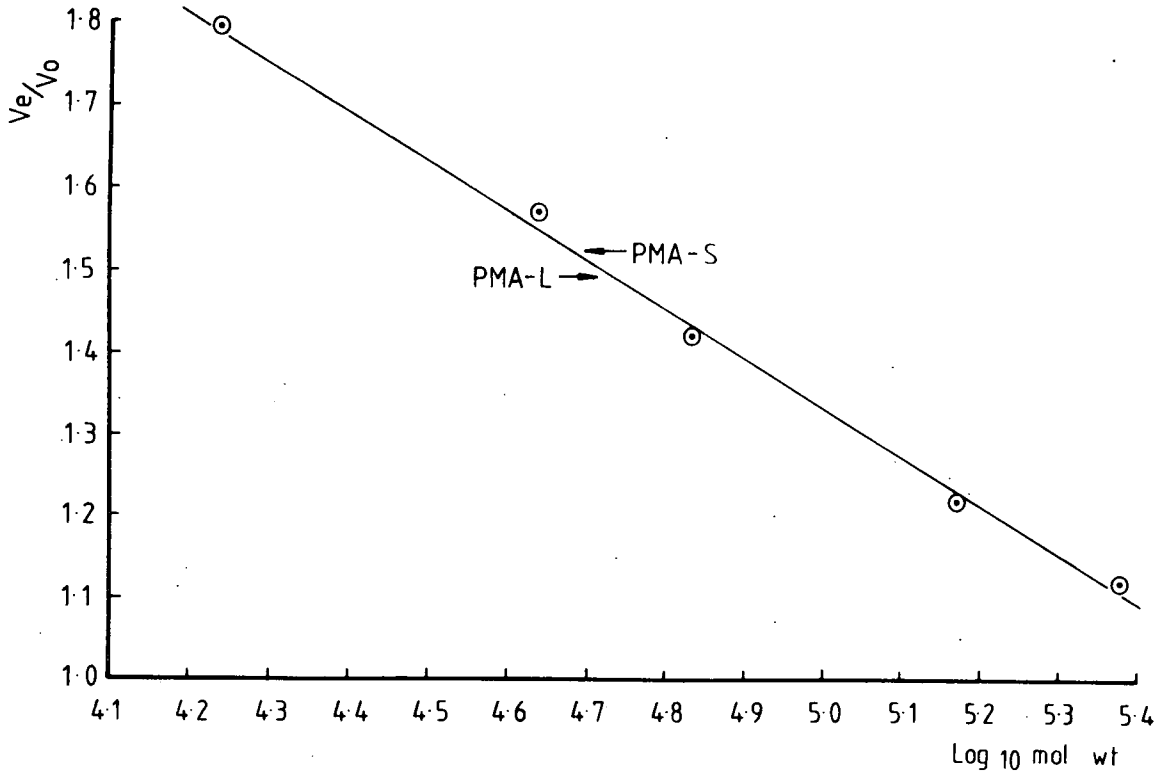

Ve/Vo
1·8
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
PMA-S
PMA-L
4·1
4·2
4·3
4·4
4·5
4·6
4·7
4·8
4·9
5·0
5·1
5·2
5·3
5·4
Log 10 mol wt

Fig. 22

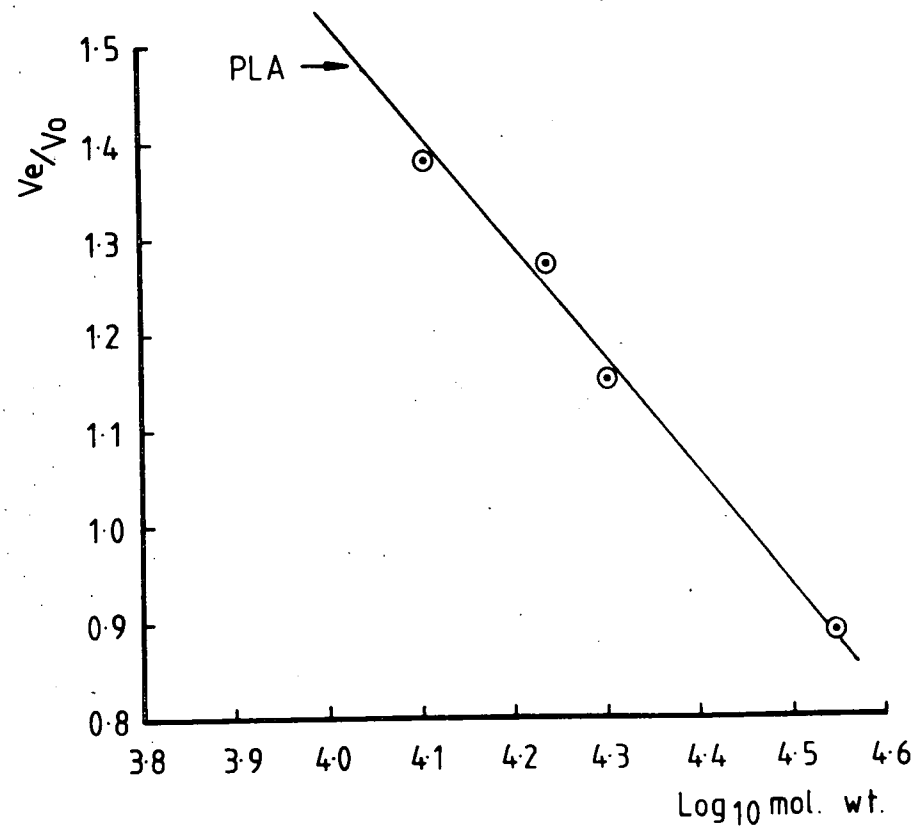

Ve/Vo
1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
PLA
3·8
3·9
4·0
4·1
4·2
4·3
4·4
4·5
4·6
Log 10 mol. wt.

FIG. 23.

Calibration curve relating the $\log_{10}$ molecular weights of standard proteins with their electrophoretic mobilities relative to bromophenol blue (10), on 12.5% SDS-polyacrylamide gels. Standard proteins in order of decreasing size, are : (1) transferrin, (2) convicilin, (3) bovine serum albumin, (4) catalase, (5) vicilin I, (6) ovalbumin, (7) lactate dehydrogenase, (8) vicilin II, (9) soyabean trypsin inhibitor, (10) ferritin, and (11) β-lactoglobulin.

FIG. 24.

Calibration curve relating the $\log_{10}$ molecular weights of standard proteins with their electrophoretic mobilities relative to bromophenol blue (10),on 17% SDS-polyacrylamide gels. Standard proteins in order of decreasing size, are : (1) transferrin, (2) convicilin, (3) bovine serum albumin, (4) catalase, (5) vicilin I, (6) ovalbumin, (7) lactate dehydrogenase, (8) vicilin II, (9) soyabean trypsin inhibitor, (10 ferritin, and (11) β-lactoglobulin.

Fig.23

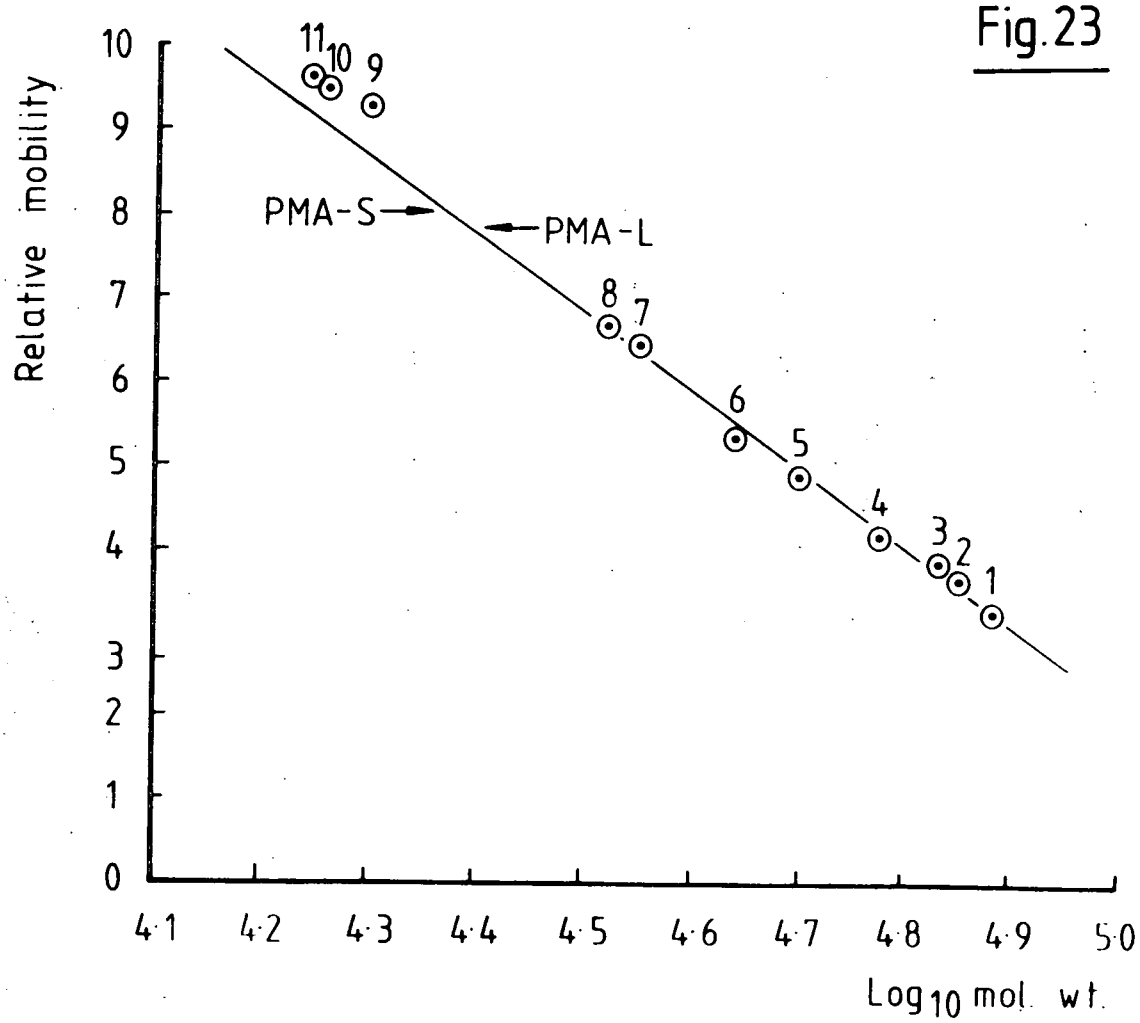

Relative mobility
10
9
8
7
6
5
4
3
2
1
0
11
10
9
PMA-S
PMA-L
8
7
6
5
4
3
2
1
4·1
4·2
4·3
4·4
4·5
4·6
4·7
4·8
4·9
5·0
Log10 mol. wt.

Fig.24

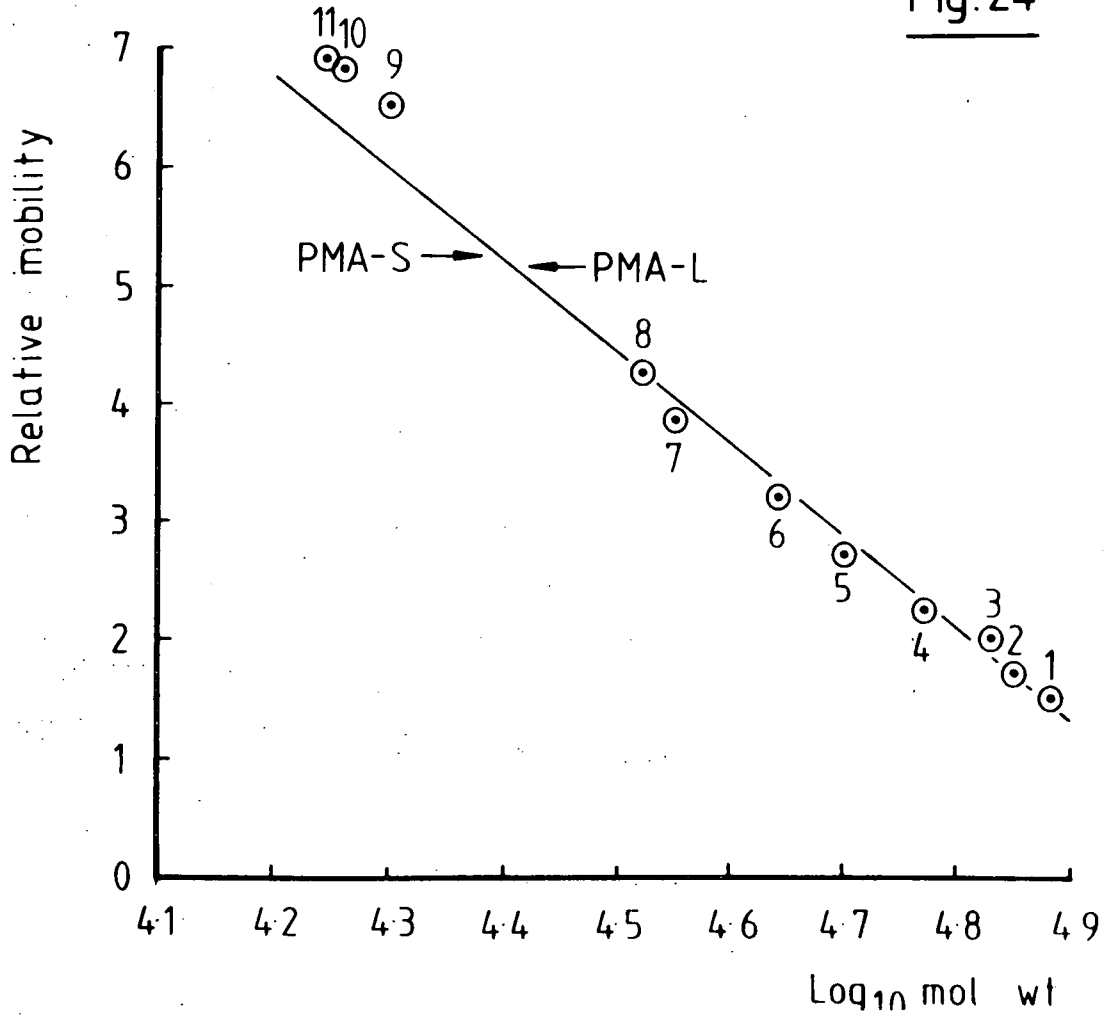

Relative mobility
7
6
5
4
3
2
1
0
11
10
9
PMA-S
PMA-L
8
7
6
5
4
3
2
1
4·1
4·2
4·3
4·4
4·5
4·6
4·7
4·8
4·9
Log10 mol wt

FIG. 25.

Determination of the subunit molecular weight(s) for PMA-L and PMA-S by 17% SDS-polyacrylamide gel electrophoresis.

Standard proteins.

Track	1	:	transferrin
"	2	:	vicilin + convicilin
"	3	:	bovine serum albumin
"	4	:	catalase
"	5	:	ovalbumin
"	6	:	lactic dehydrogenase
"	7	:	soyabean trypsin inhibitor
"	8	:	ferritin
"	9	:	β-lactoglobulin
"	10	:	PMA-S
"	11	:	PMA-L

FIG. 26.

Determination of the subunit molecular weight(s) for PMA-L and PMA-S by 12.5% SDS-polyacrylamide gel electrophoresis.

Standard proteins.

Track	1	:	transferrin
"	2	:	vicilin + convicilin
"	3	:	bovine serum albumin
"	4	:	catalase
"	5	:	ovalbumin
"	6	:	lactic dehydrogenase
"	7	:	soyabean trypsin inhibitor
"	8	:	ferritin
"	9	:	β-lactoglobulin
"	10	:	PMA-S
"	11	:	PMA-L

Fig. 25

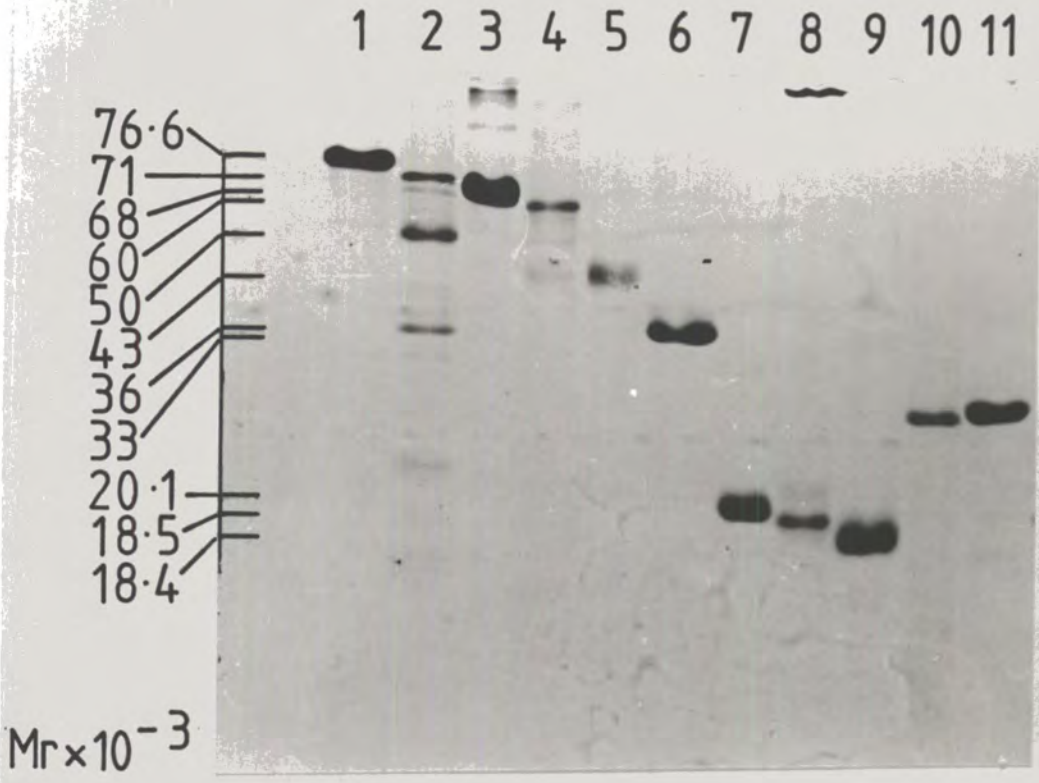
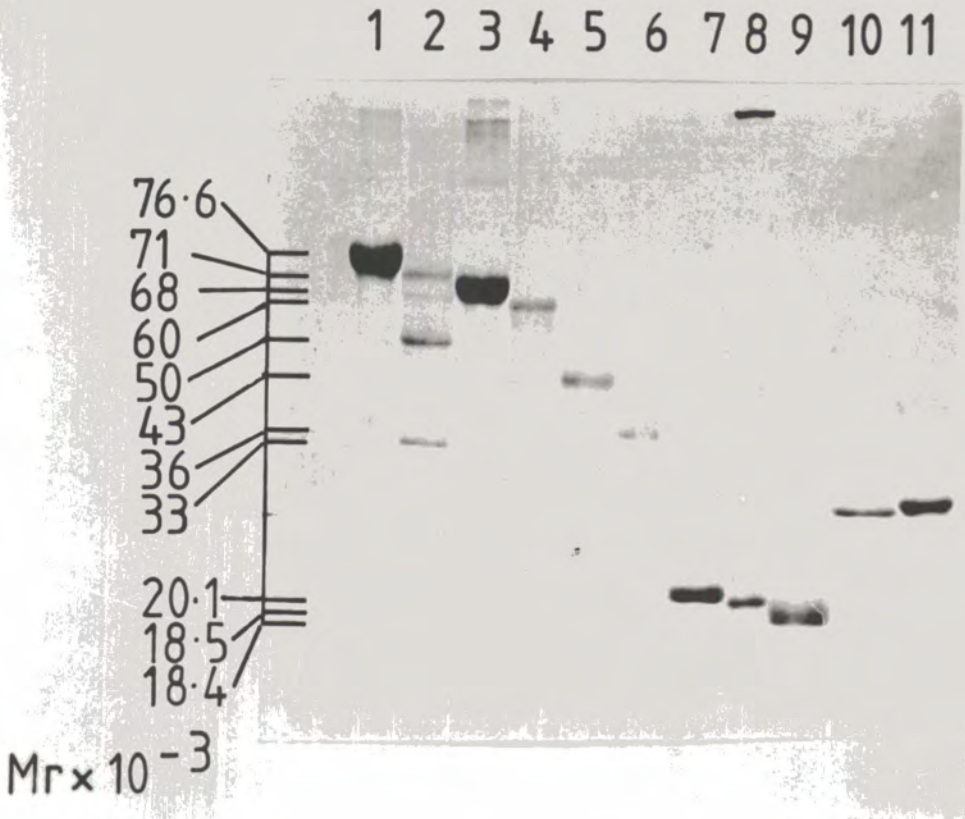

1 2 3 4 5 6 7 8 9 10 11
76·6
71
68
60
50
43
36
33
20·1
18·5
18·4
Mr×10⁻³

Fig. 26


1 2 3 4 5 6 7 8 9 10 11
76·6
71
68
60
50
43
36
33
20·1
18·5
18·4
Mr×10⁻³

(d) Subunit Mr of PLA:

After carboxymethylation the polypeptide molecular weight of PLA was estimated to have a value of 6,000 (± 300) on urea/SDS-15% polyacrylamide gels when compared to standard proteins, see Fig. 27 and Fig. 28. PLA thus seems likely to have a predominant molecular form containing two identical subunits (see discussion).

(e) Effect of 2-mercaptoethanol to calculate subunit Mr of PMA (L + S):

Total protein extract, total albumin extract, total major albumins, PMA-L, PMA-S, HMW-albumin and seed lectin were analysed by SDS-17% PAGE with and without 2-mercaptoethanol (2ME), on the same gel, (see Fig. 29). It was found that only PMA, PMA-L and PMA-S gave additional bands corresponding to a subunit Mr about 50,000 but there was no change in the band patterns of HMW-albumin and seed lectin under non-reducing conditions.

(f) Non-dissociating polyacrylamide gel electrophoresis:

Pisum sativum albumin proteins were analysed by polyacrylamide electrophoresis under non-dissociating conditions, pH 8.3. SDS and 2-mercaptoethanol were omitted from the gel, electrophoresis buffer and sample buffer, (see Fig. 30). It was found that purified preparations of PMA-L, PMA-S or total major albumins (PMA-L and PMA-S) showed variable amounts of additional bands of higher mobility, corresponding to an increase in negative charge on the proteins. This modification occurs in a discontinuous manner, i.e. there is a shift from one form to another with no intermediate forms being observed. This change probably occurs during prolonged protein preparation, i.e. purification, or on storage, since freshly prepared

FIG. 27.

Calibration curve relating the molecular weight of standard proteins with their electrophoretic mobilities relative to bromophenol blue (10), on Urea-SDS-15% polyacrylamide gels. Standard proteins in order of decreasing size, are : soyabean trypsin inhibitor, β-lactoglobulin, pea lectin (α-subunit), pea lectin (β-subunit) lysozyme, cytochrome c, and insulin α-chain (heavy chain).

Fig.27

Relative mobility
0
1
2
3
4
5
6
PLA
3·5
3·6
3·7
3·8
3·9
4·0
4·1
4·2
4·3
Log 10 mol. wt.

FIG. 28.

Determination of subunit Mr of PLA protein by Urea/SDS-15% polyacrylamide gel electrophoresis.

Standard proteins Track 1 - soyabean trypsin inhibitor

Track 2 - β-lactoglobulin

Track 3 - pea lectin

Track 4 - lysozyme

Track 5 - cytochrome c

Track 6 - insulin α-chain

Track 7 - carboxymethylated PLA

FIG. 29.

SDS-polyacrylamide gel electrophoresis of total protein extract, total albumin extract, total major albumins, PMA-S, PMA-L, HMW-albumin, isolated seed-lectin, both in reducing and non-reducing conditions.

Track No.	Sample	
1	total seed protein extract	(+ 2ME)
2	total seed albumin extract	(+ 2ME)
3	total major albumin protein	(+ 2ME)
4	PMA-S	(+ 2ME)
5	PMA-L	(+ 2ME)
6	HMW-albumin	(+ 2ME)
7	purified seed-lectin	(+ 2ME)
8	total major albumin	(- 2ME)
9	PMA-S	(- 2ME)
10	PMA-L	(- 2ME)
11	HMW-albumin	(- 2ME)
12	purified seed-lectin	(- 2ME)

Fig. 28

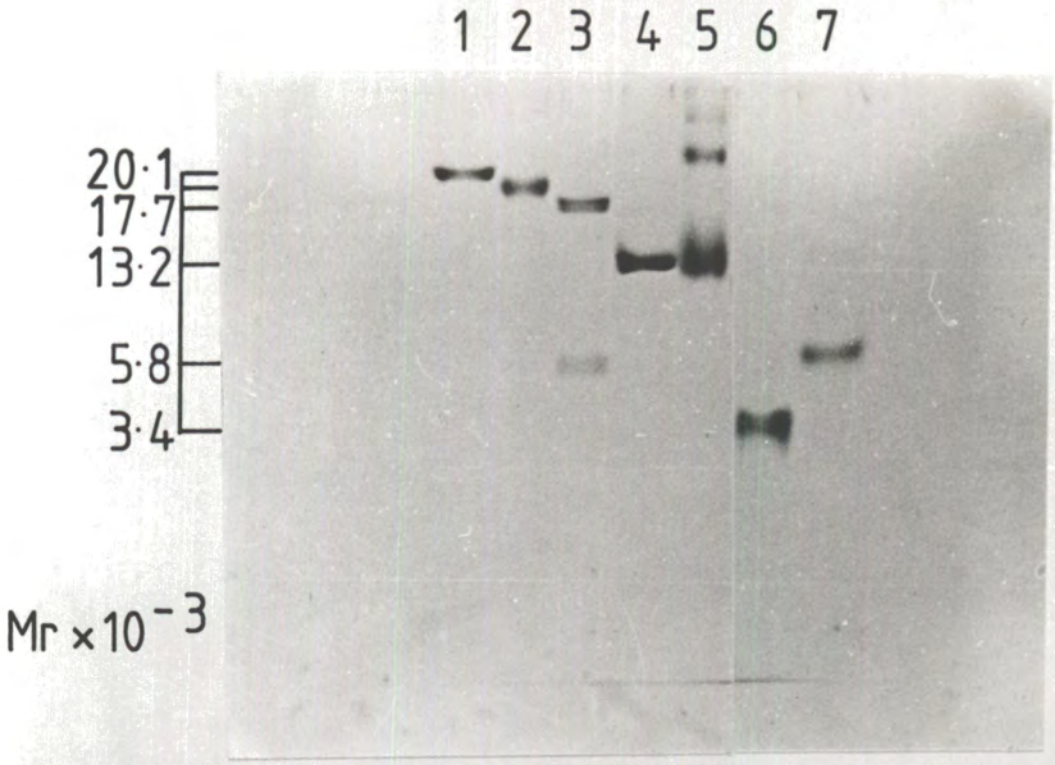
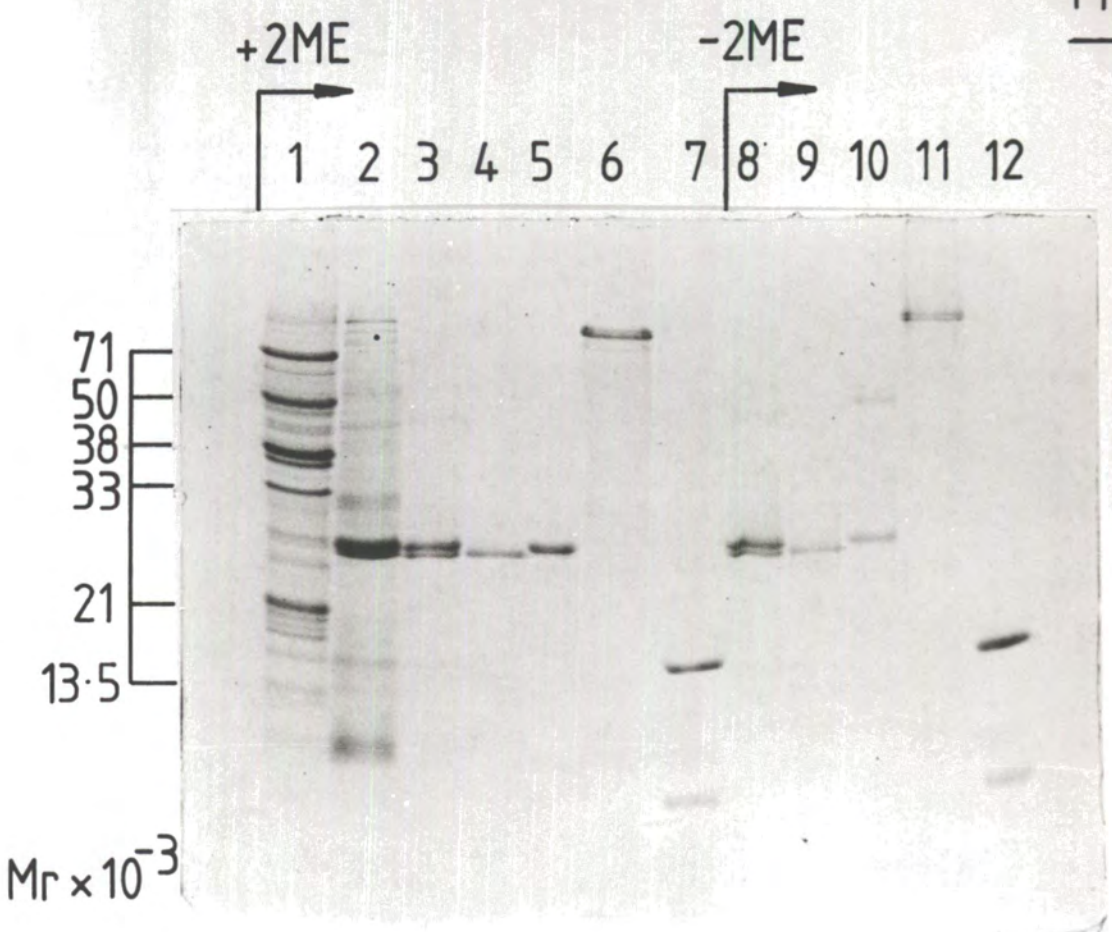

1 2 3 4 5 6 7
20·1
17·7
13·2
5·8
3·4
Mr x 10⁻³

Fig. 29


+2ME
-2ME
1 2 3 4 5 6 7 8 9 10 11 12
71
50
38
33
21
13·5
Mr x 10⁻³

PMA-L and PMA-S showed predominantly those bands present in fresh total albumin extracts and very little of the modified forms.

2. Subunit composition of albumin proteins

Subunit compositions of PMA-L and PMA-S were studied by various electrophoretic techniques as follows :-

(i) Two-dimensional polyacrylamide non-dissociating gel electrophoresis and SDS-polyacrylamide gel electrophoresis:

Two-dimensional electrophoresis of fresh total albumin extracts, was performed by combining polyacrylamide gel electrophoresis under non-dissociating conditions in the first dimension and then SDS-PAGE in the second dimension under reducing conditions, (see Fig. 31.) It was found that of the two major albumin charged components, the one of lower mobility contained only small subunits and the other of higher mobility contained only large subunits, corresponding to PMA-S and PMA-L respectively. Therefore, it was concluded that the major albumin proteins isolated contained only large (Mr 25,000) or small (Mr 24,000) subunits in the dimeric molecules.

(ii) Isoelectric focusing properties:

(a) Purified PMA-L and PMA-S were subjected to isoelectric focusing in 7.5% polyacrylamide gel slabs under both non-dissociating and dissociating conditions according to the method of Vesterberg (1975) and Gatehouse et al. (1980), using a Pharmacia flat bed apparatus (FBE 3000). Cytochrome c (pI = 10.65), myoglobin (pI = 7.3) and β-lactoglobulin (pI = 5.35) were used as standard proteins of known pI. PMA-L and PMA-S were focused to a series of very closely

FIG. 30.

Polyacrylamide gel electrophoresis of pea albumin proteins under non-dissociating and non-reducing conditions pH 8.3. The direction of electrophoresis is from the top to the bottom of the figure.

Track no.	Sample
1	total major albumin protein (PMA-L + PMA-S) stored sample. Arrowhead denote the three components (1, 2 and 3) given.
2	PMA-S, stored sample
3	PMA-S, fresh sample
4	PMA-L, stored sample
5	PMA-L, fresh sample
6	Total albumin proteins extracted from pea cotyledons, fresh sample. Arrowheads denote bands shown to be due to PMA-S and PMA-L respectively.

FIG. 31.

Two dimensional polyacrylamide gel electrophoresis of freshly prepared total albumin proteins extracted from Pisum sativum L.

First dimension: non-dissociating gel electrophoresis, non-reducing conditions.

Second dimension: dissociating (SDS) gel electrophoresis, reducing conditions.

Fig.30

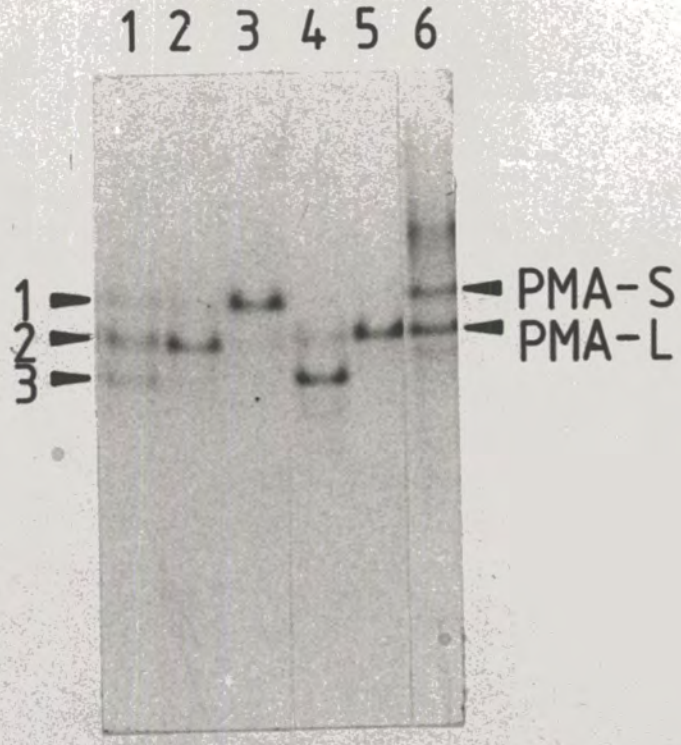

1 2 3 4 5 6
1
2
3
PMA-S
PMA-L

Fig.31

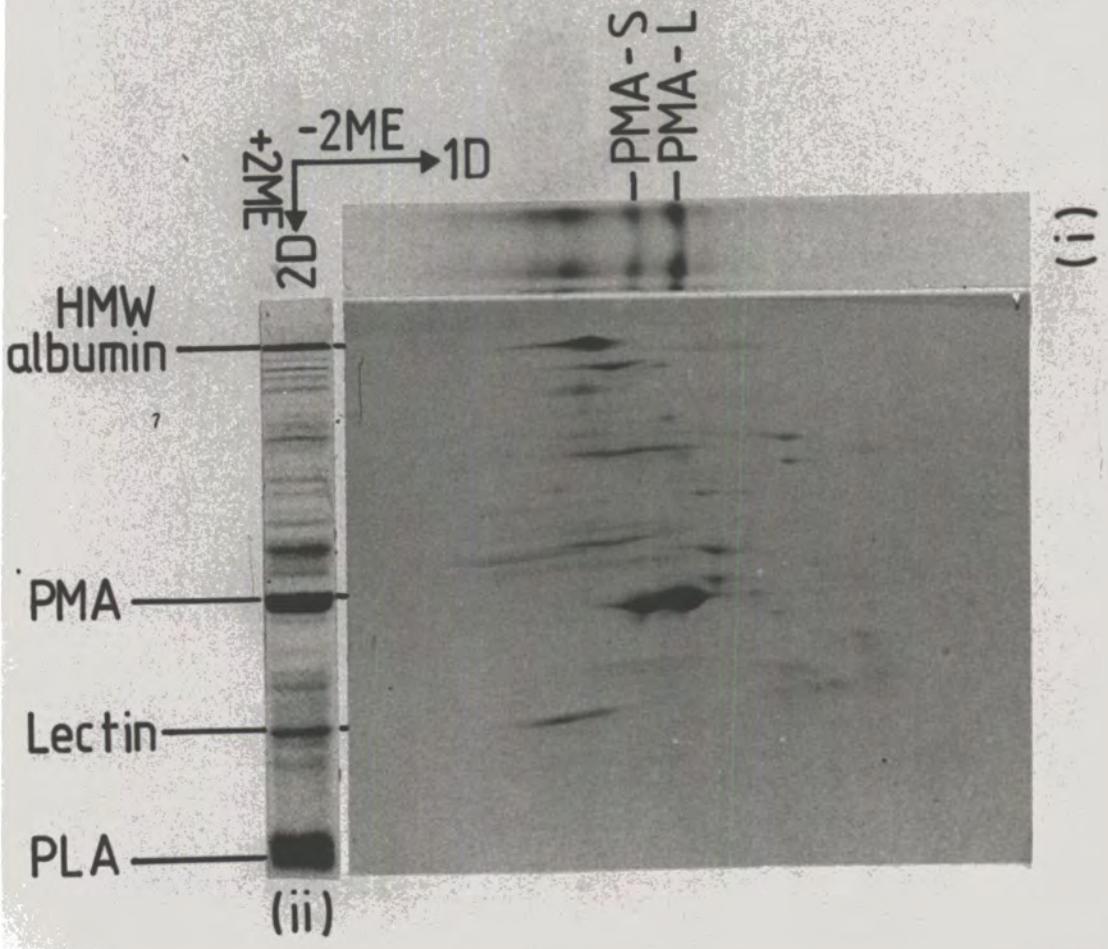

PMA-S
PMA-L
-2ME
1D
+2ME
2D
(i)
HMW
albumin
PMA
Lectin
PLA
(ii)

FIG. 32.

Isoelectric focusing of PMA proteins under dissociating conditions :-

Determination of pI values for PMA-L and PMA-S in 7.5% polyacrylamide gel slab containing 8M urea under non-reducing conditions.

Track No.	Sample
1	Myoglobin
2	Cytochrome c
3-4	β-lactoglobulin
5-6	PMA-L
7-8	PMA-S

FIG. 33.

Isoelectric focusing of PMA proteins under non-dissociating conditions :-

Determination of pI values for PMA-L and PMA-S in 7.5% polyacrylamide gel slab under non-reducing conditions.

Track No.	Sample
1	Myoglobin
2	Cytochrome c
3-4	β-lactoglobulin
5-6	PMA-L
7-8	PMA-S

Fig.32

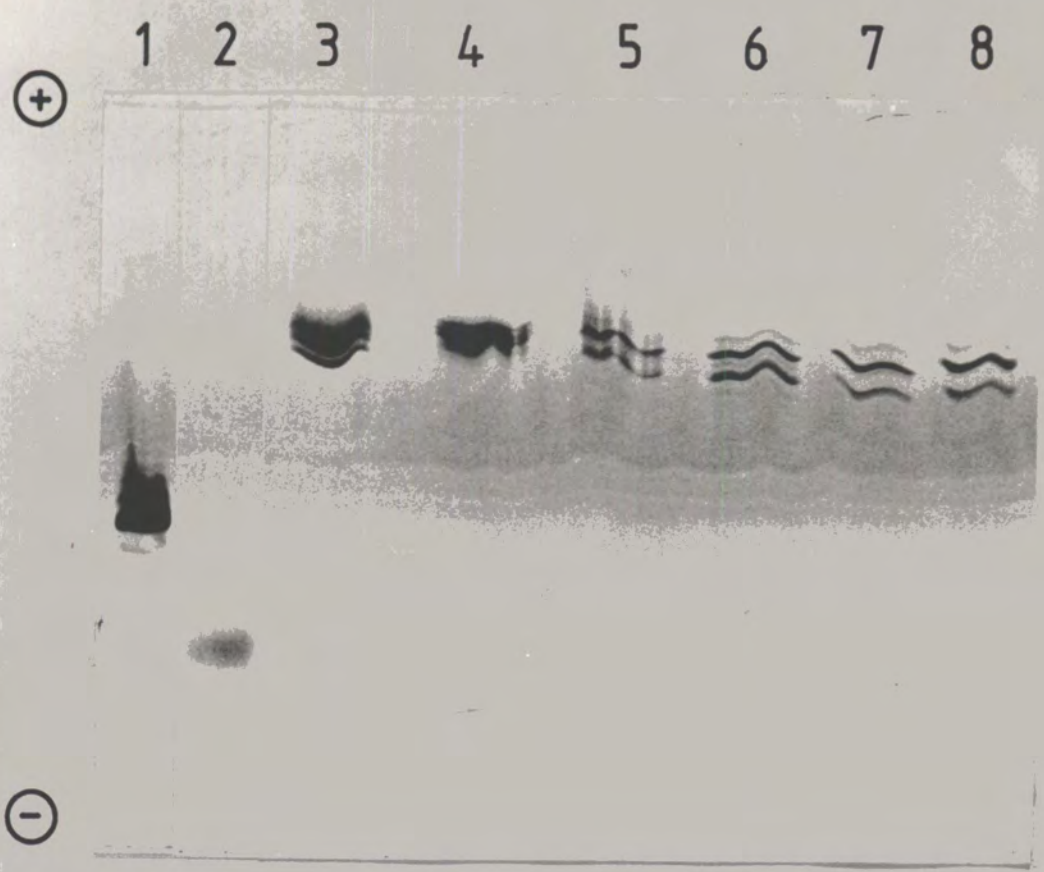
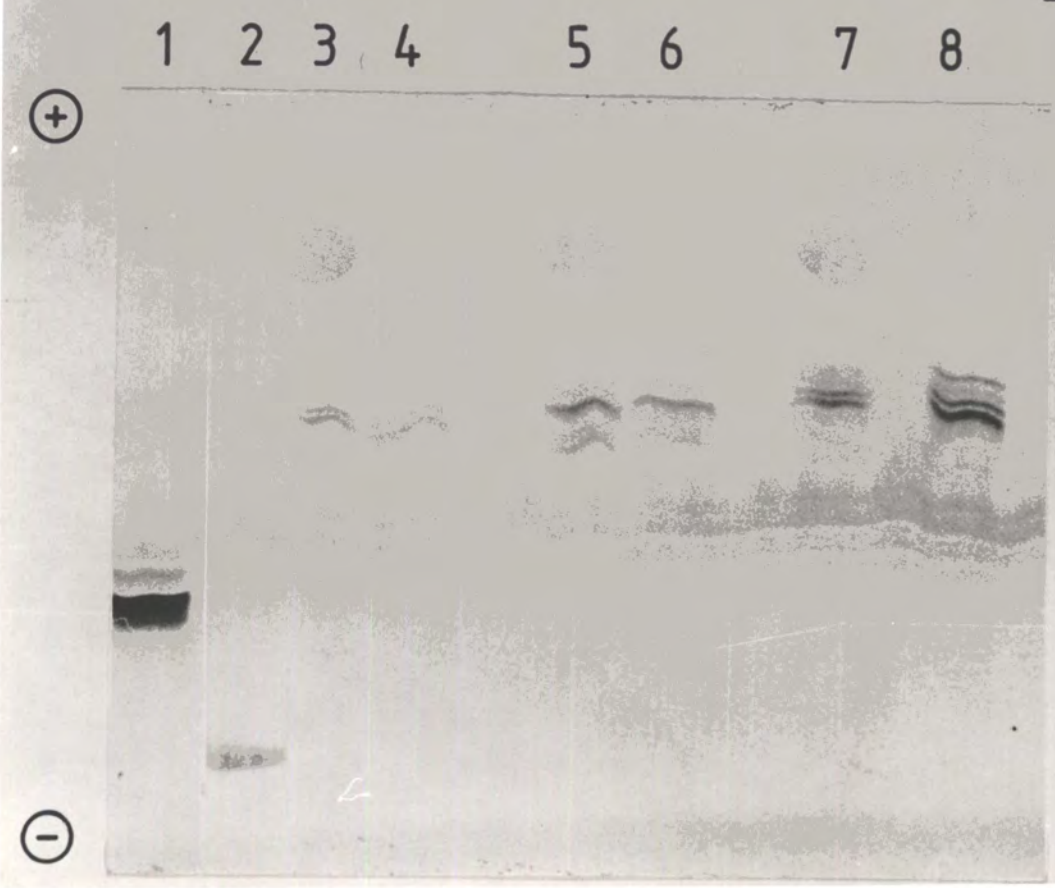


Fig.33



spaced slightly blurred bands (six to eight) indicating limited heterogeneity, in the average pI range 5.4 to 5.6, (see Fig. 32 and Fig. 33).

(b) Similarly, PLA also gave several closely spaced bands in the pI range 5.3 to 5.4, indicating limited heterogeneity.

3. Amino acid composition:

(a) For PMA-L and PMA-S proteins:

The amino acid compositions of PMA-L and PMA-S as given in Table 3, were very similar with higher levels of the sulphur amino acids, cysteine and methionine than the average for globulin proteins.

(b) For PLA protein :

The amino acid composition of PLA compared to the predicted composition from its amino acid sequence (see later), is given in Table 4, was also found to contain high levels of the sulphur containing amino acid cysteine; there was no methionine or valine in the 6,000 (± 300) Mr subunit (see discussion).

4. N- and C-terminal amino acids and N-terminal sequence determination:

(a) For PMA-L and PMA-S:

Both PMA-L and PMA-S were found to have threonine as the N-terminal, aspartic acid, asparagine and phenylalanine were released significantly as the C-terminal amino acids and both N-terminal amino acid sequences were found to be :-

Thr - Ala - Thr - Gly - Tyr - Ser - Asn -

TABLE 3

Amino acid and carbobydrate composition of PMA-L and PMA-S proteins compared with that of total seed albumins.

Amino acid data is expressed as mol/100 mol. of the total analysed and (in parentheses) as residues per subunit molecule of PMA-L and PMA-S. Residues per molecule were calculated from the average Mr for amino acids of 112 and subunit Mr values of 25,000 for PMA-L and 24,000 for PMA-S. Results are means of duplicate or triplicate determinations. Variation between determinations was less than 10%. Abbreviations used : CM - Cys, carboxymethyl cysteine; ND, not determined.

TABLE 3

AMINO ACID COMPOSITIONS OF THE MAJOR ALBUMIN PROTEINS OF PEA

	mole % (residues/mol)		Total Albumin Proteins
	PMA-S	PMA-L	
Asp	15.4 (33)	15.0 (34)	12.0
Thr*	7.5 (16)	7.2 (16)	4.5
Ser*	6.3 (14)	6.1 (14)	6.5
Glu	6.7 (14)	7.0 (16)	11.6
Gly	8.1 (17)	7.3 (16)	8.4
Ala	7.8 (17)	7.7 (17)	9.7
Val	5.5 (12)	5.6 (13)	7.5
Met	1.1 (2)	1.2 (3)	1.2
Ile	6.6 (14)	6.6 (15)	5.5
Leu	5.4 (12)	5.5 (12)	7.1
Tyr	6.6 (14)	7.2 (16)	3.9
Phe	7.8 (17)	8.4 (19)	5.1
Lys	7.5 (16)	7.9 (18)	7.9
His	1.3 (3)	1.1 (3)	2.1
Arg	4.9 (11)	5.3 (12)	4.6
CM-Cys	1.2 (3)	1.2 (3)	2.3
Pro	ND -	ND -	ND
Trp	ND -	ND -	ND

TABLE 4

Amino acid composition of PLA

Amino acid data is expressed as mol/100 mol of the total analysed and (in parentheses) as residues per subunit molecule of PLA. Residues per molecule were estimated from the average mol. wt. for amino acids of 112 and subunit mol. wt. value of 6,000 (± 300) for PLA. Results are means of duplicate or triplicate determinations. Variation between determinations was less than 10% abbreviations used : CM - Cys, carboxymethyl cysteine; ND, not determined.

TABLE 4

AMINO AND COMPOSITION OF PLA COMPARED TO PREDICTED COMPOSITION FROM THE AMINO ACID SEQUENCE

Amino acid	Residues	polypeptide (54 a.a.)
	Found	Predicted
CM - Cys	3.2	4
Asp	8.8	10
Thr*	0.8	1
Ser*	5.4	5
Glu	5.7	5
Gly	4.5	5
Ala	3.9	3
Val	0.3	0
Met	0.0	0
Ile	2.0	2
Leu	1.3	1
Tyr	2.1	2
Phe	3.5	4
Lys	5.0	5
His	2.0	2
Arg	1.4	1
Pro	ND	4
Tryp	ND	1

* Corrected for decomposition

5. Amino acid sequence determination:

(a) For PLA

(i) N- and C-terminal amino acids:

The N-terminal amino acid of carboxymethylated PLA was shown to be aspartic acid. The C-terminal amino acid determination on PLA using carboxypeptidase gave low yields of amino acids; however aspartic acid, asparagine and phenylalanine were released in significant amounts.

(ii) N-terminal sequence:

Carboxymethylated PLA was directly sequenced using the DABITC method and an N-terminal sequence 12 amino acids was determined,(see Fig. 34).

(iii) Production, separation and sequencing of peptides:

Carboxymethylated PLA was separately digested with trypsin, chymotrypsin and Staphylococcus aureus V8 protease and the resulting peptides were separated and sequenced.Complete sets of overlapping peptides were obtained which enabled the sequence of PLA to be deduced as shown in Fig. 34. Amino acid compositions of all peptides were determined semi-quantitatively as a check for the sequence assignments, and quantitative amino acid compositions were made on the C-terminal peptides and other peptides as necessary to confirm the squences (results not shown). The deduced sequence is 54 amino acids in length (Fig. 34) and contains two positions of heterogeneity : one at amino acid 45,both phe and val were found in peptides apparently not separable by h.p.l.c.,and another was at the C-terminal amino acid, where separate peptides containing asp and asn as C-terminal amino acids were isolated

FIG. 34.

Amino acid sequence of PLA. Solid lines indicate expected peptides, short arrows determined residues, and dotted arrows residues deduced from amino acid composition.

Fig. 34

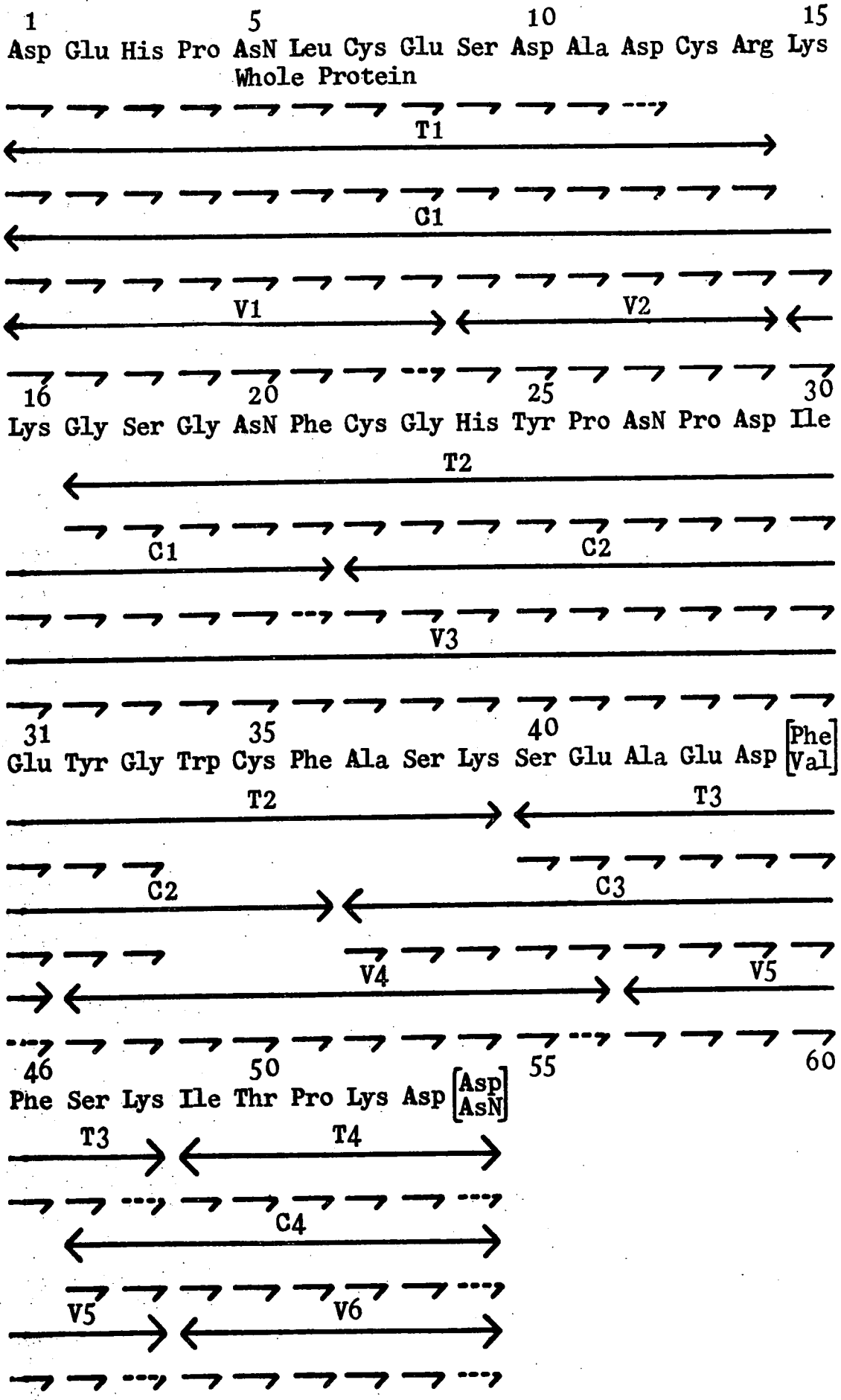

1
5
10
15
Asp Glu His Pro AsN Leu Cys Glu Ser Asp Ala Asp Cys Arg Lys
Whole Protein
T1
C1
V1
V2
16
20
25
30
Lys Gly Ser Gly AsN Phe Cys Gly His Tyr Pro AsN Pro Asp Ile
T2
C1
C2
V3
31
35
40
Glu Tyr Gly Trp Cys Phe Ala Ser Lys Ser Glu Ala Glu Asp [Phe Val]
T2
T3
C2
C3
V4
V5
46
50
55
60
Phe Ser Lys Ile Thr Pro Lys Asp [Asp AsN]
T3
T4
C4
V5
V6

FIG. 35.

Hydrophilicity profile and secondary structure prediction for PLA.

Fig.35

HYDROPHILICITY INDEX
+3·0
+2·0
+1·0
0
-1·0
-2·0
0
5
10
15
20
25
30
35
40
45
50
55
DEHPNLCESDADCRKKGSGNFCGHYPNPDIEYGWCFASKSEAEDFFSKITPKDN

in approximately equal proportions for all three enzyme digests (see discussion).

(iv) Secondary structure and hydrophilicity profile:

The amino acid sequence determined for PLA was used to produce a hydrophilicity profile for the protein, using the computer procedure of Hopp and Woods (1981) and to produce a secondary structure prediction using the computer programme of Garnier et al., (1978). The obtained results are shown in Fig. 35 (see discussion for detail).

6. Cyanogen bromide cleavage:

(a) For PMA-L and PMA-S

Analysis on SDS gels showed that cyanogen bromide cleaved both large and small subunits into three distinct fragments each showing the presence of at least two methionine residues per subunit, (see Fig. 36). The three polypeptide fragments obtained after 3 h incubation were unchanged on prolonged reaction times (6, 24, 48 h).

Using highly resolving urea-SDS-polyacrylamide gel electrophoresis comparison of these polypeptide fragments with those obtained from the total major albumin protein confirmed that one fragment (Mr 11,500) was identical in both large and small albumin subunits, whereas the other two fragments were of slightly higher molecular weight in the large major albumin subunit (Mr 8,400 and 7,200 in PMA-L and 7,600 and 6,600 in PMA-S) when compared to standard proteins, (see Fig. 37 and Fig. 38).

(b) For PLA

Cyanogen bromide cleavage of carboxymethylated PLA protein did not show the presence of any fragment for methionine residue per subunit, as there was no change in mobility of PLA on urea/SDS-PAGE after reaction with CNBr for up to 48 h, (see Fig. 39). Therefore, it was concluded that PLA protein does not contain any internal methionine residue.

FIG. 36.

SDS-Urea-polyacrylamide gel electrophoresis analysis of cyanogen bromide (CNBr) cleavage fragments from large, small and total major albumin proteins.

Track No.	Sample
1	- untreated PMA-L
2	- untreated total major albumin
3	- untreated PMA-S
4	- CNBr cleaved PMA-L after 6 h treatment
5	- CNBr cleaved total major albumin after 6 h treatment
6	- CNBr cleaved PMA-S after 6 h treatment
7	- CNBr cleaved PMA-L after 24 h treatment
8	- CNBr cleaved total major albumin after 24 h treatment

FIG. 37.

Determination of the subunit molecular weights for cyanogen bromide fragments of PMA-L and PMA-S after 24 h treatment by Urea-SDS-15% polyacrylamide gel electrophoresis.

Standard proteins	Track 1 - soyabean trypsin inhibitor
	Track 2 - β-lactoglobulin
	Track 3 - myoglobin
	Track 4 - lysozyme
	Track 5 - cytochrome c
	Track 6 - insulin
	Track 7 - CNBr cleaved PMA-L (24 h)
	Track 8 - CNBr cleaved PMA (24 h)
	Track 9 - CNBr cleaved PMA-S (24 h)

FIG. 39.

Urea/SDS-polyacrylamide gel electrophoresis analysis of CNBr cleavage fragment(s) from PLA protein.

Track No.	Sample
1	- untreated PLA
2	- carboxymethylated PLA before CNBr reaction
3	- PLA after 3 h CNBr reaction
4	- PLA after 6 h CNBr reaction
5	- PLA after 24 h CNBr reaction
6	- PLA after 48 h CNBr reaction

Fig. 36

Fig. 39

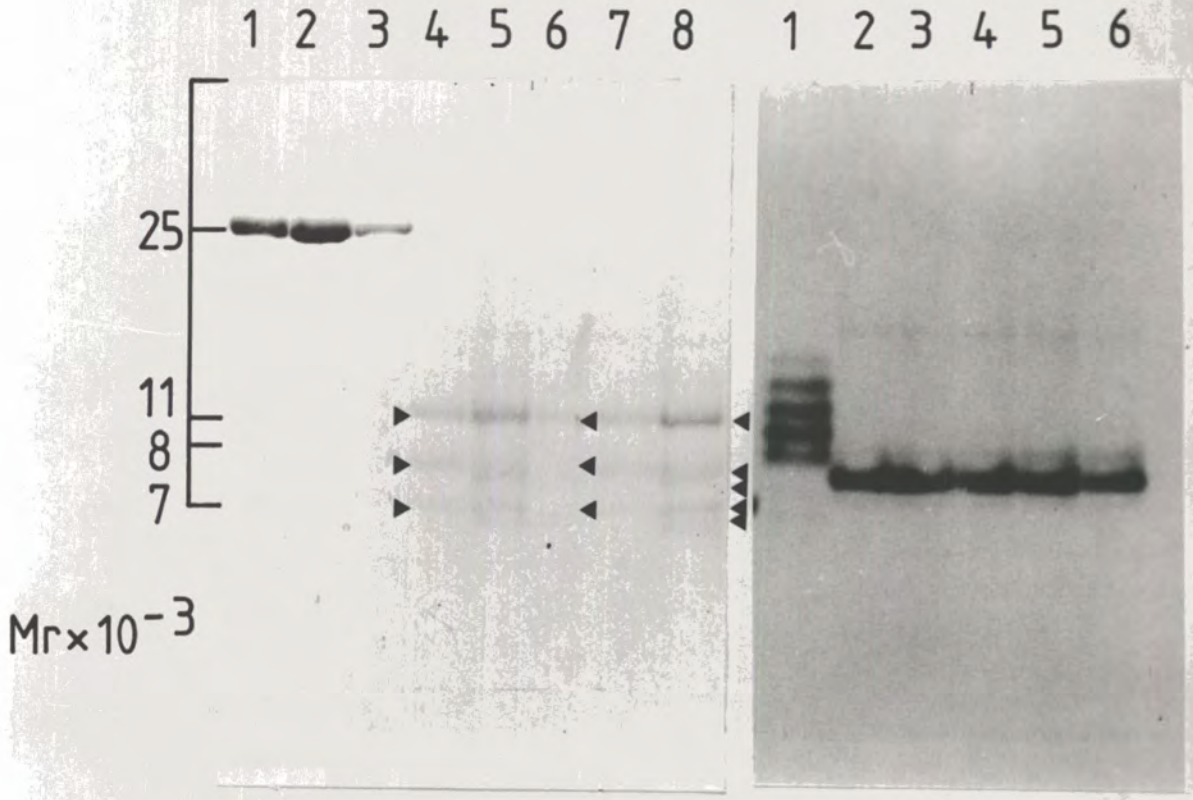


Fig. 37

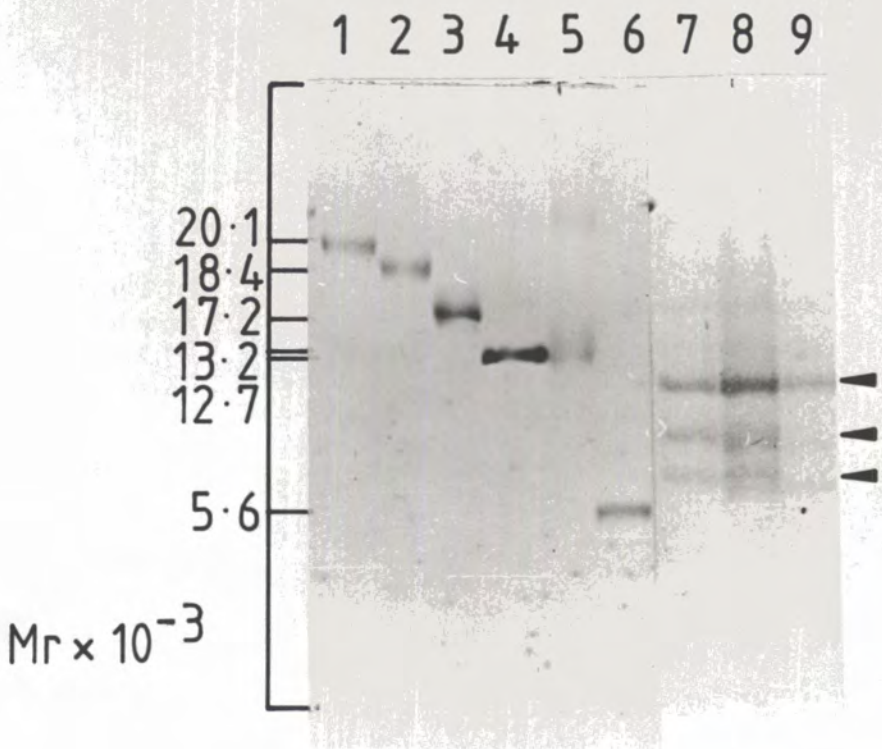
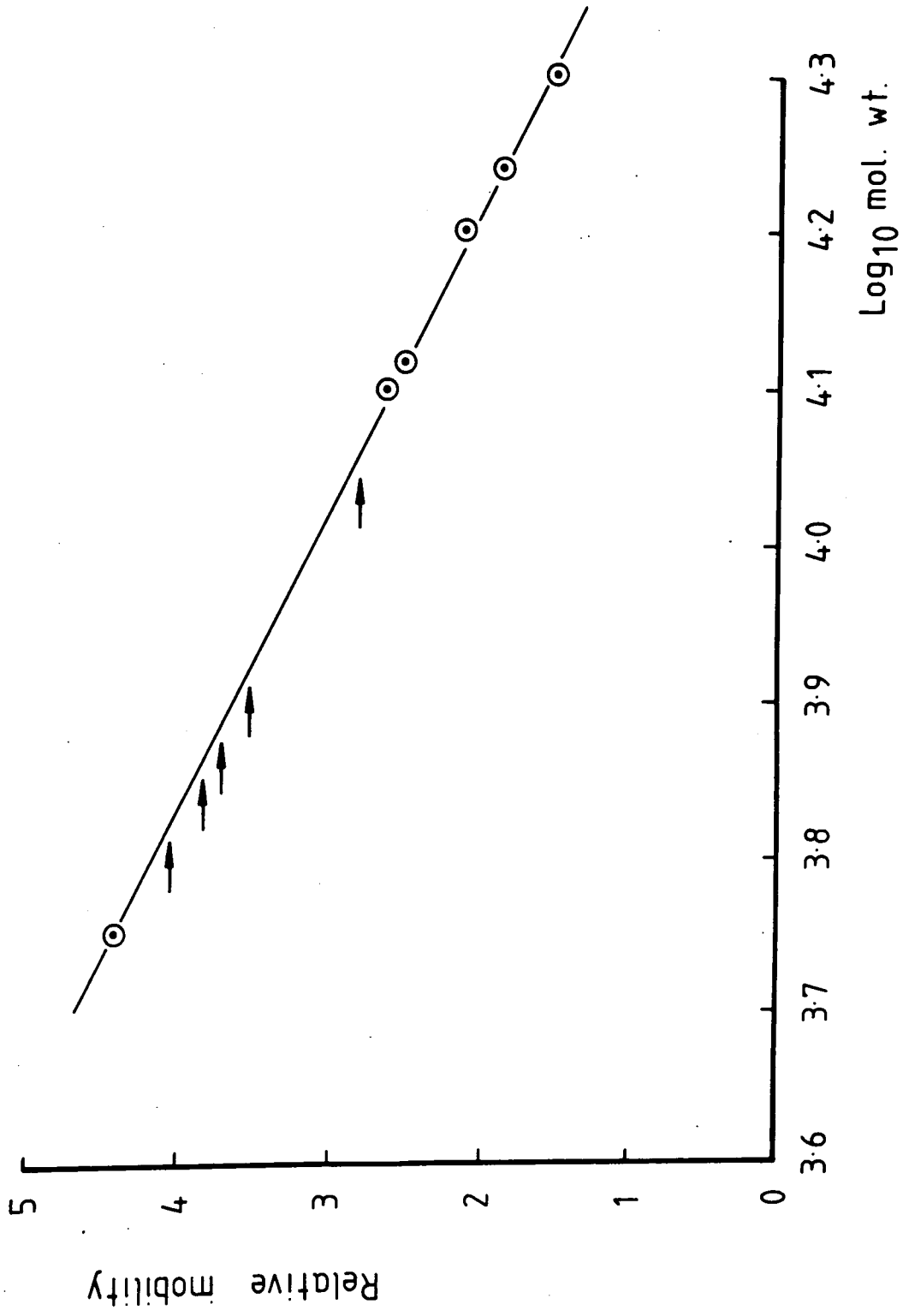


FIG. 38.

Calibration curve relating the log molecular weight of standard proteins with their electrophoretic mobilities relative to bromophenol blue (10), on 7M Urea-SDS-15% polyacrylamide gels. Standard proteins in order of decreasing size, are : soyabean trypsin inhibitor, β-lactoglobulin, myoglobin, lysozyme, cytochrome c and insulin.

CNBr polypeptide fragments = →

Fig. 38



7. Tryptic peptide maps:

Tryptic peptide maps as estimated by h.p.l.c. reverse phase chromatography elution profile were found to be similar for PMA-L and PMA-S proteins.

8. Carbohydrate analysis:

It was found that PMA-L, PMA-S and PLA contained 0.48%, 0.45% and 0.56% (w/w) sugar respectively, when assayed by the phenol-sulphuric acid method, calculated from the standard calibration curve of glucose, (see Fig. 40). These amounts were comparable with those found in a known non-glycoprotein, bovine serum albumin and were significantly less than one sugar residue per 25,000 Mr for PMA-L, 24,000 Mr for PMA-S and 6,000 (± 300) for PLA subunits.

9. Glycoprotein analysis:

It was observed that no bands of PMA-L, PMA-S and PLA stained for carbohydrate (Fig. 41), so it was concluded that none of these albumins is a glycoprotein.

10. Reaction with concanavalin A:

Concanavalin A did not react with PMA-L and PMA-S in a double immunodiffusion experiment (Gleeson and Jermym, 1977) as described by Croy et al. (1980). So it was concluded that pea seed major albumin proteins contained no carbohydrate residues.

11. Antigenic properties of PLA:

PLA did not react with antibodies raised against pea seed storage proteins, major albumin (PMA) or lectin, either by double diffusion tests or after electrophoretic transfer from SDS-PAGE ("Western" blotting).

12. Immunological cross-reactions:

Total major albumin, PMA-L and PMA-S were allowed to diffuse

FIG. 40.

Calibration curve for phenol-sulphuric acid carbohydrate assay using glucose as standard. Assays for PMA and PLA (1 mg) shown in diagram. ● = assay for HMW-albumin

FIG. 41.

Diagram of SDS-polyacrylamide gel electrophoresis of various proteins stained with Coomassie Blue and with dansyl hydrazine to detect glycoproteins. Arrowed bands were stained by dansyl hydrazine.

Track no.	Sample
1	Phaseolus globulin (+ve control)
2	Phaseolus albumin (+ve control)
3	Ovalbumin (+ve control)
4	Low molecular weight albumin
5	Legumin basic (-ve control)
6	Myoglobin (-ve control)
7	High molecular weight albumin
8	Total major albumin protein
9	PMA-S
10	PMA-L

Fig. 40

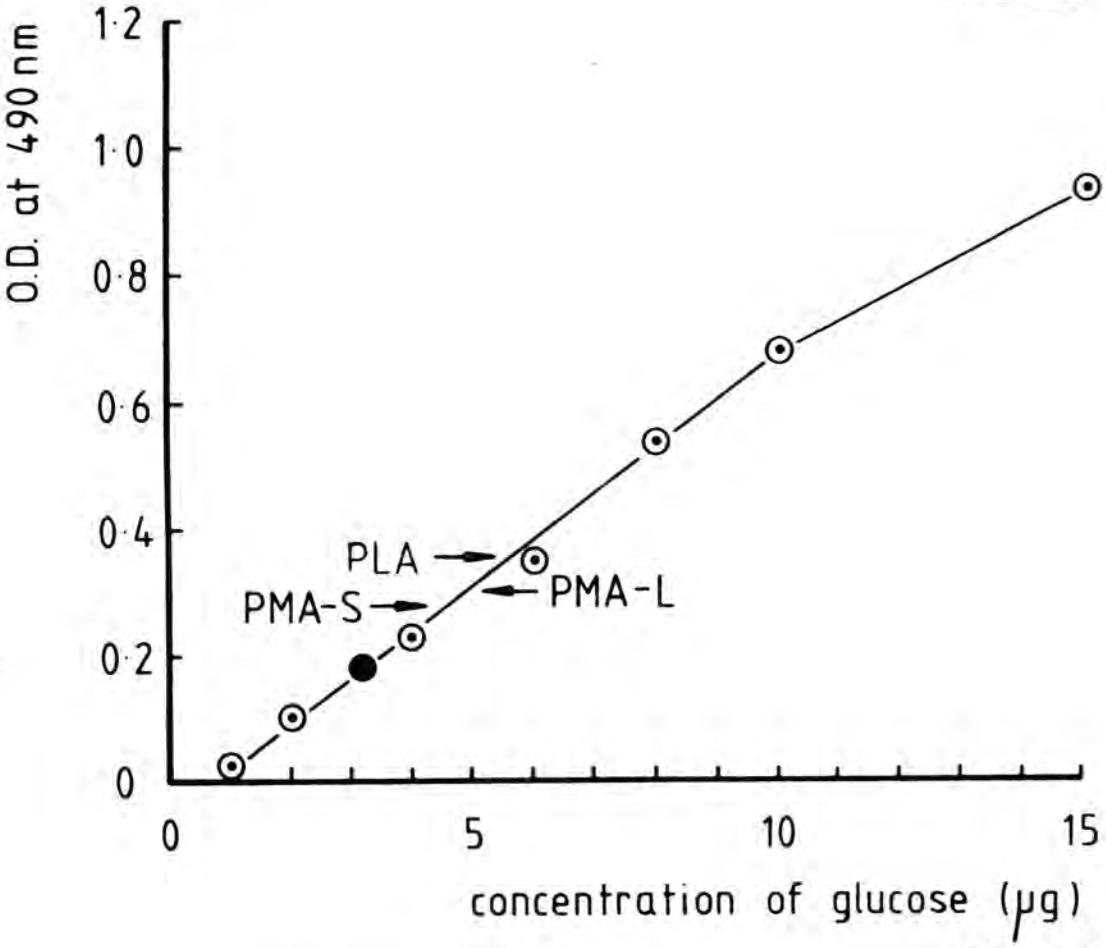
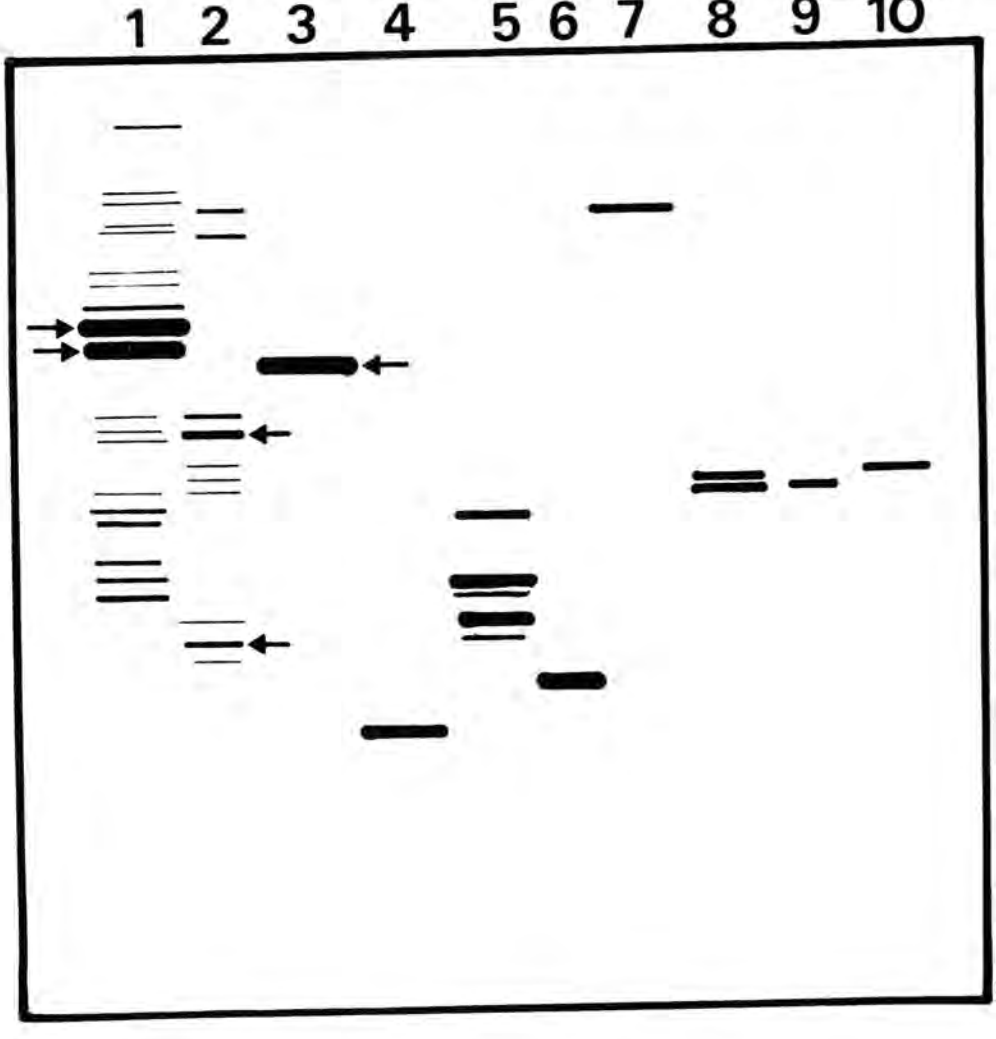

O.D. at 490 nm
1·2
1·0
0·8
0·6
0·4
0·2
0
0
5
10
15
PLA
PMA-S
PMA-L
concentration of glucose (µg)

Fig. 41


1 2 3 4 5 6 7 8 9 10

against anti-serum prepared against total major albumin proteins (PMA-L + PMA-S) in an Ouchterlony double-immunodiffusion test as described by Croy et al. (1979). Total major albumin, PMA-L and PMA-S all gave a reaction of complete identity and gave no cross-reactions with other seed proteins or with antibodies raised against other albumin proteins, e.g. anti-lectin, or globulin proteins, (see Fig. 42a).

The precipitin lines produced by total major albumin, PMA-L and PMA-S were excised and analysed by 17% SDS-polyacrylamide gel electrophoresis using a total protein extract and total albumin extract (20 mM Na-acetate buffer, pH 5.0) as standard protein preparations. Non-reducing conditions were used in order to prevent IgG heavy and light subunits interfering with the band patterns of total major albumin, PMA-L and PMA-S proteins and showed major polypeptides of Mr about 22-24,000, (see Fig. 43).

13. Haemagglutination assays:

No haemagglutinating activity was found associated with PMA-L and PMA-S proteins when assayed with 2% erythrocytes in micro-titration plates,(see Fig. 44).

14. Functional properties:

(a) Potential enzyme activities

Neither PMA-L nor PMA-S showed activity in any of the enzyme assays tested, i.e. α-, β-D-glucosidase, α-,β-D-galactosidase, α-D-mannosidase and α-amylase.

(b) Inhibitory activities

(i) Amylase inhibitory activity

None of PMA-L, PMA-S and PLA showed any significant

FIG. 42.

Immunodiffusion of a) purified pea albumin proteins and b) total albumin extracts.

a) Abbreviations used: A = anti-(major albumin) antibodies; Ale = anti-lectin antibodies; L = large major albumin protein; S = small major albumin protein; T = total major albumin proteins; Le = lectin.

b) A = anti-(major albumin) antibodies; other wells contained total albumin extracts from: (1,3,5) Pisum sativum (pea), (2) Lens culinaris (lentil), (4) Lathyrus odoratus (sweet pea), and (6) Cicer arietinum (chick pea).

FIG. 43.

SDS-polyacrylamide gel electrophoresis (non-reducing conditions) of precipitin arcs seen in Fig. 42a.

Track No.	Sample
1	Total seed protein extract (standard preparation)
2	Total seed albumin extract (standard preparation)
3	Total major albumin proteins
4	PMA-S
5	PMA-L
6	PMA-S precipitin arc
7	PMA precipitin arc
8	PMA-L precipitin arc

Fig. 42

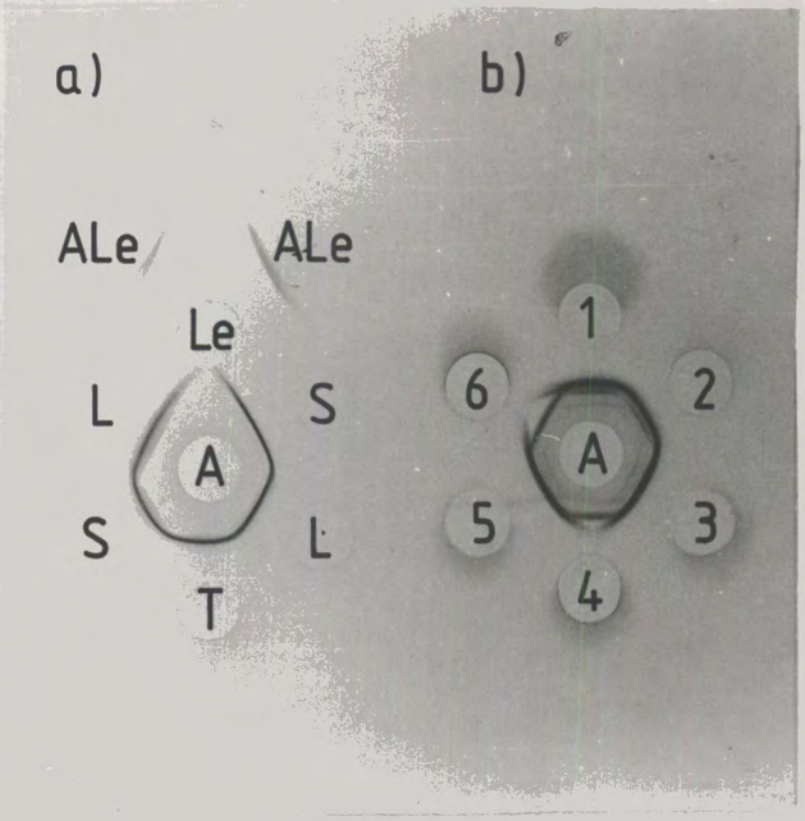


Fig. 43

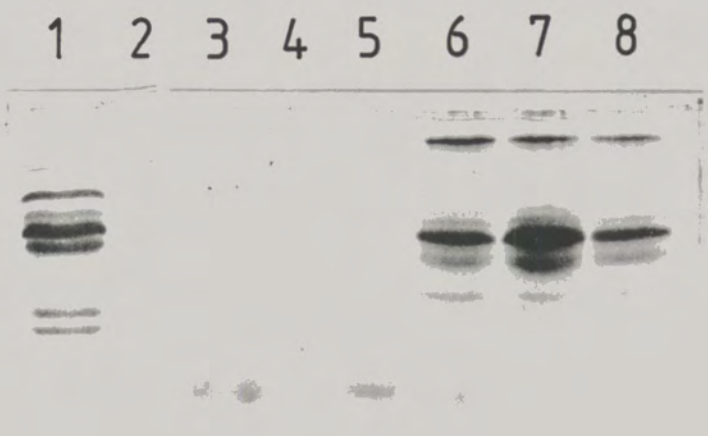
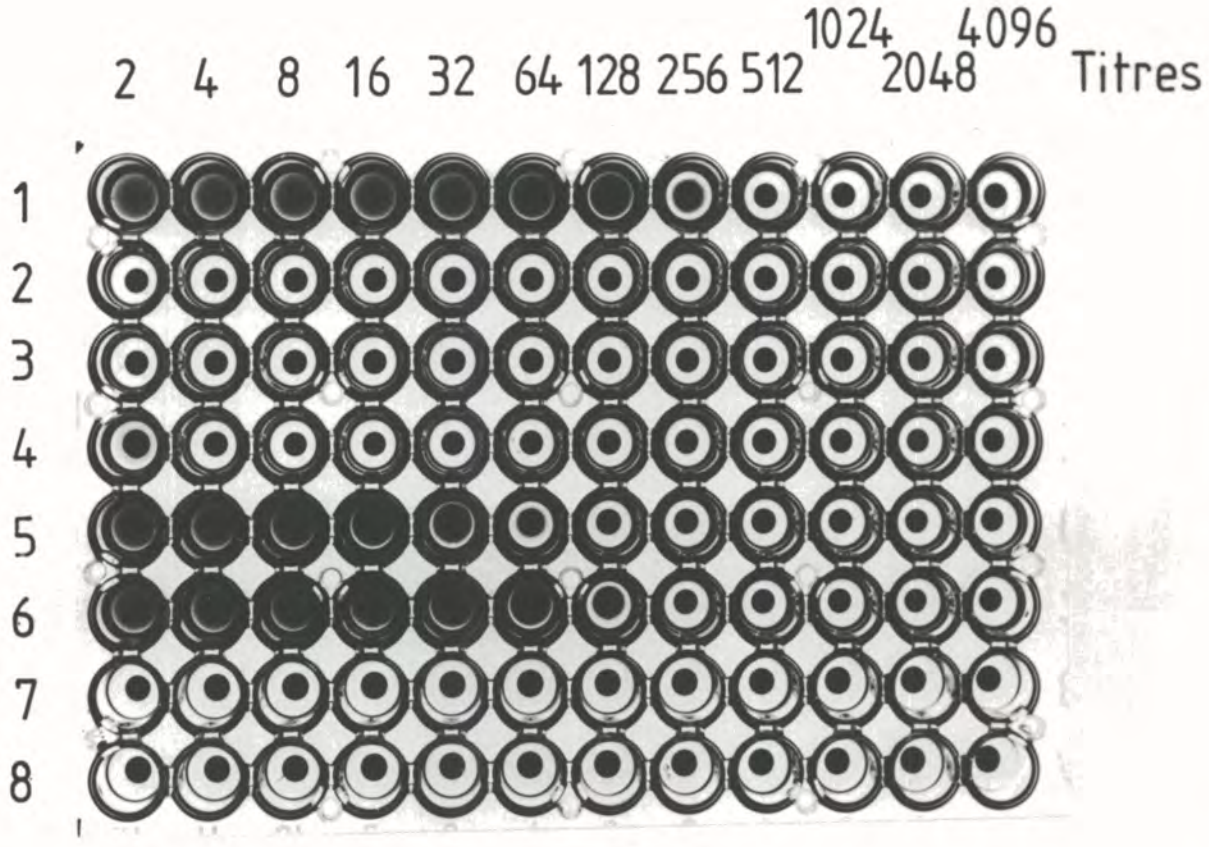


FIG. 44.

Estimation of haemagglutinating activity of purified albumin proteins and isolated pea seed lectins with serial dilution from 0.5 mg/ml to x 12 obtained in microtitration plates.

Row no.	Sample
1	isolated seed lectin (+ve control)
2	HMW-albumin
3	PMA-S
4	PMA-L
5	concanavalin A (+ve control)
6	seed lectin in PBS (containing 0.1M glucose)
7-8	2% erythrocytes in PBS (only)

Fig. 44



inhibitory activity when tested against pacreatic α-amylase.

(ii) Trypsin and chymotrypsin inhibitory activities

Purified PMA-S and PLA did not show trypsin inhibition or chymotrypsin inhibition, whereas PMA-L was found to exhibit 30% (against 50 µg sample) inhibition in a trypsin-inhibition assay and 61.0% (against 50 µg sample) inhibition in a chymotrypsin-inhibition assay relative to corresponding controls. To clarify these results a total albumin extract of pea seeds was fractionated by chromatography on DEAE-cellulose to estimate the distribution of protease inhibitors in the eluted fractions relative to PMA-L, PMA-S and PLA. Eluted fractions were assayed for inhibitory activity against trypsin and chymotrypsin (relative to their respective controls), and the pooled fractions according to uv-absorbance (280 nm) were assayed for protein composition by SDS-polyacrylamide gels electrophoresis (see Fig. 45 and Fig. 46). Three major peaks of protease inhibitor activity were eluted from the ion-exchange column; the first one did not represent any of the PMA proteins, the second one seemed to be eluted at the trailing edge of peak 7, which represented PMA-L, in the last one, eluted immediately before the PLA peak (see discussion).

15. Insect bio-assays:

The effects of various protein fractions from Pisum sativum on development of larvae of Callosobruchus maculatus (a major storage pest of many legumes) were determined, see Table 5. These results show that whereas the total albumin protein fraction when incorporated at a rate of 5%, had a significant effect upon larvae development, the effect of the total major albumin at the same concentration, was only moderate; PLA had very little effect upon development. However, when lectin was incorporated into the diet at a rate of 2.5%, 100% mortality was observed. This fraction clearly accounts for the observed toxicity of the total albumin protein.

FIG. 45.

UV-absorbance (280 nm) elution profile of total pea albumin proteins from DEAE-cellulose column. Bar and cross-hatching indicate fraction containing PLA. Inhibitory activity of an aliquot of each fraction (50 µl) against trypsin (50 µg, dash line) and chymotrypsin (50 µg, solid line) is also shown.

FIG. 46.

SDS-17% polyacrylamide gel electrophoresis of the pooled fractions according to uv-absorbance (280 nm) indicated in Fig. 45.

Track No.	Sample
1	Pooled fractions 6-10 (peak No.1)
2	Pooled fractions 12-16 (peak No.2)
3	Pooled fractions 20-22 (peak No.3)
4	Pooled fractions 23-25 (peak No.4)
5	Pooled fractions 26-28 (peak No.5)
6	Pooled fractions 29-30 (peak No.6)
7	Pooled fractions 31-34 (peak No.7)
8	Pooled fractions 36-38 (peak No.8)
9	Pooled fractions 39-42 (peak No.9)
10	Pooled fractions 45-48 (peak No.10)
11	Pooled fractions 52-54 (peak No.11)
12	Pooled fractions 55-62 (peak No.12)

Fig.45

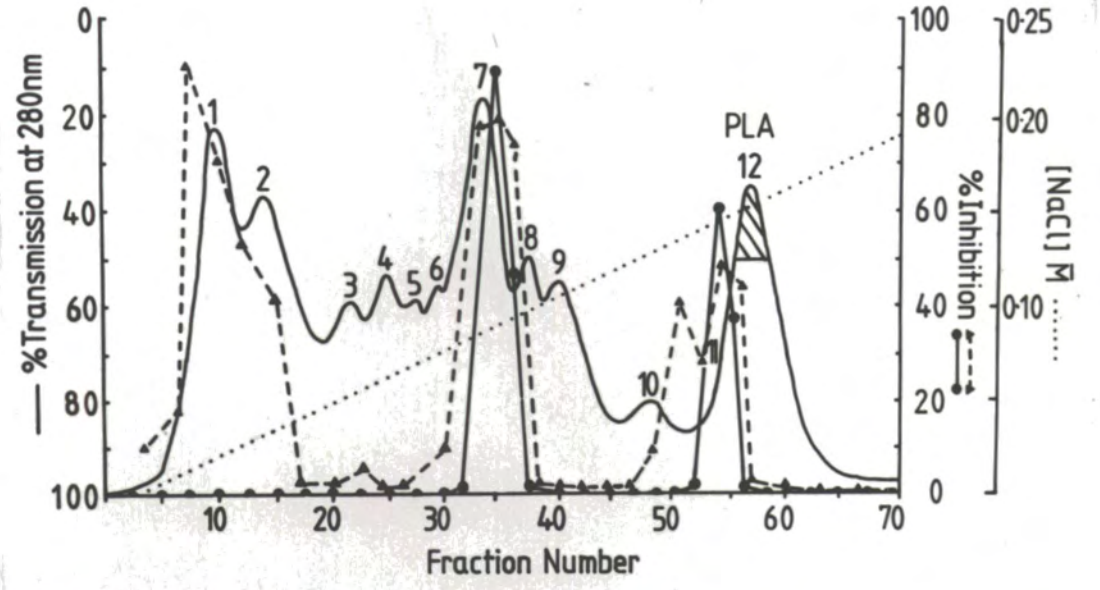

— %Transmission at 280nm
%Inhibition
[NaCl] M
PLA
Fraction Number
0
20
40
60
80
100
10
20
30
40
50
60
70
0·25
0·20
0·10
1
2
3
4
5
6
7
8
9
10
11
12

Fig. 46

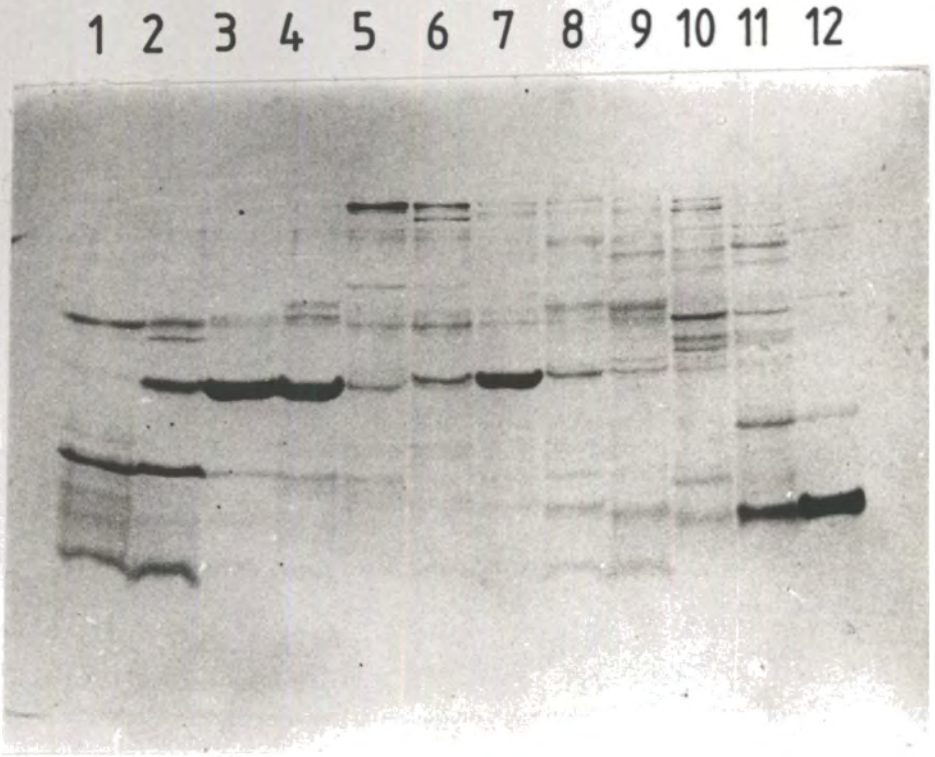

1 2 3 4 5 6 7 8 9 10 11 12

TABLE 5

Effects of various pea protein fractions on development of larvae of *Callosobruchus maculatus*.

TABLE 5

Protein fraction	% fraction (incorporated) into diet	% Adult emergence relative to control
Control	0	100
Total albumin protein	5	0
Total major albumin	5	58.5
Low mol. wt. albumin (PLA)	2.5	86.6
Pea lectin	2.5	0

C. Species distribution of pea major albumin type proteins

(a) Double immuno-diffusion experiments:

Total albumin extracts from a variety of different legume species were screened for PMA-type proteins by double immuno-diffusion experiments against anti-PMA antibodies. Lathyrus odoratus (sweet pea), Lens culinaris (lentil) and Cicer arietinum (chick-pea) were the only three species observed to show specific immuno-precipitations as judged by reactions of identity (lentil), or partial identity with (chickpea and sweet pea) PMA, (see Fig. 42b). No reaction was obtained with extracts from Vicia faba (broad bean); Glycine max (soyabean); Dolichos lablab; Cajanus cajan (pigeon pea); Phaseolus vulgaris (french bean); Phaseolus coccineus (runner bean); Vigna unguiculata (cowpea) and Vigna mungo (mungbean).

(b) Western blotting experiment:

Examination of the three (lentil, chick-pea and sweet pea) immunoprecipitating albumin extracts analysed on SDS-polyacrylamide gels showed major polypeptides of Mr ∿ 22000 - 24000, which when examined by "Western blotting" showed specific anti-PMA binding, confirming their homology to the pea major albumin.

D. Demonstration of pea major albumin type proteins in different parts of plant

Total albumin extracts from different parts of pea seeds, i.e. testa, whole cotyledon, outer-cotyledon, inner-cotyledon, radicles, hypo-cotyls, leaves, stems and roots, were also screened for PMA-type proteins by analysis on SDS-polyacrylamide gels, see Fig. 47, whole cotyledon, outer cotyledon, inner cotyledon, were found to contain the highest concentration of major polypeptides of Mr ∿ 25,000. Radicles and hypo-cotyls showed the least, when electro-blotted onto

nitro-cellulose and probed with anti-PMA IgG followed by peroxidase coupled anti-rabbit IgG, confirming their homology to the pea major albumin proteins, (see Fig.48). Testa, leaves, roots and stems did not contain any PMA type proteins, although apart from the testa, they have been shown to contain variable amounts of different albumin polypeptides, (Fig. 47).

E. Screening for variations of pea major albumin and lectin type proteins

Total albumin extracts from single cotyledons of about 200 different pea lines were analysed on SDS-polyacrylamide gel electrophoresis to demonstrate the variations of pea major albumin (PMA) or PMA (L + S), PMA-L, PMA-S, low molecular weight albumin (PLA), high molecular weight albumin (HMW-albumin) and lectin type proteins. The observed variations of these proteins in their electrophoretic banding patterns are illustrated in Figs. 49a and 49b. It was found that the bands corresponding to PMA (L + S) of subunit molecular weight 25000 were absent in line 1382; PMA-S representing band of subunit molecular weight 24000 was missing or only weakly present in line 21; the band for PMA-L of subunit molecular weight 25000 was found to be absent in lines 808, 809, 936, 1256, 1293 and 1376. No lectin bands corresponding to subunit molecular weight 17,700 and 5,800 were present in lines 1293 and 1376. The high molecular weight albumin of subunit molecular weight $\sim$ 100,000 was also found to be absent in line 59, whereas no variation of PLA representing band subunit molecular weight 6000 ($\pm$ 300) was observed in the pea lines analysed.

In addition to the standard PMA (L + S) electrophoretic banding patterns, there was one additional band of subunit molecular weight $\sim$ 23000 very close to the PMA-S band which was found to be present in lines 360, 721, 799, 806, 851, 1298, 1463, 1469, 1495, 1504, 1533, 5106,

FIG. 47.

SDS-polyacrylamide gel electrophoresis of total albumin extracts from different parts of plant.

Track no.	Sample
1	total major albumin
2	PLA
3	total albumin extract from testa
4	total albumin extract from cotyledons
5	total albumin extract from outer cotyledons
6	total albumin extract from inner cotyledons
7	total albumin extract from radicles
8	total albumin extract from hypo-cotyls
9	total albumin extract from leaves
10	total albumin extract from stems
11	total albumin extract from roots

FIG. 48.

Protein subunits of PMA type from different parts of plant transferred from SDS-polyacrylamide gels onto nitrocellulose filter paper by 'Western blotting' and detected by reaction with anti-PMA antibodies.

Track no.	Sample
1	total albumin extract from roots
2	total major albumin
3	PLA
4	total albumin extract from testa
5	total albumin extract from cotyledons
6	total albumin extract from outer cotyledons
7	total albumin extract from inner cotyledons
8	total albumin extract from radicles
9	total albumin extract from hypo-cotyls
10	total albumin extract from leaves
11	total albumin extract from stems

Fig. 47

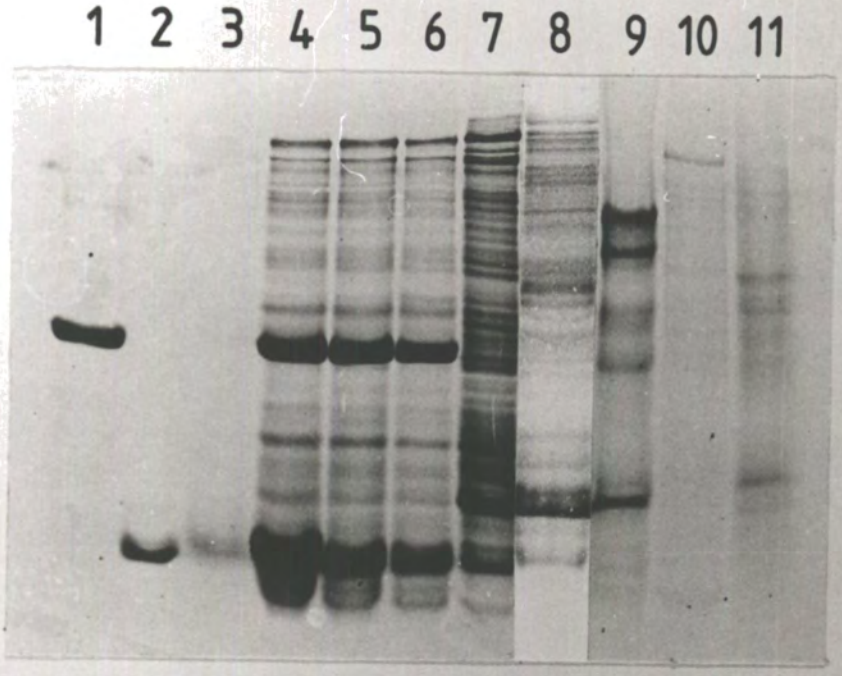
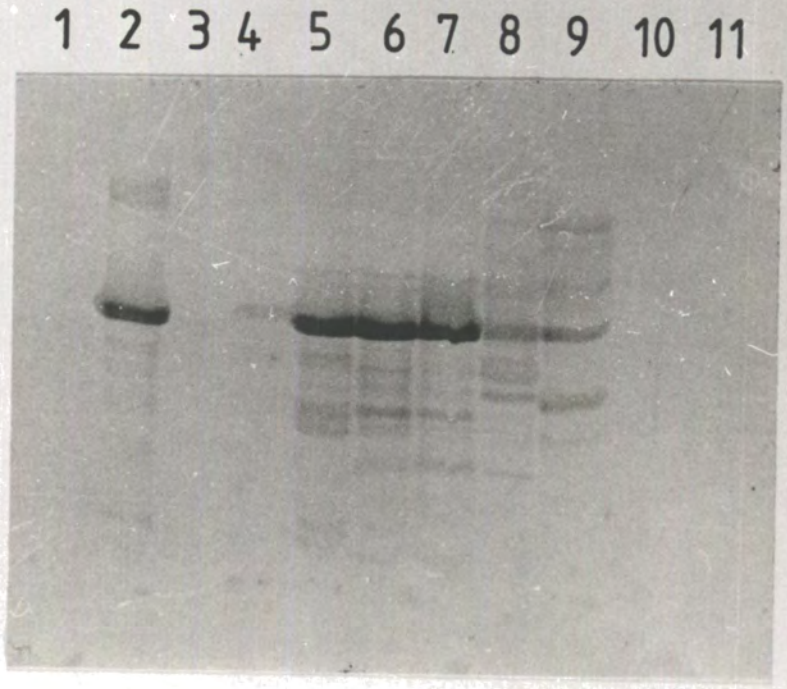


Fig. 48



5263, 5452, 5453 and 5711; similarly, two additional bands of subunit molecular weights ∿ 22000 - 23000 were also observed in line 5534.

Analysis of the total albumin extracts from most of the variant pea lines (unless otherwise stated) found on SDS-polyacrylamide gel electrophoresis were also screened for PMA type proteins by "Western blotting" experiments using anti-PMA IgG; and the results obtained are illustrated in Fig. 50. PMA-S showed specific anti-PMA binding, confirming its presence in the line 21, whereas lines 808, 809, 936, 1256, 1293 and 1376, which showed the absence of the band for PMA-L of subunit molecular weight 25000 in their electrophoretic banding patterns, also did not show any specific anti-PMA binding. It was also observed that the lines, which showed additionally one and two bands very close to PMA-S band in their electrophoretic banding patterns, also did not give any specific reaction with anti-PMA IgG, indicating their non-homology with the pea major albumin proteins.

F. Investigation of albumin proteins in germinating and developing seeds

(i) Determination of moisture content of cotyledons of germinating pea seeds:

Figs. 51 and 52 give the fresh weights and moisture content at various stages of seed germination.

(ii) Synthesis and degradation of pea major albumin type proteins:

(a) From germinating seed extracts:

Degradation of the major albumin proteins from 0, 1, 3, 5, 7, 9 and 11 days germinating seed extracts (freshly prepared) was investigated by analysis on SDS-polyacrylamide gel electrophoresis, 7M urea-SDS-15% polyacrylamide gel electrophoresis, both under reducing conditions, (see Fig. 53 and Fig. 54). Further investigation was carried out by analysis on non-dissociating-polyacrylamide gel eledtrophoresis

FIG. 49.

a) SDS-polyacrylamide gel electrophoresis, under reducing conditions, of total albumin extracts of selected variant Pisum parental lines.

Track No.	Sample
1	Standard protein mixture of pea major albumin, PLA and pea lectin
2,10	Total albumin extract of Pisum sativum L. variety Feltham First
3	Total albumin extract of Pisum line 21
4	Total albumin extract of Pisum line 809
5	Total albumin extract of Pisum line 936
6	Total albumin extract of Pisum line 1276
7	Total albumin extract of Pisum line 806
8	Total albumin extract of Pisum line 5106
9	Total albumin extract of Pisum line 5534
11	Total albumin extract of Pisum line 1376
12	Total albumin extract of Pisum line 1382

b) SDS-polyacrylamide gel electrophoresis, under reducing conditions, of total albumin extract of some selected variant Pisum parental lines.

Track No.	Sample
S	Standard protein mixture of pea major albumin, PLA and pea lectin
1	Total albumin extract of Pisum line 59
2	Total albumin extract of Pisum line 1293

FIG. 50.

Protein subunits of PMA type from selected variant pea lines transferred from SDS-polyacrylamide gels onto nitrocellulose filter paper by 'Western blotting' and detected by reaction with anti-PMA antibodies.

Track No.	Sample
1	Protein subunits of PMA type from pea line 1382
2	Protein subunits of PMA type from pea line 1376
3	Protein subunits of PMA type from pea line 5534
4	Protein subunits of PMA type from pea line 5711
5	Protein subunits of PMA type from pea line 5106
6	Protein subunits of PMA type from pea line 806
7	Protein subunits of PMA type from pea line 59
8	Protein subunits of PMA type from pea line 1276
9	Protein subunits of PMA type from pea line 936
10	Protein subunits of PMA type from pea line 809
11	Protein subunits of PMA type from pea line 21
12	Protein subunits of PMA type from Pisum sativum L.

Fig. 49a

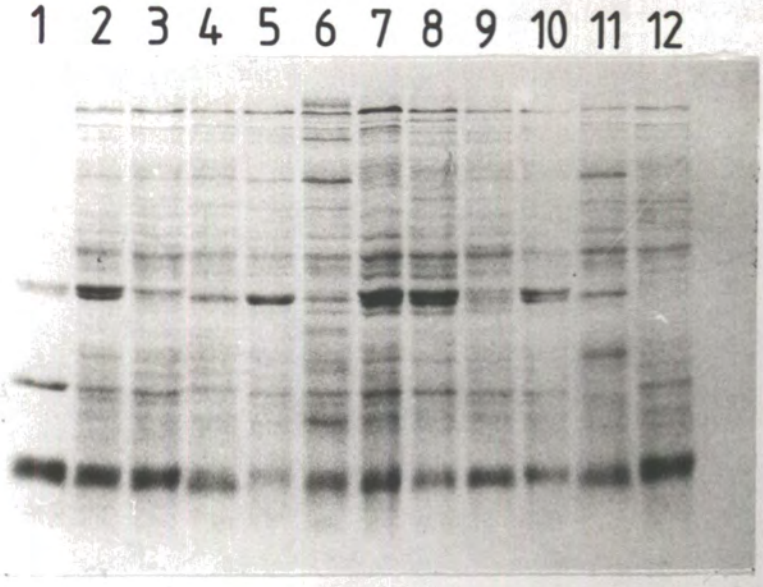


Fig. 49 b

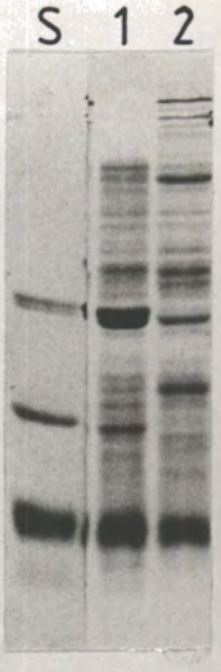


Fig. 50

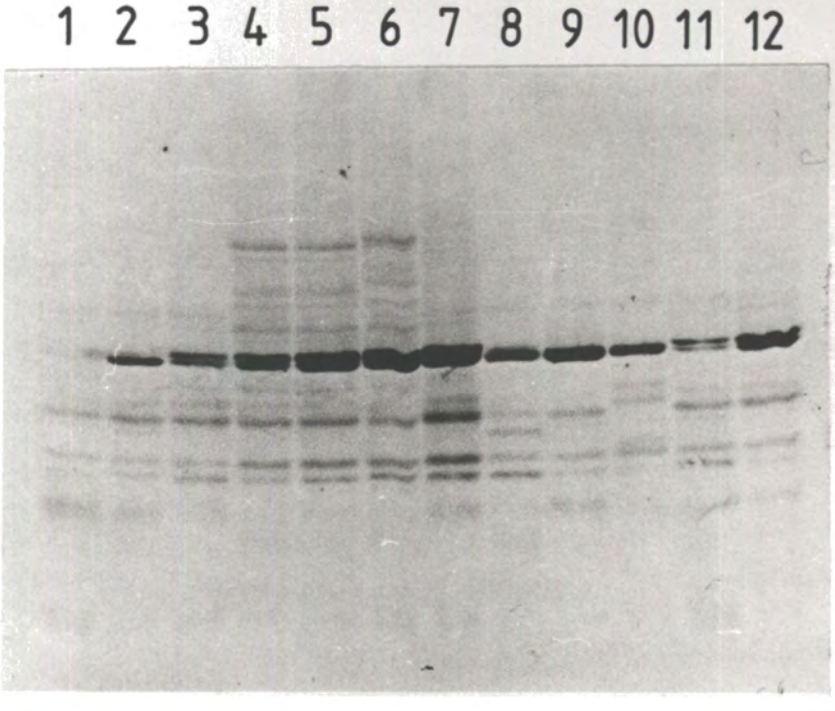


FIG. 51.

Changes in dry (--O--) and fresh (—●—) matter content per cotyledon in germinating pea seeds.

FIG. 52.

Determination of moisture (%) content of cotyledons of germinating pea seeds.

Fig.51

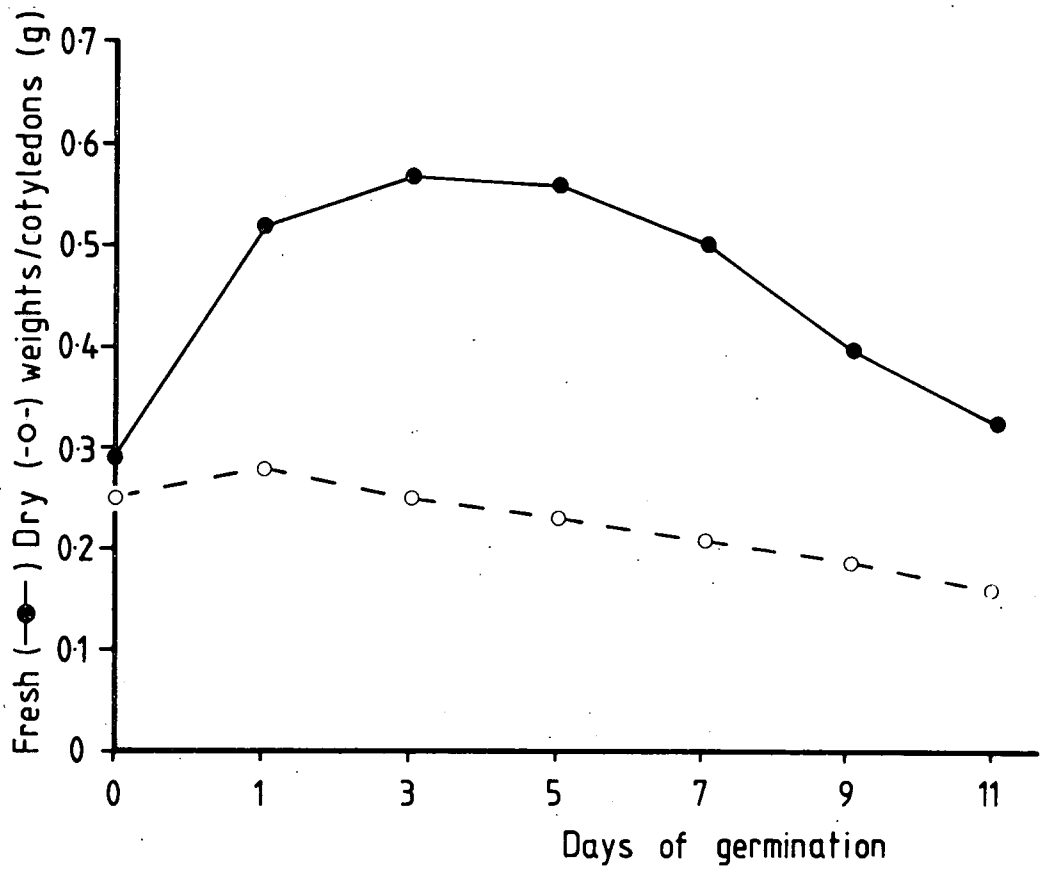

Fresh (—●—) Dry (-o-) weights/cotyledons (g)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0
1
3
5
7
9
11
Days of germination

Fig.52

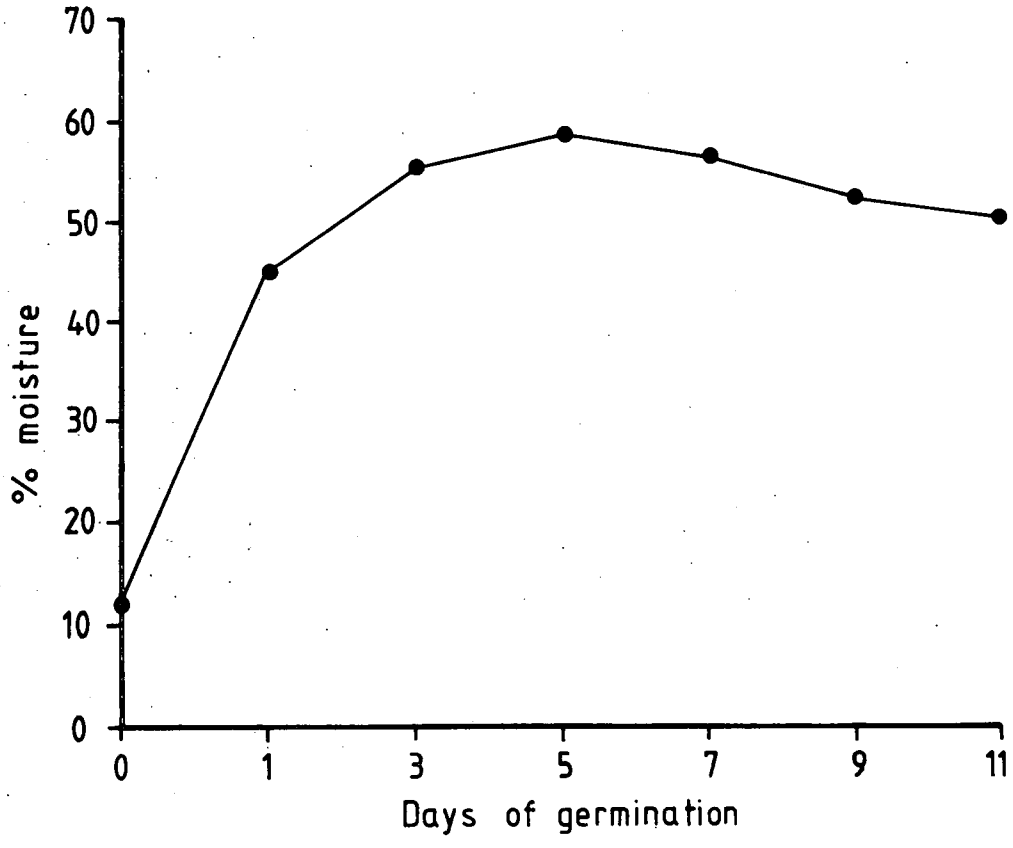

% moisture
70
60
50
40
30
20
10
0
0
1
3
5
7
9
11
Days of germination

FIG. 53.

SDS-polyacrylamide gel electrophoresis of total albumin extracts from 0-11 days germinating seeds under reducing conditions.

Standard proteins { Track 1 - Total major albumin
Track 2 - Pea lectin
Track 3 - High MW albumin
Track 4 - Total albumin extract at 0-day germination
Track 5 - Total albumin extract after 1-day germination
Track 6 - Total albumin extract after 3-days germination
Track 7 - Total albumin extract after 5-days germination
Track 8 - Total albumin extract after 7-days germination
Track 9 - Total albumin extract after 9-days germination
Track 10 - Total albumin extract after 11-days germination

FIG. 54.

Urea-SDS-polyacrylamide gel electrophoresis of total albumin extracts from 0-11 days germinating seeds under reducing conditions.

Track No.	Sample
1	Purified low mol. wt. albumin protein
2	Total albumin extract at 0-day germination
3	Total albumin extract after 1-day germination
4	Total albumin extract after 3-days germination
5	Total albumin extract after 5-days germination
6	Total albumin extract after 7-days germination
7	Total albumin extract after 9-days germination
8	Total albumin extract after 11-days germination

Fig. 53

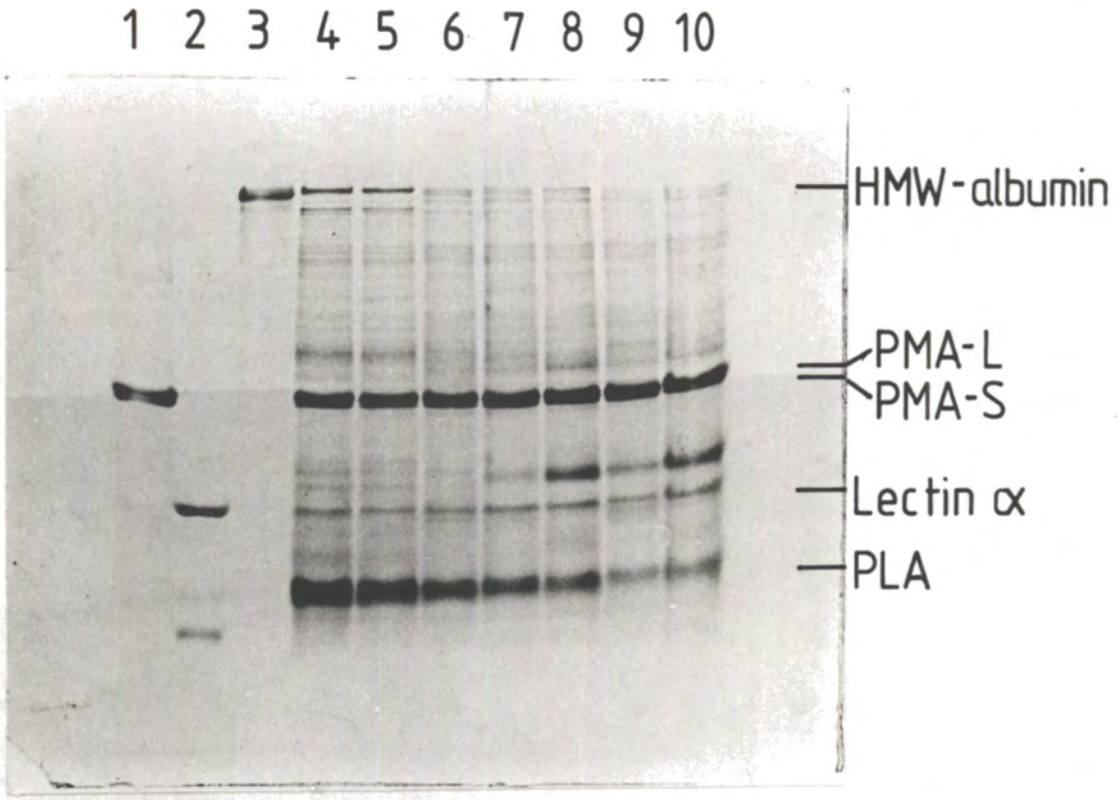

1 2 3 4 5 6 7 8 9 10
HMW-albumin
PMA-L
PMA-S
Lectin α
PLA
0 1 3 5 7 9 11 days germination

Fig. 54

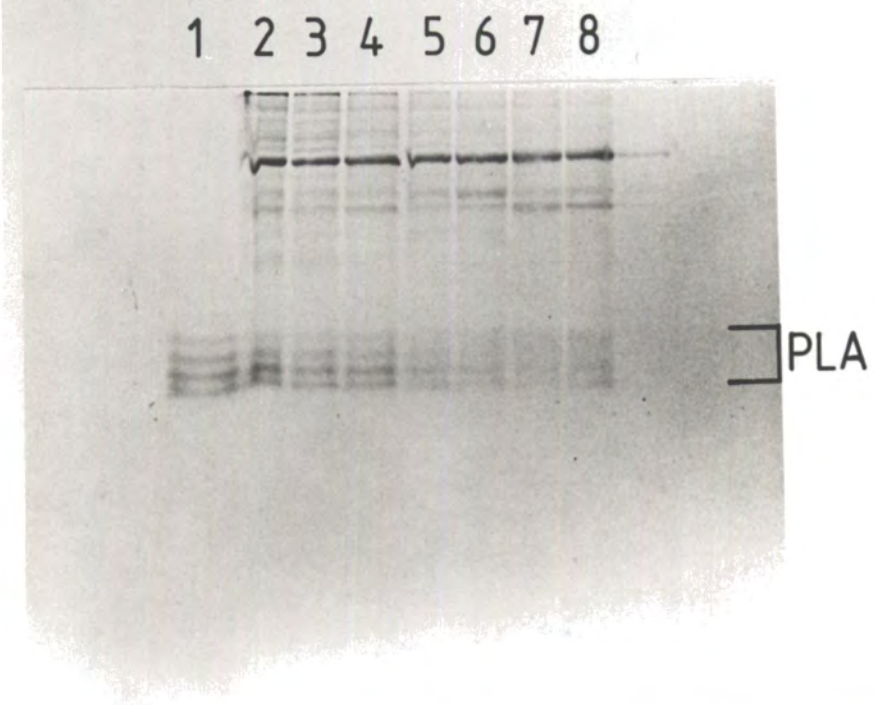

1 2 3 4 5 6 7 8
PLA
0 1 3 5 7 9 11 days germination

FIG. 55.

Non-dissociating polyacrylamide gel electrophoresis of total seed albumin extracts of Pisum sativum L. from 0-11 days of germinating seeds, under non-reducing conditions.

Track No.	Sample
1	Total seed albumin extract at 0-day germination
2	Total seed albumin extract after 1-day germination
3	Total seed albumin extract after 3-days germination
4	Total seed albumin extract after 5-days germination
5	Total seed albumin extract after 7-days germination
6	Total seed albumin extract after 9-days germination
7	Total seed albumin extract after 11-days germination

FIG. 56.

Two-dimensional polyacrylamide gel electrophoresis of a total seed albumin extract of Pisum sativum L. at 0-day germination.
First dimension: non-dissociating gel electrophoresis, non-reducing conditions.
Second dimension: dissociating (SDS) gel electrophoresis, reducing conditions.

Fig. 55


1 2 3 4 5 6 7
PMA-S
PMA-L
0 1 3 5 7 9 11 days germination

Fig. 56

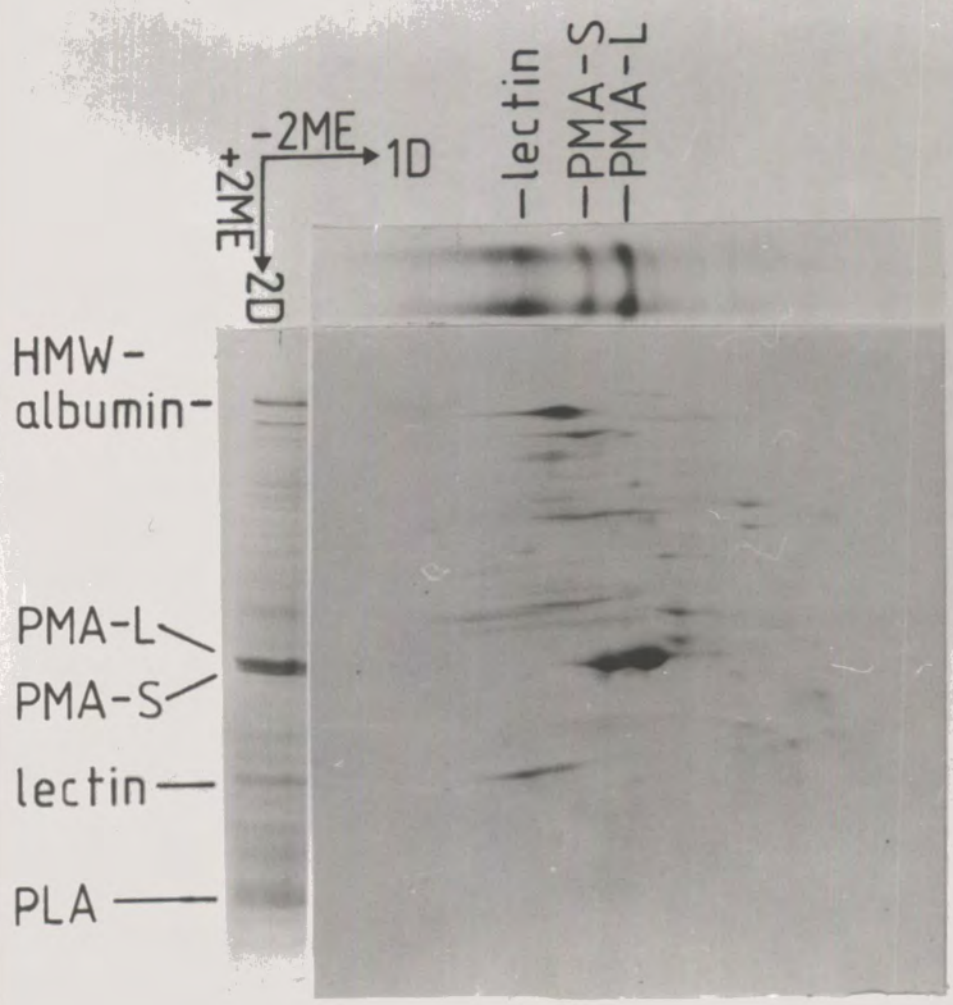

-2ME
1D
+2ME
2D
-lectin
-PMA-S
-PMA-L
HMW-albumin
PMA-L
PMA-S
lectin
PLA

FIG. 57.

Two-dimensional polyacrylamide gel electrophoresis (as in Fig. 56) of a total seed albumin extract of Pisum sativum L. after 1-day germination.

FIG. 58.

Two-dimensional polyacrylamide gel electrophoresis (as in Fig. 56) of a total seed albumin extract of Pisum sativum L. after 3-days of germination.

FIG. 59.

Two-dimensional polyacrylamide gel electrophoresis (as in Fig. 56) of a total seed albumin extract of Pisum sativum L. after 5-days of germination.

FIG. 60.

Two-dimensional polyacrylamide gel electrophoresis (as in Fig. 56) of a total seed albumin extract of Pisum sativum L. after 7-days of germination.

FIG. 61.

Two-dimensional polyacrylamide gel electrophoresis (as in Fig. 56) of a total seed albumin extract of Pisum sativum L. after 9-days of germination.

FIG. 62.

Two-dimensional polyacrylamide gel electrophoresis (as in Fig. 56) of a total seed albumin extract of Pisum sativum L. after 11-days of germination.

Fig. 57

1 day germination

Fig. 58

3 days germination

Fig. 59

5 days germination

Fig. 60

7days germination

Fig. 61

8 days germination

Fig. 62

11 days germination

under non-reducing conditions, see Fig. 55 and also by two-dimensional electrophoresis combining non-dissociating polyacrylamide gel electrophoresis under non-reducing conditions in the first dimension and SDS-polyacrylamide gel electrophoresis under reducing conditions in the second dimension, see Fig. 56, Fig. 57, Fig. 58, Fig. 59, Fig. 60, Fig. 61 and Fig. 62.

It was observed that pea major albumin (PMA) composed of PMA-L (Mr 25,000 subunit) and PMA-S (Mr 24,000 subunit), the major polypeptide components of seed albumin fraction did not show any significant change in amount during the first 11 days of germination. The low molecular weight albumin, PLA (Mr 6000± 300 subunit) was found to decline gradually after 3 days germination. There was also no change in amount for the polypeptide composition of α-chain (Mr 17,700) of lectin, whereas degradation of β-chain (Mr 5,800) could not be demonstrated, as it was not visible in any of the gels used. It was also found that among the albumin proteins, only the high molecular weight albumin (Mr 100,000 subunit) declined rapidly in amounts between 1 and 3 days, although it had not completely disappeared after 11 days germination.

(b) From developing seed extracts:

Synthesis of pea major albumin type proteins from 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 and 24 days developing seed extracts after flowering was examined by analysis on SDS-polyacrylamide gels, Figs. 63-68.

Synthesis of pea major albumin, large (Mr 25,000 subunit), low molecular weight albumin, PLA (Mr 6000 ± 300 subunits) and lectin (Mr 17,700 for α-chain and Mr 5,800 for β-chain) started in developing seeds at the same time 13 days after flowering, whereas pea major albumin, small (Mr 24,000 subunit) and HMW-albumin (Mr 100,000 subunit) seemed

FIG. 63.

SDS-polyacrylamide gel electrophoresis of total albumin extracts of developing seeds (Pisum sativum L.) from 16 to 24 days after flowering.

Track No.	Sample
1	purified total major albumin (+ve control)
2	isolated pea lectin (+ve control)
3	high molecular weight albumin (+ve control)
4	total albumin extract of mature peas (+ve control)
5	total albumin extract of peas, 24 days after flowering
6	total albumin extract of peas, 21 days after flowering
7	total albumin extract of peas, 20 days after flowering
8	total albumin extract of peas, 19 days after flowering
9	total albumin extract of peas, 18 days after flowering
10	total albumin extract of peas, 17 days after flowering
11	total albumin extract of peas, 16 days after flowering

FIG. 64.

SDS-polyacrylamide gel electrophoresis of total albumin extracts of developing seeds (Pisum sativum L.) from 9 to 17 days after flowering.

Track No.	Sample
1	total albumin extract of peas, 9 days after flowering
2	total albumin extract of peas, 10 days after flowering
3	total albumin extract of peas, 11 days after flowering
4	total albumin extract of peas, 12 days after flowering
5	total albumin extract of peas, 13 days after flowering
6	total albumin extract of peas, 14 days after flowering
7	total albumin extract of peas, 15 days after flowering
8	total albumin extract of peas, 16 days after flowering
9	total albumin extract of peas, 17 days after flowering
10	purified high molecular weight albumin (+ve)
11	purified pea lectin (+ve control)
12	purified total major albumin (+ve control)

Fig. 63

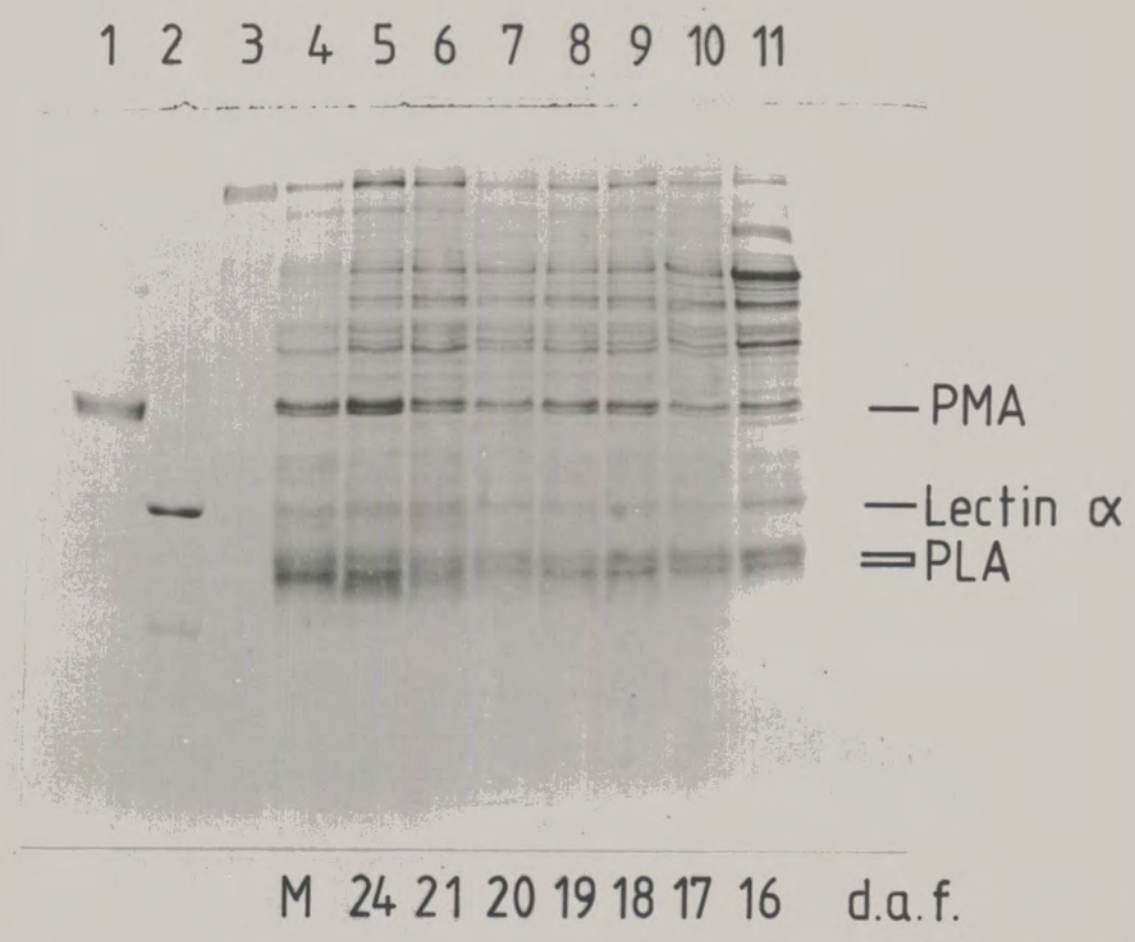

1 2 3 4 5 6 7 8 9 10 11
PMA
Lectin α
PLA
M 24 21 20 19 18 17 16 d.a.f.

Fig. 64

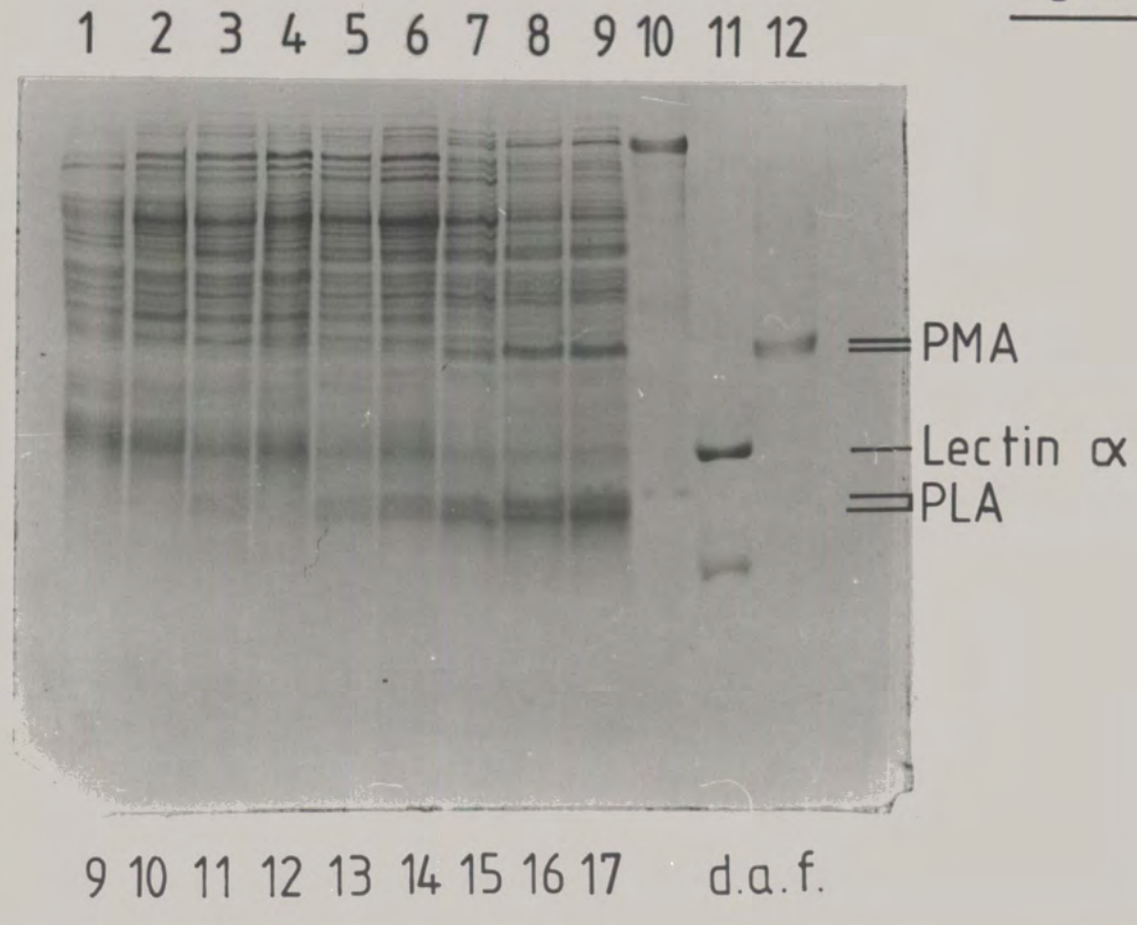

1 2 3 4 5 6 7 8 9 10 11 12
PMA
Lectin α
PLA
9 10 11 12 13 14 15 16 17 d.a.f.

FIG. 65.

Urea-SDS-polyacrylamide gel electrophoresis of total albumin extracts of developing seeds (*Pisum sativum* L.) from 16-24 days after flowering.

Track No.	Sample
1	purified low mol. wt. albumin (+ve control)
2	total albumin extract of peas, 24 days after flowering
3	total albumin extract of peas, 21 days after flowering
4	total albumin extract of peas, 20 days after flowering
5	total albumin extract of peas, 19 days after flowering
6	total albumin extract of peas, 18 days after flowering
7	total albumin extract of peas, 17 days after flowering
8	total albumin extract of peas, 16 days after flowering

FIG. 66.

Urea-SDS-polyacrylamide gel electrophoresis of total albumin extracts of developing seeds (*Pisum sativum* L.) from 9-16 days after flowering.

Track No.	Sample
1	purified low mol. wt. albumin (+ve control)
2	total albumin extract of peas, 9 days after flowering
3	total albumin extract of peas, 10 days after flowering
4	total albumin extract of peas, 11 days after flowering
5	total albumin extract of peas, 12 days after flowering
6	total albumin extract of peas, 13 days after flowering
7	total albumin extract of peas, 14 days after flowering
8	total albumin extract of peas, 15 days after flowering
9	total albumin extract of peas, 16 days after flowering

Fig. 65

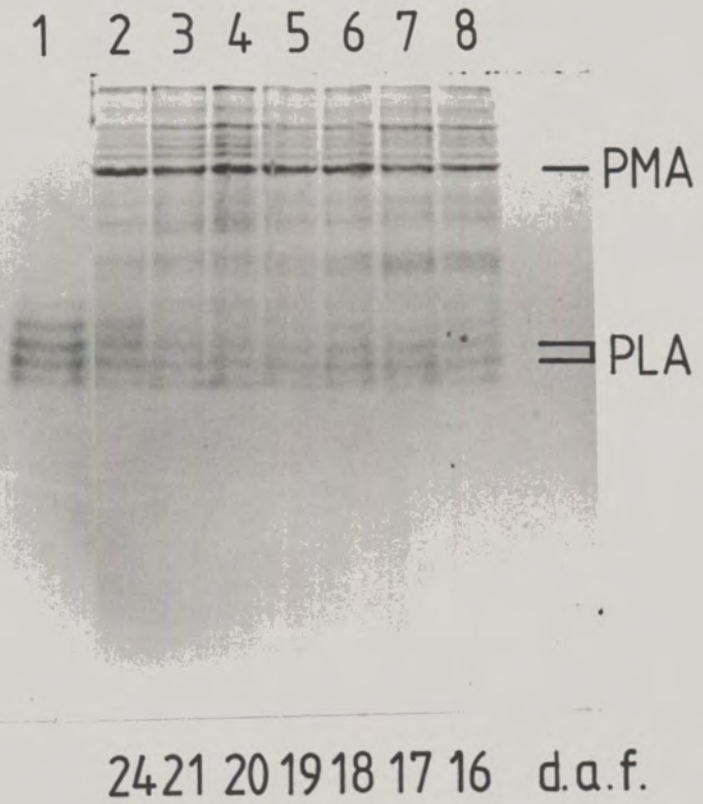

1 2 3 4 5 6 7 8
PMA
PLA
24 21 20 19 18 17 16 d.a.f.

Fig. 66

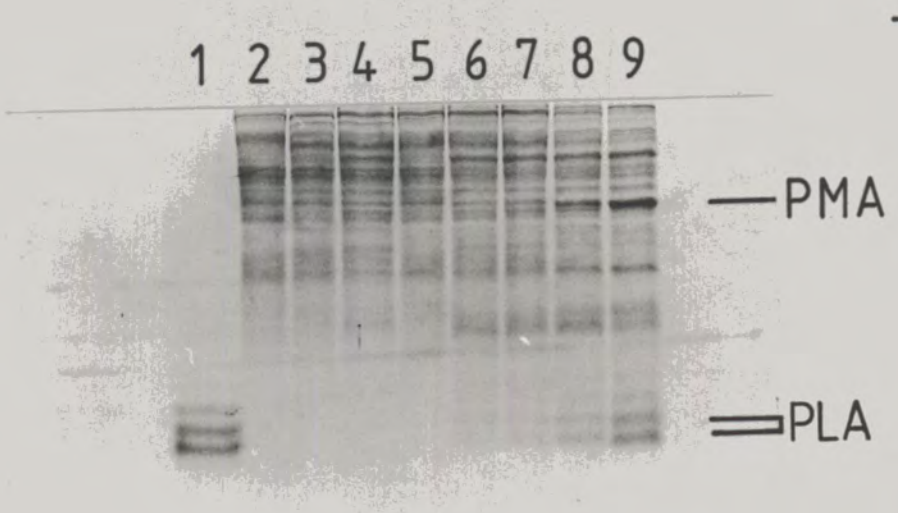

1 2 3 4 5 6 7 8 9
PMA
PLA
9 10 11 12 13 14 15 16 d.a.f.

FIG. 67.

Non-SDS-polyacrylamide gel electrophoresis of total albumin extracts of Pisum sativum from 24-16 days after flowering

Track 1 - total albumin extract of mature peas

Track 2 - total albumin extract 24 days after flowering

Track 3 - total albumin extract 21 days after flowering

Track 4 - total albumin extract 20 days after flowering

Track 5 - total albumin extract 19 days after flowering

Track 6 - total albumin extract 18 days after flowering

Track 7 - total albumin extract 17 days after flowering

Track 8 - total albumin extract 16 days after flowering

FIG. 68.

Non-SDS-polyacrylamide gel electrophoresis of total albumin extracts of Pisum sativum from 9-15 days after flowering.

Track 1 - total albumin extract of mature peas

Track 2 - total albumin extract 9 days after flowering

Track 3 - total albumin extract 10 days after flowering

Track 4 - total albumin extract 11 days after flowering

Track 5 - total albumin extract 12 days after flowering

Track 6 - total albumin extract 13 days after flowering

Track 7 - total albumin extract 14 days after flowering

Track 8 - total albumin extract 15 days after flowering

Fig. 67

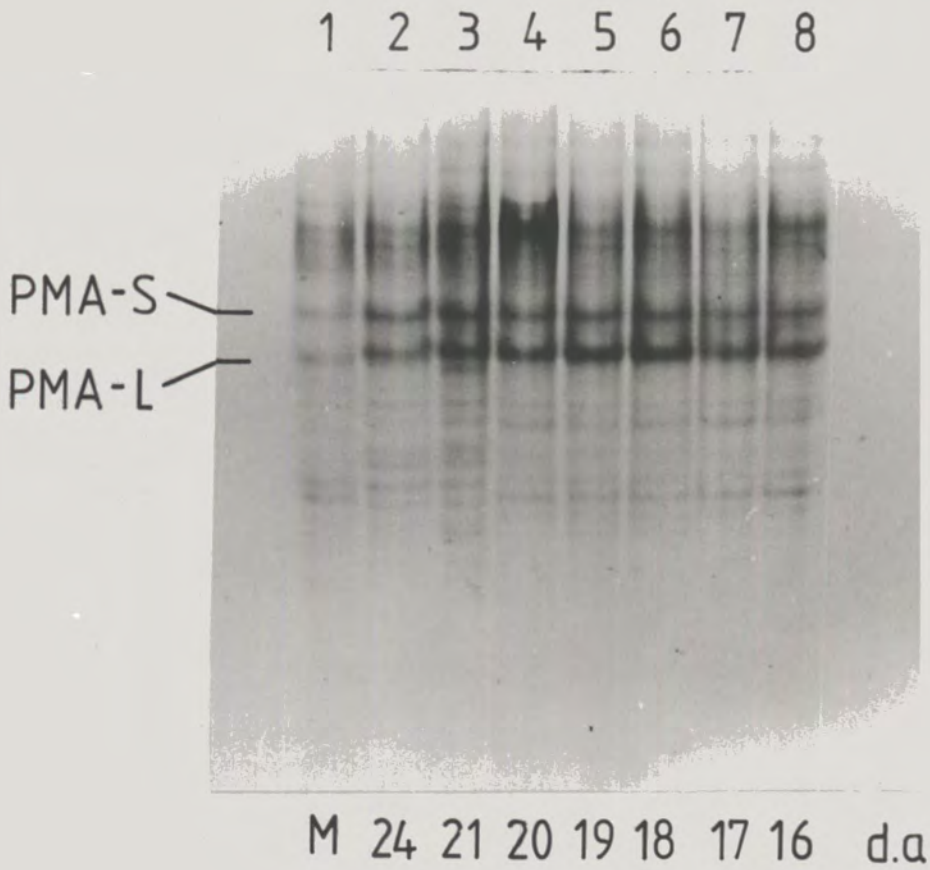

1 2 3 4 5 6 7 8
PMA-S
PMA-L
M 24 21 20 19 18 17 16 d.a.f.

Fig 68


1 2 3 4 5 6 7 8
PMA-S
PMA-L
M 9 10 11 12 13 14 15 d.a.f.

to be started 15-16 days after flowering and seeds were fully matured, 24 days after flowering.

DISCUSSION

SECTION 4

1. Purification of the major albumin proteins:

A prerequisite for all protein characterization studies is that the protein in question is homogeneous. Since the previous work of Danielson (1951, 1956), the albumin fraction from legume seeds has been regarded as consisting of enzymes, whereas the globulin fraction has been considered to constitute the storage proteins (Gatehouse et al., 1984). The preparation of these two fractions normally makes use of the differential solubility of the component proteins present in legumes. As defined by the pioneer of plant protein classification, T.B. Osborne (1924),albumins are water soluble, whereas globulins are insoluble in water. Hence a typical preparation of a pea seed albumin fraction for isolation of pea major albumin proteins involves extraction of the seed meal with solutions which fail to extract the globulin fraction (see later).

Prior to extraction, air-dried mature pea seeds(which had been stored at 4°C),were dried under vacuum at room temperature for a short time to remove superficial moisture, in order to facilitate milling. The testas of the seeds were removed before milling, to avoid difficulties in later purification or isolation stages caused by the presence of pigments, phenolics and other constituents of seed coats. It was also found necessary to defat the meal prior to extraction, to remove oils and pigments which could also interfere with the subsequent purification steps.

Milling of seeds was carried out immediately prior to extraction when possible, as it has been reported by Nash et al. (1971), and Nash and Wolf (1967) that there is a significant decrease in the amount of protein

that can be extracted on storage of soyabean meals. In this study, extractions of albumins from meals were normally carried out with low ionic strength buffer at or around pH 5.0, rather than with distilled or deionised water, since extraction of seed meals with water has been shown to extract globulins along with albumins (Grant et al., 1976; Guldager, 1978). However, the albumin fraction of seed meals is globulin free after extraction with low ionic strength Na-acetate buffer pH 5.0 (Guldager, 1978; Croy et al., 1984), and therefore 20 mM sodium or ammonium acetate buffer pH 5.0 was used as extraction buffer.

All extractions of albumin protein from air-dried, stored seed were carried out at 4°C as precautionary measure to minimize proteolytic degradation, because proteases are also extracted and are likely to be active at these pH values in seed extracts. Nevertheless, it has been shown (Casey, 1979b) that the inclusion of proteinase inhibitors such as di-isopropyl phosphorofluoridate and phenylmethylsulphonyl fluoride in the extraction of Pisum meals does not significantly alter the SDS-polyacrylamide gel electrophoresis pattern of aqueous buffer extracts. Casey (1979a) also demonstrated that increasing the extraction temperature to 25°C and prolonging the extraction time from 4 h to overnight at this temperature did not alter the composition of Pisum extracts.

The first purification step used after extraction was the fractionation of extracts by the addition of solid ammonium sulphate. This is a useful step as recommended by Danielson (1956) in the preparation of homogeneous proteins, as it serves to remove other non-protein materials from the extract, and gives rise to a crude fractionation of the proteins present. The selection of ammonium sulphate is based on the

following advantages in its use: (1) it is a very mild protein precipitating agent (2) as much as 90% protein is precipitated in its concentration range 0.33 - 1.0M, (3) it does not change the conformation of proteins even in 4M saturated solution, (4) it has very small heat of solution which means little change in temperature when dissolving the salt, and little change in solubility of the salt with the temperature of the solution, (5) it is relatively inexpensive in bulk in a suitably pure form low in heavy meals, (6) it is easily removed by dialysis or during subsequent purification steps (7). Precipitates can be redissolved immediately for further purification or stored in a relatively stable condition (4°C). Although ammonium sulphate fractionation is normally used as one of several purification steps, in some cases a pure protein can be obtained by its use alone. The method for the preparation of glycoprotein II from Phaseolus vulgaris involved only ammonium sulphate fractionation of the globulin proteins (Pusztai and Stewart (1980), Tyler (1981) and Croy (unpublished results).

A similar attempt was also carried out with the albumin fraction in this study. Ammonium sulphate fractionation at 50-90% relative saturation gave partial separation of the major albumin proteins but they were found to be contaminated by co-precipitation of small amounts of a large number of other proteins. The protein fraction precipitating from total seed albumins between 50 and 90% rel. satn. with ammonium sulphate was found to contain all or most of the major albumin proteins (PMA) composed of PMA-L (subunit Mr 25,000) and PMA-S (subunit Mr 24,000); PLA (subunit Mr 6,000 ± 300) (Fig. 2, tracks 5-8) and also pea seed lectins, in significant amounts (Fig. 2, tracks 4-5), in agreement with the results of Guldager (1978). It can be tentatively concluded that a single ammonium sulphate fractionation step is not sufficient to

isolate any of the albumin proteins in a pure form.

It was necessary to use a combination of three purification techniques in order to isolate PMA-L, PMA-S and PLA in completely pure form from the albumin fraction.

The ammonium sulphate fractionation of a total extract provided an early concentration step, and enriched both major albumin and PLA relative to the other albumin proteins.

Gel filtration on a Sephadex G-150 column led to the removal of low Mr albumin proteins and also other non-protein material associated during the first step of purification. SDS-polyacrylamide gel electrophoresis of the chromatographed fractions showed no major albumins eluted in the first peak areas, whereas the main peak (Fig. 3) consisted largely of the major albumin proteins with subunits of about Mr 25,000 (Fig. 4, tracks 5-6); in agreement with the following reports: Grant et al. (1976); Guldager (1978); Murray (1979); Jakubek and Przybylska 1979); Tyler (1981) and Matta (1981). This peak was also contaminated with small amounts of PLA (subunit Mr 6,000 ± 300). In addition, variable amounts of PLA were also eluted in the last peaks (3 and 4) (Fig. 3 and Fig. 4, tracks 7-8) but was insufficient for further purification and subsequent characterisation. A Sephadex G-75 column was therefore used to isolate PLA protein in sufficient amounts (Fig. 10, peaks 3-4; Fig. 11, tracks 3-6).

It has been suggested that other methods are also effective in fractionating the albumin proteins. These include: zonal isoelectric precipitation with acidic buffers (Tyler, 1981; Croy and others, unpublished observations); and gel filtration (Croy et al. 1980).

Pea lectins in the albumin fraction are known to be specific for glucose/mannose residues (Entlicher et al. 1970; Trowbridge, 1974),and

these were bound to the Sephadex columns, effectively removing them from the other albumin protein. Isolation of the lectins was accomplished by using 0.1M D-glucose in the column buffer (see methods section), as described by Trowbridge (1974). The eluted lectins were found to be largely free from contaminating proteins as judged by SDS-polyacrylamide gel electrophoresis (Fig. 7, track 8; Fig. 8 and Fig. 9, tracks 7-8).

Sephadex purified major albumins were completely free from lectin as judged by the absence of haemagglutination activity (Fig. 44) or reaction with anti-pea lectin antibodies (Fig. 42a). The lectin was found to agglutinate 2% erythrocytes at a minimum concentration of about 4 μg per ml under the conditions of the assay (see method section), in agreement with earlier work of Trowbridge (1974) (see later).

The molecular filtration purification step indicated that major albumin proteins are intermediate in size between HMW-albumin and PLA, this was confirmed by other methods (see later).

Resolution of the albumin protein mixture into its components by Sephadex G-150 or Sephadex G-75 chromatography was not complete since no separate peaks of individual albumin proteins were obtained. It was concluded that a single gel filtration step for the isolation and purification of individual albumin components was not sufficient; in agreement with the observations of Guldager (1978); Jakubek and Przybylska (1979), Tyler (1981) and Matta (1981).

It is known that certain molecular types do not display typical elution behaviour and are eluted earlier or later than predicted from their molecular size. This could be due to gel-solute interations and adsorption phenomena which are thought to be of two main types: (a) Electrostatic interactions, which may be due to the small number

of ionized-COOH groups on Sephadex, leading to the adsorption of some basic substances and exclusion of acid substances from the gel matrix, at low ionic strength (Gellotte, 1960). This effect is most prominant on the more tightly cross-linked Sephadex types (Janson, 1967). (b) Aromatic adsorption due to the interaction of heterocylic and homocyclic compounds with Sephadex (Janson,1967). Once again this effect is more pronounced with high density matrix Sephadex gel types and low mol. wt. solutes (Janson, 1967, Brook and Housley, 1969b). The interaction is thought to be the result of hydrogen-bonding between aromatic substituents and the hydroxy-ether linkage between dextran chains (Williams, 1972; Brook and Munday, 1970; Determan and Walter, 1968; Brook and Housley, 1969a).

However, since the proteins to be fractionated in this case were of medium mol. wt. except PLA, and did not have a high aromatic amino acid e.g. (Phe) content (Tables 3 and 4), plus the fact that all buffers used contained electrolytes, it can be assumed that anomalous elution was minimised.

Probably the other main reason for the incomplete separation of albumin proteins on the columns used was that chromatography was carried out on a preparative scale, using a large column and high sample loadings in order to obtain an enrichment and high yields of a specific protein fraction, i.e. seed major albumin. Peak broadening effects occur due to the various appreciable protein - protein interactions encountered at high protein concentrations.

The third and final purification step for the separation and preparation of the major albumins was achieved by ion-exchange chromatography on DEAE-cellulose. This form of chromatography was found to be an excellent final purification step. It was also

subsequently shown to be a convenient preliminary fractionation step of total albumin extracts.

Elution of a single protein species occurs at a specific concentration of eluting salt determined by the strength of interaction between the net -ve charge on the protein and the +ve charge on the ion exchanger. Bearing these facts in mind, PMA-S was found to have a lower level of acidic amino acids (Glu and Asp which are responsible for its -ve charge) than that of PMA-L (Table 3), and this may have been reflected in the pattern of elution from DEAE-cellulose in which PMA-S was eluted at a lower molar concentration of NaCl (0.085M) (see Fig.5, peak 2; Fig. 6 track 5) than PMA-L (0.14 M NaCl) (see Fig. 5, peak 5; Fig. 6 track 7). Similarly PLA was found to have the highest level of acidic amino acids (Glu: Asp = 5:10) of the three albumin proteins (Table 4 and Fig. 34) and was eluted at the end of the elution profile at a salt concentration of 0.16 - 0.18M (see Fig. 12, peak 9; Fig. 14, peak 10; Fig. 13 track 10; Fig. 15 track 11). PMA-L, PMA-S and PLA proteins purified by this method consisted mainly of 25,000, 24,000 and 6000 (± 300) Mr subunits respectively. Therefore this step provided a source of fairly pure PMA-L, PMA-S and PLA. Fractions from the other peaks were found to contain variable amounts of different proteins other than PMA and PLA. The other proteins were not characterised further.

PMA-L, PMA-S, the total major albumin PMA (i.e. total pooled fractions from peaks 2 and 5, (Fig. 5) and PLA were only considered in this study for subsequent analyses.

The success of the present method(s) are due largely to the very efficient extraction of the albumin proteins with low ionic-strength acid buffer, which fails to extract any detectable amounts

of globulin proteins, in agreement with the reports of Guldager (1978) and to the good resolution achieved in the final column chromatography step on DEAE-cellulose. Additionally it has been suggested that hydroxylapatite chromatography may also be effective in separation and purification of albumin proteins (Tyler, 1981; Croy et al., unpublished observations).

2. Estimation of major albumin proteins in total cotyledonary protein:

Quantitative estimations of PMA and PLA obtained by 'rocket' immunoelectrophoresis and densitometric scanning, indicate that both of these proteins are significant components of total cotyledonary protein. These results agree with the reports of Schroeder (1982), who described two albumin polypeptides (Mr ∿ 22,000 and Mr ∿ 8,000) which were abundant components of the total albumin proteins.

3. Characterisation of albumin proteins:

The Mr values of 53,000 ± 10% for PMA-L and 48,000 ± 10% for PMA-S calculated by comparison with Mrs for standard proteins run on a gel filtration column of Sephacryl S-200 (Fig. 21) are in reasonable agreement with Grant et al. (1976), who reported the presence of major albumin components with Mr 48,000 and 47,700 in both 'Century' and 'Trapper' varieties of peas. Similarly, an Mr value of 11,000 ± 10% for PLA was determined on a column of Sephadex G-50 (Fig. 22). PLA appears to be identical to a protein described as "13000 Mr" by Chandler et al. (1984) and also described by Schroeder (1984). On the other hand, Guldager (1978) claimed the presence of albumin proteins with Mr values between 15,000 and 68,000 in pea lines of K-42' (Dark skin perfection) but did not characterize these proteins. Both PMA-L

and PMA-S were eluted as single symmetrical peaks from the Sephacryl column (data not presented), indicating that there was negligible interaction with the column and that these proteins were free from contaminants, whereas PLA eluted as a peak with a small trailing shoulder from the G-50 column (data not presented) possibly suggesting an interaction with the column material, or the presence of small amounts of contaminating molecular species of lower Mr.

Subunit sizes of the major albumin proteins estimated by SDS-polyacrylamide gel electrophoresis, gave values of 25,000 for PMA-L and 24,000 for PMA-S, which agrees with the results of Grant et al. (1976) and Tyler (1981) and Matta (1981) but differ slightly from the observations made by Guldager (1978), Murray (1979), Jakubek and Przybylska (1982), who claimed a subunit Mr $\sim$ 23,000; and Schroeder (1982) who described a subunit of Mr $\sim$ 22,000. However, such estimates are consistent with dimeric molecules for both PMA-L and PMA-S proteins.

The polypeptide Mr of PLA could not be estimated accurately from conventional SDS-PAGE gels since it ran as a band of Mr < 10,000 (Fig. 13, track 10; Fig. 15 track 11) where the gel system does not resolve polypeptides well. The molecular weight was thus determined by gel electrophoresis on urea-containing gels. PLA as isolated ran as a series of four bands in this system (Fig. 39, track 1) even in the presence of 2-mercaptoethanol, but after carboxymethylation the protein gave a single band, suggesting that the multiple bands were artefacts due to -SH group interactions. The value for carboxymethylated PLA was therefore estimated from SDS-urea PAGE gels and found to be 6,000 (±300) (Fig. 27 and Fig. 28). Such estimates agree with the reports of Jakubek and Przybylska (1979) and Schroeder (1984).

When PMA, PMA-L and PMA-S are run on SDS-PAGE gels under non-reducing conditions (Fig. 29, tracks 8-10) an additional faint band with subunit Mr ∿ 50,000 is observed, in addition to the subunit bands of Mr ∿ 25,000 observed under reducing conditions. These bands possibly represent dimers of the subunit polypeptides, due to artefactual intermolecular disulphide bonding.

Analyses of fresh albumin extracts and PMA-L and PMA-S on two-dimensional gels showed that the albumin proteins PMA-L and PMA-S contained either large (Mr 25,000) or small (Mr 24,000) subunits (Figs. 30, 31) in the dimeric molecules; molecules containing one of each type of subunit were not present. Furthermore since different pea genetic lines show different proportions of large and small subunits and in some cases only single subunit types (Ragab and Croy, unpublished; Figs. 49 and 50) it seems likely that mixed dimers of large and small subunits are not formed.

Amino acid composition of the albumin protein (Table 3) shows that both PMA-L and PMA-S contained significantly higher levels of sulphur amino acids, e.g. cysteine and methionine than the globulin proteins. These results support the reports of Grant et al. (1976); Hurich et al. (1977), Jakubek and Przybylska (1979), Murray (1979) and Schroeder (1982) of the sulphur rich nature of the albumin fraction and suggest the major albumin proteins as further candidates for investigations into improving the nutritional quality of peas (Davies, 1976; Schroeder, 1982; Boulter, 1982). Furthermore, the amino acid composition of the major albumins resembled that of the total seed albumins (Table 3; Grant et al., 1976; Jakubek and Przybylska, 1979) presumably reflecting the significant proportion of the total albumin represented by the major albumins.

The amino acid composition of PLA (Table 4) and the predicted composition

from the amino acid sequence (see later) also shows that this protein contained a high level of cysteine (4 residues), but both methionine and valine were absent. The level of acidic amino acids (Asp:Glu = 10:5) was higher than that of the basic amino acids (Lys:Arg = 5:1). Proline (4) and tryptophan (1) residues were predicted in the sequence from the amino acid sequence data (Fig. 34) but were not observed on amino acid analysis (Table 3 and Table 4). Tryptophan is destroyed by acid hydrolysis, and a separate estimation for tryptophan was not carried out in this study. O-phthalaldehyde was used (as mentioned earlier, see method secton) to detect amino acids by a post-column fluorescent derivatisation system coupled to a fluorimeter. Using this technique proline was not detected as it does not give a fluorescent derivative. However, the reported amino acid composition data of pea albumin fraction shows significant proportion of both proline and tryptophan (Grant et al., 1976; Murray, 1979).

Chemical methods have been developed for cleaving polypeptide chains at specific amino acid residues. The most successful involves reaction of a polypeptide with CNBr, which cleaves peptide bonds at the carboxyl side of methionine residues. The methionine residue is converted into a C-terminal homoserine lactone residue. The number of fragments produced from a polypeptide by CNBr can be predicted from the number of methionine residues present in the chain. They can also be analysed by various electrophoretic techniques.

Confirming the methionine content of the major albumins, CNBr reactions with both large and small subunits of PMA gave three distinct fragments indicating the presence of at least two methionine residues per subunit (Fig. 36, tracks 4, 6 and 7). Since 2-3 methionine residues per subunit are predicted by the amino acid

composition (Table 3), these results are in resonably good agreement, but do not eliminate the possibility that an additional, small, CNBr fragment may also be produced. However, summation of the Mr values for the CNBr fragments agrees with the calculated subunit molecular weights of PMA-L and PMA-S. Such results, although confirming the similarity of the subunit polypeptides, indicated that the primary sequences of the two subunits may vary slightly in at least two regions but may have one large highly conserved region. Similar tryptic peptide maps were obtained in the form of h.p.l.c. elution profiles for PMA-L and PMA-S (data not presented), in confirmation of the conclusions of structural relatedness.

On the other hand, PLA protein did not produce any CNBr fragments as there was no change in mobility of carboxymethylated PLA on urea/SDS-PAGE after prolonged reaction with CNBr for up to 48 h (Fig. 39). This result confirmed the absence of methionine in agreement with the amino acid composition data (Table 4).

Prior to amino acid sequence analysis it is necessary to determine whether the protein contains more than one polypeptide chain. Provided that there are no blocked N-terminal residues (e.g. N-acetylated residues occur, but infrequently),the number of free amino-terminal residues per mole of native protein can be an indication of the number of polypeptide chains in the protein. If the polypeptide chains have no covalent cross-linkages, they can be separated by treating the protein with acid, base, or high concentration of salt or a denaturing agent. On the other hand, if they are covalently cross-linked by one or more disulphide bonds between half-residues of cystine these cross-linkages must be cleaved either by 2-mercaptoethanol or by DTT,

followed by alkylation (as described earlier).

Threonine was the only N-terminal amino acid determined in both PMA-L and PMA-S, suggesting that both proteins contain only one polypeptide chain and further confirming structural similarity, whereas aspartic acid was determined at the N-terminal of PLA and no other amino acids were observed, suggesting the sample was homogeneous and contained only one polypeptide chain.

The N-terminal amino acid sequence (Thr-Ala-Thr-Gly-Tyr-Ser-Asn-) was determined for both PMA-L and PMA-S, supporting the previous N-terminal result and indicating that the N-terminal region of the proteins showed complete homogeneity. Heterogeneity of the amino acid residue positions in the subunits could be confirmed after determining the complete amino acid sequence of PMA-L and PMA-S, which was not done in this study. However, the present amino acid sequence and composition data for PMA-L and PMA-S supports the previous conclusions of structural relatedness.

Carboxypeptidase A, one of a group of digestive enzymes, is relatively specific for removal of C-terminal residues from a polypeptide chain. The liberated free amino acid reacts with dansyl chloride or Edman reagent (PITC) to form the respective derivatives that could be identified by paper or polyamide thin-sheets providing the basic principle of identifying the C-terminal amino acid residue of a polypeptide chain. Difficulties arise, however, because the enzyme does not stop after removing the initial C-terminal residue, but continues to attack new C-terminal residues every time the chain is shortened. It is thus necessary to monitor the rate of release of amino acids. Bearing the above facts in mind, the C-terminal amino

acid determination on PMA-L, PMA-S and PLA using carboxypeptidase A gave low yields of amino acids. In all 3 cases, aspartic, asparagine and phenylalanine were released in significant amounts. This observed C-terminal heterogeneity may account for the heterogeneity of these proteins seen on iso-electric focusing (Fig. 32 and Fig. 33). It may be due to post-translational deamination of an asparagine residue (see later), or proteolytic processing.

The three enzymes used to cleave the PLA polypeptide showed their normal specificities. The deduced sequence is 54 amino acids in length (Fig. 34), giving an overall molecular weight of 6050 in reasonable agreement with the Mr deduced by urea/SDS-PAGE (Fig. 27, Fig. 28). The sequence data contain two positions of heterogeneity: at amino acid 45 both Phe and Val were found in peptides apparently not separable by h.p.l.c. Although valine was a comparatively minor amino acid [in agreement with the amino acid composition (Table 4)] at this position (about 25% that of phenylalanine), there does appear to be real heterogeneity at this position. Heterogeneity was also observed at the C-terminal amino acid, where separate peptides containing asp or asn as C-terminal amino acids were isolated in approximately equal proportions. This heterogeneity is in agreement with the direct C-terminal determination on the protein, apart from the presence of Phe in the latter, which was thus considered to be artefactual. The C-terminal heterogeneity may be due to post-translational deamidation of an asparagine residue, or may be encoded. These results may account for the heterogeneity of PLA seen on isoelectric focusing (data not presented). The amino acid composition for PLA (Table 4) was in good agreement with that predicted by the deduced sequence (Fig. 34). Furthermore, PLA sequence is not homologous

to the 2S storage protein of castor bean (Ricinus communis) (Sharief and Li, 1982) or rapeseed (Brassica napus) (Crouch et al., 1983), and therefore appears to represent a new sequence family (Fig. 69).

The secondary structure and hydrophilicity profile (Fig. 35) predicted from the determined amino acid sequence data of PLA indicated that this protein has hydrophilic regions near the N- and C-terminal, and a hydrophobic region around the central point of the polypeptide; corresponding to this, the N- and C-terminal regions are in α-helix form, whereas the central region has a high content of β-turns. There is no β-sheet conformation predicted for any region in the protein, but it seems likely that the central region of the polypeptide could form a β-sheet structure rather than continuous β-turns. The presence of 4 cysteine residues in the PLA polypeptide chain introduces a complication in that the nature and location of disulphide cross-links was not determined, and this may dictate the folding of the polypeptide chain into β-turns rather than a β-sheet structure. The N-terminal α-helix region has two cysteine residues in it, which could provide a means of disulphide linking the subunits of the dimeric molecules together, although the results of gel electrophoresis suggested that the subunits may not be covalently linked.

TCA is used as a protein precipitating agent in the determination of sugar present in glycoprotein to distinguish "free" and "bound" sugars present in the precipitated protein which are determined after hydrolysis either qualitatively by gel-liquid-chromatography (Sweely et al., 1966) by TLC or quantitatively by the phenol/H_2SO_4 acid method (Dubois et al., 1956).

Quantitative data of sugar estimation (Fig. 40) indicated that

PMA-L, PMA-S and PLA did not contain significant amounts of carbohydrate (less than one sugar residue per 30,000 Mr polypeptide) when assayed by the phenol/H_2SO4 acid method. Additionally all these proteins gave negative reactions when stained for carbohydrate after SDS-PAGE using the sensitive dansyl hydrazine method of Eckhardt et al., (1976), under conditions where known glycoproteins stained strongly (Fig. 41). Furthermore, both PMA-L and PMA-S did not show any reaction with concanavalin A. All these results suggested that these proteins are not glycoproteins.

4. Subcellular location of albumin proteins

Unlike storage proteins, the subcellular localization of albumin proteins is not clear. Despite the large yield of protein from pea protein bodies, the amount of albumin fraction recovered from them was very low (Croy et al, 1984), as reported by other groups (Konopska, 1978; Weber and Newmann, 1980; Varner and Schidlovsky, 1963) in contrast to the results obtained from subcellular fractionation of other legume seeds (e.g. those of the French bean, Phaseolus vulgaris), where the predominant albumins are located within the protein bodies (Croy, 1977; Pusztai et al., 1977; Youle and Huang, 1978; Bollini and Chrispeels, 1978). Murray (1979). More recently Croy et al. (1984) have reported that albumin proteins are predominantly present in the cytoplasm, the soluble cytosol fraction of the cotyledon cells. This is not the location for all albumin fraction proteins, however, as Weber et al. (1978) reported that the homologous lectin in Vicia faba was protein body associated. Unequivocable localization of albumin proteins can be carried out by quantitative subcellular fractionation studies and immuno-histochemical studies at the electron microscope level, as have been performed for the pea globulins (Craig and Millerd, 1981).

5. Functions of pea albumin proteins:

(a) Enzymic activities:

It is widely held that the albumins of pea seeds are mainly enzyme proteins, i.e. metabolic proteins (Gatehouse et al.,1984), e.g. it has been reported that pea cotyledons contain significant amounts of glycosidase enzymes (Neely and Beevers, 1980). Assays for enzymatic activity were made on PMA-L and PMA-S proteins, but did not reveal activity in any of the glycosidase enzyme assays tested (see results section). Enzyme activities are present in the albumin fraction, since significant α-D-mannosidase activity was observed in the fraction precipitated between 40 and 60% saturation of ammonium sulphate from the crude albumin extract (data not presented), in agreement with Neely and Beevers (1980). Although many enzymes are likely to be present in the albumin fraction very few of them would account for sufficient protein to be individually detectable in gels by the dye-binding technique commonly employed. The electrophoretic separation of pea albumin proteins does not simply reveal a multitude of fine polypeptide bands and certain major polypeptides such as PMA, PLA, high molecular weight albumin and lectins dominate the electrophoretic profile (Fig. 7).

(b) Inhibitory activities:

None of PMA-L, PMA-S and PLA had any detectable pancreatic α-amylase inhibitory activity, suggesting that none of these proteins is an α-amylase inhibitor.

Pea is known to contain protease enzyme inhibitors of low Mr (Tomé et al., 1981). Both qualitative and quantitative assessments of protease inhibitory activity of PMA-L, PMA-S and PLA against trypsin and chymotrypsin indicated that neither PMA-S or PLA was a protease

inhibitor, but PMA-L showed low levels of trypsin and chymotrypsin inhibitory activity, though it is by no means certain that this was an intrinsic activity or merely contamination. The results of an experiment in which a total seed albumin extract was chromatographed on DEAE-cellulose (Figs. 45 and 46) showed that although pea seeds contained protease inhibitors active against trypsin and chymotrypsin these inhibitors did not co-elute with PMA-L or PMA-S. The elution of one peak of inhibitor activity in the trailing edge of the PMA-L peak suggested that the previous inhibitory activity of PMA-L is likely to be accounted for by small amounts of contaminating protease inhibitor. It was thus tentatively concluded that PMA-L is not a protease inhibitor. The last major peak of protease inhibitory activity eluted immediately before the PLA peak and also contained inhibitory activity against both trypsin and chymotrypsin. However, the PLA peak was clearly separated from the inhibitors, and thus this protein too does not appear to be a protease inhibitor of the type normally found in legume seeds. Fractions containing high protease inhibitor activities contained polypeptides of low Mr similar to PLA. These polypeptides were observed in the earlier purification and are tentatively now identified as pea seed protease inbibitors. PMA-L, PMA-S and PLA are not therefore protease inhibitors.

(c) Haemagglutination activity:

Many seeds, in particular those of the legumes, contain relatively

large concentrations of phytohaemagglutinins (lectins), i.e. substances which agglutinate blood cells (Lis and Sharon, 1973; Liener, 1980; Trowbridge, 1974; Gatehouse et al., 1980), e.g. concanavalin A (conA) comprises 2-3% of the protein of jackbean (Canavalia ensiformis) (Sumner and Howell, 1936), and soyabean agglutinin(SBA)1-1.5% of the soyabean (Glycine max) protein (Liener and Pallansch, 1952). The properties of PMA-L, PMA-S and HMW-albumin, including also the isolated pea lectins and conA (as positive controls) were judged by assaying their agglutinating activity with 2% rabbit erythrocytes (see methods section). None of PMA-L, PMA-S and the high molecular weight albumin proteins had any haemagglutinating activity up to a maximum concentration 0.5 mg/ml (Fig. 44). Pea lectins showed haemagglutination down to a concentration of less than 4 µg/ml, in agreement with Trowbridge (1974). It was concluded that the physiological function of pea albumin proteins therefore, is different from that of lectins.

(d) Antimetabolic effects:

Seeds are known to contain many toxic substances, which are important factors from a nutritional point of view. Various toxic factors associated especially with legume proteins have been reported by Liener (1974a)and Boulter (1983). Low Mr proteins, many of them albumins, may play a role as an antimetabolite for seed-attacking pest, like some legume trypsin inhibitors (Gatehouse et al., 1979). The effects of various pea protein fractions were tested by a bio-assay on the development of the larvae of Callosobruchus maculatus (Table 5). Purified PLA did not show any significant toxic effect on the development of the insects. A moderate toxic effect of total major albumin fraction was obtained which indicated either a low intrinsic

toxicity or small amounts of contaminating lectins or low Mr protease inhibitors, since the fractions bio-assayed were prepared by large scale gel filtration on a column of Sephacryl S-300 (data not presented). The significant toxic effect of total albumin fraction upon larva development can not be associated with PMA either, since PMA itself was non-toxic. It was tenatively concluded that neither pea major albumin nor PLA are significantly antimetabolic in this system.

6. Homology of pea albumin proteins to those of other species

A qualitative assessment of the major albumin distribution in different legume species by immunological screening (see results section) (Fig. 42b) indicated the presence of homologous major albumin proteins in only three other legume species - lentil, chickpea and sweetpea only. This type of protein thus seems to be characteristic of Pisum species and those closely related to it, and suggests that the lentil is the most closely related species to Pisum sativum, as has been shown in similar studies with other seed proteins, e.g. legumin; Sammour and Gatehouse (unpublished work).

7. Distribution of albumin proteins in other parts of the pea plant

Preliminary screening for the presence of major albumin proteins in different parts of pea seeds (Fig. 47 and Fig. 48) indicates that only the cotyledons (both inner or outer cotyledon) contain significant amounts of the major albumin polypeptides of Mr 25,000. In addition significant amounts of HMW-albumin polypeptides (Mr 100,000), PLA polypeptide and lectin polypeptides were only found in the cotyledons in good agreement with the reports of Müller and Gottschalk (1973), who stated that more than 95% total protein is present in the cotyledon of seeds. Only small amounts of major albumin proteins are

present in radicles and hypo-cotyls. It was also concluded that testa, leaves, stems and roots do not contain significant amounts of any of the pea major albumin proteins.

8. Genetic variation in albumin proteins:

Preliminary qualitative screening by SDS-PAGE of total protein extracts from 200 different genetic lines (Fig. 49 and Fig. 50) revealed lines varying in the amounts or lacking PMA-L, HMW-albumin, PMA and lectin polypeptides, indicating that the genes responsible for their synthesis were inactive or missing in these lines. In contrast, PLA was present in all lines tested.

9. Synthesis and degradation of albumin proteins:

(a) Synthesis during seed development:

A knowledge of the sequence of accumulation and changing subunit patterns of the albumin protein fraction, during seed development, is a pre-requisite for the analysis of the developmental controls involved. This knowledge could eventually help the plant breeder formulate strategies aimed at improving seed protein yield and quality.

Pea plants were grown under controlled environmental conditions; this had two advantages: firstly seeds matured earlier than if grown under normal (natural) field conditions (Millerd and Spencer, 1974) and secondly, synthesis of the proteins (storage) in seeds, grown in this way, occurs at precise, predictable stages of seed development (Millerd and Spencer, 1974; Millerd et al., 1975; Gatehouse et al., 1981, 1982b, 1984). Seed development itself may be separated into two phases, which are related to time (days) after flowering (DAF), under controlled conditions for a given pea line (Gatehouse et al., 1984). During the first phase, which lasts from 0 to 8 DAF (Gatehouse et al., 1982b) under these conditions, and occupies about one third

of the active development of the seed; cell division occurs in the embryo but very little synthesis of storage reserves occurs. The second phase extends from 8 to 24 DAF (Gatehouse et al., 1982b), when cell division ceases and the structures of the mature seed, the cotyledons and embryonic axis, appear. Towards the end of this phase, there is a decrease in synthetic activity and the seed begins to dessicate (Gatehouse et al., 1984).

The synthesis of albumin proteins has been reported to take place prior to the synthesis of vicilin and legumin during the seed developmental stages by Beevers and Poulson (1972); Millerd and Spencer (1974); Basha (1974) and Guldager (1978). In this study developing seeds were examined at 14 stages, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 and 24 days after flowering. The stage of 33 DAF represented the completion of seed maturation (Beevers and Poulson, 1972). PMA-L, PLA and lectin were first detected at about 13 DAF.

Their accumulation was shown to be complete at 21 DAF (Figs. 63-68). The PMA-L synthesis data is supported by the '<u>in vitro</u>' synthesis observations of Ragab (personal communication), who has also described the synthesis of PMA-L at about 12 DAF. The appearance of PMA-S and HMW-albumin commenced two days later (Figs. 63-68) than the other albumin proteins; their accumulation was confirmed at 16 DAF by two-dimensional gel electrophoresis.

This qualitative assessment of the major albumin protein synthesis during seed development indicated that not all the major components of the total seed albumin fraction are synthesised simultaneously

and that the component composition varies with the developmental stages of the cotyledons, although their pattern of synthesis and accumulation are broadly similar to that of the globulin storage proteins (Gatehouse et al., 1981, 1982b). Guldager (1978) using semi-quantitative crossed-immunoelectrophoresis has reported that the synthesis of the predominant albumins commenced after the onset of legumin synthesis although the identity of these albumins in terms of subunit composition was not described. Quantitative estimation by immunoelectrophoresis would be required to confirm these present results as has been performed for the globulin proteins by Gatehouse et al., (1984).

(b) Degradation during seed germination:

As defined by Boulter and Derbyshire (1971), storage proteins function by supplying nitrogen compounds for respiration and growth during periods of intense metabolic activity. A storage protein is thus any protein present in major quantity in the seeds which is degraded in vivo following germination, thus functioning as a reserve of amino nitrogen and carbon skeletons and for which no other function may be demonstrated. By these functional criteria, legumin, vicilin and convicilin, the major globulin protein types from pea seeds, have already been shown to be storage proteins (Daneilson, 1951; Basha and Beevers, 1975, 1976; Croy et al., 1980). According to the same criteria, certain polypeptide components belonging to the albumin fraction would also appear to be storage proteins. It was observed on SDS-PAGE that the high molecular weight albumin protein was degraded rapidly following the first few days of germination (Figs. 53-62) and thus seems likely to serve as a storage protein confirming the observation of Murray (1979) for Pisum sativum.

In contrast, the breakdown of the pea major albumin proteins, PMA-L, PMA-S and the lectins during germination is quite different from that of the HMW-albumin. There was no apparent change in the amounts of PMA-L, PMA-S and lectin bands (Figs. 53-62) up to 11 days after germination. This result agrees with the observations of Guldager (1978); Murray (1979), Tyler (1981) and Jakubek and Przybylska (1982) who claimed the non-degradation of a major albumin polypeptide of Mr 22000-24000 in pea cotyledons up to 3 weeks after germination. Clearly the absence of significant degradation of such a protein, coupled with its high sulphur content (Table 3) indicates that its role is distinctly different from the storage proteins and suggests that pea major albumins and lectins may play a role as structural proteins, in an agreement with Murray (1979) and Jakubek and Przybylska (1982); although further studies are needed to test this hypothesis. The role of PLA is also not as yet clear. It is relatively sulphur rich protein (4 sulphur atoms/54 amino acid residues, c.f. legumin 9 sulphur atoms/525 amino acid residues; Lycett et al., 1984) and may therefore act as a 'sulphur storage protein' in a similar way to the role proposed for trypsin inhibitors (Pusztai, 1967) although it is not an inhibitor of trypsin or chymotrypsin as previously mentioned.

PLA appears to be identical to a protein described as "13,000 Mr" by Chandler et al., (1984) and also described by Schroeder (1984). The former authors described the accumulation and decline of the mRNA for this polypeptide during seed development which occurs in manner very similar to storage protein mRNAs. Schroeder (1984) lends support to the hypothesis that PLA functions as a 'sulphur storage protein', since its synthesis is markedly depressed in a low sulphur environment.

Careful analysis of the behaviour of this protein on seed germination will be required to confirm or deny this hypothesis. Preliminary data (Fig. 53 and Fig. 54) suggests that PLA also may not be rapidly degraded after seed germination, and persists in the cotyledons after the major storage proteins have disappeared. This result disagrees with the reports of Murray (1979) who claimed that albumin polypeptides of Mr 10,000 and Mr 8,000 present in total albumin fraction had disappeared by 9 days after seed germination.

10. Conclusion:

The failure to find any role for PMA-L, PMA-S and PLA may suggest that pea seeds have a synthetic capacity for proteins in excess of their requirements.

Since both pea major albumin and low molecular weight albumin proteins represent significant amounts of the total cotyledonary protein and are important nutritionally due to their higher levels of sulphur amino acids than that of globulins they may offer hope for useful advances in breeding to improve protein quantity in terms of yield and nutritional quality of pea seeds.

REFERENCES

Anstee, J. H. and Charnley, A. K. (1977). J. Insect Physiol. 23: 965-974.

Austin, K. F. (1965). In: "Immunological Diseases". Ed. Samter, M. pp. 221-225. Little, Brown and Co., Boston, Massachusetts.

Aykroyd, W. R. and Doughty, J. (1964). FAO Nutritional Studies No. 19, p.73. Rome.

Basha, S. M. M. (1974). Ph.D. Thesis, Grad. Coll. Univ. Oklahoma, Norman, pp. 79.

Basha, S. M. M. and Beevers, L. (1975). Planta 124: 77-87.

Basha, S. M. M. and Beevers, L. (1976). Plant Physiol. 57: 93-97.

Beevers, L. and Poulson, R. (1972). Plant Physiol. 49: 476-481.

Bell, A. A. (1981). Ann. Rev. Plant Physiol. 32: 21-81.

Blagoveschenskii, A. V. (1967). "Biochemistry of Legume Plants". Akad. Nauk. SSSR.

Bohlool, B. B. and Schmidt, E.L. (1976). J. Bacteriol. 125: 1188-1194.

Bollini, R. and Chrispeels, M.J. (1978). Planta 142: 291-298.

Booth, A. N., Robbins, D. J., Ribelin, W. E. and Deeds, F. (1960). Proc. Soc. Expt. Biol. Med. 104: 681-683.

Boulter, D. (1982). Proc. Nutr. Soc. 41: 1-6.

Boulter, D. (1983). In: "Better Crops for Food". Pitman Books, London (Ciba Foundation Symposium 97), pp. 16-27.

Boulter, D. (1984). Phil. Trans. Roy. Soc. Lond. B304: 323-332.

Boulter, D. and Crocomo, O. J. (1979). In: "Plant Cell and Tissue Culture". Eds. Sharp, W. R. et al., pp. 615-632. Ohio State University Press, Columbus.

Boulter, D. and Derbyshire, E. (1971). In: "Chemotaxonomy of the Leguminosae". Eds. Harborne, J. B., Boulter, D. and Turner, B. L., pp. 285-308. Academic Press, London.

Boulter, D., Evans, I. M. and Derbyshire, E. (1973). Qual. Plant. - Plant Foods Hum. Nutr. 23: 239-250.

Boulter, D., Evans, I. M. and Thompson, A. (1973). Proc. 1st Int. Inst. Tropical Agric. Grain Legume Improvement Workshop, Ibadan, Nigeria, 29th Oct.-2nd Nov., pp. 239-248.

Boulter, D., Thurman, D. A. and Derbyshire, E. (1967). New Phytol. 66: 27-36.

Boulter, D., Evans, I. M., Thompson, A. and Yarwood, A. (1973). Nutritional Improvement of Food Legumes by Breeding. Protein Advisory Group (PAG) of the United Nations System, United Nations, New York 10017, pp. 205-215.

Brohult, S. and Sandegren, E. (1954). In: "The Proteins", Vol. 2, Part A, pp. 487-512. Eds. Neurath, H. and Bailey, K. Academic Press, London and New York.

Brook, A. J. W. and Housley, S. (1969a). J. Chromat. 41: 200-204.

Brook, A. J. W. and Housley, S. (1969b). J. Chromat. 42: 112-116.

Brook, A. J. W. and Munday, K. C. (1970). J. Chromat. 47: 1-8.

Burr, H. K. (1975). In: "Protein Nutritional Quality of Foods and Feeds", Part 2, pp. 119-134. Ed. Friedman, M. Marcel Dekker, New York.

Byers, M., Smith, S. J. and Miflin, B. J. (1983). J. Sci. Fd. Agric. 34: 447-462.

Calvert, H. E., Lalonde, M., Bhuvaneswari, T. V. and Bauer, W. D. (1978). J. Microbiol. 24: 785-793.

Casey, R. (1979a). Biochem. J. 177: 509-520.

Casey, R. (1979b). Heredity 43: 265-272.

Chandler, P. M., Spencer, D., Randall, P. J. and Higgins, T. J. V. (1984). Plant Physiol. Submitted.

Chang, J. Y., Brauer, D. and Wittmann-Liebold, B. (1978). FEBS Lett. 93: 205-214.

Chapman, G. P., Fagg, C. W. and Peat, W. E. (1979). Z. Pflanzen Physiol. 94: 247.

Craid, S. and Millerd, A. (1981). Protoplasma 105: 333-339.

Criddle, R. S. and Willemot (1967). In: "Protides of Biological Fluids", Vol. 15, pp. 55-67. Ed. Peeters, H. Elsevier, Amsterdam.

Crouch, M. L., Tenbarge, K. M., Simon, A. E. and Ferl, R. (1983). J. Mol. Appl. Genet. 2: 273-283.

Croy, R. R. D. (1977). Ph.D. Thesis, University of Aberdeen, U.K.

Croy, R. R. D., Derbyshire, E., Krishna, T. G. and Boulter, D. (1979). New Phytol. 83: 29-35.

Croy, R. R. D., Gatehouse, J. A., Tyler, M. and Boulter, D. (1980). Biochem. J. 191: 509-516.

Croy, R. R. D., Hoque, M. S., Gatehouse, J. A. and Boulter, D. (1984). Biochem. J. 218: 795-803.

Danielson, C. E. (1949). Biochem. J. 44: 387-400.

Danielson, C. E. (1951). Acta Chem. Scand. 5: 541-544.

Danielson, C. E. (1956). Ann. Rev. Plant Physiol. 7: 215-235.

Davies, D. R. (1976). Euphytica 25: 717-724.

Dazzo, F. B. (1978). Rhizobium Newsletter 23: 21-25.

de Muelenaera, H. J. H. (1964). Nature (Lond.) 201: 1029-1030.

Derbyshire, E., Wright, D. J. and Boulter, D. (1976). Phytochem. 15: 3-24.

Determan, H. and Walter, I. (1968). Nature (Lond.) 219: 604-605.

Dubois, M., Giltes, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956). Anal. Chem. 28: 350-356.

Eckhardt, H. E., Hayes, C. E. and Goldstein, I. J. (1976). Anal. Biochem. 73: 192-197.

Entlicher, G., Kostir, J. V. and Kocourek, J. (1970). Biochim. Biophys. Acta 221: 272.

Erlanger, B. F., Kokowsky, N. and Cohen, W. (1961). Arch. Biochem. Biophys. 95: 271-278.

Evans, R. J. and Brandemer, S. L. (1967). Cereal Chem. 44: 417-426.

Evans, I. M., Croy, R. R. D., Hutchinson, P., Boulter, D., Payne, P. I. and Gordon, M. E. (1979). Planta 144: 455-462.

Figarella, C., Negri, C. A. and Guy, O. (1975). Eur. J. Biochem. 53: 457-463.

Food and Agricultural Organisation of the United Nations. Production Year Book 1970, 1971. Nutritional Studies No. 24, Rome.

Food and Agricultural Organisation of the United Nations. Production Year Book 1972, 1973. Nutritional Studies No. 26, Rome.

Fox, D. J. (1964). Ph.D. Thesis, University of Liverpool, U.K.

Fox, D. J., Thurman, D. A. and Boulter, D. (1964). Phytochem. 3: 417-419.

Fries, J. H. (1966). J. Asthma Res. 3: 209-211.

Garnier, J., Osguthorpe, D. J. and Robson, B. (1978). J. Mol. Biol. 120: 97-120.

Gatehouse, A. M. R. (1984). In: "Anti-nutritional Proteins in Plants". In Press.

Gatehouse, A. M. R. and Boulter, D. (1983). J. Sci. Fd. Agric. 34: 345-350.

Gatehouse, A. M. R., Dewey, F. M., Dove, J., Fenton, K. A. and Pusztai, A. (1984). J. Sci. Fd. Agric. 35: 373-380.

Gatehouse, A. M. R., Gatehouse, J. A. and Boulter, D. (1980). Phytochem. 19: 751-756.

Gatehouse, A. M. R., Gatehouse, J. A., Dobie, P., Kilminster, A. M. and Boulter, D. (1979). J. Sci. Fd. Agric. 30: 948-958.

Gatehouse, J. A. and Boulter, D. (1980). Physiol. Plant. 49: 437-442.

Gatehouse, J. A., Croy, R. R. D. and Boulter, D. (1980). Biochem. J. 185: 497-503.

Gatehouse, J. A., Croy, R. R. D. and Boulter, D. (1984). CRC Critical Review in Plant Sci. 1: 287-314.

Gatehouse, J. A., Croy, R. R. D., Morton, H., Tyler, M. and Boulter, D. (1981). Eur. J. Biochem. 118: 627-633.

Gatehouse, J. A., Lycett, G. W., Croy, R. R. D. and Boulter, D. (1982a). Biochem. J. 207: 629-632.

Gatehouse, J. A., Evans, I. M., Bown, D., Croy, R.R. D. and Boulter, D. (1982b). Biochem. J. 208: 119-127.

Gates, P., Yarwood, J. N., Harris, N., Smith, M. L. and Boulter, D. (1981). In: "Vicia faba, Physiology and Breeding", pp. 299-312. Ed. Thomson, R. Martinus Nijhoff, The Hague.

Gellotte, B. (1960). J. Chromat. 3: 330-342.

Glazer, A. N., Delanger, R. J. and Sigman, D. S. (Eds.) (1975). "Chemical Modification of Proteins - Selected Methods and Analytical Procedures". North Holland/Elsevier, Amsterdam.

Gleeson, P. A. and Jermym, M. A. (1977). Aust. J. Plant Physiol. 4: 25-37.

Goldstein, G. B. (1969). J. Allergy 44: 37-50.

Graham, T. A. and Gunning, B. E. S. (1970). Nature (Lond.) 228: 81-82.

Grant, D. R., Sumner, A. K. and Johnson, J. (1976). Can. Inst. Fd. Sci. Technol. J. 9: 84-91.

Gray, W. R. (1972). Meds. Enzymol. 25: 121-138.

Green, G. M. and Lyman, R. L. (1972). Proc. Soc. Exp. Biol. Med. 140: 6-12.

Guldager, P. (1978). Theor. Appl. Genet. 53: 241-250.

Gupta, Y. P. (1983). In: "Chemistry and Biochemistry of Legumes", pp. 287-327. Ed. Arora, S. K. Edward Arnold Press.

Hartley, B. S. (1960). Ann. Rev. Biochem. 29: 45-72.

Hashimoto, F., Horigome, T., Kanbayashi, H., Yoshida, K. and Sugano, T. (1983). Anal. Biochem. 129: 192-199.

Heywood, V. H. (1971). In: "Chemotaxonomy of the Leguminosae", pp. 1-30. Eds. Boulter, D. and Turner, B. L. Academic Press, London and New York.

Hopp, T. R. and Woods, K. R. (1981). Proc. Natl. Acad. Sci. USA. 78: 3824-3828.

Hurich, J., Parzysz, H. and Przybylska, J. (1977). Genetica Polonica 18: 241-252.

Jackson, P., Boulter, D. and Thurman, D. A. (1969). New Phytol. 68: 25-33.

Jaffe, W. G. (1960). Arzneim. Forsch. 10: 1012-1016.

Jaffe, W. G. (1969). In: "Toxic Constituents of Plant Foodstuffs", pp. 69-101. Ed. Liener, I. E. Academic Press, New York.

Jaffe, W. G. and Vega Lette, C. L. (1968). J. Nutr. 94: 203-210.

Jain, H. K. (1971). Indian Farming 21(8): 9-10.

Jakubek, M. F. and Przybylska, J. (1978). Pisum Genetics Assoc., Pisum Newsletter 10: 24.

Jakubek, M. F. and Przybylska, J. (1979). Pisum Genetics Assoc., Pisum Newsletter 11: 12-13.

Jakubek, M. F. and Przybylska, J. (1982). Pisum Genetics Assoc., Pisum Newsletter 14: 26-28.

Janson, J. C. (1967). J. Chromat. 28: 12-20.

Janzen, D. H., Juster, H. B. and Liener, I. E. (1976). Science 192: 795-796.

Johnston, A. W. B., Brewster, V. and Davies, D. R. (1977). Annals. Bota. 41: 381-385.

Kakade, M. L. and Evans, R. J. (1965). Br. J. Natl. 19: 269-276.

Kienholz, E. M., Jensen, L. S. and McGinnins, J. (1962). Poult. Sci. 41: 367.

Klydzhev, V. and Pleshkov, B. P. (1965). Dokl. Timiryazevsk Sel'skokhoz Akad. 115: 135.

Konopska, L. (1978). Biochem. Physiol. Pflanz. 173: 322-326.

Kunitz, M. (1945). Science, New York 101: 668-669.

Laemmli, U. K. (1970). Nature (Lond.) 227: 680-685.

Laurell, C. B. (1966). Anal. Biochem. 15: 45.

Liener, I. E. (1962). Am. J. clin. Nutr. 11: 281.

Liener, I. E. (1974a). J. Agric. Fd. Chem. 22: 17-22.

Liener, I. E. (1974b). Indian J. Nutr. Dietet. 10: 303-322.

Liener, I. E. (1980). In: "Toxic Constituents of Plant Foodstuffs", pp. 430-460, 2nd Edition. Academic Press, New York.

Liener, I. E. and Kakade, M. L. (1969). In: "Toxic Constituents of Plant Foodstuffs", pp. 7-68. Ed. Liener, I. E. Academic Press, New York.

Liener, I. E. and Pallansch, M. J. (1952). J. Biol. Chem. 197: 29.

Lis, H. and Sharon, N. (1973). Ann. Rev. Biochem. 42: 541-574.

Lis, H. and Sharon, N. (1981). In: "The Biochemistry of Plants", Vol. 6. Proteins and Nucleic Acids, pp. 371-447. Ed. Marcus, A. Academic Press, New York.

Lycett, G. W., Croy, R. R. D., Shirsat, A. and Boulter, D. (1984). Nucleic Acids Res. In Press.

Malley, A., Baecher, L., Mackler, B. and Perlman, F. (1975). J. Allergy and Clin. Immunol. 56: 282-290.

Matta, N. K. (1981). Ph.D. Thesis, University of Durham, U.K.

Matta, N. K., Gatehouse, J. A. and Boulter, D. (1981). J. Exp. Bot. 131: 1295-1307.

Matta, N. K. and Gatehouse, J. A. (1982). Heredity 48(3): 383-392.

McKee, H. S. (1962). Nitrogen Metabolism in Plants, 329. Oxford: Clarendon, 728 pp.

Merril, C. R., Goldman, D., Sedman, S. A. and Ebert, M. H. (1981). Science 211: 1437-1438.

Mestechy, J., Kraus, F. W., Hurst, D. C. and Voight, S. A. (1969). Anal. Biochem. 30: 190-198.

Miflin, B. J. and Shewry, P. R. (1980). Presented at the International Association of Plant Physiologists Conference at Calgary, Canada, July 1980, on Nitrogen and Crop Yield, pp. 1-19.

Millerd, A. (1975). Ann. Rev. Plant Physiol. 26: 53-72.

Millerd, A., Thomson, J. A. and Schroeder, H. E. (1978). Aust. J. Plant Physiol. 5: 519-534.

Millerd, A., Spencer, D., Dudman, W. F. and Stiller, M. (1975). Aust. J. Plant Physiol. 2: 51-59.

Millerd, A. and Spencer, D. (1974). Aust. J. Plant Physiol. 1: 331-341.

Mirelman, D., Galun, E., Sharon, N. and Lotan, R. (1975). Nature (Lond.) 256: 414-416.

Mirsa, B. K., Singh, S. P. and Barat, G. K. (1980). Qualitas Pl. Mater. Veg. 30: 259.

Montgomery, R. D. (1969). In: "Toxic Constituents of Plant Foodstuffs", pp. 143-157. Ed. Liener, I. E. Academic Press, New York.

Morris, G. F. I., Thurman, D. A. and Boulter, D. (1970). Phytochem. 9: 1707-1714.

Müller, H. and Gottschalk, W. (1973). In: "Nuclear Techniques for Seed Protein Improvement", pp. 235-253. Intern. Atomic Energy Agency, Vienna.

Murray, D. R. (1979). Plant, Cell and Environment 2: 221-226.

Nash, A. M. and Wolf, W. J. (1967). Cereal Chem. 44: 183-192.

Nash, A. M., Eldridge, A. C. and Wolf, W. J. (1967). J. Agric. Fd. Chem. 15: 102.

Nash, A. M., Kwolek, W. F. and Wolf, W. J. (1971). Cereal Chem. 48: 360-368.

Neely, R. S. and Beevers, L. (1980). J. Exp. Bot. 31: 299-312.

Niess, E., Ivy, C. A. and Nesheim, M. C. (1972). Proc. Soc. Exp. Biol. Med. 140: 291-296.

Odell, B. L. and Savage, J. E. (1960). Proc. Soc. Exp. Biol. Med. 103: 304.

Ory, R. L. and Sekul, A. A. (1983). In: "Seed Proteins", pp. 83-99. Phytochemical Soc. of Europe Symposia Series No. 20. Eds. Daussant, J., Mosse, J. and Vaughan, J. Academic Press, London.

Osborne, T. B. (1924). In: "The Vegetable Proteins", p.154. Longmans, Green and Co., London.

Ouchterlony, O. and Nilsson, L. A. (1978). In: "Handbook of Experimental Immunology", Vol. 1 - 'Immunochemistry', pp. 19.1-19.44. Ed. Wier, D. M. Blackwell Scientific Publications.

Pant, R., Rajagopalan Nair, C. R. and Singh, K. S. (1968). Curr. Sci. 37: 166.

Payne, J. W. (1976). In: "Chromatographic and Electrophoretic Techniques", Vol. 2, 4th edition, pp. 321-345. Ed. Smith, I. Heinemann, London.

Perlman, F. (1966). Food Technol. 20: 1438-1442.

Phadke, K. and Sohonie, K. (1962). J. Sci. Industr. Res. 21C: 155-158.

Pharmacia Fine Chemicals, Gel filtration theory of practice, pp. 1-64.

Phillips, D. E., Eyre, M. D., Thomson, A. and Boulter, D. (1981). J. Sci. Food Agric. 32: 423-432.

Protein Advisory Group Statement 22 (1973). In: "Nutritional Improvement of Food Legumes by Breeding", pp. 349-380. Ed. Milner, M. Wiley and Sons, New York.

Pusztai, A. (1967). Nutr. Abs. Rev. 37: 1-9.

Pusztai, A., Grant, G. and Palmer, R. (1975). J. Sci. Food Agric. 26: 149-156.

Pusztai, A., Croy, R. R. D., Grant, G. and Watt, W. B. (1977). New Phytol. 79: 61-71.

Pusztai, A., Clarke, E. M. W. and King, T. P. (1979). Proc. Nutr. Soc. 38: 115-120.

Pusztai, A. and Stewart, J. C. (1980). Biochim. Biophys. Acta 623: 418-428.

Rackis, J. J. (1974). J. Am. Oil Chem. Soc. 51: 161A-174A.

Reid, M. S. and Bielski, R. L. (1968). Analyt. Biochem. 22: 374-381.

Richardson, L. R. (1948). J. Nutr. 36: 451-462.

Ripa, A. and Geidans, M. (1964). Latv. PSR Zinat Akad. Vest. 8: 69.

Ryan, C. A. (1973). Ann. Rev. Plant Physiol. 24: 173-196.

Ryan, C. A. and Walker-Simmons, M. (1981). In: "The Biochemistry of Plants", Vol. 6. Proteins and Nucleic Acids, pp. 321-350. Ed. Marcus A. Academic Press, New York.

Schroeder, H. E. (1982). J. Sci. Fd. Agric. 33: 623-633.

Schroeder, H. E. (1984). J. Sci. Fd. Agric. In Press.

Segrest, J. P. and Jackson, R. L. (1972). Med. Enzymol. 28: 54-63.

Sequeira, L. (1978). Ann. Rev. Phytopathol. 16: 453-481.

Sharief, F. S. and Li, S. S.-L. (1982). J. Biol. Chem. 257: 14753-14759.

Shewry, P. R., Ellis, J. R. S., Pratt, H. M. and Miflin, B. J. (1978a). J. Sci. Fd. Agric. 29: 433-441.

Shewry, P. R., Hill, J. M., Pratt, H. M., Leggatt, M. M. and Miflin, B. J. (1978b). J. Exp. Bot. 29: 677-692.

Shewry, P. R., Field, J. M., Lew, E. J.-L. and Kasarda, D. D. (1982). J. Exp. Bot. 133: 261-268.

Shewry, P.R., Field, J.M., Faulks, A.J., Parmar, S., Miflin, B.J., Dietler, M.D., Lew, E.J-L. and Kasarda, D.D. (1983a). (in press).

Shewry, P.R., Finch, R., Parmar, S., Franklin, J. and Miflin, B.J. (1983b) Heredity, 50: 179-189.

Shewry, P. R., Miflin, B. J. and Kasarda, D. D. (1984). Phil. Trans. Roy. Soc. Lond. B304: 297-308.

Siegel, A. and Fawcett, B. (1976). Food legume processing and utilization. International Research Centre, Ottawa, Ontario, Canada.

Spencer, D. (1984). Phil. Trans. Roy. Soc. Lond. B304: 275-285.

Spies, J. R. (1974). J. Agric. Fd. Chem. 22: 30-36.

Srikantia, S. G. (1982). In: "Human Nutrition", pp. 197-207. Eds. Jukes, T. H. and Neuberger, A. Current issues and controversies. M.T.P. Press, Lancaster.

Stein, M. (1976). Qual. Plant. - Plant Fds. Hum. Nutr. 26: 227-243.

Stillmark, H. (1889). Arch. Pharmakol. Inst. Dorpat. 3: 59.

Sumner, J. B. (1926). J. Biochemistry, Tokyo 69: 435.

Sumner, J. B. and Howell, S. F. (1936). J. Bacteriol. 32: 227.

Sweely, C. C., Wells, W. W. and Bentley, R. (1966). Meth. Enzym. 11: 93-108.

Tauber, H., Kershaw, B. B. and Wright, R. D. (1949). J. Biol. Chem. 179: 1155-1161.

Thomson, J. A., Schroeder, H. E. and Dudman, W. F. (1978). Aust. J. Plant Physiol. 5: 263-279.

Tomé, D., Dhoye, O., Gaborit, T., Koslowski, A. and Valdebouze, P. (1981). Sciences des Aliments 1: 587-601.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Proc. Natl. Acad. Sci. USA. 76: 4350-4354.

Travis, J. and Roberts, R. C. (1969). Biochem. 8: 2884-2889.

Trowbridge, I. S. (1974). J. Biol. Chem. 249: 6004-6012.

Tyler, M. (1981). Ph.D. Thesis, University of Durham, U.K.

Van Etten, C. H. (1969). In: "Toxic Constituents of Plant Foodstuffs", pp. 103-142. Ed. Liener, I. E. Academic Press, New York.

Varner, J. E. and Schidlovsky, G. (1963). Plant Physiol. 38: 139-144.

Vesterberg, O. (1975). In: "Isoelectric focusing", pp. 78-113. Eds. Arbuthnott, J. A. and Beely, J. A. Butterworths and Co., London.

Von Pirquet, C. (1906). Muench. Med. Woechenschr. 53: 1457.

Wagh, P. V., Klaustermeier, D. F., Waibel, P. E. and Liener, I. E. (1963). J. Nutr. 80: 191-195.

Walsh, K. A. and Wilcox, P. E. (1970). In: "Methods in Enzymology", Vol. 19, p.33. Eds. Perlmann, G. E. and Lorand, L. Academic Press, New York.

Weber, E. and Neumann, D. (1980). Biochem. Physiol. Pflanz. 175: 279-306.

Weber, E., Manteuffel, R. and Neumann, D. (1978). Biochem. Physiol. Pflanz. 172: 597-614.

Weeke, B. (1973). In: "A Manual of Quantitative Immunoelectrophoresis", pp. 15-46. Eds. Axelsen, N. H., Krøll, J. and Weeke, B. Universitets Forlaget, Oslo.

Whatman Information Leaflet. Advanced ion-exchange cellulose, pp. 1-6.

Williams, K. W. (1972). Lab. pract. 21: 667-670.

Wilson, C. M., Shewry, P. R., Faulks, A. J. and Miflin, B. J. (1981). J. Exp. Bot. 32: 1287-1293.

Woods, K. R. and Wang, K. T. (1967). Biochim. Biophys. Acta 133: 369-370.

Youle, R. J. and Huang, A. H. C. (1978). Plant Physiol. 61: 1040-1042.

Zussman, B. M. (1966). South. Med. J. 59: 935-939.

Fig. 69. Dot-matrix comparisons of the amino-acid sequences of PLA (=PSA2S) and the 2S storage proteins of Brassica napus (BNA2S) and Ricinus communis (RCO2S). The latter two proteins are also compared to show their homology. The amino acid sequences are given in reverse order and the N-terminals of the proteins are in the lower right-hand corners of the matrices.

FIG. 69.

BNA2S

YSPGPMTKQFPCVSVQPINCVKPLH
TATQYIRSVMQQKGQQQGQQQIQQKVAKSAGKLTPCVCLPEEQHLENCCQQLLPPRQQPGQPNEMDDEFD
FEGDLTLWQQCARLHQAQQFEKRCKPIRFPGASNTADDEDFEVVTRYISANTLLFFFALTASVLFLKNAM

PSA2S

NDKPTIKSFFDEAEKSAFCWGYEIDPNPYHGCFNGSGKKRCDADSECLNPHED

PSA2S vs BNA2S....6 matches. Minimum score 72
AMINO ACID TEST WITH MATRIX SCORE

PSA2S vs RCO2S....6 matches. Minimum score 72
AMINO ACID TEST WITH MATRIX SCORE

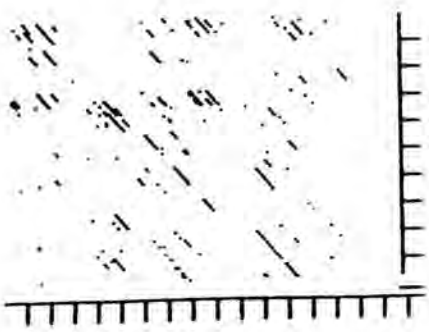
PSA2S

NDKPTIKSFFDEAEKSAFCWGYEIDPNPYHGCFNGSGKKRCDADSECLNPHED

RCO2S

FRCQTPSVGCMSPLNAATRFAEFVN
QGQLQQQQIAQRLGECRCQSQMQKLHDCCGRLSREQRRPGQGSVQQKIYEQCQRLNQQEQIQGRCGQQSP

RCO2S vs BNA2S....6 matches. Minimum score 72
AMINO ACID TEST WITH MATRIX SCORE



RCO2S

FRCQTPSVGCMSPLNAATRFAEFVN
QGQLQQQQIAQRLGECRCQSQMQKLHDCCGRLSREQRRPGQGSVQQKIYEQCQRLNQQEQIQGRCGQQSP

BNA2S

YSPGPMTKQFPCVSVQPINCVKPLH
TATQYIRSVMQQKGQQQGQQQIQQKVAKSAGKLTPCVCLPEEQHLENCCQQLLPPRQQPGQPNEMDDEFD
FEGDLTLWQQCARLHQAQQFEKRCKPIRFPGASNTADDEDFEVVTRYISANTLLFFFALTASVLFLKNAM

Biochem. J. (1984) **218**, 795–803
Printed in Great Britain

The major albumin proteins from pea (*Pisum sativum* L)

Purification and some properties

Ronald R. D. CROY, Mohammed S. HOQUE, John A. GATEHOUSE and Donald BOULTER
Department of Botany, University of Durham, South Road, Durham DH1 3LE, U.K.

(*Received 26 August 1983/Accepted 17 November 1983*)

A scheme is described for the fractionation of pea (*Pisum sativum*) albumin proteins. By using this scheme, two closely related major albumin proteins have been isolated and purified to homogeneity. The larger protein, designated PMA-L, has $M_r \sim 53000$ and consists of two 25000-M_r subunits, whereas the smaller, PMA-S, has $M_r \sim 48000$ and contains two 24000-M_r subunits. There was no evidence of mixed dimers of the two subunit sizes, despite their close homology as judged by immunological cross-reaction, amino acid composition, *N*-terminal amino acids, tryptic-peptide mapping and CNBr-cleavage products. Both proteins contained significant amounts of sulphur amino acids. The proteins were shown to be located in the soluble cytosol fraction of cotyledon cells and are not significantly degraded on seed germination. Preliminary screening indicates the presence of homologous major albumin proteins in at least three different, though closely related, legume species.

Studies on the seed proteins of pea (*Pisum sativum* L.) have largely concentrated on the predominant proteins, namely the globulin or storage-protein fraction (Gatehouse *et al.*, 1983). This fraction accounts for most (75–80%) of the total seed protein and is therefore, on a quantitative basis, of immediate importance in nutritional aspects of pea breeding. The albumin fraction, variously reported to constitute 14% (Grant *et al.*, 1976), 42% (Murray, 1979) or 15–38% (Davies, 1976) of the total seed protein, depending on the genetic line and method of albumin extraction, has long been regarded as the enzymic or metabolic protein fraction in seeds, containing small amounts of a large number of proteins (Danielson, 1956; Basha & Beevers, 1975; Boulter, 1982; Gatehouse *et al.*, 1983). Recent reports, however, now suggest that the pea albumin fraction contains a few proteins present in amounts large enough to be conveniently isolated and to be considered important nutritionally (Davies, 1976; Grant *et al.*, 1976; Guldager, 1978; Murray, 1979; Tyler, 1981). Furthermore, the levels of the sulphur amino acids in the pea albumin fraction have been reported to be disproportionately high when compared on an equal weight basis with the globulin fraction (Davies, 1976; Hurich *et al.*, 1977; Jakubek & Przybylska, 1978, 1979), making the albumin proteins even more important nutritionally. The inheritance of different types of pea major albumins and the tentative location of the major albumin gene locus has been reported (Blixt *et al.*, 1980). However, before such proteins can be profitably included in breeding programmes, it is necessary to investigate the properties and location of the individual components, as has beeen done for the globulin proteins (Gatehouse *et al.*, 1983).

Although it has been suggested (Murray, 1979) that the albumin fraction does contain storage proteins as defined by Derbyshire *et al.* (1976), certain major albumin components do not behave as storage proteins, as judged by their continued presence in germinating seeds long after storage proteins have been degraded (Murray, 1979; Guldager, 1978; Tyler, 1981; Jakubek & Przybylska, 1982). This then raises the question as to what the primary function(s) of these proteins might be. Few reports exist of comprehensive fractionation schemes for the albumins or the isolation and study of individual albumin proteins, except in the case of lectins (Trowbridge, 1974). We describe in the present paper the isolation and properties of two major albumin proteins and

Abbreviations used: SDS, sodium dodecyl sulphate; rel. satn., relative saturation; PMA-L, pea major albumin large; PMA-S, pea major albumin, small; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; h.p.l.c., high-pressure liquid chromatography; IgG, immunoglobulin fraction from serum.

report on some initial work into the elucidation of their function.

Experimental

Materials

Pea seeds (*Pisum sativium* L.), variety Feltham First, were obtained from Suttons Seeds Ltd., Reading, Berks., U.K.; seeds of other legumes (unspecified varieties) were obtained locally. Sephadex G-150, Sephacryl S-200, Blue Dextran and Pharmalyte ampholines were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K. DEAE-cellulose (DE-52) was purchased from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. and hydroxyapatite (Bio-Gel HT grade) was from Bio-Rad Laboratories Ltd., Bromley, Kent, U.K. Standard proteins were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. and BCL Ltd., Bell Lane, Lewes, East Sussex, U.K. Agarose was purchased from BRL, Cambridge, U.K. All other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K. and were of AnalaR grade wherever possible.

Methods

Fractionation of the pea albumins and isolation of the major protein components. Pea seeds were dehulled and the cotyledons milled to pass through a 365 μm-mesh sieve. The meal (50 g) was extracted twice with 20 mM-sodium acetate buffer, pH 5.0, at a ratio of 1 g of meal to 5 ml of buffer, for 1–2 h at 4°C. Extracts were clarified by centrifugation at 10000*g* for 30 min and then fractionated by $(NH_4)_2SO_4$ precipitation at 0°C. Precipitates in the ranges 0–50% and 50–90% rel. satn. were prepared, redissolved in 10 ml of 50 mM-Tris/HCl buffer, pH 7.5, and either dialysed against water and freeze-dried or used immediately for further fractionation. $(NH_4)_2SO_4$-precipitated fractions were chromatographed on a column of Sephadex G-150 (3.2 cm diam. × 56 cm, flow rate 20 ml/h) in 50 mM-Tris/HCl buffer, pH 7.5 (Fig. 1*a*). Appropriate fractions from the G-150 chromatography were pooled and applied directly to a column of DE-52 cellulose (2.5 cm diam. × 26 cm, flow rate 40 ml/h) equilibrated with 50 mM Tris-HCl, pH 7.5, and then eluted with a linear NaCl gradient (0–0.5 M, 500 ml total volume) in the same buffer (Fig. 1*b* below). Fractions eluted from the columns were analysed by SDS/polyacrylamide-gel electrophoresis and pooled accordingly. Globulin and albumin fractions were prepared from mature, developing or germinating seeds and from protein bodies as follows: total protein extracts were made from defatted seed meal or protein bodies, in 50 mM-sodium borate buffer, pH 8.0, and clarified by centrifugation. The extracts were then dialysed overnight against a large excess of 20 mM-acetate buffer, pH 5.0, at 4°C (Pusztai & Watt, 1970) and the globulin proteins, which were quantiatively precipitated, recovered by centrifugation at 40000*g* for 30 min, leaving the albumin proteins in the supernatant. All separated fractions were dialysed against distilled water and freeze-dried. Albumin protein extracts were prepared from small samples of meal (100–200 mg) from seeds of different species, by extraction with 20 mM-ammonium acetate buffer, pH 5.0 (1 ml) for 1 h at 4°C. After centrifugation at 13000*g* for 10 min the supernatants were used directly for immunodiffusion or were freeze-dried before SDS/polyacrylamide-gel electrophoresis.

M_r determinations. A column of Sephacryl S-200 (1.6 cm diam. × 40 cm, flow rate 7 ml/h), equilibrated with 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.25 M-NaCl and 0.1% NaN_3, was calibrated with standard proteins [(M_r in parentheses) catalase (240000); pea vicilin (150000); bovine serum albumin (68000); ovalbumin (43000); and myoglobin (16200)], to obtain a standard graph of V_e/V_0 against $\log M_r$. Albumin protein samples were subsequently chromatographed on the column and their molecular weights calculated from the corresponding V_e/V_0 values.

Gel electrophoresis. The subunit compositions of protein preparations were analysed by SDS/polyacrylamide-gel electrophoresis in slab gels by the methods of Laemmli (1970) and as modified by Matta *et al.* (1981) for various one-dimensional and two-dimensional techniques. M_r values for albumin protein subunits were obtained from a standard graph prepared for the following standard subunits analysed by SDS/polyacrylamide-gel electrophoresis (subunit M_r in parentheses): transferrin (76600); bovine serum albumin (68000); catalase (60000); ovalbumin (43000); lactic dehydrogenase (36000); soya-bean trypsin inhibitor (20100); ferritin (18500); β-lactoglobulin (17500); and pea seed lectin (18000). Non-dissociating electrophoresis was carried out in 8.5% (w/v)-acrylamide gels as described by Laemmli (1970), but omitting SDS and mercaptoethanol from all buffers. One-dimensional isoelectric focusing was performed in 7.5% (w/v)-acrylamide slab gels as described by Gatehouse *et al.* (1980). Urea was omitted from gels used for analysis of non-dissociated protein samples. Gels were stained for proteins with Coomassie Brilliant Blue R and for glycoproteins with the dansylhydrazine fluorescent stain as described by Eckhardt *et al.* (1976).

Amino acid analyses. Protein samples were reduced and carboxymethylated with iodoacetamide (Glazer *et al.*, 1975) before acid hydrolyses, as described by Croy *et al.* (1980), and analysed on a Variant 5060 h.p.l.c. system, with a

Micropak A.A. column (Varians; 15cm × 4mm diam.) using the Varian PCR-1 post-column derivatization and Fluorichrom detection system.

N-Terminal amino acid determination. *N*-Terminal amino acids were determined by the methods of Gray (1972) and Woods & Wang (1967).

Tryptic-peptide analyses. Tryptic peptides from carboxymethylated protein samples were prepared and analysed by h.p.l.c. using a Micropak MCH-10 (C_{18}, 10μm) column as described by Gatehouse *et al.* (1982). The peak elution profiles were compared as peptide maps.

CNBr cleavage. Albumin proteins were digested for different times (0, 3, 6, 24 and 48h) with CNBr as described by Croy *et al.* (1980) and the fragments were analysed on urea/SDS-containing gels as described by Hashimoto *et al.* (1983). Standard proteins used for estimating the M_r values for the fragments were: soya-bean trypsin inhibitor (M_r 20100); β-lactoglobulin (17500); myoglobin (16200); lysozyme (13200); cytochrome *c* (12700); and insulin (5600).

Carbohydrate analysis. Albumin proteins were precipitated and washed extensively with 10% (w/v) trichloroacetic acid and their sugar contents measured by the phenol/H_2SO_4 acid method of Dubois *et al.* (1956).

Antibody production, immunodiffusion and immunoelectrophoresis. Antibodies against purified albumin proteins were raised in New Zealand White rabbits and IgG fractions isolated from the antisera as described previously (Evans *et al.*, 1979). Immunodiffusions were performed in 1% (w/v) agarose gels as described by Croy *et al.* (1979). The identity of precipitin lines was confirmed by excision from the gel and analysis by SDS/polyacrylamide-gel electrophoresis as described by Croy *et al.* (1980). Rocket immunoelectrophoresis was carried out by the method of Weeke (1973), with antibodies (IgG) raised against purified major albumins (anti-PMA). Protein subunits were transferred from SDS/polyacrylamide gels on to nitrocellulose filters (Schleicher and Schüll) by electroblotting (Bio-Rad Trans Blot Cell). The filters were allowed to react with anti-PMA and then with peroxidase-coupled goat anti-rabbit IgG, before staining with 4-chloro-1-naphthol according to the standard 'Western blotting' protocols (Towbin *et al.*, 1979).

Haemagglutination assays. Assays were carried out by a serial-dilution method on microtitration plates with a 2% (v/v) suspension of untreated rabbit erythrocytes in phosphate-buffered saline [50mM-sodium phosphate buffer (pH7.5)/0.15M-NaCl]. Pea lectins and concanavalin A were used as standard haemagglutinins.

Protein-body isolation. All operations were carried out at 0°–4°C. Cotyledons from 20-day-old developing seeds were chilled, grated into homogenization buffer [0.5M-sucrose/50mM-sodium phosphate buffer, pH7.5 (buffer A)], and stirred gently for 5–10min to release the organelles. The homogenate was strained through muslin, centrifuged for 5min at 300*g* to remove starch grains and protein bodies pelleted by centrifugation for 10min at 10000*g* and gently resuspended in buffer A. Samples of the homogenate, or of protein bodies, were layered on to 20ml linear (30–90% (w/v)-sucrose gradients and centrifuged in an MSE Prepspin 65 ultracentrifuge for 15h at 60000*g* (MSE 3 × 25ml swing out rotor). Gradients were unloaded through an ISCO-UV monitor and fractions collected according to the A_{280} profile. Only the two major protein fractions from the gradients, corresponding to the total soluble (cytosol) proteins (top of the gradient) and the protein bodies [density (ρ) = 1.3g/ml] were analysed for protein composition. Total soluble proteins and protein-body proteins were fractionated into globulins and albumins as described previously, and freeze-dried (Pusztai & Watt, 1970)

Enzyme activities. Albumin proteins were tested for potential enzyme and inhibitor activities as follows: α- and β-D-glucosidases, α- and β-D-galactosidases and α-D-mannosidase were assayed using the *p*-nitrophenyl derivatives of the corresponding sugars according to the methods of Anstee & Charnley (1977). Amylase and amylase inhibitors were estimated by the method of Mestechy *et al.* (1969). Trypsin inhibitors were estimated by the method of Erlanger *et al.* (1961).

Concanavalin A. Precipitation reactions of the major albumins with concanavalin A were performed in 1% (w/v) agarose gels as described by Croy *et al.* (1980).

Results and discussion

Purification of major albumin proteins

The scheme described in the Experimental section has been used to purify two major pea albumin proteins to homogeneity, as judged by SDS/polyacrylamide-gel electrophoresis. Each consists of single-M_r subunits.

The protein fraction precipitating from total seed albumins between 50 and 90%-rel.-satn. $(NH_4)_2SO_4$ contained all or most of the major albumin proteins. This fraction was subsequently chromatographed on a Sephadex gel-filtration column. SDS/polyacrylamide-gel electrophoresis of the chromatographed fractions showed that the main peak (Fig. 1*a*) consisted largely of the major albumin proteins (subunit M_r about 25000). Pea seed lectins, also precipitating in this $(NH_4)_2SO_4$ fraction, were bound to the Sephadex, and the purified major albumins were completely free from

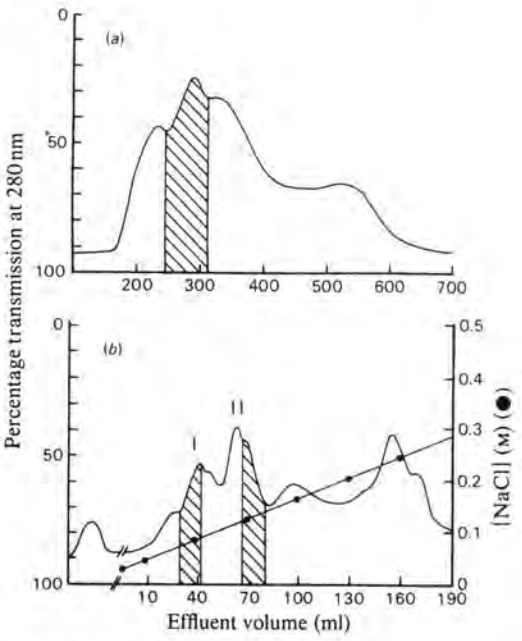


Fig. 1. *U.v.-absorbance (280nm) elution profiles of albumin proteins from (a) Sephadex G-150 and (b) DEAE-cellulose columns*

Cross-hatched areas indicate fractions pooled for further purification and analysis. Peaks I and II in (*b*) contained the small and large major albumin proteins respectively.

lectin as judged by the absence of haemagglutination activity or reaction with anti-(pea lectin) antibodies (Fig. 5*a* below).

Separation and purification of the major albumins was achieved by chromatography on DEAE-cellulose. SDS/polyacrylamide-gel analysis (Fig. 2*a*) of fractions in the leading edge of the first large peak (I) eluted from the column (Fig. 1*b*) contained major albumin protein consisting only of the small subunits (subunit M_r 24000) (Fig. 2*a*, track 5), whereas the trailing edge of the second large peak (II) (Fig. 1*b*) containing major albumin consisting only of the larger subunits (subunit M_r 25000) (Fig. 2*a*, track 6). For simplicity the large and small *Pisum* major albumins were termed PMA-L and PMA-S respectively. Other fractions from the two peaks contained variable amounts of large and small subunits. PMA-L, PMA-S and the total major albumin proteins (total pooled fractions from Peaks I and II, Fig 1*b*) were used in the subsequent analyses (Fig. 2*a*). Total major albumin preparations showed traces of contaminating proteins absent from PMA-L or PMA-S (Fig. 2*a*, track 4). The success of the present method is due largely to the very efficient extraction of the albumin proteins with low-ionic-strength acidic buffer, which fails to extract any detectable amounts of globulin proteins, in agreement with the reports of Guldager (1978). Quantitative data obtained by

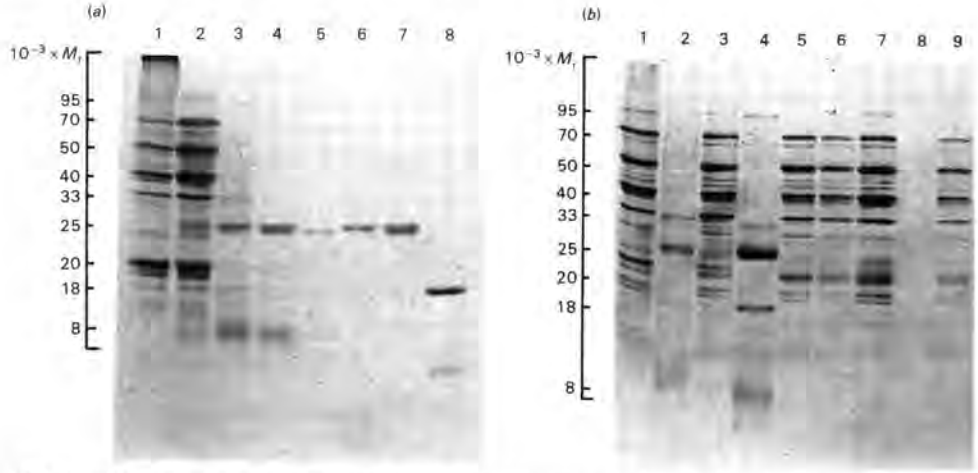


Fig. 2. *SDS/polyacrylamide-gel analyses of isolated fractions as described in the Experimental section*

(*a*) Purification of the major albumin proteins: track 1, total seed globulin proteins; track 2, total seed proteins; track 3, total seed albumin proteins; track 4, total major albumin proteins; track 5, small major albumin protein; track 6, large major albumin protein; track 7, equal amounts of purified large and small major albumin proteins, mixed and co-electrophoresed; track 8, purified pea lectin. (*b*) Subcellular fractions from developing pea cotyledons purified on sucrose density gradients. Track 1, total seed proteins; track 2, total seed albumin proteins; track 3, total soluble proteins from the top of the gradient; track 4, albumin proteins from the top of the gradient; track 5, globulin proteins from the top of the gradient; tracks 6 and 9, globulin proteins from protein bodies; track 7, total protein-body proteins; track 8, albumin proteins from protein bodies.

rocket immunoelectrophoresis indicates that the major albumins may represent as much as 2% of the dry weight of the seed, i.e. 8–10% of the total cotyledonary protein (results not shown).

M_r and subunit compositions

Chromatography of the major albumin proteins on Sephacryl S-200 and comparison of V_e/V_0 values with those for standard proteins gave M_r values of $53000 \pm 10\%$ for PMA-L and $48000 \pm 10\%$ for PMA-S. Subunit M_r values for these proteins, estimated from SDS/polyacrylamide-gel electrophoresis, were 25000 for PMA-L and 24000 for PMA-S. Such estimates are consistent with dimeric molecules for the major albumin proteins.

The major albumin proteins isolated contained only large (M_r 25000) or small (M_r 24000) subunits (Fig. 2*a*, tracks 5 and 6) in the dimeric molecules, suggesting that molecules containing one of each type of subunit are not present. In support of this suggestion, analysis of fresh albumin extracts or total protein extracts by non-dissociating polyacrylamide-gel electrophoresis (Fig. 3, track 6), and on two-dimensional gels combining this technique with SDS/polyacrylamide-gel electrophoresis (results not shown), indicated two major albumin components, one of low electrophoretic mobility, containing only small subunits (Fig. 3, track 3), and the other of higher mobility, containing only large subunits (Fig. 3, track 5). However, purified preparations of PMA-L, PMA-S, or total major albumins, examined by polyacrylamide-gel electrophoresis, showed variable amounts of additional bands of higher mobility, corresponding to an increase in negative charge in the proteins. This modification occurs in a discontinuous manner, i.e. there is a shift from one form to another with no intermediate forms being observed (cf. Fig. 3, tracks 2 and 3, 4 and 5). It is apparent that this change occurs during prolonged protein purification or on storage, since freshly prepared PMA-L and PMA-S show predominantly those bands present in fresh total albumin extracts and very little of the modified forms (cf. Fig. 3, tracks 3, 5 and 6). The modification may be analogous to that observed by Matta & Gatehouse (1981) in pea legumin β-subunits on storage, and is possibly due to chemical or enzymic deamidation of amino acid amide residues. These results are thus not inconsistent with a homodimer structure for PMA-L and PMA-S.

Two-dimensional electrophoresis, combining isoelectric focusing under dissociating conditions and SDS/polyacrylamide-gel electrophoresis, showed that each subunit size class consists of several (six to eight) charge forms (Fig. 4), as has been shown for several other pea proteins (Krishna *et al.*, 1979; Gatehouse *et al.*, 1980, 1981). Since the

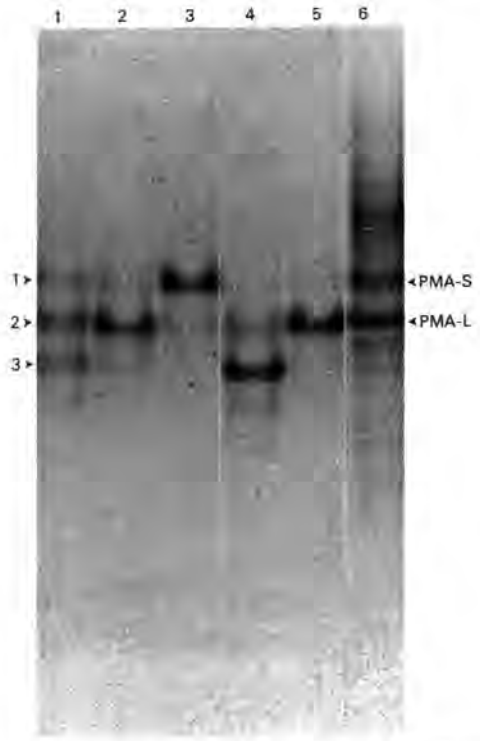


Fig. 3. *Polyacrylamide-gel electrophoresis of pea albumin proteins under non-dissociating conditions, pH 8.3*
The direction of electrophoresis is from the top to the bottom of the Figure. Track 1: total major albumin protein (PMA-L + PMA-S); stored sample; arrowheads denote the three components (1, 2, 3). Tracks 2 and 3: PMA-S, stored sample (track 2) and fresh sample (track 3). Tracks 4 and 5: PMA-L, stored sample (track 4) and fresh sample (track 5). Track 6: total albumin proteins extracted from pea cotyledons (fresh sample); arrowheads denote bands shown to be due to PMA-S and PMA-L on analysis by SDS/polyacrylamide-gel electrophoresis in the second dimension (results not shown).

subunit arrays of PMA-L (Fig. 4*c*) and PMA-S (Fig. 4*b*) closely resemble those in the total seed albumins (Fig. 4*a*), it seems likely that they are genuine charge isomers and not artefacts due to the charge modification discussed above.

Characterization of PMA-L and PMA-S

Although subunits of PMA-L and PMA-S do not associate together *in vivo*, the two proteins are structurally very similar. PMA-L and PMA-S gave a reaction of complete identity in immunodiffusion against anti-(total major albumin) antibodies (Fig. 5*a*) and gave no cross-reactions with other seed proteins or with antibodies raised against other albumin proteins (e.g. anti-lectin; Fig. 5*a*) or globulin proteins.

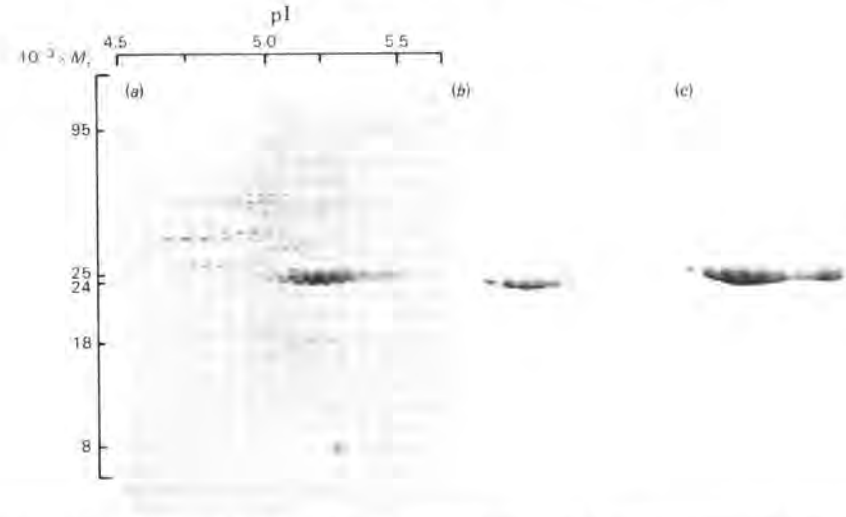


Fig. 4. *Two-dimensional gel analyses of albumin proteins combining isoelectric focusing in urea gels in the first dimension and SDS/polyacrylamide-gel electrophoresis in the second dimension*
(*a*) Total seed albumin proteins; (*b*) small major albumin protein; (*c*) large major albumin protein.

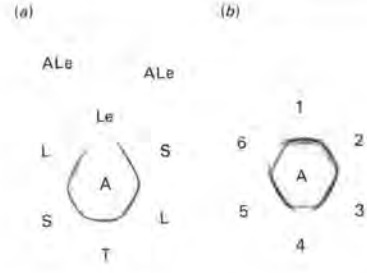


Fig. 5. *Immunodiffusion of* (*a*) *purified pea albumin proteins and* (*b*) *total albumin extracts*
(*a*) Abbreviations used: A, anti-(major albumin) antibodies; ALe, anti-lectin antibodies; L, large major albumin protein; S, small major albumin protein; T, total major albumin proteins, Le, lectin. (*b*) A, anti-(major albumin) antibodies; other wells contained total albumin extracts from: (1, 3, 5) *Pisum sativum* (pea), (2) *Lens cularis* (lentil), (4) *Lathyrus odoratus* (sweet pea), and (6) *Cicer arietinum* (chickpea).

The amino acid compositions of both proteins (Table 1) were very similar, with higher levels of the sulphur amino acids (cysteine and methionine) than the average for globulin proteins. These results support the reports of Grant *et al.* (1976), Hurich *et al.* (1977), Jakubek & Przybylska (1979) and Murray (1979) of the sulphur-rich nature of the albumin protein fraction and suggest the major albumin proteins as further candidates for investigations into improving the nutritional quality of peas (Davies, 1976; Boulter, 1982).

Table 1. *Amino acid and carbohydrate compositions of large and small major albumin proteins compared with that of total seed albumins*
Amino acid data is expressed as mol/100 mol of the total analysed and (in parentheses) as residues per subunit molecule of PMA-L and PMA-S. Residues per molecule were calculated from the average M_r for amino acids of 112 and subunit M_r values of 25000 for PMA-L and 24000 for PMA-S. Results are means of duplicate or triplicate determinations. Variation between determinations was less than 10%. Abbreviations used: CM-Cys, carboxymethylcysteine; ND, not determined.

	Composition (mol/100 mol)		
	PMA-S	PMA-L	Total Albumin Proteins
Asp	15.4 (33)	15.0 (34)	12.0
Thr*	7.5 (16)	7.2 (16)	4.5
Ser*	6.3 (14)	6.1 (14)	6.5
Glu	6.7 (14)	7.0 (16)	11.6
Gly	8.1 (17)	7.3 (16)	8.4
Ala	7.8 (17)	7.7 (17)	9.7
Val	5.5 (12)	5.6 (13)	7.5
Met	1.1 (2)	1.2 (3)	1.2
Ile	6.6 (14)	6.6 (15)	5.5
Leu	5.4 (12)	5.5 (12)	7.1
Tyr	6.6 (14)	7.2 (16)	3.9
Phe	7.8 (17)	8.4 (19)	5.1
Lys	7.5 (16)	7.9 (18)	7.9
His	1.3 (3)	1.1 (3)	2.1
Arg	4.9 (11)	5.3 (12)	4.6
CM-Cys	1.2 (3)	1.2 (3)	2.3
Pro	ND	ND	ND
Trp	ND	ND	ND
Carbohydrate	0	0	ND

* Corrected for decomposition.

CNBr cleaves both large and small subunits into three distinct fragments showing the presence of at least two methionine residues per subunit (Fig. 6, tracks 4, 6 and 7). Since two to three methionine residues per subunit are predicted by the amino acid composition (Table 1), these results are in reasonably good agreement, but they do not eliminate the possibility that an additional, presumably small, CNBr fragment may also be produced. The three polypeptide fragments obtained after 3h incubation were unchanged on prolonged reaction times (6, 24 and 48h). By using highly resolving urea/SDS gels, comparison of these polypeptide fragments with those obtained from the total major albumin confirmed that one fragment (M_r 11500) was coincident in both large and small major albumin subunits, whereas the other two fragments were of slightly higher M_r in the large major albumin subunit (M_r 8400 and 7200 in PMA/L and M_r 7600 and 6600 in PMA-S; Fig. 6, tracks 4 and 6). Summation of the M_r values for the CNBr fragments agrees with the calculated subunit M_r values. Such results, although confirming the similarity of the subunit polypeptides, indicated that the primary sequences of the two subunits vary slightly in at least two regions but may have at least one large highly conserved region.

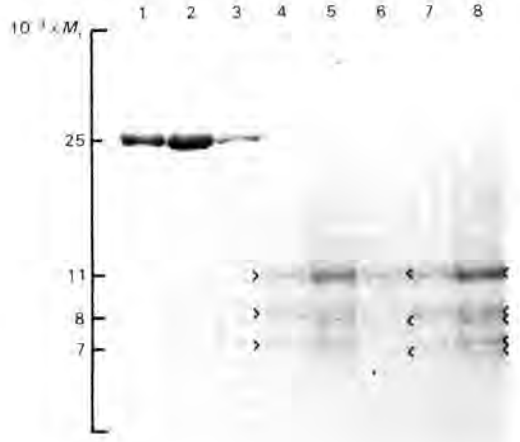


Fig. 6. *SDS/urea/polyacrylamide gel analysis of CNBr cleavage fragments from large, small and total major albumin proteins*

Tracks 1, 2 and 3, untreated large, total and small major albumin proteins; tracks 4 and 7, CNBr-cleaved large major albumin (6 and 24h treatment); tracks 5 and 8, CNBr-cleaved total major albumins (6 and 24h treatments); track 6, CNBr-cleaved small major albumin (6h treatment).

Tryptic peptide maps in the form of h.p.l.c. elution profiles were similar for PMA-L and PMA-S, reinforcing the above conclusions of structural relatedness. Both PMA-L and PMA-S were found to have threonine as the *N*-terminal amino acid.

Neither of the major albumin proteins is a glycoprotein as judged by: chemical analysis (Table 1); running known glycoproteins (and non-glycoproteins for comparison) on SDS/polyacrylamide gels followed by glycoprotein-specific staining (Croy *et al.*, 1980); and the absence of any reaction with concanavalin A (Gleeson & Jermym, 1977).

Degradation and subcellular location of the major albumins

The breakdown of the major albumins during germination is quite different from that of the globulin storage proteins. The pea globulins, legumin, vicilin and convicilin, were all rapidly degraded during the first few days of germination, whereas PMA-L, PMA-S and the lectins were all maintained longer than seven days after germination (results not shown; Tyler, 1981). This result agrees with the observations of Murray (1979) and Jakubek & Przybylska (1982), who described the continued presence of a major albumin polypeptide of $M_r \sim 22000$–24000 in pea cotyledons up to 20 days after germination, and of Guldager (1978), who described significant amounts of albumins and lectin remaining after the disappearance of vicilin and legumin.

Clearly the absence of significant degradation of such a protein, coupled with its high sulphur content, indicates that its role is different from that of the storage proteins. This has similarly been suggested by Murray (1979) and Jakubek & Przybyska (1982), although the suggestion of a structural function made by the latter authors seems unlikely in view of the protein's high aqueous solubility. In an effort to elucidate its function we have carried out preliminary localization studies by subcellular fractionation. The results strongly support a cytoplasmic (the soluble fraction of the cytosol) location for the major albumins, since SDS/polyacrylamide-gel analysis of the total albumin proteins isolated from the soluble cytoplasmic fraction at the top of the density gradients showed the albumin polypeptides (PMA-L, PMA-S, lectin and others) present as the main components (Fig. 3*b*, track 4). By contrast, no such polypeptides were observed in the albumin fraction from protein bodies, even at high sample loadings (Fig. 2*b*, track 8). Despite the large yield of protein from the protein bodies, the amount of albumin fraction recovered from them was very low, as reported by other groups (Konopska, 1978; Weber & Newmann, 1980; Varner & Schidlovsky, 1965) and differ from the

results obtained from subcellular fractionation of other legume seeds (e.g. those of the French bean, *Phaseolus vulgaris*), where the predominant albumin proteins are located within the protein bodies (Croy, 1977; Pusztai *et al.*, 1977; Youle & Huang, 1978; Bollini & Chrispeels, 1978). The present results support the suggestions of Murray (1979) of a cytoplasmic site for the pea albumins, but are at variance with the reports of Weber *et al.* (1978), who reported that the homologous albumin lectin in broad bean (*Vicia faba*) was protein-body-associated. In the present study the major storage proteins were all mainly present in the protein-body fraction (Fig. 2*b*, tracks 6 and 9), with lesser amounts of released globulins present in fractions from the top of the gradient (Fig. 2*b*, track 5). Thus it is likely that most of the isolated protein bodies are intact. Croy (1977) and Pusztai *et al.* (1977), who used a similar system, were able to show that a significant redistribution of *Phaseolus vulgaris* protein-body albumin proteins did not take place. This makes the present results unlikely to be an artefact, since it is improbable that all the major albumin proteins and lectins would have been quantitatively released from the protein bodies. However, unequivocable localization of these proteins awaits quantitative subcellular-fractionation studies and immuno-histochemical studies at the electron-microscopic level as have been performed for the pea globulins (Craig & Millerd, 1981).

Assays for functional properties

The cytoplasmic location of PMA-L and PMA-S suggests some kind of metabolic or anti-metabolic role for these proteins. However, neither PMA-L nor PMA-S showed activity in any of the enzyme assays tested (α- and β-D-glucosidases, α- and β-D-galactosidases, α-D-mannosidase, amylase). Furthermore, neither protein exhibited inhibition in amylase-inhibition assays with porcine pancreatic amylase or trypsin-inhibition assays with bovine trypsin, and no haemagglutinating activity was associated with the proteins. The role of the major albumin proteins therefore remains unknown.

Species distribution of pea major albumin type proteins

Total albumin extracts from a variety of different legume species were screened for PMA-type proteins by immunodiffusion against anti-PMA IgG. *Lathyrus oderatus* (sweet pea), *Lens culinaris* (lentil) and *Cicer arietinum* (chick-pea), were the only three species to show specific immunoprecipitations as judged by reaction of identity (lentil) or partial identity (chick-pea and sweet pea) with PMA (Fig. 5*b*).

No reactions were obtained with extracts from *Vicia faba*, *Glycine max* (soya-bean), *Dolichos lablab* (horse gram), *Cajanus cajar* (pigeon pea), *Phaseolus vulgaris*, *Phaseolus coccineus* (runner bean), *Vigna unguiculata* (cowpea) and *Vigna mungo* (mung bean). Analysis of the three immunoprecipitating albumin extracts on SDS/polyacrylamide gels showed major polypeptides of $M_r \sim 22000$–24000, which when examined by 'Western blotting' showed specific anti-PMA binding, confirming their homology with the pea major albumin (results not shown). This type of protein thus seems to be characteristic of *Pisum* and closely related species, and suggests that the lentil is the most closely related species to *Pisum sativum*, as has been shown with other seed proteins (e.g. legumin; R. Sammour, unpublished work).

We thank Mrs. Philippa Brown, Mr. Paul Preston, Mr. Dave Bown and Mr. Russell Swinhoe for excellent technical assistance, Dr. M. Tyler for the use of unpublished results, Dr. A. M. R. Gatehouse for carrying out trypsin-inhibition assays, and Dr. H. Hirano, who carried out two-dimensional gel analyses. M. S. H. thanks the British Council for a Technical Co-operation Training Award.

References

Anstee, J. H. & Charnley, A. K. (1977) *J. Insect Physiol.* **23**, 965–974
Basha, S. M. M. & Beevers, L. (1975) *Planta* **124**, 77–87
Blixt, S., Pryzybylska, J. & Zimniak-Przybylska, Z. (1980) *Genet. Pol.* **21**, 153–161
Bollini, R. & Chrispeels, M. J. (1978) *Planta* **142**, 291–298
Boulter, D. (1982) *Proc. Nutr. Soc.* **41**, 1–5
Craig, S. & Millerd, A. (1981) *Protoplasma* **105**, 333–339
Croy, R. R. D. (1977) Ph.D. Thesis, University of Aberdeen
Croy, R. R. D., Derbyshire, E., Krishna, T. G. & Boulter, D. (1979) *New Phytol.* **83**, 29–35
Croy, R. R. D., Gatehouse, J. A., Tyler, M. & Boulter, D. (1980) *Biochem. J.* **191**, 509–516
Danielson, C. E. (1956) *Annu. Rev. Plant Physiol.* **7**, 215–236
Davies, D. R. (1976) *Euphytica* **25**, 717–724
Derbyshire, E., Wright, D. & Boulter, D. (1976) *Phytochemistry* **15**, 3–24
Dubois, M., Giles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
Eckhardt, H. E., Hayes, C. E. & Goldstein, I. J. (1976) *Anal. Biochem.* **73**, 192–197
Erlanger, B. F., Kokowsky, N. & Cohen, W. (1961) *Arch. Biochem. Biophys.* **95**, 271–278
Evans, I. M., Croy, R. R. D., Hutchinson, P., Boulter, D., Payne, P. I. & Gordon, M. E. (1979) *Planta* **144**, 455–462
Gatehouse, J. A., Croy, R. R. D. & Boulter, D. (1980) *Biochem. J.* **185**, 497–503
Gatehouse, J. A., Croy, R. R. D., Morton, H., Tyler, M. & Boulter, D. (1981) *Eur. J. Biochem.* **118**, 627–633

Gatehouse, J. A., Lycett, G. W., Croy, R. R. D. & Boulter, D. (1982) *Biochem. J.* **207**, 629–632
Gatehouse, J. A., Croy, R. R. D. & Boulter, D. (1983) *CRC Crit. Rev. Plant. Sci.* in the press
Glazer, A. N., Delange, R. J. & Sigman, D. S. (eds.) (1975) *Chemical Modification of Proteins — Selected Methods and Analytical Procedures*, North-Holland/Elsevier, Amsterdam
Gleeson, P. A. & Jermym, M. A. (1977) *Aust. J. Plant Physiol.* **4**, 25–37
Grant, D. R., Sumner, A. K. & Johnson, J. (1976) *Can. Inst. Food Sci. Technol. J.* **9**, 84–91
Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
Guldager, P. (1978) *Theor. Appl. Genet.* **53**, 241–250
Hashimoto, F., Horigome, T., Kanbayashi, H., Yoshida, K. & Sugano, T. (1983) *Anal. Biochem.* **129**, 192–199
Hurich, J., Parzysz, H. & Przybylska, J. (1977) *Genet. Pol.* **18**, 241–252
Jakubek, M. F. & Przybylska, J. (1978) *Pisum Genet. Assoc. Pisum Newsl.* **10**, 24
Jakubek, M. F. & Przybylska, J. (1979) *Pisum Genet. Assoc. Pisum Newsl.* **11**, 12
Jakubek, M. F. & Przybylska, J. (1982) *Pisum Genet. Assoc. Pisum Newsl.* **14**, 26–28
Konopska, L. (1978) *Biochem. Physiol. Pflanz.* **173**, 322–326
Krishna, T. G., Croy, R. R. D. & Boulter, D. (1979) *Phytochemistry* **18**, 1879–1880
Laemmli, U. K. (1970) *Nature (London)* **238**, 680–685
Matta, N. K. & Gatehouse, J. A. (1981) *Phytochemistry* **20**, 2621–2623
Matta, N. K., Gatehouse, J. A. & Boulter, D. (1981) *J. Exp. Bot.* **131**, 1295–1307
Mestechy, J., Kraus, F. W., Hurst, D. C. & Voight, S. A. (1969) *Anal. Biochem.* **30**, 190–198
Murray, D. R. (1979) *Plant Cell Environ.* **2**, 221–226
Przybylska, J., Blixt, S., Hurich, J. & Zimniak-Przybylska, Z. (1977) *Genet. Pol.* **18**, 27–38
Pusztai, A. & Watt, W. B. (1970) *Biochim. Biophys. Acta* **207**, 413–431
Pusztai, A., Croy, R. R. D., Grant, G. & Watt, W. B. (1977) *New Phytol.* **79**, 61–71
Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
Trowbridge, I. S. (1974) *J. Biol. Chem.* **249**, 6004–6012
Tyler, M. (1981) Ph.D. Thesis, University of Durham
Varner, J. E. & Schidlovsky, F. (1963) *Plant Physiol.* **38**, 139–144
Weber. E. & Newmann, D. (1980) *Biochem. Physiol. Pflanz.* **175**, 279–306
Weber, E., Manteuffel, R. & Newmann, D. (1978) *Biochem. Physiol. Pflanz.* **172**, 597–614
Weeke, B. (1973) in *A Manual of Quantitative Immunoelectrophoresis* (Axelsen, N. H., Kroll, J. & Weeke, B., eds.), pp. 15–46, Universitets Forlaget, Oslo
Woods, K. R. & Wang, K. T. (1967) *Biochim. Biophys. Acta* **133**, 369–370
Youle, R. J. & Huang, A. H. C. (1978) *Plant Physiol.* **61**, 1040–1042