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

Immunoelectrophoretic Analysis of Seed Proteins from *Pisum sativum* L.

P. Guldager
Department of Genetics, The Royal Veterinary and Agricultural University, Copenhagen (Denmark)

Summary. Soluble proteins of pea seed were investigated by quantitative immunological methods. Vicilin, legumin, pea seed lectin (PEA), 26 albumins and a globulin (B_1) were detected and observed during seed development, germination and under different extraction and fractionation procedures. Vicilin and legumin were found to be immunologically distinctly different. Legumin was found to be comprised of two similar proteins, Legumin species I and II. Vicilin, but no legumin, was detected in the embryonic axis.

Three albumins, B_1 and PEA were found to be synthesized after the onset of legumin synthesis.

Among the pea lines investigated, one line exhibited distinct differences with respect to the albumins and PEA.

Some observations indicate that PEA might interact with other seed proteins of pea.

Key words: *Pisum sativum* – Seed proteins – Seed development – Germination – Quantitative immunoelectrophoresis

Introduction

The study of proteins from legume seed is important as a basis for quantitative and qualitative improvements of legume seed production. Seed proteins are an extremely, and increasingly, important component of nutrition for both humans and animals. From a nutritional point of view most legume seeds are deficient in sulfur amino acids mainly due to a low content of these amino acids in the globulin fraction of the storage proteins (Jackson et al. 1969).

Further, legume seed proteins seem to offer an attractive system for the study of differential gene expression in eucaryotes. Storage proteins in legumes are tissue-specific and they are synthesized in relatively short periods during development of the cotyledons (Millerd 1975). The biochemistry and biosynthesis of legume seed proteins have recently been reviewed by Millerd (1975) and Yarwood (1977). Both authors mention the existing limitations in the characterization of legume seed proteins.

In the mature seed of pea, about 25% of the dry weight is protein. About 95% of the protein is found in the cotyledons (Müller and Gottschalk 1973). Saline soluble globulins constitute the main fraction of pea seed proteins and consist of vicilin (mol. wt. about 186.000 Dalton, isoelectric point pH 5.5 (Danielsson 1949 1950)) and legumin (mol. wt. about 400.000, isoelectric point pH 4,7 (Danielsson 1949; Johnson & Richards 1962))

The vicilin of pea is reported to be heterogenuous (Basha 1974). The water soluble proteins are jointly named albumins (Millerd 1975). The ratio of globulins to albumins is found to be 1,4:1 (Basha 1974).

In the present study the saline soluble proteins of pea seed are investigated mainly by quantitative immunological methods. The validity of crossed immunoelectrophoresis for investigation of storage proteins in pea is elucidated. The observations are possibly valid for the study of most saline soluble proteins in legumes. Manteuffel and Scholz (1975) have studied vicilin purification in *Vicia faba* by the same method.

The effects of different extraction and fractionation methods, heat treatments and pH shifts on saline soluble proteins were monitored in the present study by crossed immunoelectrophoresis. This method was also used to elucidate varietal differences in pea and to study the synthesis of pea seed proteins during seed development and the depletion of proteins in the germinating seed.

Material and Methods

Plant Material

The standard pea line used for most of the present investigations is designated K-42 and originates from the line Dark Skin Perfection obtained from commercial sources (S 66, Ohlsens Enke, Copenhagen). *Pisum sativum* var. 'elatius', Line L 1293, originally col-

0040-5752/78/0053/0241/$ 2.00

lected from the Jordan valley, was obtained from Dr. Blixt, The Pisum Gene Bank, Weibullsholm, Sweden. Fifty different pea lines (not listed) were selected to represent maximum genetic variation with respect to seed protein content. Equal weights of seed material from each line were included in a mixture of seed proteins used for antiserum production.

Seed of *Vicia faba* (Erfordia), *Vicia sativum* (Chenonceaux), *Phaseolus vulgaris* (Carlos Favorite and Weibulls Stella) *Glycine max* (Fiskeby V), *Lupinus lúteus* (Weiko III) was obtained from P. Flengmark Roskilde State Research Station on Plant Science, Roskilde, Denmark.

Plants grown for developmental studies were grown in soil under greenhouse conditions. Germination was performed on filter paper at 20° in an incubator in darkness.

Extraction, Heat Treatments and pH Shifts

Dry mature seeds were ground in a cyclone sample mill (Udy Analyzer Co.). One g of the pea meal was extracted for 1 h at 4° in 10 ml 50 mM Tris-HCl buffer containing 0.1 M NaCl at pH 8.0 (standard buffer). The extract was clarified by centrifugation (12.000 g) for 20 minutes. This extraction procedure is referred to as standard extraction in the following text. Extractions were also made using glass distilled water instead of standard buffer and by the standard buffer containing 1 M instead of 0.1 M NaCl. Extraction was further undertaken at 0° (standard buffer) and at various pH levels, with and without 0.1 M NaCl in the buffer: 2.3 (50 mM Sodium acetate-HCl buffer); 3.7, 4.6, 4.7, 5.0, 5.3, 5.5, 5.6, 6.0, (50 and 100 mM Sodium acetate-acetic acid buffers) and 9.0 (50 mM tris HCl buffer). Standard extracts were heated in a water bath to 60°, 75°, 90° and 100° each for both 5 minutes and 1 hour. Standard extracts were further titrated by 1 N HCl or 1 N NaOH to pH 2.3, 4.7, 10.0, 11.0, 11.5, 11.6, 11.8, 12.1 and 12.6, and incubated at 4° for 3 hours prior to immunoelectrophoresis. The same pH shifted series were after incubation at 4° for 3 h, readjusted to pH 7.5 and incubated for 16 h at 4° before application to immunoelectrophoresis. Imbibed seeds, germinated seeds and parts dissected from the seed were disintegrated by use of a mortar and quartz sand with 3 ml standard buffer per gram fresh weight. The extracts were stored at 4° and protected against microbial growth by 15 mM sodium azide.

Quantitative Immunoelectrophoresis

The crossed immunoelectrophoresis, tandem crossed immunoelectrophoresis, fused rockets and intermediate gel electrophoresis used were in accordance with Axelsen et al. (1973), with minor modifications.

The agarose was type HSA from Litex, Denmark. 1,25% (w/v) agarose was applied to glass plates 82 X 82 mm forming a 1,6 mm deep layer. The buffer was 50 m Mol Tris/citric acid of pH 8,6. The voltage in the 1st dimension was 7.0 volt/cm (45 min.) and in the 2nd dimension 2.5 volt/cm (16 h). The plates were stained for 10 min. in 0,25% Coomassie Brilliant Blue R 250, 45% ethylalcohol, 10% acetic acid and 45% demineralized water. The gels were subsequently destained 10 min. in the same solution as for staining but without the dye. In the immunoelectrophoreograms (IEGs) shown (except fused rockets) the anode is to the right in the 1st dimension and at the top in the 2nd dimension.

Three antisera were used against total saline soluble seed proteins (abPI), legumin (abL) and vicilin (abV). The antiserum abPI consists of a mixture of antisera against pea line K 42 (4 rabbits) K 7 (4 rabbits) and against a mixture of 50 different pea lines (5 rabbits). Pooling of these 3 sera was made as no significant qualitative differences were detected. Injections in rabbits were made using equal volumes of Freunds adjuvans and crude protein from standard extractions of dry seed, 10 mg protein pr. ml. The injections and bleedings were made through the courtesy of Dr. J.V. Spärck at the Immunological Laboratory, Statens Seruminstitut, Copenhagen. The specific antisera abL and abV, were made on the basis of the curretage of precipitation arcs from crossed immunoelectrophoresis of ab-PI and legumin and vicilin, respectively, in collaboration with Thorkild Bøgh-Hansen, Protein Laboratory, University of Copenhagen. (Publication in preparation).

Fractionation, Gel Filtration and Mol. Wt. Determination

Preparation of albumin, vicilin and legumin fractions was made in accordance with Danielsson (1949). After ammonium sulphate precipitation albumins and globulins are separated by dialysis against water, which precipitates the globulins. The globulins are further separated by means of isoelectric precipitation.

For gel filtration ultrogel AcA 34 (LKB, Sweden) was packed in a 750 X 16 mm column and run at a flow rate of 12 ml per h using the extraction buffer at room temperature for partial fractionation of crude protein. Elution profiles were monitored with a UV cord (LKB). Protein sampled at the peaks in the elution profile were investigated by fused rockets and crossed immunoelectrophoresis.

Molecular weights of the main fractions were determined from the linear regression between log mol. wt. and ml eluate established by gel filtration ($r^2 = 0.98$) of the following standard proteins (combithec, Boehringer Mannheim): Ferritin (540.000), Catalase (240.000), Aldolase (158.000), Albumin (bovine) (67.000), Albumin (hen) (45.000), Chymotrypsinogen A (25.000) and Cytochrome c (12.500).

Isolation of Pea Seed Lectin (PEA)

(PEA is the adopted abbreviation for the commercial available product of pea seed lectin isolated according to Trowbridge (1973) by E.Y. Laboratories, San Mateo, U.S.A.). PEA was, with minor modifications, isolated in accordance with Trowbridge (1973). Standard extracts were precipitated with 80% ammonium sulfate. The precipitate was dissolved in the extraction buffer and applied to a Sephadex g 100 column, 50 X 16 mm, at room temperature (flow rate 18 ml per h). PEA retained by the Sephadex was instantly released from the Sephadex by elution with D(+)-Mannose (0,1 M) dissolved in extraction buffer.

Standard extracts were treated in order to influence the binding specificity of PEA. Standard buffer containing 0.2 M EDTA-Na or 3% D(+)-Mannose(w/v) or a mixture of 0.01 M Mg Cl_2, 0.01 M $MnCl_2$ and 0.01 M Ca $(NO_3)_2$ was used for extraction in order to abolish and enhance the binding specifity of PEA (Paulova et al. 1971).

[^{35}S] in vivo Labelling and Immunoradioauthography

15-20 cm shoots carrying 3-5 developing pods were cut and placed in a 10 ml solution of Knops medium containing 500μCi per ml [^{35}S] sulfur (SIS.1, sulphate, Amersham, England) for 8 hours. This was followed by a chase of Knops medium for 60 hours before the seeds were extracted by the standard procedure.

IEGs containing [^{35}S] labelled proteins were covered by X ray film Kodak X-omat RP, XRPI and exposed for 50 days.

Results

Immunoelectrophoresis

A crossed immunoelectrophoreogram (IEG) of a standard extract of the line K 42 (Dark Skin Perfection) is shown in Figure 1a.

The arc pattern in Figure 1a is dominated by a few major proteins. Legumin (L) and vicilin (V) were identified from fractions prepared in accordance with Danielsson (1949) by tandem crossed immunoelectrophoresis and by determination of the molecular weight of L and V (see later). Pea seed lectin (PEA) was similarly identified with lectin prepared by affinity chromatography in accordance with Trowbridge (1973). The three arcs, designated A_1-A_3 (Figure 1a), were found in the water soluble albumin fraction of the Danielsson fractionation and thus classified as albumins. The proteins designated B_1 and B_2 were found only in the legumin fraction (see later). The mentioned precipitation arcs were all visible on IEGs made from the extraction from 1/100 of the pea meal (3 mg) from one pea seed

Crossed immunoelectrophoresis has been found to be a quantitative method for the analysis of various proteins (Axelsen et al. 1973). The relation between amount of protein applied and the precipitation arc area is shown in Figure 2 for vicilin and legumin. The arc area was measured as the area enclosed by the arc and intersept between the 1st and the 2nd dimension gels.

The antiserum formed against total standard extract, ab PI, contained antibodies against most or all proteins present in the standard extracts of K 42, apart from low molecular weight proteins (<12.000 Dalton). Supernatants from crude standard extracts precipitated with antiserum PI did not show any visible unexplainable bands in polyacrylamide geld (not shown). The specific antisera against vicilin and legumin (abV and abL) were tested by inserting intermediate gel strips of these sera. Figures 6a and 6b indicate the specificity of the sera. Vicilin and legumin reacted purely with their corresponding antiserum in the intermediate gel strips. No signs of immunological cross reactions could be detected. The specific antisera were used to distinguish vicilin and legumin in mixtures of seed proteins.

A number of pea lines were tested against antiserum abPI. One line, L 1293, was found to differ quantitatively in missing at least two albumins, A_1 and A_2, and PEA, as judged from IEG's (Fig. 1b).

Other legume species were also tested against antiserum abPI: *Vicia faba, Vicia sativa, Phaseolus vulgaris, Glycine max* and *Lupinus luteus*. Figures 1c and 1d illustrate the precipitation arcs formed by *Vicia faba* and *Phaseolus vulgaris*. *Vicia faba* and *Vicia sativa* showed particularly strong reactions to abPI, indicating similarities in

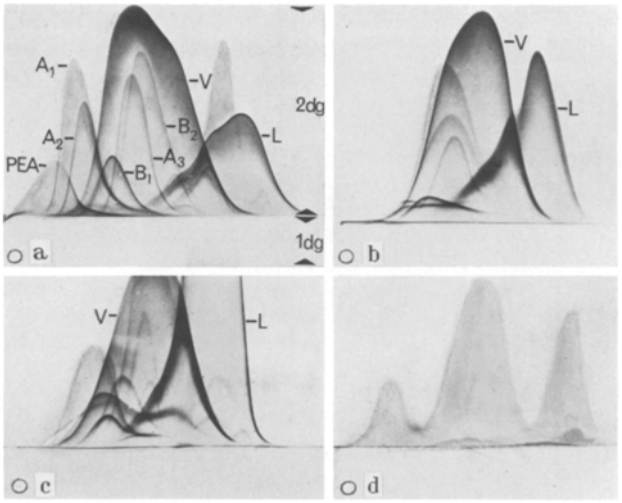

Fig. 1a-d. Immunoelectrophoreograms (IEGs) on seed of *Pisum sativum, Vicia faba* and *Phaseolus vulgaris* 1 dg = 1st dimension gel; 2 dg = 2nd dimension gel. V = vicilin; L = legumin; PEA = pea seed lectin; A_1-A_3 = Three albumins; B_1 and B_2 = Two globulins. Samples of seed extract were applied to the punched hole (Application well) in the 1st dimension agarose in the left downwards corner. In the 1st dimension, electrophoresis proteins were partially separated along a horizontal line to the right where the anode was attached. In the 2nd dimension electrophoresis, the anode was applied along the upper line of 2nd dimension gel containing antiserum. As the proteins (antigens) met their corresponding antibodies in the 2nd dimension gel, precipitation arcs were formed where the antigen concentration during electrophoresis reached a certain level specific to the antigen-antibody in question. **a** 5μl of standard extract from seeds of pea line K 42 (standard extraction) against antiserum abPI, 6.4% agarose (vol/vol), **b** Pea line L 1293 as 1 a. A_1, A_2 and PEA are missing, compared to K 42 (1 a), **c** *Vicia faba* as 1 a. Several seed proteins seem to have similar antigenic structure in *Pisum* and *Vicia*, **d** *Phaseolus vulgaris* as 1 a. Only few precipitation arcs were formed

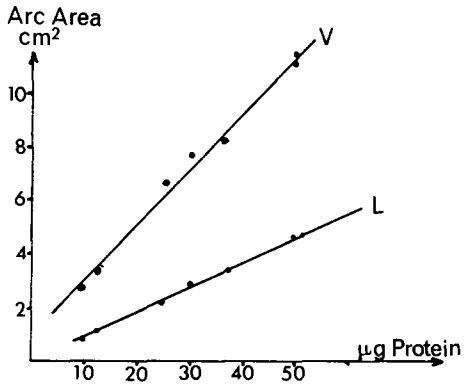

Fig. 2. Relationship between protein concentration and arc areas for vicilin and legumin. V = vicilin (r^2 = 0.98); L = legumin (r^2 = 0.99) The protein applied to crossed immunoelectrophoresis was in the form of various dilutions of standard extracts. Arc areas were measured at IEGs as the area enclosed by the arc and the intersept between the 1st and 2nd dimension gels

the structure of the main protein in these species of *Vicia* to those present in *Pisum sativum.*

Vicilin

The vicilin fraction prepared according to Danielsson (1949) from the line K 42 was contaminated with unidentified proteins (Fig. 3a). Gel filtration of this preparation yielded a fraction of vicilin without any contaminating proteins detectable on IEGs (Fig. 3b).

The mol. wt. of vicilin was determined by means of gel filtration. On the basis of gel filtration of unfractionated standard extracts from line K 42, the mol. wt. of vicilin was found to be 154,600 Dalton. The elution profile and the corresponding fused rockets are shown in Figures 4a and 4b. When subjected to gel filtration, the vicilin from the Danielsson's fractionation exhibited a mol. wt. of 166,600 Dalton.

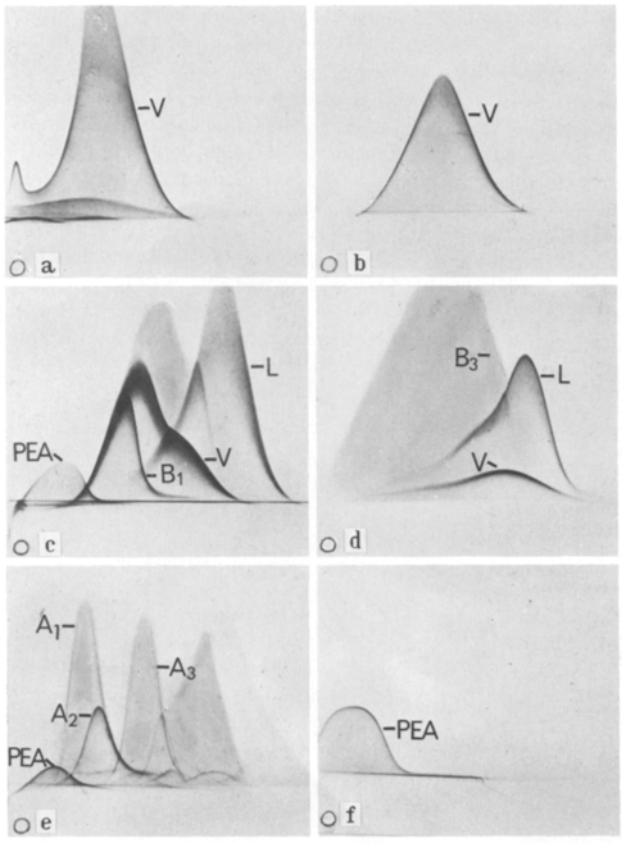

Fig. 3a-f. IEGs on Danielsson fractionation and gel filtration of standard extracts from line K 42. V = vicilin, L = legumin, PEA = pea seed lectin. A_1-A_3 = 3 albumins. B_1, B_2 = 2 globulins. **a** Vicilin fraction (Danielsson 1949), **b** Gel filtration of the vicilin fraction, (Fig. 3a), **c** Legumin fraction (Danielsson 1949), **d** Gel filtration of the legumin fraction, (Fig. 3c), **e** Albumin fraction (Danielsson 1949), **f** Lectin from gel filtration of standard extract 5µl sample were applied and run against 6.4% abPI in all tests

In order to elucidate the sensitivity of vicilin to variable conditions of handling, different extraction conditions, pH shifts and heat treatments were applied (see methods). With the presence of 0.1 M NaCl in the extraction buffer, vicilin could be extracted equally well from pH 2.3 to pH 9. Without the presence of NaCl, vicilin was not extractable at pHs below 5.5 (ionic strength of the buffer was 0.015). Extraction with glass distilled water yielded a high proportion of vicilin (pH in the extract was 6.6) (Fig. 5a). By titration of standard extracts with NaOH and subsequent immunoelectrophoresis, vicilin was found to be partly denaturated or dissociated at pH 11.6 (Fig. 5c). At pH 11.8 no immunological response could be detected. Samples titrated to 11.6 and subsequently readjusted to pH 7.5 yielded a stronger immunological reaction for vicilin than the 11.6 sample. Standard extracts

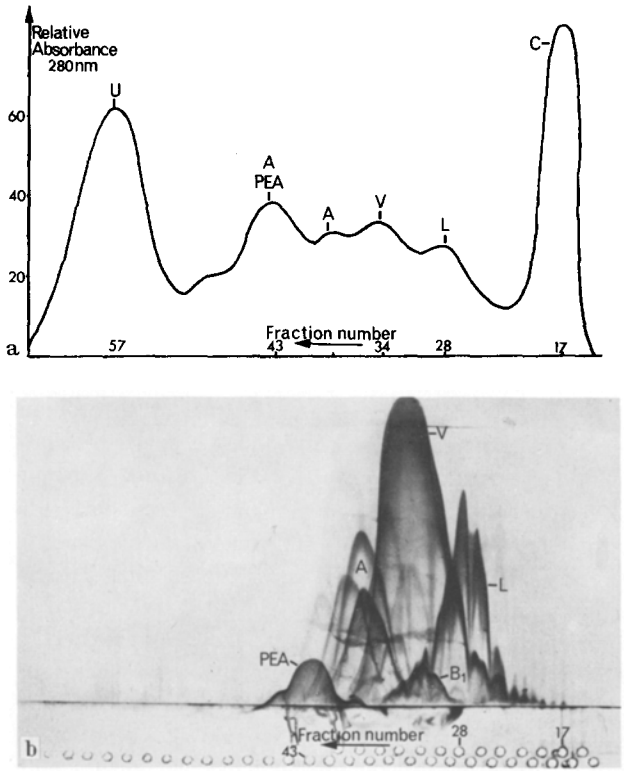

Fig. 4a and b. Eluation profile (Fig. 4a) and corresponding fused rockets (Fig. 4b) of fractions from gel filtration of standard extracts from seeds of line K 42. U = unidentified low mol. wt. material <4000 Dalton. A = unidentified albumins. PEA = pea seed lectin. V = vicilin. B_1 = a globulin. L = legumin. C = unidentified high mol. wt. material (supposedly chromatin material, ratio of absorbance 280/260 nm was 1,0). **a** Eluation profile (75 cm column, ultrogel AcA 34) monitored at 280 nm (by a UV cord), **b** The corresponding fused rockets. Fractions collected from gel filtration were applied in application wells punched in a row seen along the lower line of the IEG. Before electrophoresis (anode upwards), fractions from neighbouring wells were by diffusion allowed to fuse. Ten µl of each fraction were applied and run against 6,4% abPI

heated at 75° up to 1 h, showed a relative high immunological response for vicilin (Fig. 5e). At 90° for 5 minutes the vicilin seemed completely denaturated or dissociated as no antigenic reaction occurred.

Legumin

The legumin fraction of K 42 prepared according to Danielsson (1949) was heavily contaminated with vicilin, PEA and other proteins (Fig. 3c). Even following gel filtration of this fraction, legumin was still contaminated by vicilin and some other protein designated B3 (Fig. 3d). The mol. wt. for legumin was determined as described for vicilin. The mol. wt. was found to be 427,000 and 423.600 Daltons for the unfractionated standard extracts and the legumin fraction from Danielsson's fractionation, respectively.

The arc of legumin was often observed on IEGs to be divided into two closely running lines indicating that legumin might comprise more than one protein. Figure 6 shows that legumin comprises two proteins designated legumin species I and II (LsI, LsII). The Figures 6c and 6d show a legumin fraction from a gel filtration of a standard extract of K 42 in crossed immunoelectrophoresis with intermediate gels containing specific antisera against vicilin and legumin, respectively. Legumin is in both cases seen to be divided into two peaks. The arrows (sp) in Figure 6 points to the downward continuation of the arc from the right peak (LsII), indicating differences in the antigenic structure of left and right peak proteins. LsI and LsII show differences in electrophoretic mobility. The values relative to the Bromophenol blue front in the 1st dimension electrophoresis were respectively 0.37 and 0.57. The mol. wts of the two legumin proteins are presumably similar as the mutual magnitude of the two peaks was constant in the fractions from the gel filtrations containing legumin. The observation reported was confirmed on the basis of gel filtration of the legumin fraction from Danielsson's fractionation (not shown).

In contrast to vicilin, legumin could not be extracted at low pH values. The stability at high pH values was

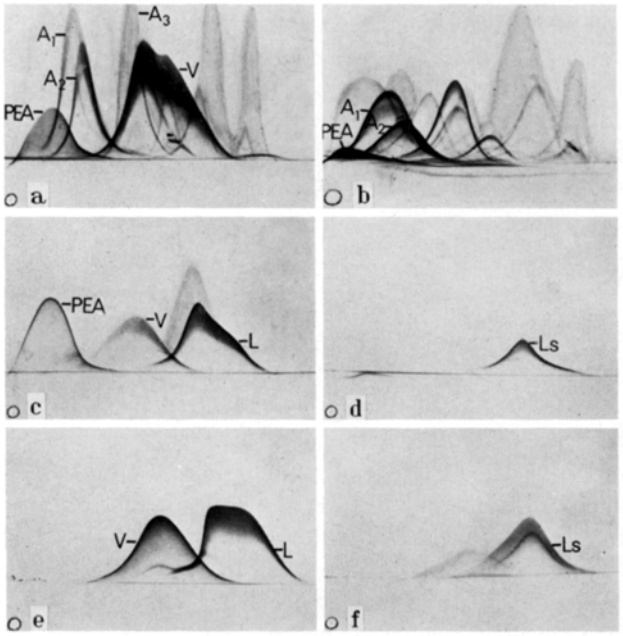

Fig. 5a-f. IEGs on water extraction, acid extraction and on the effect of pH shift and heat treatments of standard extracts from line K 42. V = vicilin. L = legumin. PEA = lectin. Ls = 'reminant' legumin (further explanation in text and by Figure 1). **a** Proteins extracted with glass distilled water (pH in the extract were 6.6), **b** Extraction at pH 5.0 without addition of NaCl (100 mM sodium acetat/acetic/acid buffer), **c** Standard extract titrated to pH 11.6 prior to electrophoresis, **d** Standard extract titrated to pH 12.1, **e** Standard extract heated to 75° for 1 hour, **f** Standard extract heated to 100° for 1 hour. Following the various treatments the samples were centrifugated and 5µl of supernatant was applied and run against 6.4% abPI except 5 b (12.8% abPI)

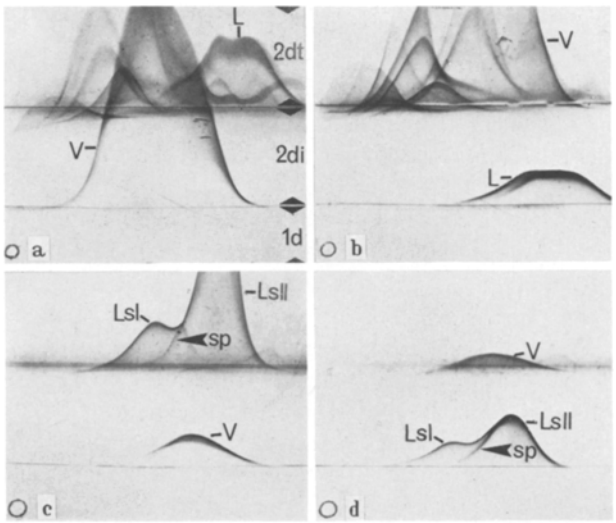

Fig. 6a-d. IEGs from crossed immunoelectrophoresis with intermediate gel of K 42 and of an impure legumin fraction of K 42. 1 d = 1st dimension gel. 2 di = 2nd dimension intermediate gel. 2 dt = 2nd dimension top gel. V = vicilin. L = legumin. LsI = legumin species I. LsII = legumin species II. sp = arc spur. **a** K 42 (5µl). 2 di contained 6.4% antivicilin serum, abV, 2 dt contained 6.4% anti pea seed serum, abPI, **b** K 42 (5µl). 2 di contained 6.4% antilegumin serum abL, 2dt contained 6.4% abPI, **c** Impure legumin (10µl) (fraction from gel filtration) 2 di: 6.4% abV; 2 dt: 6.4% abPI, **d** Impure legumin (10µl) (fraction from gel filtration) 2 di: 6.4% abL; 2 dt: 6.4% abPI

The pure vicilin and legumin arcs in the intermediate gels of Figures 6 a and 6 b, respectively, indicate that the specific antisera are not contaminated and that cross reaction between legumin and vicilin could not be detected. The two peaks and the spurs (sp) in Figures 6 c and 6 d indicate the existence of two related, but antigenic different legumin proteins LsI and LsII

higher than for vicilin, as was the resistance to heat treatments. Legumin could be extracted in only small amounts at pH 4.7 in the presence of 0.1 M NaCl. Extraction with glass distilled water yielded no legumin (Figure 5a). The antigenic reaction of legumin was not significantly affected by titration up to pH 11.8 and even at pH 12.1 a considerable antigenic reaction was observed (Fig. 5d). In contrast, titration to pH 2.3 completely abolished the antigenic reaction. Legumin was unaffected when heated at 75° (1 h) and even after 1 hour at 100° a considerable antigenic response was observed (Fig. 5f). The proteins responsible for antigenic reaction after titration to pH 12.1 and heating to 100° were by means of tandem immunoelectrophoresis found to be immunologically identical.

PEA

PEA was found both in the albumin and legumin fractions from the Danielsson fractionation. Pure PEA was prepared according to Trowbridge (1973). No signs of contamination could be detected in IEGS of this preparation. Immunological pure PEA was further obtained as a late fraction in gel filtration of standard extracts (Fig. 3f). PEA is known to bind to or complex with certain glycoproteins. The exact function of PEA in pea seed is not known.

PEA might interact with glycoproteins of the saline soluble seed proteins. Paulova et al. (1971) have reported that EDTA decreases or abolishes binding specificity of PEA while Mn^{2+} and Ca^{2+} ions enhance agglutination by PEA. PEA binds specifically to D-mannose. The PEA concentration in the extracts was not influenced by either EDTA, Mn^{2+}, Ca^{2+} or D-mannose, as judged from IEGS.

PEA was found in all extraction procedures applied at pH between 2.3 ans 9.0, with and without the presence of NaCl. Titration with NaOH showed antigenic reactions up to pH 11.8. Around pH 11.0 to 11.6 the concentration of PEA increased about 100% compared to the original standard extracts, as judged from arc areas (Fig. 5c).

Albumins

In the albumin fraction from the Danielsson procedure 14 different proteins were detected. Three of these were designated $A_1 - A_3$ (Fig. 1a). Gel filtration of the albumin fraction separated the albumins into groups of 3-5 proteins. The mol. wt. for the albumins was found to range between 15,000 and 68,000 Dalton.

Seventeen albumins were detected by extraction from the pea seed by means of glass distilled water but these extracts also contained vicilin (Fig. 5a). Albumins free of globulins could be extracted at pH 5.0 without salt (Fig. 5b) (sodium acetate-acetic acid buffer of ionic strength 0.03). By this last extraction method, 24 different proteins were detected.

As for vicilin, legumin and lectin, the observed albumins seem not to be sensitive to moderate temperatures during extraction or to the storage of the extracts for shorter periods. IEGS of extracts prepared at 0° and incubation of standard extracts at 37° for 22 hours showed arc patterns identical to the fresh standard extracts. By heating standard extracts to 60° most albumins started to denature or dissociate in contrast to the globulins. Most of the albumins were also much more sensitive to pH shifts than the globulins. Only a few unidentified albumins could stand extreme pH values (pH 2.3 or pH 11.0).

Other Proteins

The protein designated B_1 in Figure 1a was found in the legumin fraction from the Danielsson procedure. On the basis of gel filtration the mol. wt. was found to be about 200,000 Dalton. The protein was not extractable by glass distilled water and was not found in the albumin fraction. Another high mol. wt. protein, designated B_3 in Figure 3d, was detected in fractions from gel filtration of the legumin fraction. The mol. wt. for this protein was also about 200,000 Dalton. The protein designated B_2 (Fig. 1a) was not identified in the fractions from gel filtration. B_2 should probably be found in the fractions containing albumins of a mol. wt. below 100,000 Dalton.

Synthesis of Pea Seed Proteins

Steps in the accumulation of seed proteins in line K 42 are illustrated in Figure 7. The figure illustrates the appearance and further accumulation of specific proteins. The best criteria for the determination of developmental stages of the seed and the time of synthesis of seed proteins was, in accordance with Millerd et al. (1974), found to be for fresh weight of the seed rather than time after flowering or pod size.

Synthesis was further observed by means of [^{35}S] in vivo labelling. Figures 7e and 7f show the IEG and the corresponding immunoradioauthograph of seed with a fresh weight of 0.5 g. Labelling was attempted for 5 earlier development stages (0.01 g to 0.40 g) but without detectable incorporation. Only the initiation of legumin synthesis could be detected on the immunoradioauthographs produced from the early stages.

The chronology of protein synthesis was found to be as follows: 22 different proteins were detected in the early stages when the seed weighs only 0.07 g to 0.18 g. At around 0.25 g fresh weight initiation of vicilin synthesis was detected. At around 0.30 g initiation of legumin

synthesis was detected. It seems that legumin synthesis was stopped or slowed for a period up to 0.50 g at which stage the full amount of legumin in mature seed was quickly reached.

The albumins most outstanding in the standard IEG of mature seed (Fig. 1a: A_1-A_3, B_1-B_2 and PEA) were found to be synthesized at a late stage when the seed weight reached about 0.50g or more. The immunoradioauthograph (Fig. 7f) indicates that albumins A_1-A_3, B_2 and legumin contains sulfur amino acids and that synthesis of these albumins takes place around 0.5 g of fresh weight. Incorporation of [^{35}S] into sulfur amino acids was not detected for vicilin and PEA. This is in agreement with the previously reported lack of sulfur amino acids in PEA (Entlicher et al. 1970) and with the reported low content of these amino acids in vicilin (Jackson et al. 1969).

During seed development the accumulating vicilin and legumin form arcs which are symmetrical in shape and without shoulders. The arcs formed by vicilin and legumin from mature seed are not symmetrical indicating that modifications of these proteins occur during development of the seed.

Germination

Steps in the depletion of seed proteins of line K 42 are illustrated in Figure 8. The presence of proteins of the mature seed was observed in the cotyledons and in the embryonic axis for 12 days.

Figure 8a shows that the embryonic axis (the top 1.5-2 mm of the axis cut from imbided seed at 24 h) contains many of the proteins of the mature seed but no legumin. The observation was confirmed by use of the specific antisera abV and abL. A few days after germination none of the seed proteins could be detected in the fast growing embryonic axis (stem and radicals).

In the cotyledons smaller changes were observed in the first 5 days from the onset of germination. Around day 3

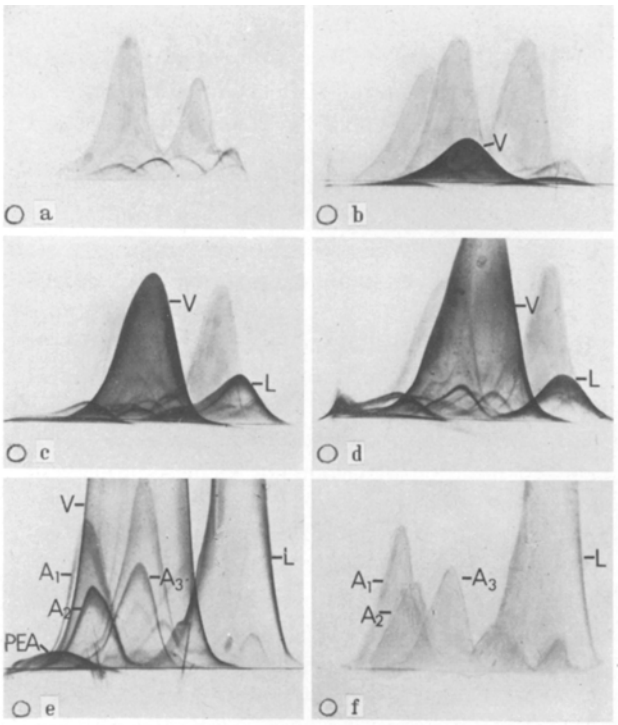

Fig. 7a-f. IEGs on various stages of seed development (line K 42). V = vicilin. L = legumin. PEA = lectin. A_1-A_3 = three albumins. **a** Extract from an early developmental stage of pea seeds. Seed fresh weight was 0.09 g. Twenty-two albumins were detected. None of the globulins were synthesized before the fresh weight passed about 0.20 g, **b** Freshweight 0.25 g, 20µl extract. Synthesis of vicilin has started, **c** Freshweight 0.33 g, 15µl extract. The content of vicilin increases. Synthesis of legumin has started, **d** Freshweight 0.49 g, 15µl extract. Further increase of vicilin. Legumin synthesis seems temporarily stopped, **e** Freshweight 0,50 g, 15µl extract. Synthesis of PEA, A_1-A_3. Apart from the globulin B_1 most proteins visible on IEGs of mature seed were found at this stage. This sample was [^{35}S] in vivo labelled, **f** Immunoradioauthography of the IEG in Figure 7 e. The albumins A_1-A_3, several minor albumins, and legumin are seen to incorporate [^{35}S] at this stage. In all IEGs the antiserum was 12.8% abPI

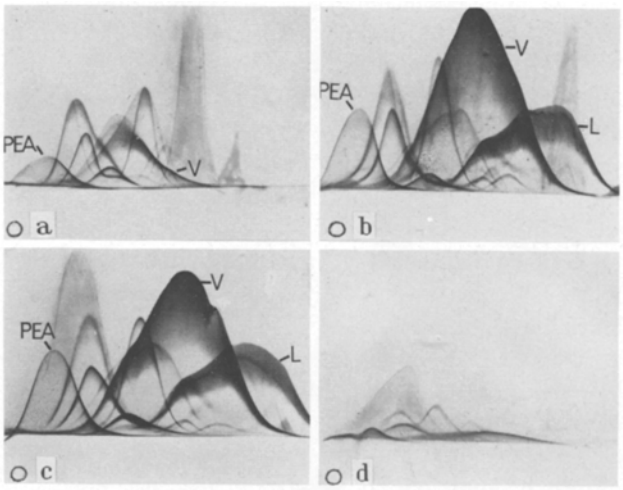

Fig. 8a-d. IEGs on proteins extracted from embryonic axis and on extracts from germinating cotyledons (line K 42). V = vicilin. L = legumin. PEA = pea seed lectin. **a** The 1.5-2.0 mm apex of the embryonic axis cut from seeds imbibed for 24 hours and extracted at 0° (standard extraction). Legumin is missing. **b** Proteins extracted from the cotyledons 5 days after the onset of germination. The amount of fresh weight material to extraction buffer was kept constant (see methods). The amount of PEA is seen to be increased, **c** As b, but 8 days after onset of germination, **d** As b, but 10 days after. A sudden and nearly complete breakdown of storage proteins occurs 10-12 days after the onset of germination. 15µl extract was applied against 12.8% abPI

the concentration of PEA increased about 100% as judged from the arc area. At day 5 the shape of the vicilin arc changed, with the disappearance of the shoulder of the right side of the vicilin arc. Eight days after the onset of germination the concentrations of legumin and vicilin were still relatively high (Fig. 8c). Ten to twelve days after the onset of germination vicilin and legumin virtually disappeared while PEA and some albumins still were present, although in decreased amounts.

Discussion

The dominating proteins of pea seed, vicilin and legumin were found to be immunological distinctly different. This is not in accordance with previously reported findings. Kloz and Turkova (1963) found a 30-40% serological cross-reaction between vicilin and legumin from pea. Jackson, Boulter and Thurman (1969) on the basis of tryptic digestion reported, that there are considerable portions of the amino acid sequence of vicilin and legumin which are the same or very similar. These findings may be due to vicilin contamination of the legumin fraction in the process of isoelectric precipitation as observed with the fractionation procedure according to Danielsson (1949) in the present investigation.

Mutual contamination of the vicilin and legumin fractions might further explain the discrepancy between the mol. wt. as determined by Danielsson (1949), 186,000 for vicilin and 331,000 Dalton for legumin, and the present findings, about 160,000 for vicilin and 425,000 Dalton for legumin. Johnson and Richards (1962) reported the mol. wt. of pea legumin to be 398,000 ± 15,000 based on sedimentation and diffusion measurements. Their investigations are the most comprehensive reported and the mol. wt. estimate is thus possibly the most accurate available for legumin. The method of gel filtration can only yield rough estimates of mol. wt. for the complex proteins vicilin and legumin.

Vicilin and legumin in the mature seed of pea seem to be proteins of a complex structure. The precipitation arcs of both proteins were asymetrical with several shoulders. Heterogenity of vicilin from pea, based on observation of diffuse banding on polyacrylamid gels, has previously been reported by Basha (1974).

In the present study, legumin is shown to be comprised of two proteins, LsI and LsII. These proteins were found to differ in electrophoretic mobility and to have a high degree of common antigenic structure, although differences were detectable. As both antigenically reactive legumin proteins have a molecular weight of about 425,000 Dalton, it is likely that LsI and LsII are found in the extract as two independent proteins in the relative amounts 1:2. There is no evidence that legumin dissociates during the procedure of electrophoresis.

Johnson and Richards (1962) have reported that pH values higher than 10 and heating to more than 90° transforms legumin into a slow sedimenting material of low mol. wt. The antigenic reaction of samples heated to 100° or titrated to pH 12.1 is thus possibly a result of reassociation of subunits during sample application and electrophoresis (at pH 8.6, 10°). For vicilin, but not for legumin, the pH shift of standard extracts: pH 8.0→pH 11.6→pH 7.5 yielded a higher antigenic reaction than pH 8.0→11.6, indicating that the process of reassociation may continue beyond the conditions given by the applied procedure of electrophoresis. By means of sedimentation analysis Grant & Lawrence (1964) have observed an apparent reassociation of dissociated vicilin when, by dialysis, the pH was changed from 10 to 7.

The observed synthesis of albumin prior to the synthesis of vicilin and legumin confirms results reported by Beevers & Poulson (1972), Millerd and Spencer (1974) and Basha (1974). Twenty-two different proteins were detected prior to the onset of vicilin synthesis in the present study by use of antiserum abPI. As abPI was produced on a basis of dry mature seed, other proteins might be present at this stage but undetectable by the method applied.

The three albumins (A_1-A_3) and the globulin B_1, that in the present study were found to be synthesized after the onset of legumin synthesis, may represent different storage proteins.

The depletion of vicilin and legumin prior to depletion of a number of albumins during germination confirms results reported by Basha (1974) and Basha and Beevers (1976). These authors have reported a change in the component composition of vicilin and legumin during germination which might be in accordance with the change in arc shapes observed in the present investigation. Still the reported change in component composition of vicilin and legumin was based on electrophoresis of SDS-dissociated proteins and might thus reflect the presence of depletion products of the globulins which could escape detection with the antiserum applied in the present investigation.

The observed presence of vicilin and a lack of legumin in the embryonic axis indicates that vicilin, but not legumin, might be transferred, perhaps on the form of subunits, from the cotyledons to the axis during seed development. Another possibility is that vicilin, but not legumin, is synthesised in the embryonic axis. The observation was made by extraction at 0° of seed imbided for 24 h. It seems unlikely that either specific proteolytic activity or transport of large amounts of vicilin from the cotyledons during the 24 hours of imbibation should be the reasons for the exclusive presence of vicilin in the embryonic axis.

Some of the saline soluble seed proteins might be bound to, or complex with, PEA. The concentration of PEA increased about 100% during germination compared

to the amount of PEA present in extracts of imbibed mature seed. An increase of the same order of magnitude was observed by titrating standard extracts to about pH 11.3. These observations might be due to release of PEA from one or more glycoproteins dissociating during germination and by a shift to basic pH. The increase of PEA by a pH shift of standard extracts indicates that PEA might interact with saline soluble proteins. This hypothesis could not be confirmed by EDTA treatments which according to Paulova et al. (1971) can decrease or abolish the binding capacity of PEA.

The observed difference in content of albumins (A_1 and A_2) and PEA between pea line K 42 and L 1293 is probably under genetic control. An analysis of seed grown under different environmental conditions confirmed the mentioned qualitative differences. Varietal differences have been reported in seed proteins from various lines of pea (Grant and Lawrence 1964; Hynes 1968; Davies 1976; Przybylska et al. 1977). These observations were made with the extracted proteins in dissociating systems and are hence not immediately comparable to the present analysis of undissociated proteins.

The interspecific immunochemical similarities observed for *Pisum sativum, Vicia faba* and *Vicia sativa* and the dissimilarities towards *Phaseolus vulgaris, Glycine max* and *Lupinus* are in accordance with the results reported by Kloz and Turkova (1963). The arc shapes and the electrophoretic position of vicilin and legumin in *Vicia faba* were similar to those observed by Manteuffel and Scholz (1975).

Quantitative immunological methods, particularly different forms of crossed immunoelectrophoresis as described by Axelsen et al., (1973) proved, in connection with the use of specific antisera, useful tools for the study of saline soluble seed proteins. The small amount of protein required (less than 1% of a single pea seed) makes the method particularly useful for both quantitative and qualitative investigations of protein content in different tissues at different developmental stages as well as for sampling where the living seed must survive the sampling procedure. The methods might also be useful for the analysis of specific quantitative genetic differences of seed proteins between different varieties.

Acknowledgment

Kirsten Henriksen is thanked for her skilful technical assistance. Professor Knud W. Henningsen is thanked for advice and help during the work and for linguistic correction of the manuscript. Dr. Thorkild Bøgh-Hansen is thanked for advice on quantitative immunoelectrophoresis and for the supply of specific antisera. Dr. J.V. Spärck is thanked for the supply of the main antiserum used. The work was in part supported by The Danish Natural Science Research Council and The Carlsberg Foundation.

Literature

Axelsen, N.H.; Krøll, J.; Weeke, B.: A manual of quantitative immunoelectrophoresis, methods and applications. Oslo: Universitetsforlaget. pp. 169. (1973)

Basha, S.M.M.: Protein metabolism in the cotyledons of *Pisum sativum* L. during seed development and germination. (Ph.D. thesis). Grad. Coll. Univ. Oklahoma, Norman. pp. 79 (1974)

Basha, S.M.M.; Beevers, L.: The development of proteolytic activity and protein degradation during the germination of *Pisum sativum* L. Planta. **124**, 77-87 (1975)

Beevers, L.; Poulson, R.: Protein synthesis in cotyledons of *Pisum sativum* L. Plant Physiol. **49**, 476-481 (1972)

Danielsson, C.E.: Seed globulins of the *gramineae* and *leguminosae*. Biochem. J. **44**, 387-400 (1949)

Danielsson, C.E.: An electrophoretic investigation of vicilin and legumin from seeds of peas. Acta Chem. Scand. **4**, 762-771 (1950)

Davies, D.R.: Variation in the storage proteins of peas in relation to sulphur amino-acid content. Euphytica **25**, 717-724 (1976)

Entlicher, G.; Koštiř, J.V.; Kocourek, J.: Studies on phytohemagglutinins. III. Isolation and characterization of hemagglutinins from the pea (*Pisum sativum* L.). Biochim. Biophys. Acta. **221**, 272-281 (1970)

Grant, D.R.; Lawrence, J.M.: Effects of sodium dodecyl sulfate and other dissociating reagents on the globulins of peas. Arch. Biochem. Biophys. **108**, 552-561 (1964)

Hynes, M.J.: Genetically controlled electrophoretic variants of a storage protein in *Pisum sativum*. Austr. J. Biol. Sci. **21**, 827-829 (1968)

Jackson, P.; Boulter, D.; Thurman, D.A.: A comparison of some properties of vicilin and legumin isolated from seeds of *Pisum sativum, Vicia faba* and *Cicer arietinum*. New Phytol. **68**, 25-33 (1969)

Johnson, P.; Richards, E.G.: The study of legumin by depolarization of fluorescence and other physicochemical methods. Arch. Biochem. Biophys. **97**, 260-276 (1962)

Kloz, J.; Turková, V.: Legumin, vicilin and proteins similar to them in the seeds of some species of the *viciaceae* family (a comparative serological study). Biol. Plant. **5**, 29-40 (1963)

Manteuffel, R.; Scholz, G.: Studies on seed globulins from legumes. Biochem. Physiol. Pfl. **168**, 277-285 (1975)

Millerd, A.: Biochemistry of legume seed proteins. Ann. Rev. of Plant Physiol. **26**, 53-72 (1975)

Millerd, A.; Spencer, D.: Changes in RNA-synthesizing activity and template activity in nuclei from cotyledons of developing pea seeds. Austr. J. Plant Physiol. **1**, 331-341 (1974)

Millerd, A.; Spencer, D.; Dudman, W.F.; Stiller, M.: Growth of immature pea cotyledons in culture. Austr. J. Plant Physiol. **2**, 51-59 (1974)

Müller, H.; Gottschalk, W.: Quantitative and qualitative situation of seed proteins in mutants and recombinants of *Pisum sativum*. In: Nuclear techniques for seed protein improvement, pp. 235-253. Vienna: Intern. Atomic Energy Agency 1973

Paulová, M.; Entlicher, G.; Tichá, M.; Koštiř, J.V.; Kocourek, J.: Studies on Phytohemagglutinins. VII. Effect on Mn^{2+} and Ca^{2+} on hemagglutination and polysaccharide precipitation by phytohemagglutinin of *Pisum sativum* L. Biochim. Biophys. Acta **237**, 513-518 (1971)

Przybylska, J.; Blixt, S.; Hurich, J.; Zimniak-Przybylska, Z.: Comparative study of seed proteins in the genus *Pisum*. I. Electrophoretic patterns of different protein fractions. Genetica Polonica. **18**, 1. 27-38 (1977)

Trowbridge, I.S.: Mitogenic properties of pea lectin and its chemical derivatives. Proc. Nat. Acad. Sci. (USA) **70** (12), 3650-3654 (1973)

Yarwood, A.: Biosynthesis of legume seed proteins. In: Plant Proteins (ed. Norton, G. pp. 41-54. London: Butterworths 1977

Received September 22, 1978
Communicated by D. von Wettstein

P. Guldager
Department of Genetics
Royal Veterinary and Agricultural University
Bülowsvej 13
DK – 1870 Copenhagen V (Denmark)