THE EXPRESSION OF LEA PROTEINS IN *PISUM SATIVUM* (PEA) SEEDS

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ABSTRACT

The experiments reported on here investigated the relationship between desiccation characteristics and the presence/absence of heat stable proteins in embryos of *Pisum sativum* (pea). Heat stable proteins seemed to be more concentrated when drying occurred in tissue that was sensitive to desiccation and were even found after viability had been lost. This may lend support to earlier views that concentrations of certain proteins have to be high enough for efficient protection and/or the presence of heat stable proteins alone is insufficient to confer desiccation tolerance.

It was also shown that embryos that were slowly dried retained higher viability than those that were quickly dried. These results were found to be the opposite of those for recalcitrant seeds/embryos. Thus it is suggested that the interpretation of studies using sensitive tissue of orthodox seeds as a model for truly recalcitrant tissue, should be treated with some caution.

INTRODUCTION

Recent studies have shown that seeds of many species acquire the ability to tolerate considerable water loss during the late stages of their development. These seeds are referred to as orthodox seeds (Chin and Roberts, 1980). There has been much interest in the mechanism of their desiccation tolerance. It has been shown that the mRNA's coding for a set of proteins (termed LEA for late embryo abundant [Dure *et al.*, 1989] or dehydrins [Close *et al.*, 1989]) are expressed at high levels during the late stages of development, coincident with the onset of tolerance (Baker *et al.*, 1988; Dure *et al.*, 1989; reviewed by Kermode, 1990; Farrant *et al.*, 1993; Vertucci and Farrant, 1994). These messengers are degraded during imbibition and germination and it has been suggested that LEA proteins play a role in preparing the embryo for dehydration and/or preventing cellular disruption on rehydration (reviewed by Kermode, 1990).

Messenger RNA's for LEA proteins have also been shown to be induced in response to dehydration of seedling tissues (Mundy and Chua, 1988; Reid and Walker-Simmons, 1993). These proteins are robust (tolerant of heating up to 80° C) and hydrophilic and thus are potentially good candidates in the protection against desiccation damage (Dure *et al.*, 1989: Kermode, 1990). It has thus been suggested that LEA proteins serve as membrane protectants and/or stabilisers of the subcellular environment in the dry state (Close *et al.*, 1989; Dure *et al.*, 1989; Lane 1991; Dure, 1993).

To date, these suggestions have been largely based on the observation of changes in mRNA levels. However, there have been some studies on the proteins themselves which support their role in the mechanism of desiccation tolerance. Blackman *et al.* (1991) have shown that several proteins accumulate during the onset of desiccation tolerance and in response to slow drying of premature soybean seeds. These decline during germination when desiccation tolerance is lost. Farrant *et al.* (1992; 1993) have shown that there is no similar accumulation of proteins in the desiccation sensitive (recalcitrant) seeds of *Avicennia marina*. Furthermore, Reid and Walker Simmonds (1993) have shown that mRNA alone is insufficient to confer tolerance in wheat seedlings and that proteins themselves must be present for tissues to survive a period of desiccation. Recently, Close *et al.* (1993), using an antibody generated to an anti-dehydrin consensus region, have demonstrated the presence of dehydrin-like proteins in the orthodox seeds of barley, wheat and maize and in dehydrated orthodox seedling tissues of several species. However, this technique also demonstrated their presence in unstressed tissues of barley.

Thus while there appears to be an interdependence between the response to drying and the presence of LEAs, it is not clear to what extent these proteins are involved in the mechanism of desiccation tolerance. It has also not been demonstrated that these proteins are universally present in orthodox seeds.

In the present study the relationship between desiccation characteristics and the presence/absence of LEAs was investigated for the orthodox seeds of *Pisum sativum* (pea). The timing of

loss of desiccation tolerance during germination was determined by monitoring the viability of seed which had been redried after being allowed to germinate for different time intervals. The presence of LEAs was tested for before and after the loss of tolerance.

The heat tolerance of LEA proteins can be exploited as a suitable way of identifying their presence in a complex mix of proteins by heating the sample (Kermode 1990; Blackman *et al.* 1991; 1992). The non-coagulated, heat stable proteins can then be separated by gel electrophoresis. This method has been used in the present study. Unheated protein samples were also subjected to electrophoresis in order to ensure that the appearance of new proteins was not due to changes in molecular mass as a consequence of the heating process. Most studies reporting the presence of LEAs, dehydrins or their mRNAs have used whole seeds. In the present study the presence of LEAs / dehydrins was investigated for in both axes and cotyledons separately.

It has been shown that rate of drying can affect viability characteristics in desiccation sensitive seeds. Rapid drying (flash drying [Berjak *et al.* 1990]) of the isolated axes allowed drying to considerably lower moisture contents than did slow drying of whole seeds (Berjak *et al.*, 1989; Pammenter *et al.*, 1991). It has been suggested that rapid drying of such desiccation-sensitive tissues was successful, not because the tissues were inherently tolerant of drying, but rather because the drying time was so rapid that subcellular damage was unable to accumulate to lethal levels (Pammenter *et al.*, 1991). It is

not known whether orthodox seed embryos and seeds, which have lost their desiccation tolerance, will respond to different rates of drying in a similar manner to recalcitrant embryos. The effect of rate of drying was tested on pea seeds before and after the loss of tolerance and the presence of LEAs was tested for immediately after drying and in tissues rehydrated for 24 hours after drying.

MATERIALS AND METHODS

PLANT MATERIAL.

Seeds of *Pisum sativum* cv. Greenfeast were used in all of the experiments. In order to prevent imbibitional damage, seeds were slowly pre-hydrated for 1 hour on damp blotting paper prior to imbibition. In order to ascertain at what stage during germination the seeds had lost their desiccation-tolerance, seeds were imbibed for different intervals (12, 36, 48, 60 hrs) prior to redrying to their original moisture content at room temperature (23° C).

SLOW DRYING OF WHOLE SEEDS.

For each imbibitional stage seeds were slowly dried back to their original moisture content (11.3 g H_2O/g dry mass) by placing them evenly spaced in a laminar flow cabinet. Viability and moisture content were assessed at time intervals 0, 4, 10, 24, 34, 48 hrs by which time the seeds had reached their original moisture content.

RAPID (FLASH) DRYING OF EMBRYONIC AXES.

Seeds were pre-hydrated as described above. After imbibition the seeds were removed from the water and the lengths of their axes were measured as a rough indicator of how far germination had proceeded. Axes of similar length were selected for the experiment. The rapid drying of embryonic axes followed Pammenter *et al's* (1991) method: Air was passed at room temperature (approximately 22° C) through two fish tank air

diffusers in parallel evenly spaced at the bottom of a plastic box 10 cm long, 10 cm wide and 4 cm deep. Excised embryonic axes were placed on fine-mesh nylon supported 2 cm above the air stones and removed after the required drying time, i.e. 20, 40, 60, 120, 160, 200, 280, 320, 360, 690 minutes until the original moisture content of the axis was reached. At each time interval, moisture content (measured as described below for whole seeds) and viability was assessed as described below.

Viability assessment of seeds (40 seeds).- Seeds were sown into seed trays containing a vermiculite mixture. The emergence of shoots was taken as an indication of retained viability. Based on the time taken for the control to yield 100% viability (i.e. 7 days), the experiment was terminated after 15 days which allowed some time for late germinations.

Moisture content assessment (10 seeds).- Moisture content of axes and cotyledons (separately) was determined gravimetrically by drying at 110° C for 48 h and expressed on a dry mass basis.

Viability assessment of axes (10 axes).- The viability of the axes was assessed in tissue culture. Axes were germinated on a medium which contained Murashige and Skoog (1962) macro- and micronutrients (see Appendix I), 5% sucrose, 0.1% caseinhydrolysate and 0.8g/l agar. The medium was adjusted to pH 5.6-5.8. 5 ml of medium was poured into separate boiling tubes and autoclaved for 20 minutes.

After the flash-drying treatment, axes were sterilized in 2% sodium hypochlorite for 10 minutes, washed 3 times in sterile

and distilled water and then aseptically transferred into the growth medium.

The tubes were kept in a growth room at 20° C and a photoperiod of 16 hrs light and 8 hrs dark cycle. After 1 week the number of shoots was recorded and the experiment terminated.

PROTEIN STUDIES.

Based on the results obtained from the viability studies, peas were imbibed for 12 (prior to loss of tolerance) and 60 (after loss of tolerance) hours. Proteins were extracted from: 1. cotyledons and axes (separately) of hydrated undried peas 2.a) axes flash dried to their original moisture content b) axes flash dried to their original moisture content and rehydrated for 24 hrs on growth medium 3.a) cotyledons and axes slowly dried to their original moisture content b) cotyledons and axes slowly dried to their original moisture content and rehydrated for 24 hrs on moist filter paper.

Protein extraction.- Approximately 0.1 g of lyophilized plant material was ground in a pre-chilled mortar. The material was filled into Eppendorf tubes and suspended in 500 μl of extraction buffer [50 mM Tris, 0.1 M KCl, 2 mM Phenylmethylsulfonyl fluoride (PMSF), pH 7.0] and kept on ice for 10 min. Samples were centrifuged at 4° C at 14000 rpm in a Beckman Model T2-21 for 20 minutes. The supernatant was retained and separated into two aliquots. One was heated to select for heat stable proteins and the other served as a control (total protein). Aliquots were stored at -80° C until used. All separations were performed in

duplicate.

Preparation of heat stable proteins.- Aliquots were heated for 10 min at 80° C, and centrifuged at 10000 rpm for 10 min to remove heat coagulated proteins. 100 µl of the supernatant was suspended in an equal volume of SDS buffer [In 50 ml: 10 ml glycerol, 5 ml 2-mercaptoethanol, 20 ml 10% SDS, 12.5 ml Stacking gel buffer [60 g/L Tris, pH 6.8]. All samples were heated at 100° C for 2 min in order for the SDS buffer to associate with the proteins. Samples were cooled to room temperature, 5 µl of bromothymyl blue added (which served as the tracking dye) and 10-30 µl of proteins were loaded and separated as described below. The flow chart below depicts the steps of the protein extraction:

Flow chart for protein extraction.

0.1g in 500µl extraction buffer

↓

centrifuge at 14000 rpm, 20 min

↓

keep supernatant

↓	↓
350 µl (heat stable)	150 µl (unheated control)
heat at 80° C, (10 min)	add equal amounts of sample to SDS buffer
centrifuge at 10000 rpm, (10 min)	heat for 2 min at 100° C
keep supernatant	load 20 - 30 µl
take out 100 µl	
add 100 µl of SDS buffer	
heat for 2 min at 100° C	
load 20 - 30 µl	

Protein separation.- 1-Dimensional SDS-Polyacrylamide gel electrophoresis, was used to separate the seed proteins (Laemmli, 1970). Molecular weight standards, combi thek marker (Boehringer Mannheim GmbH, Germany), which labels molecular weights from 20kD - 340kDa, were included in the gel run. 10 µl of each of the 8 molecular weight markers were added to 5 µl of bromothymyl blue and heated at 100° C for 2 min.

Preparation of gels.- The resolving and stacking gels were prepared as follows:

Resolving gel.

10ml Monomer [58.4g Acrylamide, 1.6g Bis acrylamide, water to 200ml]; 7.5ml Resolving gel buffer [36.3g Tris, water to 200ml, pH 8.8]; 3.0ml 10% SDS; 12.05ml H_2O; 66µl APS [10% Ammonium persulfate]; 10µl TEMED.

Stacking gel.

1.33ml Monomer; 2.5ml Stacking gel buffer [6.0g Tris, water to 100ml, pH 6.8]; 0.1ml 10% SDS; 6.1ml H_2O; 50µl APS; 5µl TEMED.

The solutions were degased before APS and TEMED were added. The APS was made up fresh for each run.

Loading of gels.- Volumes between 10µl to 30µl of protein, which had been determined in a trial run were loaded. A duplicate of each gel was made for the analysis by Western blot. Each of these were run in duplicate.

Running gel buffer [1L: 3g Tris, 14.4g Glycine, 10ml of 10% SDS] was added to the wells and to the buffer tank.

Gels were cooled to 4° C and run for approximately 5 hours

at a constant current of 30 A per gel.

Gels were then stained for 2 hours in 0.1% coomassie blue stain solution [500mls: 0.5g coomassie blue dissolved in 50% methanol and 10% acetic acid], and then destained in destain solution [10% acetic acid, 5% methanol].

Protein concentration.- Proteins were precipitated from the supernatant by adding 5 volumes of 0.1 M ammonium acetate in methanol and kept at -20° C overnight. The protein obtained after centrifugation was washed four times with acetate and freeze dried. These were redissolved in 50 µl of extraction buffer and 50 µl of SDS buffer (2x conc.). Samples were heated and separated as described previously.

WESTERN BLOT.

1. Transfer to nitrocellulose.

Proteins were transferred to nitrocellulose paper (Schleicher & Schuell) pore size 0.45 µm using a transfer buffer (25 mM Tris-HCl 0.192 M glycine 20 % methanol pH 8.3).

Electrophoresis was allowed to procede for 1.5 hours at 0.8 A after which the nitrocellulose paper was placed on a sheet of Whatman 3MM paper and allowed to dry.

2. Visualization of the transferred protein.

The dry blot was incubated in 10 mls blocking buffer (50 mM phoshate, 150 mM NaCl pH 7.4 (PBS), 5% skimmed milk powder (Nestle), 100 µl of 5% sodium azide) for 30 minutes at room temperature after which antibody was added to give a dilution of 1:50. This was further incubated for 2 hours at room

temperature.

The blot was washed 3 times with PBS, 0.05% Tween, washed once with PBS and incubated for 60 minutes in PBS containing the goat-v-rabbit 2nd antibody (coupled to alkaline phosphatase), 5 % milk powder and 100 µl 5 % sodium azide.

The blot was washed 3 times with PBS, 0.05 % Tween and once with 10 mM Tris-HCl, 150 mM NaCl pH 7.4.

The blot was incubated in 10 mls of 100 mM NaCl 100 mM $MgCl_2$ 100 mM Tris-HCl pH 9.5. After addition of 50 µl of NBT (at 0.5 g/10 mls 70 % DMF) and 50 µl of BCIP (at 0.5 g/10 mls 100 % DMF), incubation was continued until appearance of colour, then washed and air dried.

RESULTS

Physical observations of axes after imbibition for 12, 48 and 60 hours.

For up to 12 hours imbibition the radicle had not yet protruded from the seed coat (Table 1). After that time the length of the radicle increased and reached up to 0.6 - 1.8 cm. By 60 hours imbibition the axes had further elongated and reached up to 2.5 cm and shoots were visible.

Table 1. Lengths of axes after different length of imbibition.

Imbibition (hrs)	Length of radicle (cm)	Remarks
12	0.0	axes not yet protruded
48	0.6 - 1.8	shoot not yet visible
60	1.5 - 2.5	shoot visible

Response of orthodox seeds to drying after different imbibition treatments.

The hydrated seeds reached their original dry weight between 24 and 48 hours (results not shown). At these moisture levels viability for the 12 hours imbibed seeds did not drop below 93 %, suggesting that desiccation tolerance had not been lost. With the progress of germination (36, 48 and 60 hours imbibition) the seeds became increasingly sensitive to drying (Fig. 1). After 36 hours imbibition only 86 % viability was retained and drops further to 53 % after 48 hours imbibition. After 60 hours

imbibition viability was greatly reduced, but only lost once the seeds had been dried back to their original moisture levels (Fig. 1).

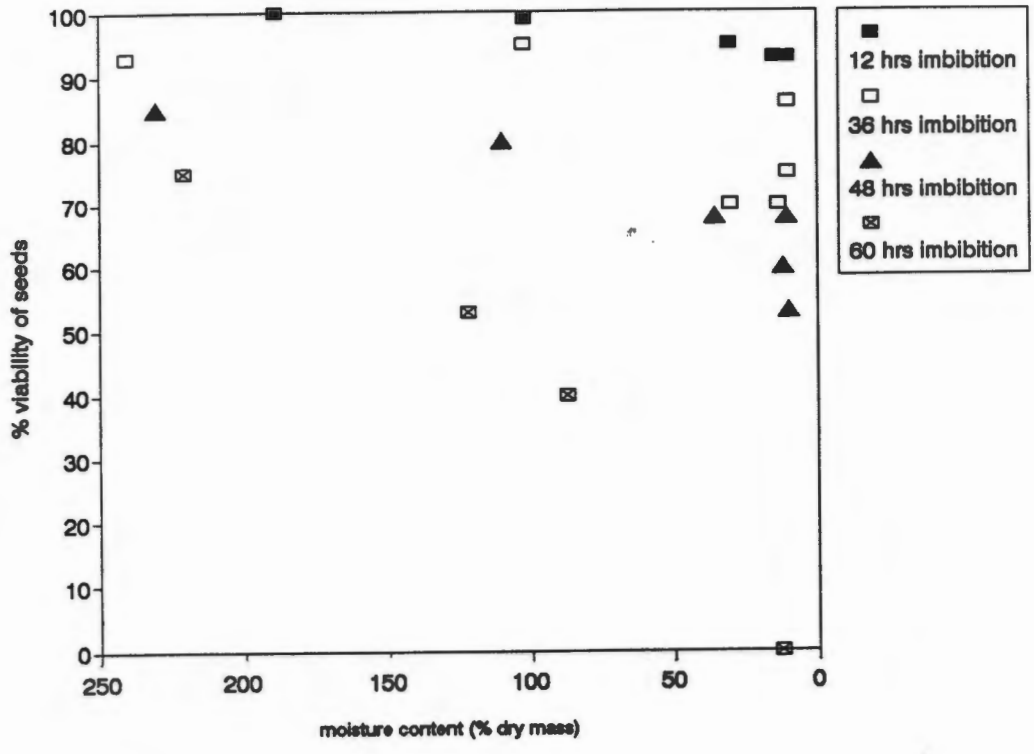

Fig. 1. Loss of viability of slow-dried seeds of *Pisum sativum* is shown to be related to a decrease in water content. With longer periods of imbibition, i.e. 12, 36, 48 and 60 hours, viability is progressively reduced.

Response of orthodox seeds to the rate of drying.

The time of drying of axes to their original moisture content was much shorter than for whole seeds (Table 2). For the 12 hour imbibition treatment after 160 minutes of rapid drying the axes had regained their original moisture content (13.0 g H_2O/ g dry weight). Axes imbibed for 48 and 60 hrs required a much longer time of drying, i.e. 11 ½ hours until their original moisture content was re-established (Table 2).

Table 2. Flash drying of axes of *Pisum sativum* (pea). Viability (V in %) and moisture content (MC in g H_2O/g dry mass) shown after different lengths of drying time (minutes) for the different imbibition (i.e. 12, 48, 60 hours) treatments.

Time	12 hrs imbibed		48 hrs imbibed		60 hrs imbibed	
(min)	V	MC	V	MC	V	MC
0	90	220.4	80	374.1	80	766.0
20	90	130.8	80	312.0	-	-
40	90	70.6	70	212.6	20	507.2
60	100	27.8	10	181.5	20	344.6
120	90	18.8	10	140.1	20	158.1
160	90	13.0	50	89	20	133.7
200			50	76.2	20	79.5
280			50	56.1	30	61.1
320			30	42	20	60.0
360			30	35.1	10	59.8
690			0	15.2	0	14.9

As for whole seeds, viability was retained in the 12 hour imbibition treatment, when the radicle had not yet protruded from the seed coat (Table 1, 2). With the progress of germination, the axes became increasingly sensitive to the loss of moisture (Table 1, Fig. 2).

At 56.1 g H_2O/ g dry weight, 50 % of the axes retained viability when imbibed for 48 hours, whereas at similar moisture contents viability was reduced to 10 % in the 60 hour imbibition treatment (Table 2, Fig. 2). When dried back to their original moisture contents viability was completely lost.

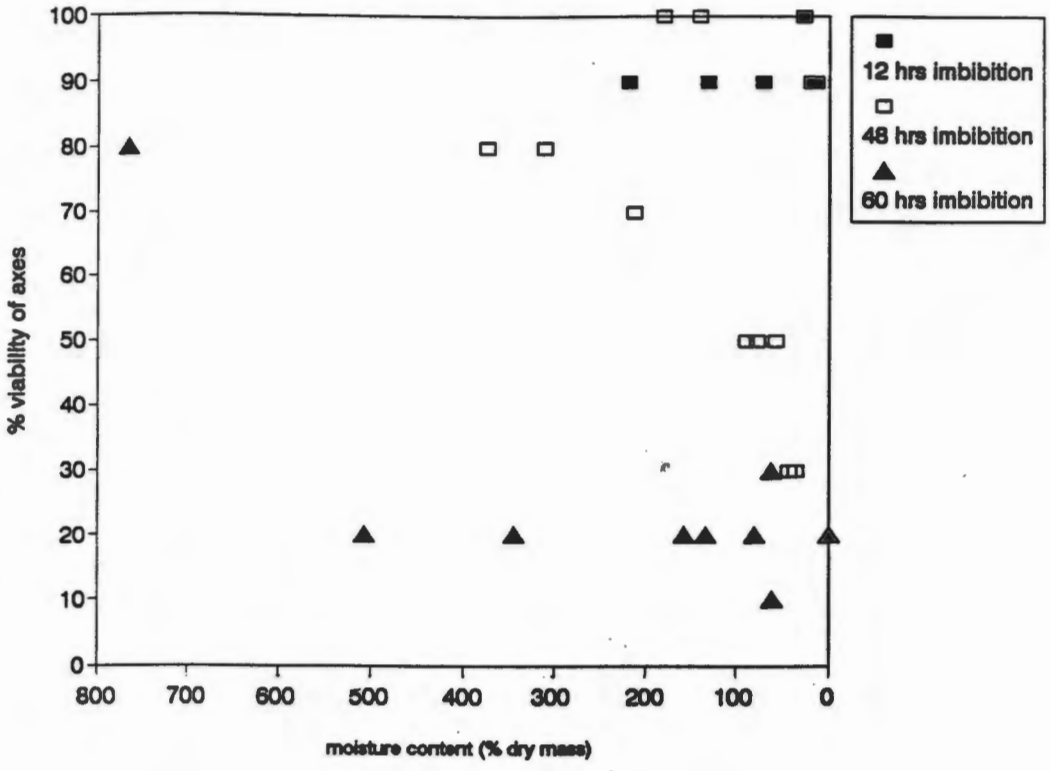

Fig. 2. Loss of viability of fast-dried axes of *Pisum sativum* is shown to be related to a decrease in water content. With longer periods of imbibition, i.e. 12, 48 and 60 hours, viability is progressively reduced.

Figure 3 gives a comparison of the effect of rate of drying on the viability. It is shown that slow drying of whole seeds allowed viability retention to lower moisture contents than when axes were rapidly dried. By 60 hours imbibition both fast and slow drying lead to the loss of desiccation tolerance when dried to original moisture levels.

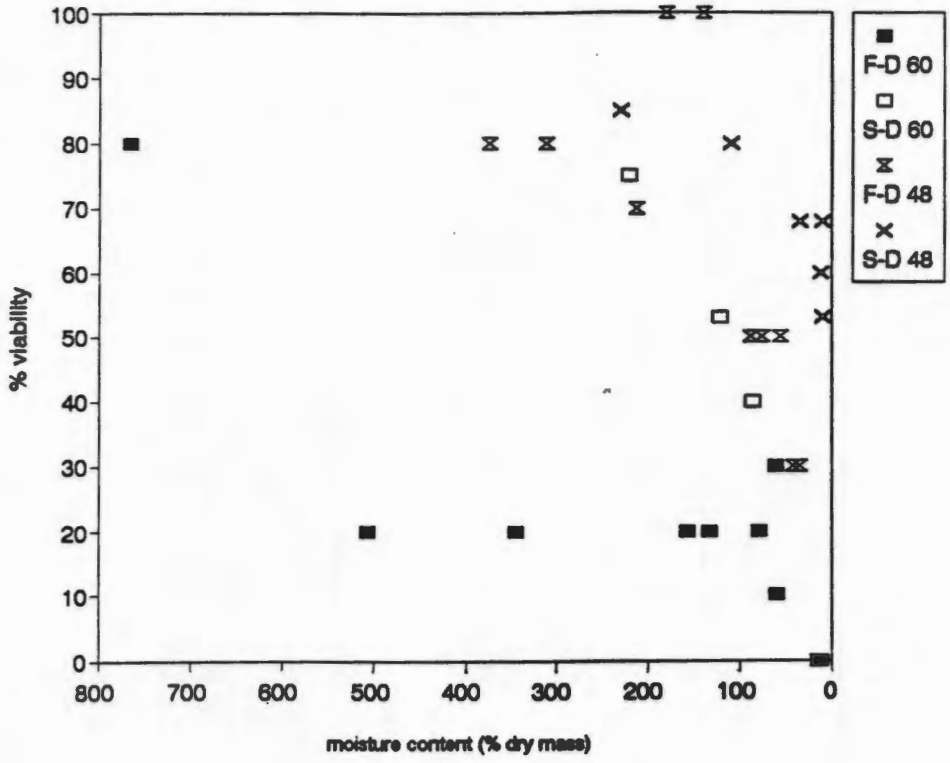

Fig. 3. Effect of rate of drying on the viability of seeds and axes of *Pisum sativum* which have been imbibed for 48 and 60 hours. F-D, fast-dried; S-D, slow-dried.

Role of proteins.

After the points during germination at which pea seeds lost tolerance were defined, the relationship of desiccation tolerance to the SDS gel electrophoretic profile of heat stable proteins of pea axes and cotyledons was examined. Representative SDS-PAGE preparations of heat stable proteins from slowly dried and fast dried axes (A) and cotyledons (C) are shown in Figures 4 to 8. Separations of control (unheated) proteins are given in the appendix II (Figures 9 to 12) and show that no breakdown of proteins from the heating process occurred.

Figure 4 is a control gel showing separation of non-dried proteins from axes and cotyledons imbibed for 12 and 60 hours. On drying (slow) of the axes, heat stable proteins became more intense relative to the non dried controls (compare for example Fig. 5 L (=lane) 1 , which was 12 hours imbibed and dried, with Fig. 4 L1, 12 hours imbibed undried axes).

Upon drying some protein bands seem to have disappeared, although it is not clear whether they completely disappeared or were present at low concentration (compare Fig. 4 and 5). However, major protein bands, indicated with arrows, ranging in molecular mass approximately between 28 and 320 kDa, were present in both the dried and non dried samples. The decrease or even lack of some of the proteins in the dried sample suggest that these cannot be crucial for survival, since after 12 hours imbibition drying did not lower viability (Fig. 1).

After rehydration of the 12 hour imbibed and dried axes there was no change in the pattern of protein present nor in their intensity (compare L1 and L2, Fig. 5).

By 60 hours imbibition, heat stable proteins have almost disappeared in the non-dried sample, whereas on drying the heat stable proteins show up stronger (compare Fig. 4 L2 and Fig. 5 L3), possibly as a response to the water deficit. Although protein bands were present (Fig. 5, L3), they were fainter than in the 12 hour imbibition treatment. [The arrows on the photograph indicate the presence of faint bands on the gel, which did not come out on the photograph].

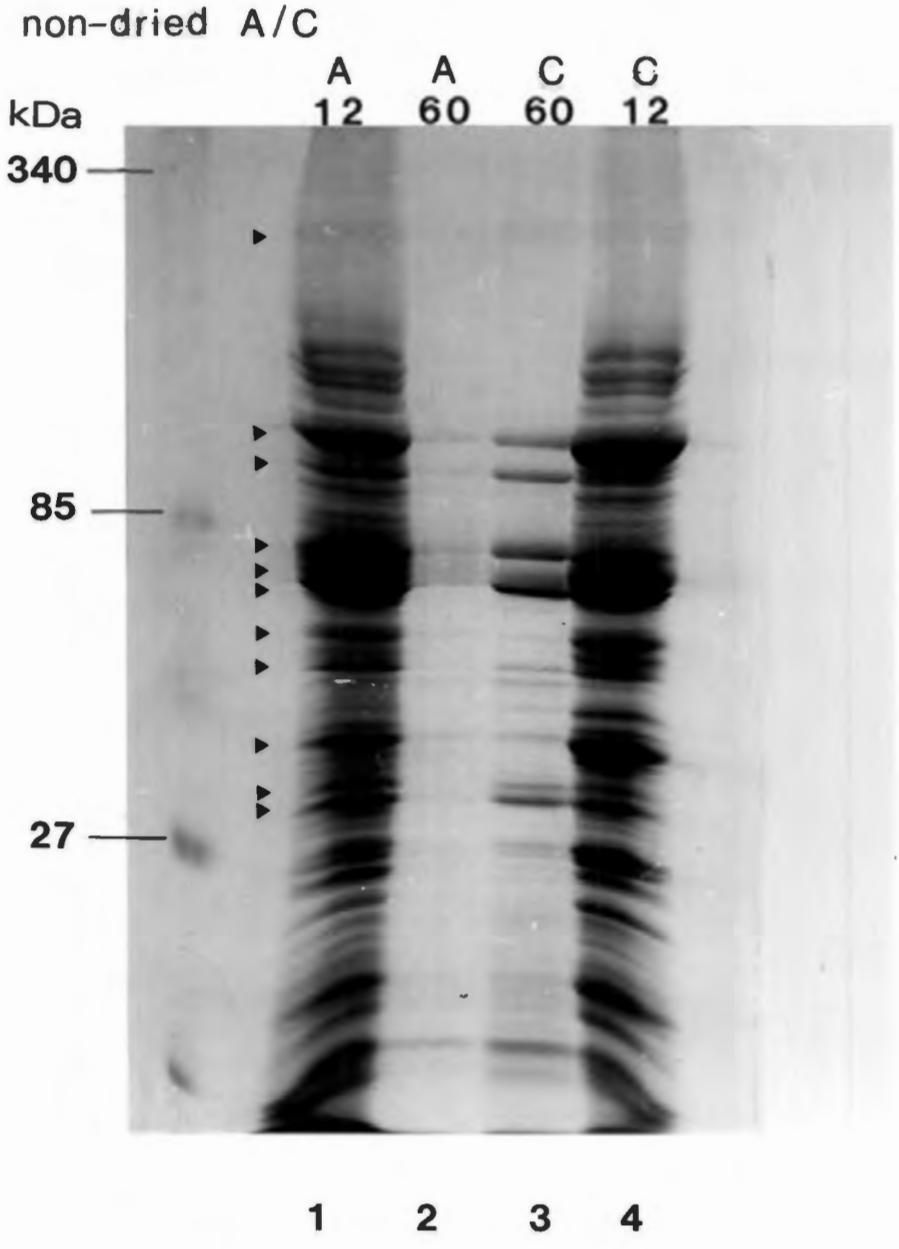

Fig. 4. Separation of heat stable proteins of non-dried axes (A) and cotyledons (C) of *Pisum sativum*, imbibed for 12 and 60 hours. Axes imbibed for 12 hours (lane 1) and 60 hours (lane 2); cotyledons imbibed for 60 hours (lane 3) and 12 hours (lane 4). Molecular masses of markers are indicated (left-hand margins). Major protein bands which are present in both the non-dried and dried samples are indicated with an arrow.

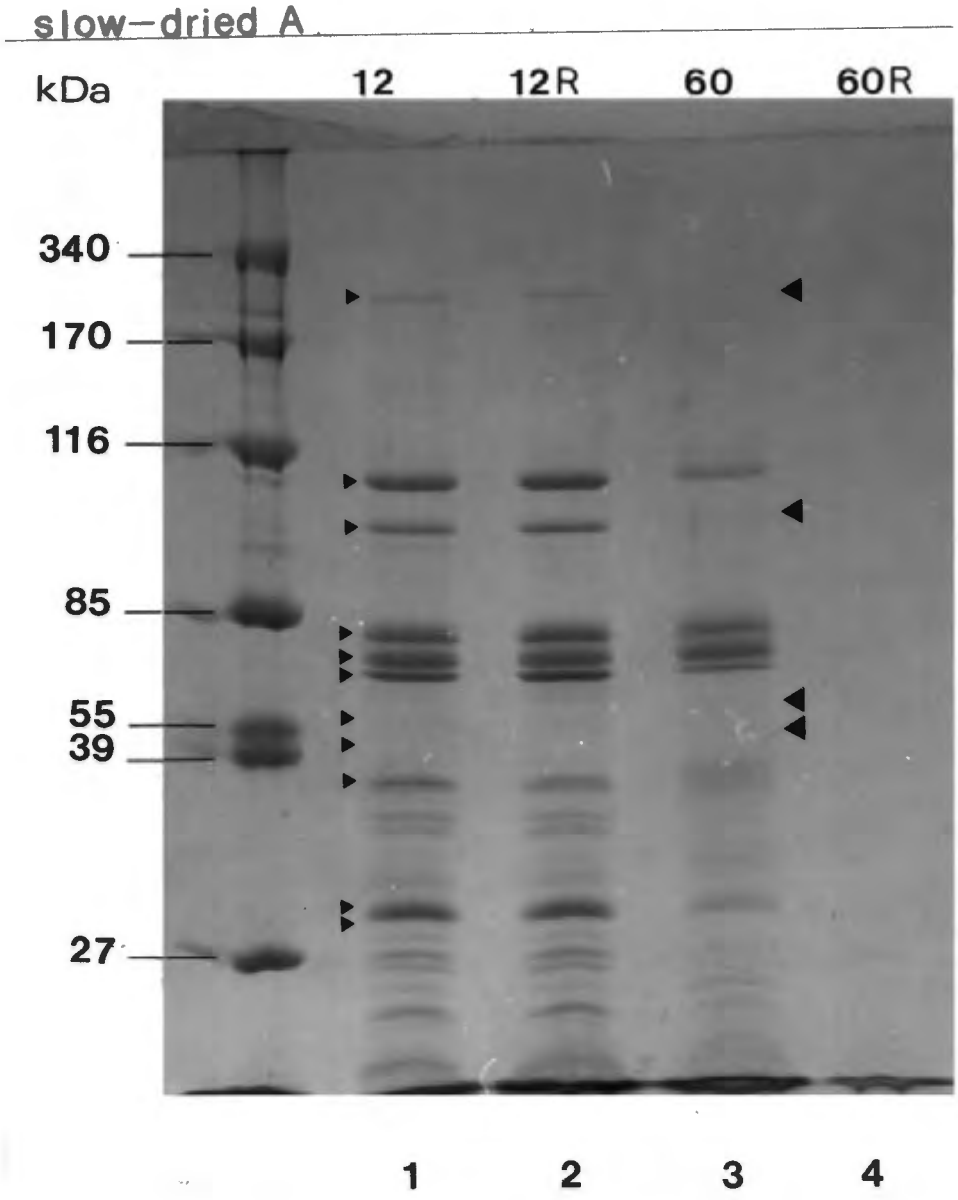

Fig. 5. Separation of heat stable proteins of slow-dried and rehydrated (R) axes (A) of *Pisum sativum*, imbibed for 12 and 60 hours. Dried axes imbibed for 12 hours (lane 1) and rehydrated (lane 2); dried axes imbibed for 60 hours (lane 3, arrows indicate bands that were visible on the gel, but which did not come out on the photograph) and rehydrated (lane 4). Molecular masses of markers are indicated (left-hand margins). Major protein bands which are present in both the non-dried and dried samples are indicated with an arrow.

There was no difference in the protein profile or apparent concentration in the 12 hour imbibed samples which had been either slow or fast dried (compare Fig. 5, 6). Proteins indicated with an arrow were present in both samples. Also by 60 hours imbibition, where tolerance to desiccation has been lost (Fig.1, 2), slow or fast dried seeds showed no differences in proteins (Fig. 7A L1 and L3).

On rehydration of the 60 hour imbibed and dried samples, the protein concentration was too low to be detected (Fig. 7A L2 and 4). To confirm the absence or presence of these proteins another extraction was performed in which proteins were concentrated (see materials and methods). This showed that proteins were still present, but the concentration was so low that heat stable proteins identified in the previous gels did not show up (Fig. 7B). This is possibly due to too much background protein, i.e. the heat stable proteins were not separated well enough from the coagulated non-heat stable proteins. The low concentration or even loss of some of these proteins was coincident with the loss of tolerance (Fig. 1, 2; Table 2).

The cotyledons had large quantities of heat stable proteins (Fig. 4 L4). Drying and rehydration after 12 hours imbibition did not reduce the amount of these proteins (Fig. 8 L1).
By 60 hours imbibition protein content of the cotyledons has decreased considerably (Fig. 4, L3). This suggests that these proteins are probably storage proteins, which were used in the germination process. Upon drying (Fig. 8 L3) and rehydration (Fig. 8 L4) the bands appeared fainter, suggesting further

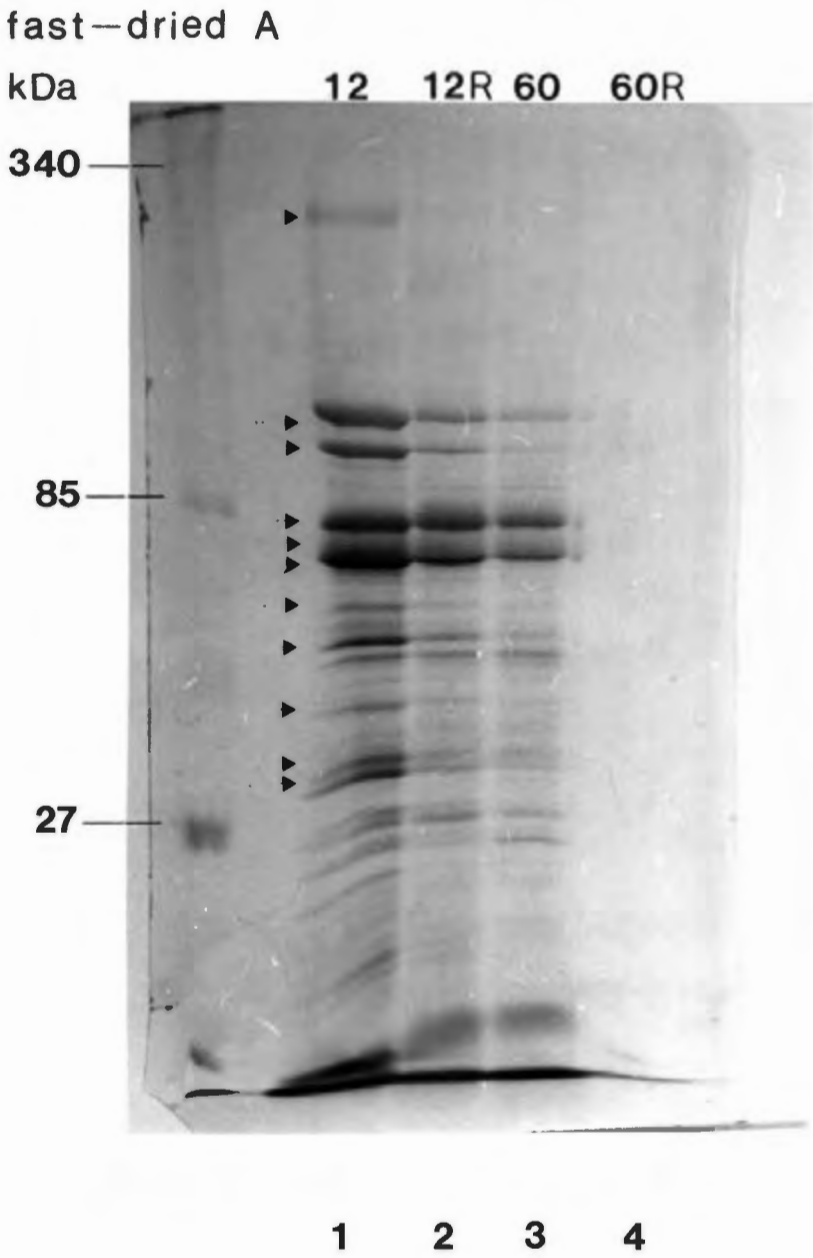

Fig. 6. Separation of heat stable proteins of fast-dried and rehydrated (R) axes (A) of *Pisum sativum*, imbibed for 12 and 60 hours. Dried axes imbibed for 12 hours (lane 1) and rehydrated (lane 2); dried axes imbibed for 60 hours (lane 3) and rehydrated (lane 4). Molecular masses of markers are indicated (left-hand margins). Major protein bands which are present in both the fast and slow dried samples are indicated with an arrow.

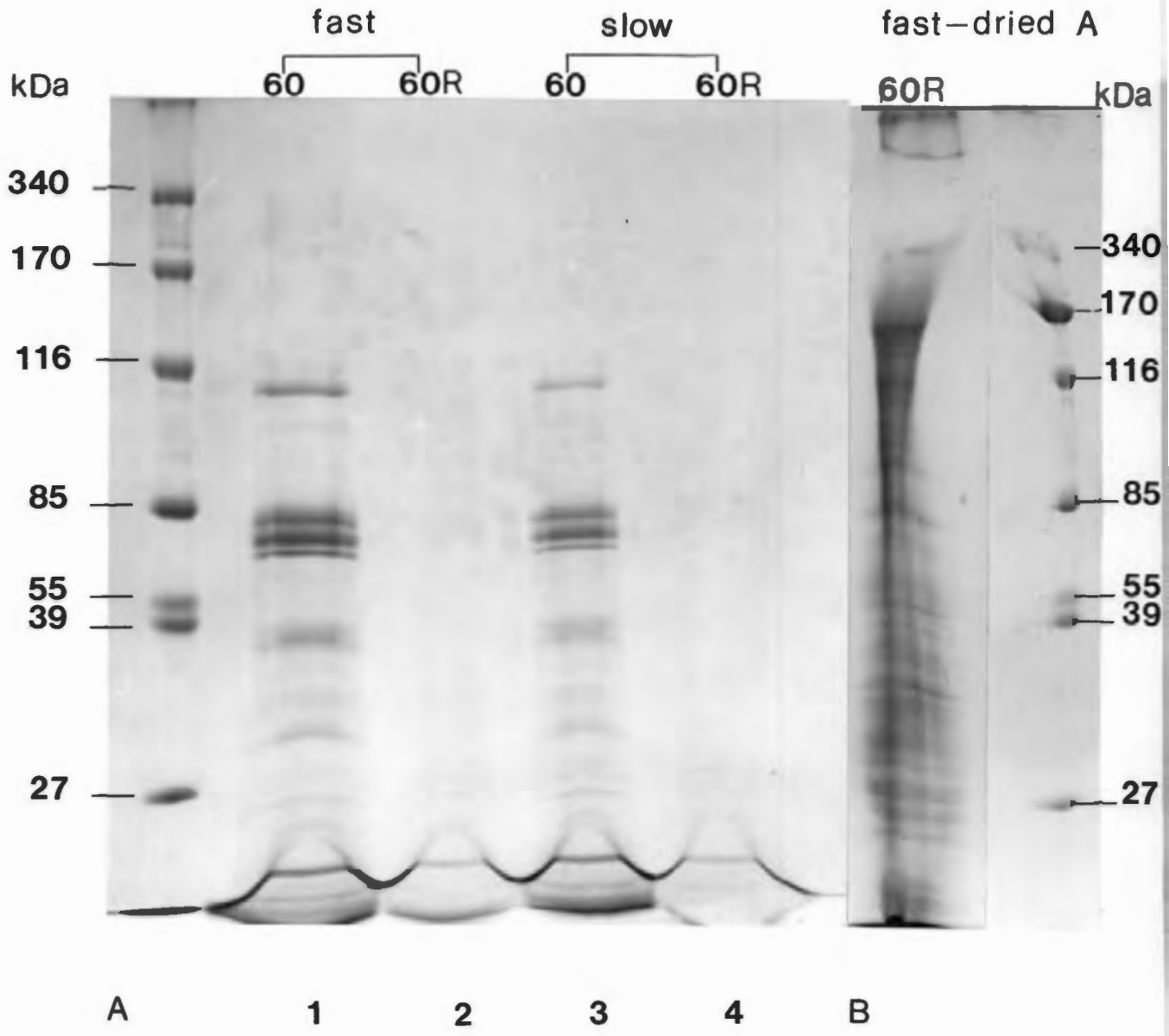

Fig. 7. A. Comparison between the separation of heat stable proteins and the rate of drying of axes (A) of *Pisum sativum*, imbibed for 60 hours. Fast-dried (lane 1), fast-dried & rehydrated (R) (lane 2), slow-dried (lane 3), slow-dried & rehydrated (lane 4).

B. Separation of concentrated heat stable proteins of fast-dried & rehydrated axes of *Pisum sativum*. Molecular masses of markers are indicated.

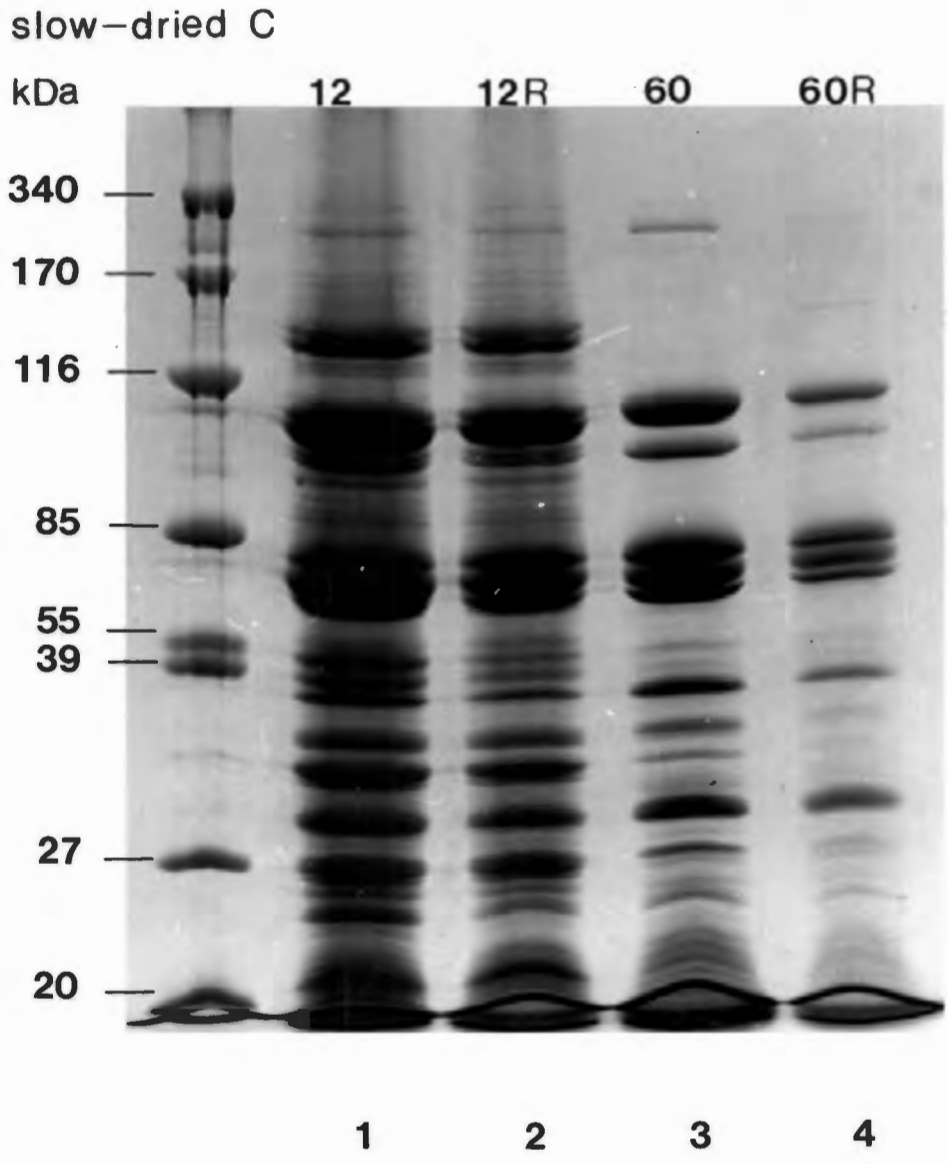

Fig. 8. Separation of heat stable proteins of slow-dried and rehydrated (R) cotyledons (C) of *Pisum sativum*, imbibed for 12 and 60 hours. Dried cotyledons imbibed for 12 hours (lane 1) and rehydrated (lane 2); dried cotyledons imbibed for 60 hours (lane 3) and rehydrated (lane 4). Molecular masses of markers are indicated (left-hand margins).

breakdown for use in the germination or even repair.

With the Western blot technique it was intended to confirm whether any of these heat stable proteins had dehydrin-like characteristics. The antibody used was, however, not purified sufficiently to detect the presence of dehydrins.

DISCUSSION

The so-called maturation or LEA proteins which accumulate during the final stages of seed maturation have been suggested to be important in the development of desiccation tolerance (Baker *et al.*, 1988; Dure *et al.* 1989) and may also play a role in the protection against desiccation damage (Dure *et al.*, 1989; Kermode, 1990).

The experiments reported on here investigated the relationship between desiccation characteristics and the presence/absence of heat stable proteins in seeds and axes of *Pisum sativum* (pea). Up to the first 12 hours from the start of imbibition, which was also the time at which elongation of the axis occurred (Table 1), the desiccation-rehydration treatment was without any deleterious effect (Fig.1, 2). Thereafter, however, the recovery of the dried seeds and axes declined and was lost after 60 hours imbibition when dehydrated to the original moisture content (Fig. 1, 2; Table 2). Dasgupta *et al.* (1982) found a similar phenomenon in bean seeds, which lost viability after 12 hours imbibition, which was also the time at which elongation of the axes occurred.

The protein studies showed that heat stable proteins in the non dried axes, imbibed for 12 hours, almost disappeared after 60 hours imbibition (Fig. 4 L1 and L2). These characteristics, i.e. heat stability, their increase towards the end of maturation and degradation during germination, suggests that these are LEA-like proteins (Dure *et al.*, 1989; Kermode, 1990; Bewley and Oliver, 1991). However, because Western blots did not work, it was not

possible to confirm that these were dehydrins. Thus they will be referred to only as LEA-like or heat stable proteins.

In the 12 hours imbibed seeds, drying reduced the amount of some LEA-like proteins in the axes, although major proteins (indicated with an arrow) were present in both samples (compare Fig. 4 L1 and Fig. 5 L1). There appeared to be no difference in concentration between the proteins that were present in the dried axes and those in the non dried axes. If there were a difference, the methods used in the present study were not sensitive enough to detect them. In addition, it is also not clear whether those proteins which were no longer visible on the gel had vanished completely or were only reduced in their concentration. In either case, these proteins cannot be critical for survival, since at 12 hours imbibition drying does not lower desiccation tolerance (Fig. 1).

By 60 hours imbibition the heat stable proteins had almost disappeared in the non dried axes (Fig. 4 L2). However, they seemed to be more concentrated on drying (Fig. 5 L3), but were in lower concentrations than in the dried axes imbibed for 12 hours (compare Fig. 5 L1 and L3). The re-appearance of these proteins seems to confirm earlier views that there is a correlation between the response to drying and the presence of dehydrins (e.g. Blackman *et al.*, 1991). However, this proposition is now somewhat uncertain, since it was shown for barley seedlings that certain dehydrins are present in some tissues whether or not the seedlings were stressed (Close *et al.*, 1993). The uncertainty of the role of these dehydrins is intensified by the fact that the presence (although protein bands

were much fainter than for the 12 hours imbibition treatment) of these LEA-like proteins after 60 hours imbibition did not confer desiccation tolerance (Fig. 1).

There are several possible explanations for this observation: If proteins are involved in desiccation tolerance, the concentrations of a particular dehydrin(s) may not be sufficient for protection (Dure, 1993; Finch-Savage *et al.*,1994; Vertucci and Farrant, 1994). This hypothesis may lend support to Dure (1993), who deduced from the molecular structure of a dehydrin its possible role as an ion carrier. Such a capacity for ion sequestration is meaningful in the cell only if sufficient quantities are present (Dure 1993). High quantities of this protein were shown to be present in dehydrated cotton embryos. In the case of the pea, further study is clearly needed to reveal whether the concentration of any of the dehydrins is important in conferring protection for desiccation.

It has been suggested by a number of authors that the presence of dehydrins alone is insufficient to confer desiccation tolerance (Blackman *et al.*, 1992; Finch-Savage *et al.*, 1994; Vertucci and Farrant, 1994). Blackman *et al.* (1991) showed that desiccation tolerance in seeds of soybean was induced when seeds underwent a slow-drying regime but not when they were held at high humidity, despite the presence of high levels of maturation proteins in both samples. Their inability to confer tolerance independently requires one to look for alternative explanations (Blackman *et al.*, 1991).

Vertucci and Farrant (1994) suggest that as seeds develop they become increasingly tolerant of drying because they progressively acquire certain molecules (such as sugars and LEA proteins) and

alter their physical characteristics (vacuolation is reduced and the amount of structured/glass-like water is increased). All characteristics need to be present for a seed to be entirely tolerant of drying. Lack of one or more of these characteristics (or lack of the required "degree" of a characteristic) leads to greater sensitivity to drying. On germination these characteristics progressively disappear, and so desiccation tolerance is progressively lost. These suggestions have been confirmed by Finch-Savage *et al.*(1994), who have shown that desiccation intolerant seeds of the temperate trees *Quercus robur*, *Aesculus hippocastanum* and *Acer saccharinum* have some dehydrin homologues. Thus the presence of these alone is insufficient to confer tolerance.

In the present study, after 60 hours of germination the axes had reached quite far along the germination pathway (Table 1) and by this stage some utilization of stored protein must have occurred. This was also suggested by decreased protein levels in the cotyledons (Fig. 4 compare L3 and L4; Fig. 8 compare L1 and L3). Thus increased vacuolation must have occurred, which could have added to the increased sensitivity to drying.

The presence of LEA-like proteins in temperate and some tropical/subtropical species such as *Camellia sinensis* and *Castanospermum australe* (Farrant *et al.* in press) also raises the question whether the LEA-like proteins found in these species are different to those found in orthodox species. It was demonstrated by Close *et al.* (1993) that each of the barley cultivars that they investigated, contained a unique dehydrin

pattern (defined by SDS-PAGE and immunoblotting). It is unknown whether some dehydrins are more efficient than others and this may be interesting to investigate in future studies.
It has also been discovered that proteins with similar physical characteristics to the dehydrins play a role in cold stress (Neven *et al.*, 1993). These facts suggest that such proteins function in response to stresses other than just dehydration.
It is evident that screening for the presence of LEA-like proteins alone, attempted in the present study with SDS-PAGE protein separation and the Western blot technique, is insufficient to make any statements about how desiccation tolerant a species is (Finch-Savage *et al.*, 1994; Farrant *et al.*, in press).

Whereas much attention has been paid to the role of dehydrins or LEA-like proteins as protectors (Close *et al.*, 1989; Dure *et al.*, 1989) and/or as stabilizers of the subcellular structures in the dry state (Lane 1991; Dure 1993), far less attention has been paid to what role they may play on rehydration or even in repair. In the present study protein profiles for the dried and dried & rehydrated state were compared (Fig. 5, 6, 7). After 12 hours imbibition (Fig. 5) no difference in protein concentration between the dried and the dried & rehydrated axes could be detected. The present study may therefore support earlier views such as Kermode's (1990), who suggested that dehydrins may function in preventing disruption of tissues on rehydration. If there are differences between dried and dried & rehydrated axes, the methods used here were not sensitive enough to detect them.

After 60 hrs imbibition (Fig. 7), LEA-like proteins were barely detectable and only when the protein sample had been concentrated (Fig. 7B) were they visible. However, in the dried state protein bands are still clearly visible. The present results therefore suggest that LEAs are not resynthesized in late germination. Therefore, whatever their role, they cannot perform it after a certain stage in germination.

It has to be pointed out that here only desiccation tolerance and its loss were investigated, which may be of limited informational value. Therefore the above hypothesis needs to be verified with a more sensitive method, investigating particularly those points during germination where embryos have lost their desiccation tolerance but are tolerant of some water loss. A suitable way to achieve this would be with radioactive labels added to a growth medium on which rehydrating embryos are placed. If dehydrins are involved in repair, newly made LEAs should be detected in the axes.

To investigate further the relationship between desiccation tolerance and the presence/absence of heat stable proteins, seeds and axes of *Pisum sativum* were dried at different rates. Previous studies of desiccation sensitive seeds have shown that rapid drying of isolated axes allowed drying to much lower moisture contents than did slow drying of whole seeds (Pammenter *et al.*, 1991). This has been attributed to the possibility that rapid drying of such sensitive tissues prevented subcellular damage accumulating to lethal levels (Pammenter *et al.*, 1991). In contrast it was shown in the present study that orthodox seeds, once they have lost their desiccation tolerance, show a

greater number of survivors when slow-dried (Fig. 3). This demonstrates that desiccation sensitive tissue of orthodox seeds functions inherently differently to tissue of recalcitrant seeds. Therefore orthodox seed tissue, which has lost its tolerance to desiccation, cannot be used as a model for studying the genetics and physiology of recalcitrant seeds or should at least be treated with some caution in the interpretation of the results.

The greater rate of survival of the slowly dried seeds may be attributed to the fact that during slow drying LEA-like proteins have time to accumulate, which is not possible in the fast dried axes. In the present study, however, only two points, i.e. the point of tolerance (12 hours imbibition) and the point where tolerance was lost (60 hours imbibition, at original moisture contents) were correlated with the protein profiles. In order to test the above hypothesis it is necessary to look at protein synthesis during the progressive drying and rehydration of tolerant (12 hours imbibition) and sensitive (60 hours imbibition) samples. Since also by the same imbibition treatment viability appears to depend on the moisture content (Table 2), different stages in the drying process should be investigated. If LEA-like proteins are involved in the protection of subcellular structures, protein levels should be higher in the slowly dried sample.

CONCLUSION

In the present study the relationship between desiccation characteristics and the presence/absence of LEA-like proteins was investigated for the orthodox seeds and seed embryos of *Pisum sativum* (pea).

Loss of tolerance to desiccation coincided with axis elongation after 12 hours of imbibition. Imbibition for longer periods resulted in reduced viability upon redrying and viability was completely lost after 60 hours of imbibition.

The nature of the proteins, i.e. heat stability, the accumulation towards the end of pre-shedding development and the decline in concentration with the progress of germination suggest them to be LEA-like proteins.

No difference in protein concentration could be detected between dried and non dried axes as long as tolerance to desiccation was undiminished. However, heat stable proteins seemed to be more concentrated when drying occurred in tissue that was sensitve to desiccation, i.e. after 60 hours of imbibition. It was tentatively suggested that this may be a response to the drying treatment, although viability had been lost completely at that stage. It is possible that concentrations of some of the heat stable proteins were too low for sufficient protection (Dure 1993). Rehydration resulted in loss of these proteins, suggesting their breakdown during rehydration. This inability to replace them may be related to their increased sensitivity. Another possibility is that the presence of heat stable proteins alone is insufficient to provide protection and that other

mechanisms have to be involved. These findings are in accordance with studies by Farrant and Vertucci (in press) and Finch-Savage *et al.*(1994). In the present study it appears likely that extensive vacuolation, confirmed in the cotyledon studies, was a factor leading to the complete loss of desiccation tolerance.

Slow drying of whole seeds showed that viability retention was higher than when seeds were dried quickly. This result was found to be the opposite of that known in recalcitrant seeds (Pammenter *et al.*, 1991). Consequently it is suggested that the interpretation of studies using sensitive tissue of orthodox seeds as a model for truly recalcitrant tissue, should be treated with some caution.

It has been suggested that if heat stable proteins play a role in protection, the accumulation of heat stable proteins may only (or to a large extent) be possible in the slow-dried axes. In contrast, in the fast-dried axes time is too short for this to occur. The present study could not provide an answer to this question, since only proteins of seeds dried to original moisture contents at tolerant (12 hours imbibition) and intolerant (60 hours imbibition) were investigated. Future studies should therefore focus on proteins synthesized during drying and rehydration of variously imbibed axes.

APPENDIX I.

Murashige and Skoog medium.

100 ml of stock 1 (16.5g/L NH_4NO_3, 19.0g/L KNO_3); 10 ml of stock 2 (37g/L $MgSO_4.7H_2O$, 2.23g/L $MnSO_4.4H_2O$; 0.86g/L $ZnSO_4.7H_2O$, 0.0025g/L $CuSO_4.5H_2O$); 10 ml of stock 3 (33.3g/L CaCl, 0.083g/L KI, 0.0013g/L CoCl, 0.0025g/L $Na_2.MoO_4.2H_2O$); 10 ml of stock 4 (17g/L KH_2PO_4, 0.62g/L H_3BO_3); 100 ml of stock 5 (0.2785g/L $FeSO_4.7H_2O$, 0.3725g/L Na_2EDTA); 10 ml of stock 6 (10g/L Inositol, 0.2g/L Glycine, 0.05 Nicotinic Acid, 0.05g/L Pyridoxine HCL, 0.01g/L Thiamine HCL); 5% Sucrose; 0.1% Caseinhydrolysate; and 0.8g Agar.

APPENDIX II.

SDS-PAGE analysis of non-heated controls.

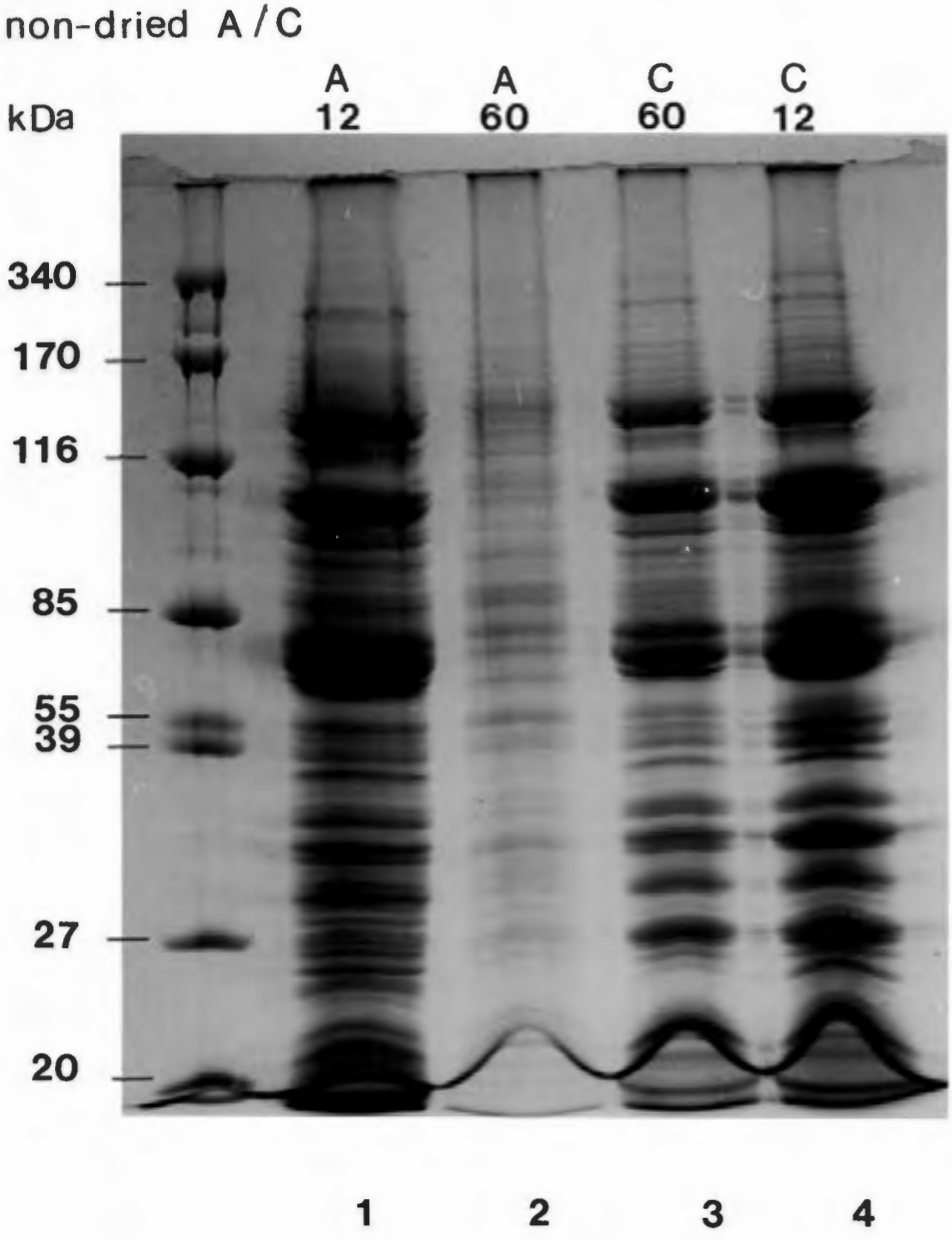

Fig. 9. Separation of control (unheated) proteins of non-dried axes (A) and cotyledons (C) of *Pisum sativum*, imbibed for 12 and 60 hours. Axes imbibed for 12 hours (lane 1) and 60 hours (lane 2); cotyledons imbibed for 60 hours (lane 3) and 12 hours (lane 4). Molecular masses of markers are indicated (left-hand margins).

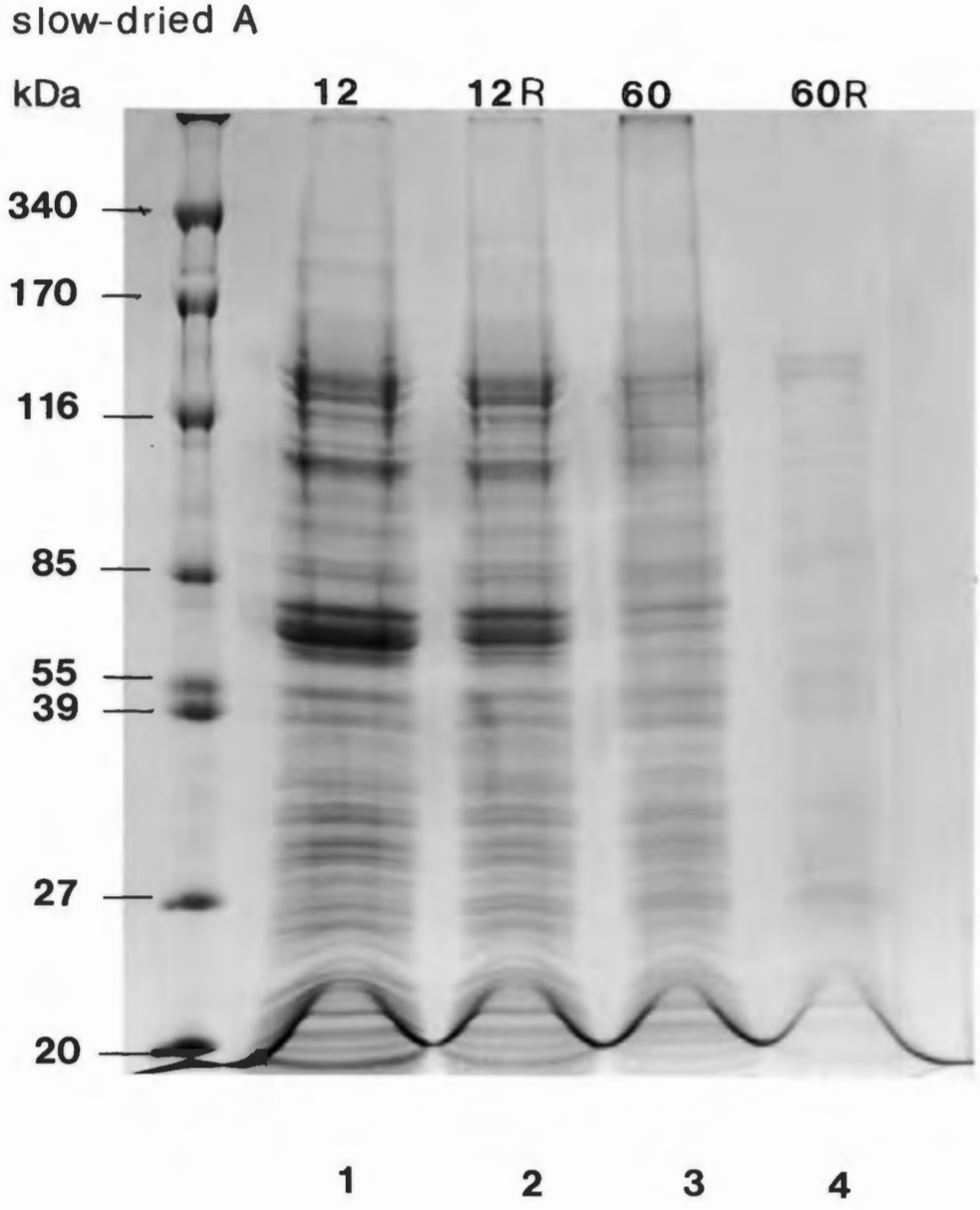

Fig. 10. Separation of control (unheated) proteins of slow-dried and rehydrated (R) axes (A) of *Pisum sativum*, imbibed for 12 and 60 hours. Dried axes imbibed for 12 hours (lane 1) and rehydrated (lane 2); dried axes imbibed for 60 hours (lane 3) and rehydrated (lane 4). Molecular masses of markers are indicated (left-hand margins).

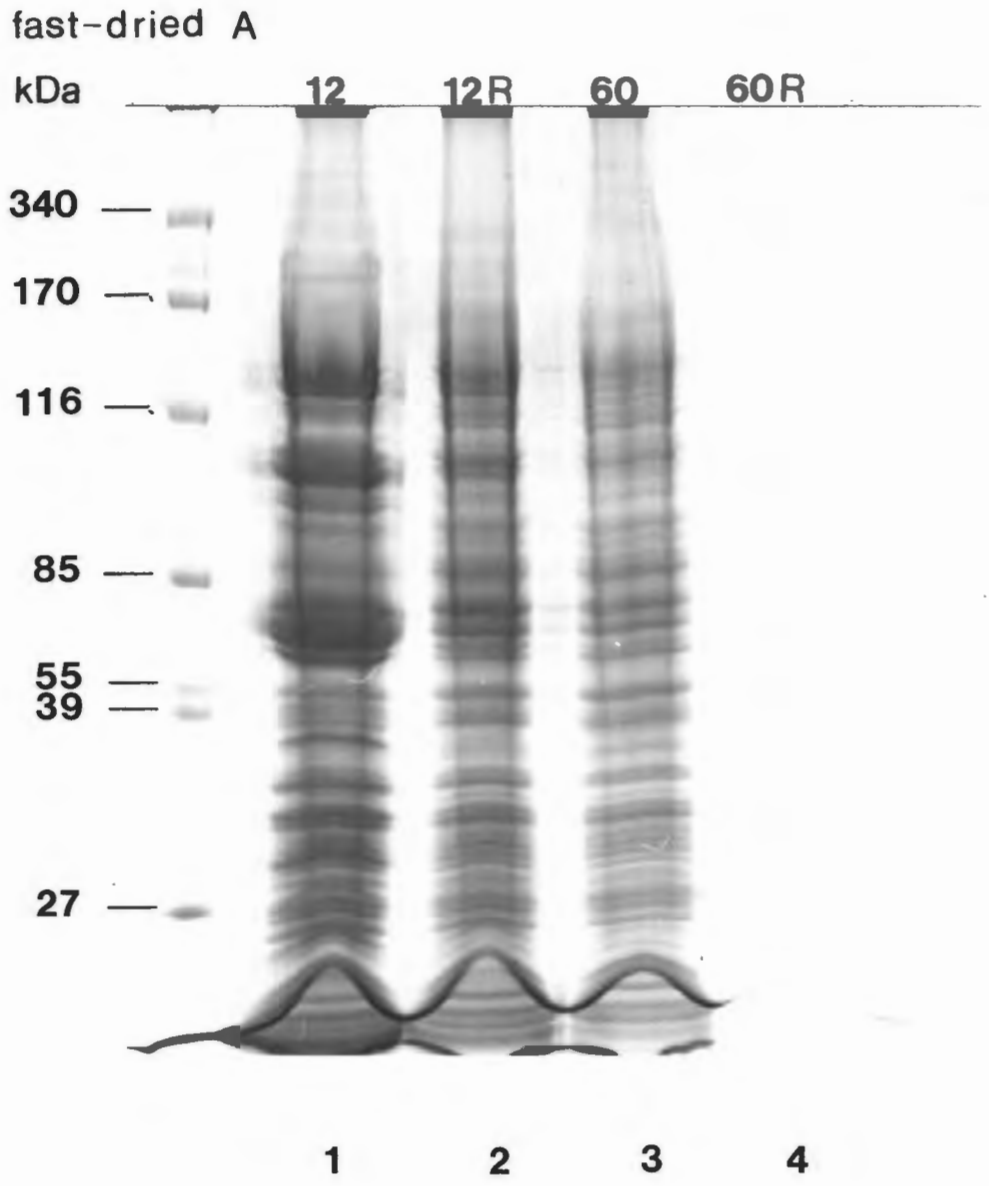

Fig. 11. Separation of control (unheated) proteins of fast-dried and rehydrated (R) axes (A) of *Pisum sativum*, imbibed for 12 and 60 hours. Dried axes imbibed for 12 hours (lane 1) and rehydrated (lane 2); dried axes imbibed for 60 hours (lane 3) and rehydrated (lane 4). Molecular masses of markers are indicated (left-hand margins).

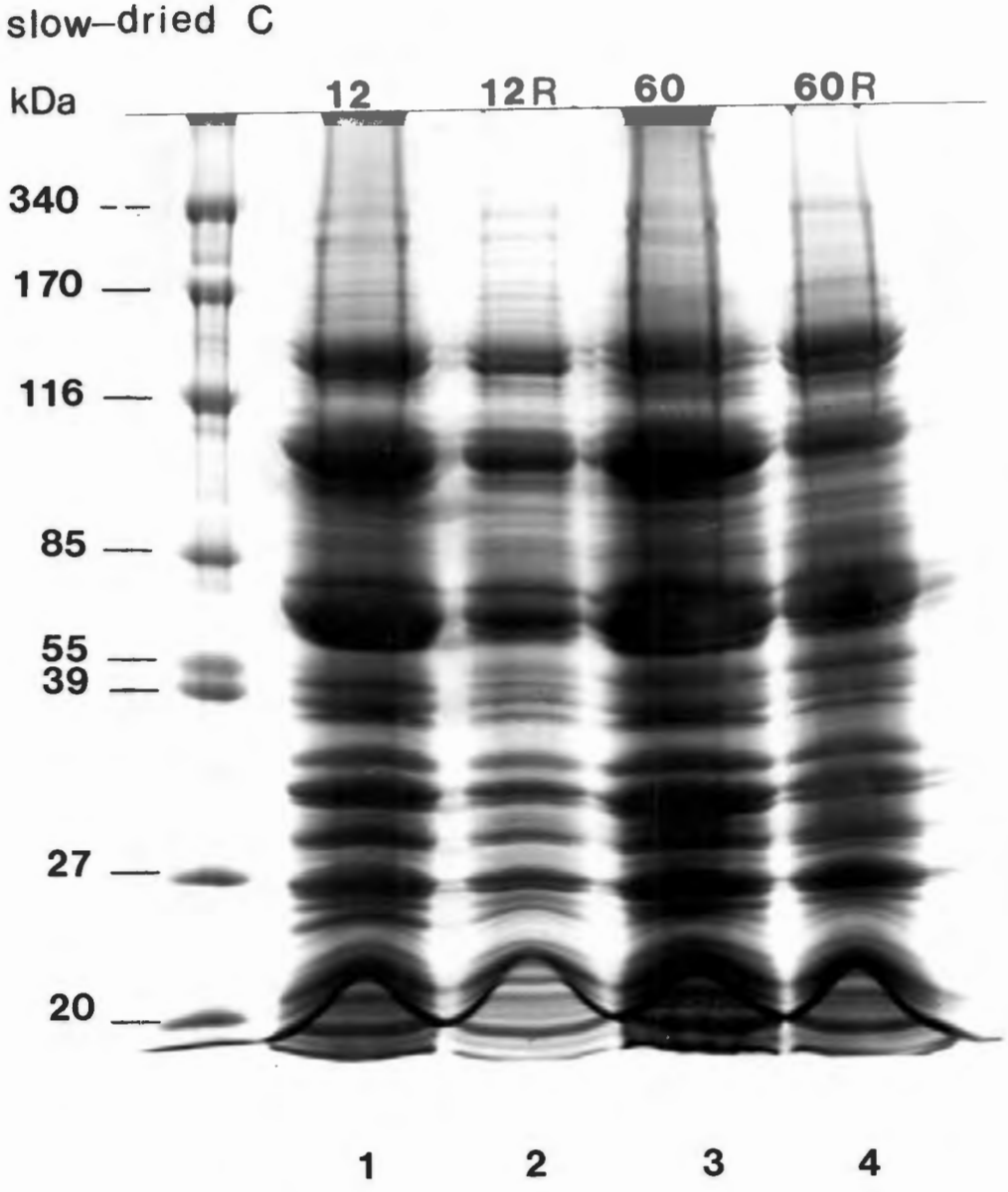

Fig. 12. Separation of control (unheated) proteins of slow-dried and rehydrated (R) cotyledons (C) of *Pisum sativum*, imbibed for 12 and 60 hours. Dried cotyledons imbibed for 12 hours (lane 1) and rehydrated (lane 2); dried cotyledons imbibed for 60 hours (lane 3) and rehydrated (lane 4). Molecular masses of markers are indicated (left-hand margins).

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