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DEGRADATION OF STORAGE PROTEINS IN GERMINATING SEEDS

ANDREI D. SHUTOV and IOSIF A. VAINTRAUB

Laboratory of Protein Chemistry, V. I. Lenin State University of Kishinev, Kishinev 277003, U.S.S.R.

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Key Word Index—Angiospermae; storage proteins; proteolytic enzymes; seed germination; proteolysis.

Abstract—The criteria for the participation of proteases in the mobilization of storage proteins during seed germination are formulated. The proteases that satisfy these criteria, namely the acid cysteine endopeptidases, serine carboxypeptidases and neutral aminopeptidases, are reviewed. The possibility of other seed proteases participating in storage protein degradation is discussed. The course of 11S and 7S storage protein degradation through the action of endogenous and exogenous proteases is described. The 11S and 7S proteins are modified during the early stages of proteolysis and the effects of these modifications on the regulation of breakdown are examined. A scheme for 11S protein degradation in germinating seeds is presented.

INTRODUCTION

The degradation of storage proteins during seed germination has been studied for a long time, the starting points being the establishment of protein degradation and the detection of proteolytic activity in seeds. Since then, physiological studies of protein degradation proper have provided the temporal pattern, histochemical and cytological characteristics of the process which leads eventually to free amino acids [1, 2]. Until recently, enzymic aspects of storage protein degradation remained unclear since almost all studies dealt with proteolytic enzymes in seeds irrespective of function [1, 3–5]. These investigations, however, revealed a multiplicity of seed proteases and the existence of a system of proteases responsible for protein degradation.

Significant advances in the subject were brought about during the last decade by the use of new approaches. For each enzyme, it is necessary to establish its particular contribution to protein degradation and the natural substrates need to be studied *in vitro*. The realization of such approaches obviously requires purification of seed proteases, or at least their separation from each other. These achievements have been discussed in several recent reviews [2, 6–9]. In this review an attempt is made to systematize the available data on the proteases participating in protein breakdown and to provide a generalized scheme for storage protein degradation in germinating seeds.

SOME GENERAL OBSERVATIONS ON THE PROCESS OF STORAGE PROTEIN DEGRADATION IN GERMINATING SEEDS

The seeds of the majority of dicotyledons and of some monocotyledons comprise in the main legumin-like 11S proteins and in some cases vicilin-like 7S proteins [10]. The 11S and 7S proteins are localized in protein bodies. The main stages of their degradation take place within protein bodies and vacuoles that are formed after their fusion in germinating seeds [1, 11–13]. The internal pH of these organelles is slightly acid. In castor beans (*Ricinus communis*), e.g., the pH value within protein vacuoles is 5.7–5.9 [14]. Thus, these pH values are characteristic of the main steps of 11S and 7S protein degradation.

Unlike other flowering plants, in most of the cereals the typical storage proteins are prolamins and glutelins located in the starchy endosperm [15]. According to Mikola, in germinated barley (*Hordeum vulgare*) grains the starchy endosperm is converted into a cell-free formation devoid of any compartmentation with pH values close to 5 [9, 16].

At the onset of germination quantitative and qualitative changes in the proteins cannot be observed for some time, even in a completely imbibed seed [2, 4, 7, 17]. Breakdown starts non-uniformly in different parts of the reserve tissue at an increasing rate [2, 4, 6, 7]. Thus, the onset of degradation is not directly related to water uptake but is determined by other subsequent processes. This conclusion is in line with the evidence indicating the role of embryo axes in protein breakdown [2, 7] and the lack of autolytic function in protein bodies of ungerminated seeds [2, 4, 7, 12, 18–20].

During seed germination 11S and 7S proteins undergo qualitative changes (modification) [7, 21]. These changes occur at the early stages of germination and then further progress. The process of modification will be dealt with in detail below. The physiological effects comprise a greater solubility [22–25] and susceptibility to the action of some endogenous proteases which are unable to hydrolyse protein of ungerminated seeds [21, 26, 27].

ENZYMES RESPONSIBLE FOR STORAGE PROTEIN MOBILIZATION

Apart from proteases responsible for storage protein hydrolysis, seeds evidently contain proteolytic enzymes performing other functions. Thus, the participation of a

protease in storage protein mobilization must be proved. Only the proteases whose participation in protein degradation is more or less well established, as well as proteases from other seeds, which are clearly homologous, will be considered here.

The proteases that make up the enzymatic machinery of storage protein degradation must meet the following criteria:

1. Ability to hydrolyse the storage protein of resting seeds or any of the products of their degradation during germination (from high molecular weight products to dipeptides).
2. Lack of temporal or spatial hindrance to the action of protease on its expected natural substrate, i.e. location of the enzyme and the substrate in the same cell compartment.
3. Ability to be active in the milieu of the corresponding cell compartment (pH, redox potential, absence or low level of inhibitory activity).

If the compartmentation in the storage tissue disappears during germination [9, 16], this must be taken into consideration. High activity and an increase in activity during seed germination may also indicate with a high probability the participation of a protease in the degradative process, but these criteria are not always met.

It is not necessary to insist that all three criteria are met to prove that a protease takes part in seed protein degradation, since they are interlinked. Thus, the presence of a protease in the protein bodies of germinating seeds is strong evidence of its participation in the hydrolysis of at least intermediate peptides in these organelles. Its pH optimum in a slightly acid range is also characteristic and indicates that it is probably localized in the protein bodies and therefore takes part in the mobilization of the seed protein.

Proteinases participating in the initial stages of degradation are of greatest interest for the elucidation of the mechanism of this process. Their substrates comprise the proteins of ungerminated seeds and those that have been modified during germination. Both have a specific structure that affects their susceptibility to proteolytic attack. The identification of these proteinases requires the fulfilment of the two first criteria, i.e. it involves the study of proteinase action on the natural substrates.

In the overwhelming majority of relevant studies only the storage proteins of resting seeds were considered to be natural substrates. However, we know that 11S and 7S proteins undergo modification during the early stages of seed germination (see above). Thus, it is only for a very short time that the proteins of dry seeds represent substrates of seed proteinases. It is obvious, therefore, that both proteins from ungerminated seeds and the modified proteins of germinated seeds must be used as natural substrates, but such studies are as yet very few [26, 28–30].

Peptide products of prolonged protein degradation, i.e. the natural substrates of the proteases responsible for the final stages of mobilization process, are devoid of the specific structures peculiar to the proteins themselves. Therefore the identification of such proteases can be based on synthetic substrates.

Proteinases hydrolysing the storage proteins of ungerminated seeds (proteinases A)

The first proteinase to be considered here is mung bean (*Vigna radiata*) cysteine proteinase ('vicilin peptidohydrolase') purified by Baumgartner and Chrispeels [31] as it was found to conform with all the three criteria of participation in seed protein mobilization. Vicilin peptidohydrolase acts on the 7S protein of ungerminated seeds of mung bean and is able to perform extensive hydrolysis [31]. It is absent from the ungerminated seeds and appears no later than on the third day of germination as a result of axis-dependent [32] *de novo* synthesis [33]. The transport mechanism of vicilin peptidohydrolase from the point of synthesis (the rough endoplasmic reticulum) to the protein bodies has been described [34–36].

The pH optimum of vicilin peptidohydrolase (with its own storage protein as substrate) is 5.1 and is close to the pH inside the protein bodies. A specific protein inhibitor of vicilin peptidohydrolase is located outside the protein bodies and therefore cannot inhibit its hydrolytic effects [19]. Low molecular weight sulphydryl compounds and protein disulphide reductases may contribute to the maintenance of the seed cysteine proteinases (including vicilin peptidohydrolase) in the active reduced form. Such reductases were detected in pea (*Pisum sativum*) seeds [37] and wheat (*Triticum aestivum*) grains [38].

The activity of vicilin peptidohydrolase in different parts of the cotyledons develops unevenly in a strict correlation with a simultaneous degradation of the storage proteins of the same cotyledon parts [18]. Thus, there is a spatial and temporal relationship between the appearance of vicilin peptidohydrolase and the onset of protein degradation. These data as well as its ability to act on the storage proteins of ungerminated seeds prove that vicilin peptidohydrolase initiates the mobilization of the proteins in mung beans.

Three other proteinases of this group were purified and studied: proteinases A of vetch (*Vicia sativa*) [28, 39, 40] and wheat [41], as well as corn (*Zea mays*) proteinase P-1a [42–44]. The properties of these enzymes and vicilin peptidohydrolase are similar (Table 1).

All the four proteinases hydrolyse their own storage proteins isolated from ungerminated seeds. They are not found in dry seeds and appear after the onset of germination and their activity increases during the breakdown of storage protein, the exhaustive hydrolysis of which results in the formation of short peptides. Evidently these enzymes have a common function, i.e. they initiate storage protein mobilization and participate in their further hydrolysis.

The similarity of the properties and functions of these proteinases indicates they are homologous. These proteinases are found in the seeds of plants of phylogenetically distant families (Fabaceae and Poaceae) and it is very likely therefore that the enzymes of this type are characteristic of many (possibly of all) flowering plants.

It is reasonable to introduce a common name for these proteinases, as has been done for other groups of related proteinases from animal or microbial sources. The most appropriate name seems to be 'proteinase A' plus the name of the corresponding plant. It reflects to a certain extent the important role these proteinases play in the process of protein mobilization.

A temporal and spatial correlation between the development of an SH-dependent acid endopeptidase and protein degradation in different parts of the cotyledons was shown in germinating seeds of cowpea (*Vigna unguiculata*) [45]. An SH-dependent endopeptidase appears in germinating seeds of castor bean [22]. It is localized in vacuoles formed after the fusion of protein bodies [46] and hydrolyses the 11S protein from un-

Table 1. Some properties of cysteine proteinases A of mung bean (vicilin peptidohydrolase [31]), vetch [39, 40], corn (proteinase P-1a [42–44]) and wheat [41]

Property	Proteinase A			
	Mung bean	Vetch	Corn	Wheat
pH optimum according to enzyme effect on endogenous protein	5.1	4.6	4.5	4.2
Molecular mass	23000	20–25000	21000	22000
Isoelectric point (pH)	3.75	< 5*	2.3	n.d.
Substrate specificity†	n.d.	Glu, Tyr, Leu;[1] Asp[2]	Glu, Tyr, Leu, Ala, Gln;[1] Asp[2]	n.d.
The mean number of residues in the peptides obtained by exhaustive hydrolysis of the SP	3‡	4	n.d.	3

n. d. = not determined.

*Bound by DEAE-cellulose at pH 5 and ionic strength ≤ 0.15.

†Amino acids whose carboxyl groups are involved into the peptide bonds split; 1, proved; 2, possible.

‡Calculated from the hydrolysis curve [31].

germinated seeds with a pH optimum equal to 5.0 [47]. Evidently these proteinases initiate the degradation of storage protein and belong to the proteinase A group. Into the same group can be obviously placed the cysteine endopeptidase with molecular mass 22 000 and isoelectric point below 5 which was partially purified in our laboratory from sunflower (*Helianthus annuus*) seeds. This enzyme appears in germinating seeds and is able to hydrolyse *in vitro* the 11S protein of ungerminated seeds at optimal pH of 5.7. Proteinases which are presumably of the proteinase A type have been reported also in germinating seeds of some other legumes [48–51] and of cereals [52–54].

The synthesis of at least an initial amount of proteinase A probably uses free amino acids present in mature seeds where 1.8–5.6 mg of free amino acids per gram have been reported [55–57]. An approximate estimate based on published data [40, 41] shows that even at the highest level of activity of proteinases A of vetch and wheat their synthesis requires less than 10 μg of amino acids per gram of ungerminated seeds. Considerably smaller amounts of those enzymes are evidently sufficient for the initiation and early stages of protein degradation. Thus, no other amino acid pool is necessary for the synthesis of these proteases which perform the major breakdown of storage protein and of other hydrolases [see 9, 16].

Hydrolysis of storage proteins by proteinases A and by some exogenous proteinases in vitro. *Modification of* 11S *and* 7S *proteins in germinating seeds*

When proteinases A act on native 11S and 7S proteins of ungerminated seeds the high molecular mass residue undergoes some regular transformations. The same or very similar changes have been observed using trypsin and some other exogenous proteinases. This similarity indicates some aspects of 11S and 7S protein structure are significant during their hydrolysis. Thus, the effects of proteinases A and of exogenous proteinases on storage proteins will be described and discussed together.

The following consecutive events occur during the proteolysis of 11S and 7S proteins:

(1) The first step of 11S protein proteolysis consists of a split-off of a limited number (one or two) TCA-soluble peptides. This step results in a sharp increase in the susceptibility of 11S protein to some endogenous proteases, which are not able to hydrolyse the native protein. This was shown in studies of proteinase A [27] and trypsin [21] effects on vetch 11S protein. Similar changes in susceptibility to proteolytic attack were observed during the partial hydrolysis of the 7S protein of kidney beans (*Phaseolus vulgaris*) by trypsin [58].

(2) Further proteolysis of 11S proteins leads to consecutive increase in their negative charge [21, 59–61]. The negative charge of vetch 11S protein hydrolysed by trypsin was shown to increase due to the removal of short basic peptides [27]. The partial proteolysis of 7S proteins will also raise their negative charge [62–64].

(3) After the initial steps of trypsin hydrolysis, solubilization of pumpkin (*Cucurbita moschata*) 11S protein occurs [65], which is evidently caused by the increase of its negative charge. Probably the initial proteolysis of other SP brings about an increase in solubility as well.

(4) In the course of the proteolysis there is a stepwise reduction of the molecular mass of acid subunits of 11S proteins and the formation of the intermediates to be split further to fragments with a molecular mass from 10 000 to 18 000 [60, 61, 65–67]. In all the works cited above, no changes in the molecular mass of basic subunits of 11S proteins were detected.

Proteinase A [31, 48] and trypsin [68, 69] also reduce stepwise the molecular mass of polypeptide chains in 7S proteins.

Ultracentrifugation [64] and gel filtration [60, 61, 70] revealed no dramatic changes in molecular mass during the initial proteolysis of 11S proteins. The fragments formed by the hydrolysis of acid subunits are retained in the partially hydrolysed molecule by non-covalent linkages [60, 61, 67] and some of them by disulphide bonds as well [60, 61]. No essential changes in the molecular mass of 7S proteins were observed during their initial proteolysis either [64, 69].

As was noted above, the modification of 11S and 7S proteins occurs at the onset of germination. The changes observed in these proteins are the same as those observed during proteolysis *in vitro*: they become susceptible to the action of some endogenous proteases which are unable to attack the native protein of ungerminated seeds [26–30];

their negative charge [27, 29, 49, 51, 71–73] and solubility [22–25] increase; fragmentation of subunits [24, 48, 51, 73–76] occurs without any dramatic lowering of molecular mass of the whole molecule [51, 72, 73, 75, 77]. The modification of the 11S and 7S proteins in germinating seeds is caused therefore mainly by limited proteolysis. The only exception known is their partial deamidation [71, 72, 77]: the hydrolysis *in vitro* of vetch 11S protein does not affect the content of amide N in the high-molecular weight residue (modified storage protein) [63]. The deamidation of protein in germinating seeds may result from the action of a specific enzyme [78]. The modification is accompanied by conformational changes [72, 77].

The exhaustive *in vitro* hydrolysis of storage proteins by proteinases A leads to their complete breakdown to peptides, mainly short peptides (di- and tripeptides) [31, 40, 41]. Free amino acids were not detected and if liberated are present in insignificant quantities. Large peptides were detected as intermediates during the extensive hydrolysis of 11S and 7S proteins by trypsin [60, 64] and proteinases A [39, 61].

The consecutive events described indicate that the proteolysis of 11S and 7S proteins involves a 'zipper' mechanism [79]. The latter is triggered off by the presence of susceptible sites in protein molecules; therefore different proteinases bring about similar changes. SDS-electrophoresis fails to distinguish between the fragments of acid subunits formed during the hydrolysis of vetch 11S protein by trypsin and by proteinase A [61]. On the contrary, the effect of the same enzyme on 11S proteins from seeds of different plant species may produce different fragments of acid subunits, as was observed in trypsin-hydrolysed 11S proteins of soya bean (*Glycine max*) [66] and vetch [60]. These differences, however, are of minor character; in general the changes of all tested 11S proteins during *in vitro* proteolysis as well as in germinating seeds are similar.

When trypsin acts on vetch [60] and soya bean [66] 11S proteins the 'zipper' process proceeds with a relatively high rate and it is completed shortly after the onset of proteolysis. During the further hydrolysis the molecular mass of the residual protein remains unchanged [60], which is characteristic of a one-by-one mechanism [79]. The relative rate of the 'zipper' hydrolysis of vetch 11S protein by proteinase A is significantly slower. As a consequence the one-by-one process predominates during all stages of proteolysis and protein breakdown is over before the 'zipper' process is completed [61]. The mixed type of proteolysis with the prevailing one-by-one process is apparently characteristic of the 11S proteins of other plants and of 7S proteins since essential changes of the molecular mass of the high molecular weight residue were not observed even after a considerable drop of its weight content in the hydrolysates [64].

As was noted above, the molecular mass of basic subunits of 11S proteins remains unchanged during hydrolysis. They are hydrolysed therefore exclusively by a one-by-one mechanism.

Proteinases and carboxypeptidases hydrolysing the storage proteins modified during seed germination

Proteinase B. In germinating vetch seeds proteinase B was detected, which was inactive toward storage proteins from ungerminated seeds but was able to hydrolyse the same proteins after their modification either during germination [28] or by limited proteolysis by proteinase A [27]. A homogeneous preparation of this enzyme was obtained [80]. The molecular mass of proteinase B is 38000 (SDS-electrophoresis data). The analysis of *N*-terminal amino acids in the A and B chains of insulin treated with proteinase B indicates that the only bonds to be split are peptide bonds formed by the carboxyl group of asparagine [80].

The pH optimum of proteinase B (5.6) is close to the pH inside the protein bodies. During germination the activity of proteinases A and B increases in parallel [28]. The participation of proteinase B in storage protein mobilization is thus very probable.

Vetch 11S protein becomes susceptible to proteinase B hydrolysis after one or two short peptides are split off by proteinase A [27]. The rate of hydrolysis by proteinase B is enhanced by the more prolonged action of proteinase A [61]. A similar rate of increase of 11S protein hydrolysis by proteinase B is observed in the course of its modification in germinating seeds [27].

Regardless of the extent of the previous effects of proteinase A, proteinase B hydrolysis of vetch 11S protein involves almost exclusively the one-by-one mechanism [61]. Proteinase B can therefore completely split this protein to peptide products. A considerable part of the latter consists of large peptides [80]. This is consistent with the presumed narrow specificity of this enzyme. Proteinase B does not hydrolyse the di- and tripeptides, but is able to act on some of the larger peptide products of the extended hydrolysis of vetch 11S protein by proteinase A [80]. Evidently the natural substrates of proteinase B may also be represented by the intermediate peptide products of storage protein hydrolysis by proteinase A.

Modified storage proteins which are the necessary substrates for the identification of proteinases B have been used in only a few studies, and vetch proteinase B is the only enzyme of this type which has been purified and partially characterized. There is some evidence that proteinase B occurs in other seeds. A study of their properties indicate that the thiol-dependent enzymes of the seeds of squash (*Cucurbita maxima*) [58], buckwheat (*Fagopyrum esculentum*) [30] and of sunflower [unpublished data; this laboratory] are proteinases B. These proteinases can only be detected in germinating seeds. They do not act on the 11S proteins of ungerminated seeds but hydrolyse modified *in vivo* 11S proteins at optimal pHs of 5.0 (buckwheat proteinase) and 5.7 (proteinases of squash and sunflower). The presence of proteinases B in the seeds of species belonging to four unrelated families (Fabaceae, Cucurbitaceae, Polygonaceae, Asteraceae) suggests that this type of enzyme may be generally present in dicotyledonous seeds.

A cysteine proteinase was recently isolated from germinating kidney beans [81]. Like the vetch proteinase B this enzyme proved to be highly specific for an asparagine side chain. Its molecular mass (23000) is however characteristic of proteinases A. The action of this purified proteinase on natural substrates has not been studied, so it is not possible to say whether or not it belongs to the proteinase B group.

Carboxypeptidases. Carboxypeptidases of the seeds of soya bean [82, 83] and cotton (*Gossypium hirsutum*) [84, 85] and grains of barley [86–92], wheat [93, 94] and rice (*Oryza sativa*) [95, 96] have been purified and characterized. Carboxypeptidases of mung bean [31] and vetch

[58] (the latter earlier named protease C [21, 28] have been partially purified. Carboxypeptidase activity (towards *Z*-dipeptides) was found in the seeds of all the plants studied [5].

The following general features characterize seed carboxypeptidases: the presence of serine in the active site; pH optimum within the range of 4–6; low specificity. It seems probable that seed carboxypeptidases and the well characterized acid serine carboxypeptidases from other plant tissues and yeast cells represent a large homologous family of enzymes [97]. The molecular weights of seed carboxypeptidases, however, vary greatly. Barley grains, e.g. contain carboxypeptidases with molecular weights of 43 000–170 000 [92].

Seed carboxypeptidases are located in the protein bodies of dicotyledons [28, 36, 46] and in the starchy endosperm of germinating cereals [92, 95, 98, 99]. The simultaneous presence of several carboxypeptidases is probably typical of seeds. More detailed information has been obtained in barley where five carboxypeptidases are present [92]. Wheat [93], rice [95] and soya beans [82] contain at least two carboxypeptidases.

The resting seeds of mung bean [74] and vetch [28] contain carboxypeptidases whose activity remains almost constant during germination. For other plants of the same family (kidney bean [100] and soya bean [82]) and for castor bean [22], carboxypeptidase activity increases in germinating seeds. One of the two rice carboxypeptidases is present in the resting grains and decreases during germination [95]. The activity of the other rice carboxypeptidase [95], and that of corn [99] and barley [101] carboxypeptidases rise during germination. The same increase was detected in germinating wheat (*Triticum aestivum*) [102]. Later, however, the same authors found similar levels of carboxypeptidase activity in resting and germinating grains of several varieties of two cultivated wheat species (*T. aestivum* and *T. durum*) [103].

In mung beans the increase in carboxypeptidase activity is due to another enzyme or enzymes which are not inactivated by the inhibitor of serine proteases [74]. On the other hand at least some of the carboxypeptidases are possibly synthesized *de novo*; this appears to be true in germinating cotton seeds [84]. There is some evidence of a *de novo* synthesis of at least one carboxypeptidase in barley (marker substrate *Z*-Phe-Ala) [104].

Vetch carboxypeptidase does not act on the proteins of ungerminated seeds [27]. The first step of protein modification transforms them into substrates susceptible to carboxypeptidase attack [21, 27]. In the course of further modification the rate of their hydrolysis *in vitro* by carboxypeptidase increases [27]. This is evidently caused by further 'zipper' proteolysis of storage proteins leading to the emergence of new *C*-terminal sequences which are due to the fragementation of polypeptide chains of 11S and 7S proteins and supposedly to their conformational changes.

Proteases in resting kidney bean [29] and squash [58] seeds, apparently carboxypeptidases, hydrolyse endogenous modified storage proteins *in vivo* but fail to act on the same proteins in ungerminated seeds. Several authors report the susceptibility of the proteins of resting seeds to the action of carboxypeptidases [31, 90, 93]. Nevertheless, they agree that protein degradation does not occur [103, 105] or is severely limited [90] in the absence of an endopeptidase.

The direct action of carboxypeptidases on high-molecular weight substrates is not of great importance *in vivo* due to the hydrolysis of storage proteins by proteinases A and B, which brings about the emergence of relative high molar concentrations of peptides. Evidently, the latter are the main natural substrates of seed carboxypeptidases [16, 31, 90, 103]. Since seed carboxypeptidases do not act on dipeptides and the hydrolysis of tripeptides is very slow [5, 9], it is the larger peptides that are the main natural substrates of carboxypeptidases. The extent of carboxypeptidase participation in the hydrolysis of intermediate peptide products of endopeptidase-split storage protein is evidently determined by the ratio between the rates of their hydrolysis by carboxypeptidases and endopeptidases. This ratio may greatly vary in the germinating grains of different cereals [106], and probably in the seeds of other plants as well, as in the seeds of one and the same plant at different stages of germination.

Aminopeptidases and dipeptidases responsible for the final stages of storage protein degradation

Only endopeptidases and carboxypeptidases are found in protein bodies [8, 97] and these enzymes are not able to complete the breakdown to free amino acids. Apparently dipeptides and possibly tripeptides produced by the joint action of proteases located in protein bodies are first transported and then hydrolysed by aminopeptidases and dipeptidases to amino acids [97, 107]. Since aminopeptidases or dipeptidases do not occur in the barley starchy endosperms, the final stages of breakdown must therefore be located outside the endosperm [9, 16]. Thus the principal and the final stages of protein degradation are evidently separated in space. The proteases which may perform the final stages of degradation comprise neutral aminopeptidases ('arylamidases') [97], basic aminopeptidases and dipeptidases [9] and probably some others.

Neutral aminopeptidases. Enzymes of this type hydrolysing β-naphthylamides and *p*-nitroanilides of phenylalanine and leucine have been purified and partially characterized in the seeds of barley [108], pea [109], vetch [107, 110] and apricot (*Prunus mume*) [111, 112]. The four enzymes are inhibited by *p*-chloromercuribenzoate have pH optima close to 7, similar molecular weights (55–66 000) and specificity (they hydrolyse hydrophobic dipeptides [108–110, 112]. They are apparently homologous.

Enzymes that can also be classified as neutral aminopeptidases (they hydrolyse the arylamides of different amino acids at pHs close to neutral) have been found in the seeds of many plants: legumes [74, 100, 113–116], other dicotyledons [22, 117, 118] and cereals [119, 120]. The seeds may contain simultaneously two [22, 113, 120], three [108, 109] or more [121, 122] β-naphthylamidases with different specificities capable of hydrolysing the derivatives of most amino acids (proline included). Some of these enzymes may be different at the active site (complete inhibition by 1,10-phenanthroline [109, 114]) as compared to the isolated aminopeptidases described above. It should be noted that not all the isolated neutral aminopeptidases can be unambiguously classified as cysteine proteases [109, 111].

These neutral aminopeptidases are located in the cytoplasm [36, 107, 114, 117]. Neutral aminopeptidases can be detected in resting seeds and their activity is constant [113, 115, 119, 120] or decreases

[74, 100, 110, 113, 114, 116, 120] during germination. The analysis of the hydrolysis of peptide products of protein degradation by vetch neutral aminopeptidase [107] shows that its residual activity exceeds that needed for the cleavage of all bonds corresponding to its substrate specificity during germination. Neutral aminopeptidases may also increase in activity in germinating seeds [22, 118]. In germinating seeds of flowering plants neutral aminopeptidases of varying specificities apparently form a system of enzymes capable of splitting to amino acids any short peptides formed during protein breakdown.

Basic aminopeptidases and dipeptidases. Two such enzymes from barley grains have been purified and characterized: an aminopeptidase hydrolysing di- and oligopeptides at pH 8.5–10.5 [123] and a metallo-dependent dipeptidase acting on the neutral dipeptides at pH 8–9 [124]. Another barley peptidase was found to hydrolyse the Ala-Pro marker substrate in an alkaline medium [9]. Barley basic peptidases probably participate in the final stages of protein degradation [9, 16].

Enzymes similar to barley basic peptidases also occur in the seeds of dicotyledons: peanut (*Arachis hypogaea*) [113], kidney bean [125] and probably squash [126, 127]. However, the localization of basic peptidases in these plants is unknown. Their high pH optima obscure their site of action and possible participation in the hydrolysis of peptide products of 11S and 7S protein degradation [9, 125]. Apart from the neutral and basic aminopeptidases and dipeptidases described above, other incompletely characterized enzymes capable of hydrolysing di- and oligopeptides may take part in the final stages of the protein breakdown [see 121, 128–130 and papers cited in the review 131].

The above results show that the proteases performing protein breakdown in the seeds of phylogenetically unrelated flowering plants are very similar. Moreover, in a number of investigations Scotch pine (*Pinus sylvestris*) seeds proved to contain proteases characteristic of the seeds of flowering plants; two acid cysteine proteinases appear during germination [132, 133], serine carboxypeptidase [134], neutral aminopeptidase [135] and basic peptidases [136]. Thus a similar enzyme machinery is probably responsible for the degradation of storage proteins in the seeds of all higher plants.

THE PATHWAYS OF DEGRADATION OF 11S PROTEINS IN GERMINATING SEEDS

Legumin-like 11S proteins are the most common form of storage protein in the seeds of flowering plants and they have been conserved during the course of evolution [10, 137, 138]. This is probably because of their role in regulating protein mobilization through the 'zipper' process during seed germination [10, 137]. A generalized scheme of 11S protein degradation based mainly on the investigations of vetch seeds is represented in Fig. 1 and discussed below. A preliminary and less detailed variant of this scheme has been described earlier [58] and is reproduced in reviews [2, 139].

The degradation of 11S protein from ungerminated seeds (1) is initiated by proteinase A that splits off one or two short peptides (1 → 2). This is the first high-rate step of the 'zipper' hydrolysis of 11S Protein. Further changes of the 11S protein are brought about by the continued 'zipper' proteolysis: the formation of high molecular weight

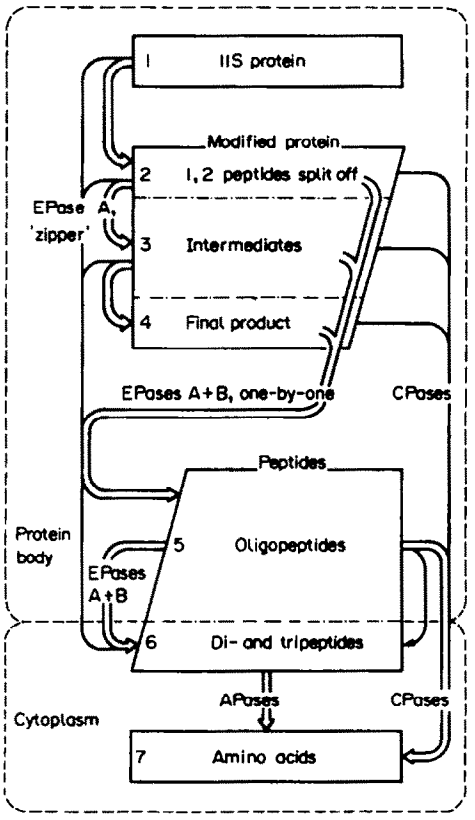

Fig. 1. The pathways of 11S protein degradation in germinating seeds. EPases = endopeptidases (proteinases); CPases = carboxypeptidases; APases = neutral aminopeptidases.

intermediates (3) and subsequently that of a residue (4) with a molecular mass 10–15% lower than the molecular mass of the initial 11S protein. This residue (4) is the final high molecular weight product of the 11S protein transformations. During all the stages of the 'zipper' process, the 11S protein is also subject to the one-by-one hydrolysis by both proteinases A and B and to the attack of carboxypeptidases. The relative contribution of the latter in the hydrolysis of high molecular weight substrates (2–4 → 7) is apparently small.

The peptide products of degradation are mainly the result of the one-by-one proteolysis. The analysis of the vetch 11S protein hydrolysis by proteinase A [61] shows that when two thirds of the protein disappear, the 'zipper' process accounts for only about 5% of peptides formed. Evidently this value is lower *in vivo* since proteinase B contributes to the hydrolysis only by one-by-one mechanism.

Both 'zipper' and one-by-one proteolysis may lead to the formation of di- and tripeptides (1–4 → 6), but the major products of proteolysis evidently comprise at first a wide range of intermediate oligopeptides (5). Their hydrolysis to free amino acids (7) may follow two extreme pathways: (a) the splitting-off of amino acids from large peptides by carboxypeptidases (5 → 7) until tri- and possibly dipeptides remain; (b) the breakdown of large peptides by proteinases A and B mainly to di- and tripeptides (5 → 6) which are transported from the protein

bodies and further hydrolysed by aminopeptidases and possibly by the other enzymes of this group (6 → 7). The relative importance of these two pathways is determined by the ratio of the activities of proteinases A and B on the one hand and carboxypeptidases on the other. At the first stages of the protein mobilization, when the synthesis of proteinases A and B is only starting and their activity is still low, free amino acids are mainly due to the action of carboxypeptidases already present in ungerminated seeds.

The analysis of data in the previous sections and the scheme show that the following factors regulate 11S protein mobilization in germinating seeds.

1. The resistance of the 11S protein to the action of the proteases located in the protein bodies of resting seeds, which prevents premature hydrolysis in developing seeds and probably during accidental short-term swelling.
2. *De novo* synthesis of proteinase A; appearance of proteinase B activity (apparently as a result of synthesis *de novo* as well); *de novo* synthesis of some carboxypeptidases.
3. The limited hydrolysis of 11S protein by proteinase A, which results in its susceptibility to the action of proteinase B and carboxypeptidases.
4. The subsequent 'zipper' hydrolysis of 11S protein by proteinase A which does not directly contribute significantly to its degradation but speeds up the action of carboxypeptidases and the one-by-one hydrolysis by proteinase B and probably by the proteinase A.
5. A change in the ratio of the activity of proteinases and carboxypeptidases that controls the relative contribution of carboxypeptidases and aminopeptidases (dipeptidases) to the final stages of protein degradation.

The degradation of 7S proteins has been much less studied. The available data, however, indicate general similarities in degradation pattern of 7S and 11S proteins. The 'zipper' proteolysis of 7S proteins and resultant changes in their susceptibility to the action of proteases of resting seeds have been detected. The hydrolysis by a one-by-one mechanism probably occurs as well. The further breakdown of the peptides formed is determined only by the level of the activity of the proteinases responsible for this process and is therefore sure to be similar to 11S protein degradation.

The course of cereal protein degradation is still unclear. However, the participation of the proteinases of the A group in the first stages and important role of carboxypeptidases in the following stages is evident [9, 41, 103].

THE POSSIBILITY OF OTHER MECHANISMS OF STORAGE PROTEIN MOBILIZATION. ASPARTYL AND METALLO-DEPENDENT PROTEINASES

Practically all the suggested mechanisms of storage protein mobilization other than those described in the previous section are based on the assumption that the initial stages are performed by the proteases of the ungerminated seeds that exist either in an active form or as an inactive complex with specific protein inhibitors. In the first case proteolysis must begin immediately after seed swelling; in the second, the onset of proteolysis is due to the inactivation of the inhibitors.

Reliable experimental proof of the ability of the proteases of ungerminated seeds to act *in vivo* on the unmodified seed proteins must meet the above-formulated criteria of protease participation in protein degradation, which is a much more difficult task than it may seem.

The enzyme preparations must not contain admixtures of proteinases localized outside the protein bodies, since among those may be found some that act on the storage proteins of ungerminated seeds, but have no contact with them *in vivo*. Even a slight action of such proteinases will be considerably amplified since they will modify the proteins and make them susceptible to the proteases contained in the protein bodies of resting seeds.

Storage proteins from ungerminated seeds used as substrates must be native. Small changes in structure caused by heat or other denaturation or by chemical modification may greatly increase their susceptibility to proteolysis. A slight action of bacterial proteinases in case of bacterial contamination may also cause structural modification [21]. These preparations must not contain admixtures of other proteins. The latter may comprise easily hydrolysable proteins whose compartmentation protects them from the action of proteases localized in protein bodies.

The ability of active proteinases in the ungerminated seeds to perform the initial stages of degradation *in vivo* claimed by a number of authors [140–144] is probably a result of non-compliance with the above-mentioned requirements. An analysis of the literature [2, 4, 7, 12, 20, 21, 105] also demonstrates that there are no trustworthy data proving this hypothesis. There is also no satisfactory explanation of the stability of storage proteins during the early stages of seed germination.

Protein inhibitors of proteinases, including those inhibiting the activity of endogenous enzymes, are found in the seeds of flowering plants [145–147]. However the participation of protein inhibitors in the regulation of proteolysis has no serious experimental justification [2, 6–9, 20, 148].

None of these hypotheses is convincing; yet it is not possible to exclude the participation of the proteinases of dry seeds in the initial stages of degradation. New information has been obtained recently from the discovery of aspartyl and metallo-dependent proteinases in dry seeds.

Aspartyl proteinases

Completely pepstatin-inhibited proteinases with pH optima in the range of 2–4 have been found in dicotyledons [149–151], cereals [54, 152, 153] and Scotch pine [132, 133]. They are evidently aspartyl proteinases. Aspartyl proteinases are already present in resting seeds. At germination their activity remains constant during the entire period of protein breakdown [54, 132]. The acid pH optimum suggests the localization of aspartyl proteinases inside the protein bodies and, therefore, their participation in degradation.

There are no reliable data confirming the ability of aspartyl proteinases to hydrolyse native storage proteins of ungerminated seeds. Aspartyl proteinases of buckwheat [151] and cucumber (*Cucumis sativus*) [150] act on 11S proteins at pH 3.0–3.2, but under these conditions 11S proteins are partially dissociated [10], which may probably affect their susceptibility to proteolysis. Incubation of soya bean proteins with the crude preparation of endogenous aspartyl proteinase did not

alter their electrophoretic behaviour [149]. Thus the assumed action of aspartyl proteinases at the early stages of seed germination (prior to the appearance of proteinases A) [9] is questionable. Aspartyl proteinases may however participate in digestion of modified proteins.

Metallo-dependent proteinases

Metallo-dependent proteinases were found in the seeds of dicotyledons: pumpkin [154], buckwheat [155], soya bean [149] and jackbean (*Canavalia ensiformis*) [156]. A similar enzyme in barley grains has been reported [157]. The buckwheat proteinase was partially purified [155]. Metallo-dependent proteinases of dicotyledons hydrolyse the storage proteins of ungerminated seeds *in vitro* and the changes observed are similar to those occurring during the initial stages of breakdown in germinating seeds (increase of the negative charge and fragmentation of the acid subunits of 11S proteins) [149, 154, 155]. These results suggest that metallo-dependent proteinases initiate protein mobilization [154, 155]. However, the changes of the 11S proteins during proteolysis are mainly determined by the structure of this substrate, the specificity of proteinase being of minor importance; similar fragments can be formed due to the action of a variety of proteinases.

The subcellular localization of seed metallo-dependent proteinases has not been studied. Their pH optima are, however, neutral [154] or slightly basic (pH 8.0–8.3 [149, 155, 156]) and differ from those of proteases and other enzymes characteristic of protein bodies. The action of soya bean metallo-dependent proteinase on 11S and 7S proteins was displayed by the appearance of subunit fragments after 24 hr incubation of the crude seed extract, but this effect was not observed when the swelled seeds were incubated during the same period [149]. These results do not agree with the localization of metallo-dependent proteinases in protein bodies either (at least in an active form). A protein inhibitor of endogenous metallo-dependent proteinase has been partially purified in buckwheat seeds [158]. However, its localization and changes in activity during seed germination are unknown as yet. Thus, there is insufficient evidence that metallo-dependent proteinases initiate protein mobilization.

CONCLUSION

The suggested scheme of 11S protein degradation is a simplified approximation of the *in vivo* process. In addition to proteinases A and B other endopeptidases (e.g. aspartyl proteinases) may participate at least in the splitting of intermediates of protein hydrolysis in the protein bodies. Little is known about the composition of the carboxypeptidases of dicotyledons and their effect on the natural substrates. Our knowledge of the properties and the role of the basic aminopeptidases and dipeptidases from dicotyledons is still poor.

Future investigations must evidently include simulation of the main stages of storage protein degradation inside protein bodies by simultaneous action of endopeptidases and carboxypeptidases *in vitro*, their ratios being close to those encountered *in vivo* at different stages of this process. The quantitative assessment of the two parallel pathways of free amino acid formation as a function of the endopeptidase–carboxypeptidase ratio and the study of peptide intermediates of degradation as the main substrate of carboxypeptidases are of interest. Progress in the determination of the primary structure of 11S and 7S proteins (for references see [2]) makes it possible to localize the fragmentation sites of their polypeptide chains during the 'zipper' proteolysis.

Many other aspects of storage protein degradation are still unclear or little studied. The course of mobilization in cereals and of minor storage globulins in dicotyledons is unknown. The possible existence of parallel mechanisms of degradation involving proteinases from ungerminated seeds needs further testing. Finally, of great interest is the elucidation of the mechanisms of mobilization in the axis, where this process takes place at the earlier stages of germination [7] and may differ from that in the main storage tissues.

The hypothesis that the enzyme machinery responsible is similar in all higher plants is a very attractive one. It is supported by most reliable data available. However, more detailed studies of the structure and properties of similar proteases from different plants is needed to prove their homology.

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