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DERLEME/**REVIEW**

PROTEIN DEPOSITION AND MOBILIZATION IN SEEDS

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ABSTRACT

Cereal and Leguminous plant's seeds take a large place of human food consumption. The germinating seed depends on its reserve material till the photosynthetic system develops. Storage proteins deposited in the seed during the maturation and have a purpose to provide free amino acids to the growing seedling. The present knowledge on protein storage during the seed development and enzymes involved in mobilization of the storage proteins has been summarized.

Key words: Storage protein, Protein mobilization, Seed germination

TOHUMLARDA PROTEİN DEPOLANMASI VE MOBİLİZASYONU

ÖZ

Tahıl ve baklagil tohumları insan beslenmesinde önemli bir yer tutmaktadır. Çimlenmekte olan bir tohum fotosentetik sistem gelişene kadar kendi içerdiği depo maddelerine bağımlıdır. Depo proteinler tohum olgunlaşması sırasında biriktirilirler ve büyümekte olan fideye amino asit sağlama amaçlıdırlar. Tohum gelişimi sırasında protein depolanması ve depo protein mobilizasyonunda rol alan enzimler konusundaki mevcut bilgiler özetlenmiştir.

Anahtar Kelimeler: Depo protein, Protein mobilizasyonu, Tohum çimlenmesi

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1. INTRODUCTION

Dry seed has a slow metabolism. Following the water uptake, some changes occur within the chemical composition of the seed; hydrolysis of storage reserves, mobilization of reaction products and synthesis of new molecules from the products (Mayer and Poljakoff-Mayber, 1989). According to FAO estimate, cereal and leguminous plant's seeds take a large place of human food consumption (70 % from cereal and leguminous seeds, 30% from animals) (FAO, 1970). Animal proteins being more expensive, people in developing countries virtually depend on seed protein alone for their entire protein requirement (Mandal and Mandal, 2000).

A leguminous seed characterized by high content of storage proteins, the total protein content is over 20 per cent; in most cases that ratio is among 7-15 in cereal seeds (Vitale and Bollini, 1995). The animal and plant proteins are different in terms of their nutritional characteristics. The legume proteins are deficient in sulphur containing amino acids methionine and cysteine, whereas plant proteins are generally deficient in lysine and tryptophan (Mandal and Mandal, 2000; Vitale and Bollini, 1995).

The protein content of the seeds can be classified as storage proteins and housekeeping proteins. The housekeeping proteins are responsible for keeping normal cell metabolism. Storage proteins are non enzymatic and have a purpose of providing sulphur and nitrogen source during germination and formation of a new plant (Mandal and Mandal, 2000). Deposition of storage proteins starts during seed maturation. Storage proteins deposited in organelles called protein bodies (Vitale and Bollini, 1995). Protein bodies can be found at embryonic axis and cotyledons of dicots and in endosperm, if endosperm exists for storage (Dalling, 1986).

The seed uses its own reserves until the photosynthesis starts. The proteolytic cleavage of the reserves can be separated to three main stages: First, the initial hydrolysis of the reserves for the synthesis of proteolytic enzymes. Next the subsequent hydrolysis to provide free amino acids for growing seedling. And at the end, the hydrolysis of the depleted storage tissue for providing the last part of amino acids before photosynthesis starts (Mayer and Poljakoff-Mayber, 1989; Daussant et al., 1983).

Storage protein mobilization has been predominantly investigated in seeds of cultivated plants such as grain legumes, rape, sunflower and cereals which during domestication underwent selection for increased protein accumulation in the cotyledon storage tissues, as in dicotyledonous seeds or in the endosperm as in cereal grains (Müntz et al., 2001).

1.1 Protein Storage during the Seed Development

The storage tissue of a seed can contain carbohydrates, proteins and lipids. Storage proteins, carbohydrates and lipids are synthesized during seed development (Millerd, 1975). Some seeds can contain mineral reserves as phosphate.

Proteins comprise one of the major important storage materials which accumulate high amounts during the second stage of seed development. This stage is preceded by the zygote development stage and followed by the desiccation stage in which seeds undergo preparation of dormancy. Storage proteins first appear in wheat endosperm about 10 days after anthesis. The embryo differentiates into embryonic axis and cotyledons during the cell division phase. Development of the endosperm and cotyledons is different at this stage. In monocots, the endosperm becomes the main storage tissue while in most dicots endosperm has only transient role and the cotyledons undertake the reserve function. During the second phase, cell expansion takes place, the embryo enlarges and there is a rapid synthesis and accumulation of food reserves. Generally the protein content suddenly increases within a few days during this period. Monocot seeds have specialized storage tissue, the endosperm (triploid) or perisperm (diploid). The developmental processes than become slower and desiccation begins which prepare the seed for dormancy. The seed loses more than 90 % of its water content, RNA and protein synthesis terminates and embryonic dormancy begins. Although metabolic processes and *de novo* protein synthesis drops dramatically during this stage, late embryogenesis associated proteins (LEA) which may be stress proteins produced to protect the seed tissue against desiccation, are synthesized during this stage. Dormancy ends in seed germination when seed storage proteins are used up for the seedling development. The duration of different stages varies with the species and environmental factors (Mandal and Mandal, 2000). The subcellular repositories for the majority of storage proteins in reserve tissues of mature seeds are protein bodies. These organelles are unit membrane-bound and commonly spherical in shape, having a diameter of 0.1-25 μm. Both size and the presence of inclusions such as phytin containing globoids or proteinaceous chrystalloids (Tully and Beevers, 1976) (Figure 1).

The protein bodies of cereal seeds present in aleurone layer and starchy endosperm. The starchy endosperm cells contain globoid protein bodies which are

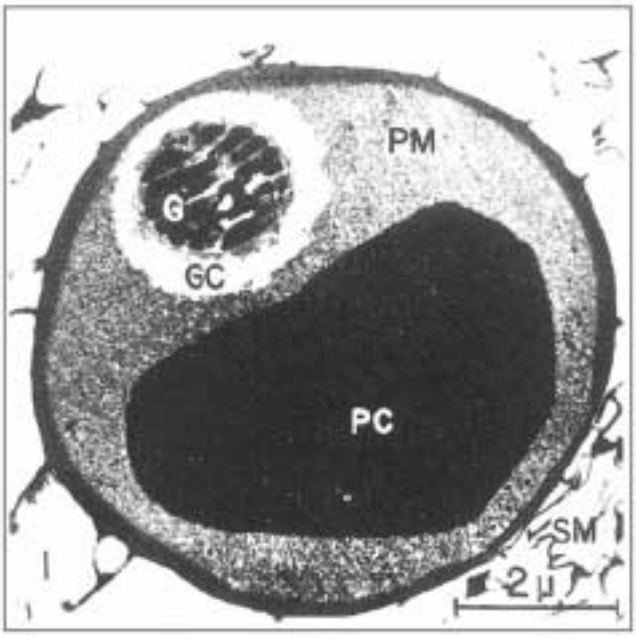


Figure 1. Electron microscopic view of a protein body from *Ricinus communis* endosperm. G: globoid, GC: globoid cavity, PC: protein chrystalloid, PM: protein matrix, SM: spherosom membranes (Tully and Beevers, 1976).

2-5 μm or bigger in diameter. These protein bodies are the centre of prolamin accumulation. As well as, cereal seeds can contain glutelin or globulin in their starchy endosperm cells (Bewley and Greenwood, 1990; Müntz, 1998).

Storage proteins are synthesized on the rough endoplasmic reticulum and transported to Golgi apparatus via tubular endoplasmic reticulum connections. The storage proteins are sorted and packed into Golgi derived vesicles and transported to the vacuole/protein body compartment. After the fusion of the vesicles with tonoplast, the storage protein is discharged into the vacuolar lumen. Evagination of the tonoplast around the concentrations of storage proteins results in the formation of virtually mature protein bodies (Bewley and Greenwood, 1990) (Figure 2).

Environmental factors such as temperature and plant nutrition can effect the storage protein accumulation (Jenner et al., 1991). Increased nitrogen supply may or may not increase storage protein synthesis depending upon the species or time of application during the development. The most dramatic effects of nutritional factors have been observed in legume and cereal seeds deprived of sulphur (S), potassium or phosphorus. In relation to the first of these elements, the synthesis of proteins which are normally rich in S-containing amino acids are severely affected by S deficiency. In S deficient pea seeds, total protein is reduced by 20 %, legumin decreases almost completely and the total albumin fraction is reduced by 35 %. In contrast, the relative level of vicilin, which does not contain S containing amino acids, increases by some 40 % (Bewley and Greenwood, 1990).

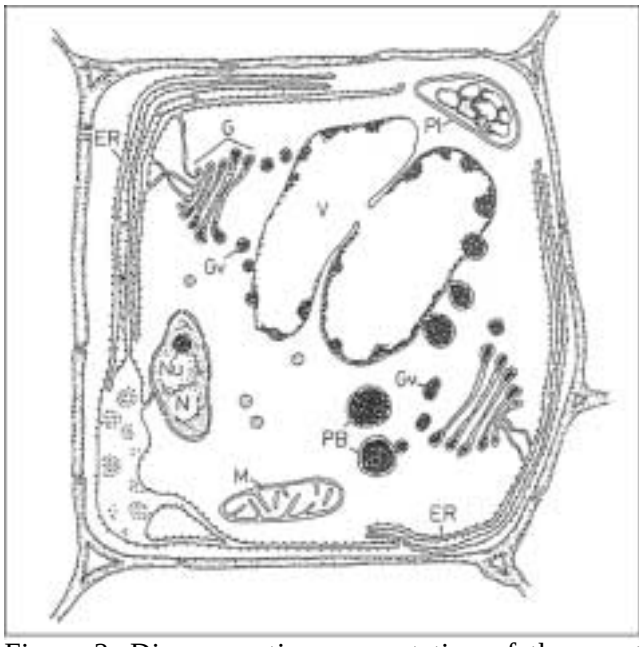


Figure 2. Diagrammatic representation of the events involved in protein body formation in storage parenchyma cells of legume seeds. Storage proteins are synthesized on the rough endoplasmic reticulum and transported to the Golgi apparatus via tubular smooth endoplasmic reticulum connections. The storage proteins are sorted and packed into Golgi derived vesicles and transported to the vacuole/protein body compartment. Fusion of the vesicles with the tonoplast, illustrated on the left of the vacuole, discharges the storage protein into the vacuolar lumen. Evagination of the tonoplast around concentrations of storage proteins, seen on the right of the vacuole, results in the formation of virtually mature protein bodies. Continued vacuolar subdivision gives rise to numerous protein bodies. ER: Endoplasmic reticulum, G: Golgi, Gv: Golgi derived vesicle, M: Mitochondrion, N: Nucleus, Nu: Nucleolus, Pl: Plastid, V: Vacuole (Bewley and Greenwood, 1990).

1.2 Characteristics of Plant Storage Proteins

Osborne (1924) classified the storage proteins as four groups according to their solubilities:

1. Albumins: Soluble in water, neutral lipids and slightly acidic pH.
2. Globulins: Not soluble in water but soluble in salt solutions.
3. Glutelins: Not soluble in water or salt, soluble in weakly acidic or alkaline solutions.
4. Prolamins: Soluble in ethanol.

According to their sedimentation characteristics in sucrose gradients dicotyledonous storage proteins are classified into 11S, 7S and 2S proteins. Whereas 11S and 7S proteins belong to globulins, the 2S proteins comprise globulins as well as albumins. The 11S and

7S globulins are named legumin-like or legumins and vicilin-like or vicilins, in accordance to predominating storage globulins in pea and faba beans (Müntz, 1996). Legumin holoproteins, which have a molecular weight of a 300-400 kDa, are composed of six nearly identical subunits. Vicilin holoproteins which have molecular weight of a 110-190 kDa are composed of two subunits (Dalling, 1986; Müntz, 1996).

Storage proteins are also deposited in bulb, bark or parenchyma tissues of plants. They are protected against premature proteolytic activity with several mechanisms. The most important protection mechanism is deposition in protein bodies. Most of the storage proteins undergo limited proteolysis after transferred into protein storage vacuole. This processing probably changes the conformation of proteins from one that is capable of transport to one that can be effectively deposited (Müntz, 1996; 1998). Some seeds can contain the low molecular weighted proteins such as 2-3S proteins (e.g. grape seeds), some others can contain high molecular weighted albumins (in Helianthus, more than 60 % of the total protein). Some seeds contain unusual type of albumins such as proteolytic inhibitors or lectins (Bewley and Greenwood, 1990).

1.3 Mobilization of Storage Proteins

Plant proteinases hydrolysing the seed storage proteins are classified into four groups according to catalytic mechanism in their active site (Bewley and Black, 1982).

1. Serine proteinases: Contain serine amino acid in their active site, e.g. Chymotrypsin and trypsin
2. Cysteine proteinases: Contain free –SH group in their active site, e.g. papain, ficin and bromelin
3. Metalloproteinases: Need metal ions as cofactor and inhibited by metal chelators, e.g. from buckwheat seeds (Belozersky et al. 1990), from sorghum (Sorghum bicolor L. Moench) (Macedo et al., 1999).
4. Acid proteinases: Active in acidic pH, e.g. animal pepsin and rennins

Proteinases are classified more specifically according to their substrates (Bewley and Black, 1982):

1. Endopeptidases: Hydrolyse the internal bonds of polypeptides and produce smaller peptides
2. Aminopeptidases: Cleave the terminal amino acid of the free amino terminus of the peptide chain
3. Carboxypeptidases: Cleave the terminal amino acid of the carboxyl terminus of the peptide chain

Proteinases are also divided as endo- and exopeptidases (Dalling, 1986).

Endopeptidases: Their pH optima range from 3.5 to 6.5. They can hydrolyse the animal proteins such as haemoglobin and casein as well as, both native and partially degraded plant storage proteins. They have relatively low molecular weight (approx. 20-40 kDa) and sulphydryl dependent. They are apparently found in protein bodies of the vacuoles derived from the fusion of the protein bodies of germinated seeds. The enzyme activities are presumed to increase during germination and seedling development due to *de novo* synthesis.

The exopeptidases are comprised of three subgroups:

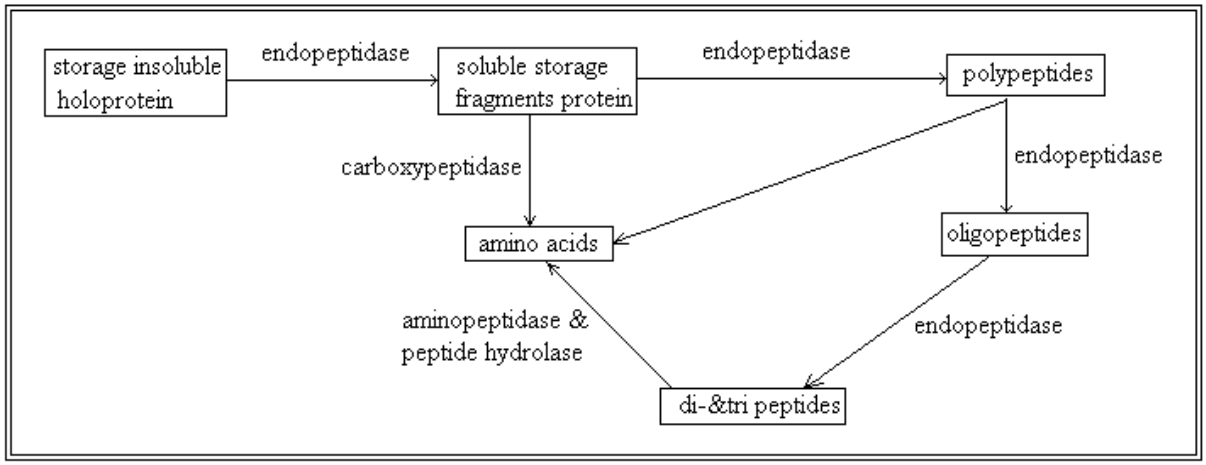
Acid carboxyptidases: They are serine dependent and the pH optima ranges from 5 to 6. The carboxypeptidases are capable of releasing at significant rates all 20 of the natural amino acid residues when present at the carboxyl terminus of peptides or proteins. Their activity increases during germination.

Arylamidases: They are characterized by their ability to catalyse the hydrolysis of the amide bond between the carboxyl group of an amino acid and the amino group of an aromatic amine. They generally have the pH optima in range of 6.5 to 8.5 with molecular weight of 60-70 kDa. These enzymes generally fall into three groups on the basis of their substrate specifities i.e. those hydrolysing the arylamides of (1) arginine, (2) proline and (3) neutral and aromatic amino acids such as leucine, alanine and phenylalanine. The arylamidases generally decline or remain relatively constant in activity during germination.

Alkaline peptidases: They have the alkaline pH optima (7.5 to 10) that hydrolyse simple peptide substrates, but are inactive against typical arylamidase substrates. While present in significant levels in both quiescent and germinating seeds.

The storage proteins are hydrolysed to amino acids by a number of proteinases. The storage tissues of germinated seeds can contain several of these proteinases. The large oligomeric proteins are initially subjected to limited proteolysis to more soluble polypeptide fragments by endopeptidases. The further degradation is continueing by endopeptidases (mostly carboxypeptidases) and endopeptidases. The final stages of degradation from small oligopeptides to amino acids may occur outside of the protein bodies and involve aminopeptidases (exopeptidases) and peptide hydrolases, e.g. dipeptidases (Müntz et al., 1985). For those proteins stored within the cytoplasm, the whole enzyme complement is presumably present therein. The liberated amino acids may be reutilized for protein synthesis or undergo deamination for providing carbon skeletons for respiration. The sequence of events can be outlined as follows (Bewley and Grenwood, 1990):

The limited proteolysis is essential for the initiation of storage protein breakdown. The triggering endopeptidase may be present in an inactive state with their substrates inside the protein bodies or they may be newly synthesized during seed germination. The structural changes induced by the initial cleavages of the storage proteins result in conformation changes that open them to further degradation. In storage tissue



cells, this is accompanied by the formation of the vacuole from the protein bodies (Müntz, 1996). The quiescent dry seed contains a number of proteolytic enzymes in addition to storage proteins. Proteolysis of the storage proteins does not occur in the mature desiccating seed for one or more of the following reasons (Dalling, 1986):

1. The native storage protein does not serve as substrate for the proteolytic enzymes present
2. The enzyme(s) and storage proteins are localized in different compartments of the cytoplasm
3. Desiccation has reversibly inactivated the enzymes

Some authors took into consideration to different criteria for classification of the enzymes responsible for degradation of storage proteins during the germination. For example, Shutov and Vaintraub (1987) classified enzymes as proteinase A and B that active in different stages of germination. Proteinase A is responsible of triggering the mobilization of storage proteins and active during the first stages of germination. Proteinase B is act at the later stages of germination and responsible for hydrolysing the modified proteins. The activation of reserve proteins in storage tissues seems to be under the hormonal control of the embryonic axis. Cotyledons detached from the axis before imbibition exhibited no increase in endopeptidase activity and globulin breakdown.

The proteolytic enzymes are present with storage proteins in protein bodies. After synthesized *de novo* in membrane bound polyribosomes, they transfered to protein bodies during germination (Baumgartner and Chrispeels, 1977).

Reserve proteins are stored in two distinct regions in the cereal grain: in the aleurone grains of the aleurone layer (up to 30 % of total seed protein) and in the protein bodies of the starchy endosperm. Mobilization of the aleurone proteins is the result of the activity of *de novo* synthesized proteinases. These enzymes are mostly unidentified and activated by hormonal (gibberellic acid) stimulation. The amino acids produced may be recycled to produce more hydrolytic enzymes which are released into the non-living starchy endosperm. Since protein hydrolysis and protein (enzyme) synthesis occurs concurrently in the same cells of the aleurone layer, the two processes must be separated. Hydrolysis of the proteins occurs within the aleurone grains (protein bodies) and the resultant peptides and amino acids are released to the protein synthetic sites in the cytoplasm. Excess amino acids diffuse into the embryonic axis for use by the developing seedling. The cells of the starchy endosperm are non-living at maturity, and hence the supply of enzymes for protein mobilization comes either from the aleurone layer, following *de novo* synthesis or by activation of enzymes already present within the dry seed (Bewley and Greenwood, 1990).

Conversely to cereals, storage proteins are present in both cotyledons and embryos in legume seeds (Vigil and Fang, 1995; Schlereth et al., 2001). However, the research on the role of embryonic axis on protein mobilization still continues and the mods of enzymatic breakdown of storage proteins during legume seed germination are not yet clearly understood. Two hypotheses have been proposed concerning the axial control of this process. First, the growing axis may act as a sink, which draws off the products of reserve mobilization, and its excision leads to an accumulation of proteolytic end products (Chin et al., 1972; Kern and Chrispeels, 1978; Davies and Chapman, 1979b; Mitsuhashi et al., 1984). Second, the growing axis may produce plant growth substances, which stimulate the synthesis of hydrolytic enzymes for reserve mobilization in the cotyledons. The effects of plant growth regulators may be specific to species and also to cultural variety. Gibberellins or cytokinins are thought to regulate this process in dicots (Allen et al., 1984; Munoz et al., 1990; Nandi et al., 1995; Yoshida and Hirasawa, 1996). And in a similar manner gibberellins arising from the embryo, influence reserve mobilization especially in the endosperm of cereals (Jacobsen and Varner, 1967; Yomo and Varner, 1973).

The transportation of amino acids released by the different proteolytic enzyme activities from cotyledons to embryonic axis has been investigated. In vetch (*Vicia sativa*) seed a slight initial decrease in

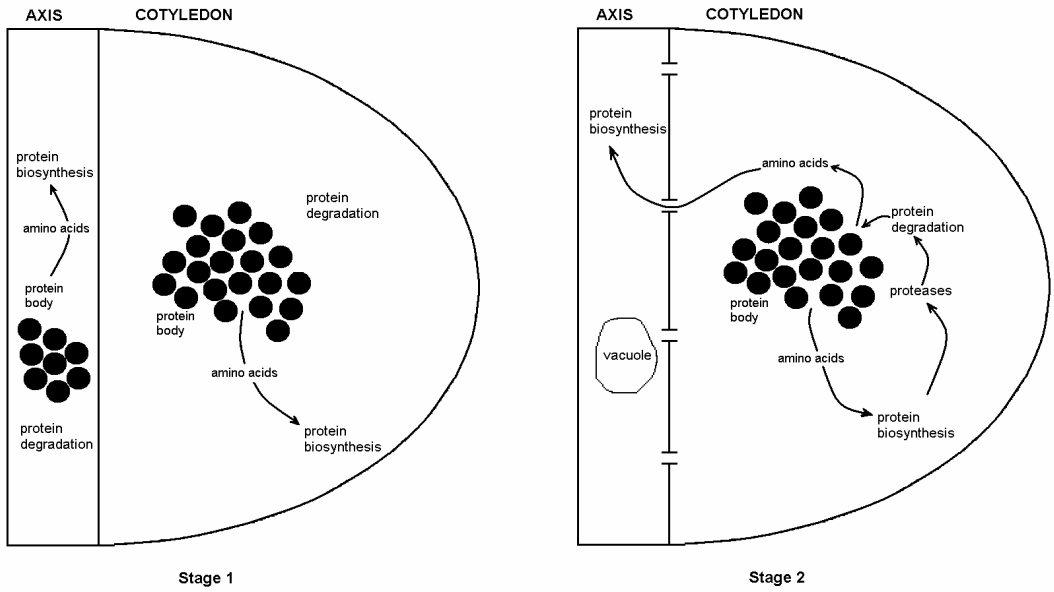


Figure 3. Storage protein mobilization in embryonic axis and cotyledons during germination (stage 1) and after germination (stage 2). In stage 1, endogenous sources supply amino acids for protein biosynthesis in both organs whereas, in stage 2, the cotyledons are the source of amino acids for the embryonic axis which now acts as a sink (Schlereth et al., 2000).

the amount of total free amino acids occurs at the beginning of imbibition which is attributed to leakage from the axis. In vetch and garden bean seeds prevascular strands are among the first tissues where protein mobilization is initiated during germination. While protein breakdown proceeds the prevascular strands are transformed into vascular bundles (Müntz et al., 2001). In the post germination period these bundles enable a fast transport of amino acids from breakdown in the cotyledons into the growing axis. Later during germination the amount of free amino acids remains unchanged as did the amount of total axis protein. No net import or export of amino acids was measured (Figure 3) (Schlereth et al., 2000).

The mobilization of the protein reserves can be regulated by growth substances released from the embryonic axis. The effect of the removal of embryonic axis and the regulation of protein mobilization by growth regulators released from the embryonic axis has been studied many times. Embryo detached from the cotyledons and effects of growth regulators in the absence of the embryo was examined. For example, a protease synthesized *de novo* after treatment with GA_3 in *Hordeum vulgare* (Jacobsen and Varner, 1967), proteolytic activity is stimulated after GA_3 treatment in the absence of the embryo in *Pisum sativum* cotyledons (Yoshida and Hirasawa, 1996). Barduche et al. (1999) reported that GA_3 can stimulate the protein mobilization and reverse the inhibition of Abscisic acid (ABA) in *Anadenanthera peregrina* cotyledons. In *Phaseolus vulgaris* cotyledons, GA_3 is not effective whereas BA promoted the hydrolytic activity (Metivier and Paulio, 1980). And also cytokinins released from the embryo induce the protein mobilization. These promotive effects of cytokinins on protein mobilization have been reported for various species. But the effect can change due to the type of cytokinins and plant species. For instance, Benzyladenine (BA) was more effective than Kinetin on protein mobilization in the absence of the embryo in *Lupinus luteus* seeds (Nandi et al., 1995). BA is also effective on stimulating the protein mobilization of *Helianthus annuus* cotyledons (Allen et al., 1984). Munoz et al. (1990) reported the stimulation of protein mobilization by Zeatin in *Cicer arietinum* cotyledons. Hormone applications are not effective on the protein mobilization in some species. For example, both ABA and GA_3 were ineffective on the protein mobilization *Pisum sativum* seeds (Malek, 1987). Kırmızı (2003) pointed out that both growth promoting (BA, IAA, GA_3) and inhibiting (ABA) substances were ineffective on protein mobilization in *Vicia faba* seeds.

The number and activity status of the proteases during germination period have been studied. Mikola and Koehlemainen (1972) determined that the eight different proteases effective during germination of *Hordeum vulgare* L. seeds, the three of them were carboxypeptidase, the other three were aminopeptidase and the remaining two were dipeptidase. Mitsuhashi and Oaks (1994) reported that endopeptidase activity could be existed at least 17 different types in *Zea mays* L. endosperm. They were classified the enzymes detected into four groups based on their time of appearance. The first group was present in dry endosperm and then disappeared after the imbibition, the second group was active during the first 2-3 days and then disappeared, the third group was rise in activity throughout the germination and the fourth group was appeared after the day 3rd and has the constant activity during the germination. The activities of different proteoytic enzymes during germination of *Zea mays* L. (San Segundo et al., 1990) have also been indicated. An aspartic proteinase was found

in ungerminated cotyledons, cysteine and serine proteinases were appeared during the germination, and the carboxypeptidases were active at beginning and last stages of germination.

The proteolytic activities are also investigated in storage organs of geophyte plants. The proteolytic enzymes of garlic (*Allium sativum* L.) and onion (*Allium cepa* L.) bulbs before and after the germination have been studied by Lin and Yao (1995a and b). They reported that endopeptidase, carboxypeptidase or some aminopeptidase activities were higher in dormant onion bulbs than those of germinated ones. Although garlic and onion belong to the same genus, their proteolytic activity patterns are not the same. For example, the casein hydrolysing activity of garlic bulbs was higher after germination than before germination, while the opposite was observed in onion bulbs. Authors also found that, haemoglobin-hydrolysing activity or some aminopeptidase activities of dormant garlic bulbs are higher than those of germinated ones. According to Lin and Yao (1995a, b), proteolytic activity in dormant garlic bulbs may be due to two possible reasons. The first possibility is that these enzymes catalyze the protein turnover needed for the high rate of protein synthesis in developing bulbs, rather than a high net rate of protein breakdown during germination. The second possibility is that some of these proteinase activities are involved in a defence mechanism.

Degradation of storage proteins during seed germination can be monitored by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electhrophoresis). Differences among the storage proteins of different lines or varieties can also be monitored by electrophoretic techniques (Hussain et al., 1988; Korochko and Bewley, 2000; Ahmed et al., 1995). The differences among the seed proteins can be attributed to different phenotypic characters (Hussain et al., 1988). According to Korochko and Bewley (2000), the seed proteins of 27 different *Medicago sativa* L. varieties which are represented with five subspecies showed great homogeneity.

Some of the proteases have been purified and characterized by many researchers; such as dipeptidase from barley (Sopanen, 1976), proteinolytic enzyme complex from pea (Yang and Malek, 1991), cysteine proteinase from *Vigna* (Yamaoka et al.,1990), cysteine endopeptidase from wheat (Kuroda et al, 1997), acid protease from soybean (Tan-Wilson et al., 1996), leucine aminopeptidase from soybean (Mikkonen and Mikola, 1986), two endopeptidases from *Vigna* (Mitsuhashi et al, 1986), cysteine endopeptidase from barley (Koehler and Ho, 1988), cysteine endopeptidase from soybean (Seo et al., 2001), aspartic proteinase from *Helianthus* (Yamaoka et al., 1990).

2. CONCLUSION

Complete breakdown of storage proteins is the result of massive *de novo* proteinase formation and the combined action of endo- and carboxypeptidases inside the protein bodies and vacuoles in the storage tissue cells of mid germination of seeds. Both amino acids and short peptides are transported from the vacuolar compartment into the cytoplasm where amino- and dipeptidases degrade them further. The amino acids then used to meet metabolic demands in the storage tissue itself like the biosynthesis of several reserve degrading hydrolases and to meet the demands imposed by the major site of protein biosynthesis in the germinating seed and growing seedling, the embryo axis.

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