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Anita Mikkonen

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OCCURRENCE AND PROPERTIES OF PROTEOLYTIC ENZYMES IN GERMINATING LEGUME SEEDS

Anita Mikkonen

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Storage proteins in legume seeds are almost totally located in the protein bodies of the cotyledonary cells. The proteins are hydrolyzed during germination into free amino acids which are transported to the growing seedling for use in protein synthesis. This hydrolysis is catalyzed by proteolytic enzymes, both proteinases and peptidases.

Cotyledons of resting kidney bean (*Phaseolus vulgaris* L.) contained low activities of neutral aminopeptidases (marker substrate Leu-ß-NA) and acid carboxypeptidases (marker substrate Z-Phe-Ala) but high activities of two alkaline peptidases: an aminopeptidase liberating most rapidly Leu and Ala residues from N-termini of di- and oligopeptides optimally near pH 9 and a dipeptidase hydrolysing Ala-Gly with a pH optimum at 8.5. Both of these alkaline peptidases were separated from neutral aminopeptidases by ion exchange and gel permeation chromatography. The purified aminopeptidase had a large molecular weight, 360 000 which distinguishes it from all the other peptidases known in legume seeds. It apparently belongs to the universal group of enzymes called leucine aminopeptidases.

Subcellular fractionation studies carried out by ultracentrifugation in aqueous and non-aqueous media showed that the alkaline peptidases of resting beans were located in the cytosol. Most of the carboxypeptidase activity was associated with protein bodies (70%) and a minor but significant proportion (30%) with the cell wall fraction.

The activities of naphthylamidase and alkaline peptidase decreased during germination, whereas the carboxypeptidase and endoproteinase activities increased, reaching their maximal values when the mobilization of nitrogen was highest. However, during the the period of rapid mobilization of nitrogen, the alkaline peptidase and carboxypeptidase activities were at approximately the same level, indicating that the alkaline peptidases may also have an important role in the mobilization of reserve proteins.

These results suggest that the alkaline peptidases may complete the hydrolysis of oligopeptides which are produced initially within the protein bodies by acid proteinases and carboxypeptidases and passed to the cytosol. The free amino acids formed are then transported to the growing seedling.

Key words: Proteolytic enzymes, alkaline peptidases, subcellular fractionation, germination, legume seeds.

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ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I Mikkonen, A. & Mikola, J. 1986: Separation and partial characterization of two alkaline peptidases from cotyledons of resting kidney beans, *Phaseolus vulgaris*. - Physiol. Plant. 68: 81-85.
https://doi.org/10.1111/j.1399-3054.1986.tb06599.x

II Mikkonen, A., Begbie, R., Grant, G. & Pusztai, A. 1986: Intracellular localization of some peptidases and α-mannosidase in cotyledons of resting kidney bean, *Phaseolus vulgaris*. - Physiol. Plant. 68: 75-80.
https://doi.org/10.1111/j.1399-3054.1986.tb06598.x

III Mikkonen, A. 1986: Activities of some peptidases and proteinases in germinating kidney bean, *Phaseolus vulgaris*. - Physiol. Plant. 68: 282-286.
https://doi.org/10.1111/j.1399-3054.1986.tb01927.x

IV Mikkonen, A. 1990: Purification and characterization of leucine aminopeptidase from kidney bean cotyledons. - Manuscript
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Contents

Abbreviations:

BANPA, α–N–benzoyl-D,L-arginine-β–naphthylamide

BAPA, α-N-benzoyl-D,L- arginine-β-nitroanilide

pCMB, p-chloromercuribenzoate

DFP, diisopropyl fluorophosphate

EDTA, ethylenediaminetetraacetic acid

ER, endoplasmic reticulum

GA_3, gibberellic acid 3

pHMB, p-hydroxymercuribenzoate

M_r, relative molecular mass

NA, naphthylamide

NEM, N-ethylmaleimide

PAGE, polyacrylamide gel electrophoresis

PMSF, phenylmethylsulfonyl fluoride

SDS, sodium dodecyl sulphate

TCA, trichloroacetic acid

TNBS, 2,4,6-trinitrobenzenesulphonic acid

Z, N-benzyloxycarbonyl

1. Introduction

Mobilization of storage proteins is an important part of nitrogen metabolism in all higher plants. When senescence occurs, the bulk of the proteins of decaying organs are broken down and the resulting free amino acids are transported to the growing parts of the plant. During the development of seeds, leaf proteins are hydrolysed and the resulting amino acids are transported to be used in the synthesis of the storage proteins of the seeds. These storage proteins of cereals, legumes, etc. are important protein sources in both human and domestic animal nutrition.

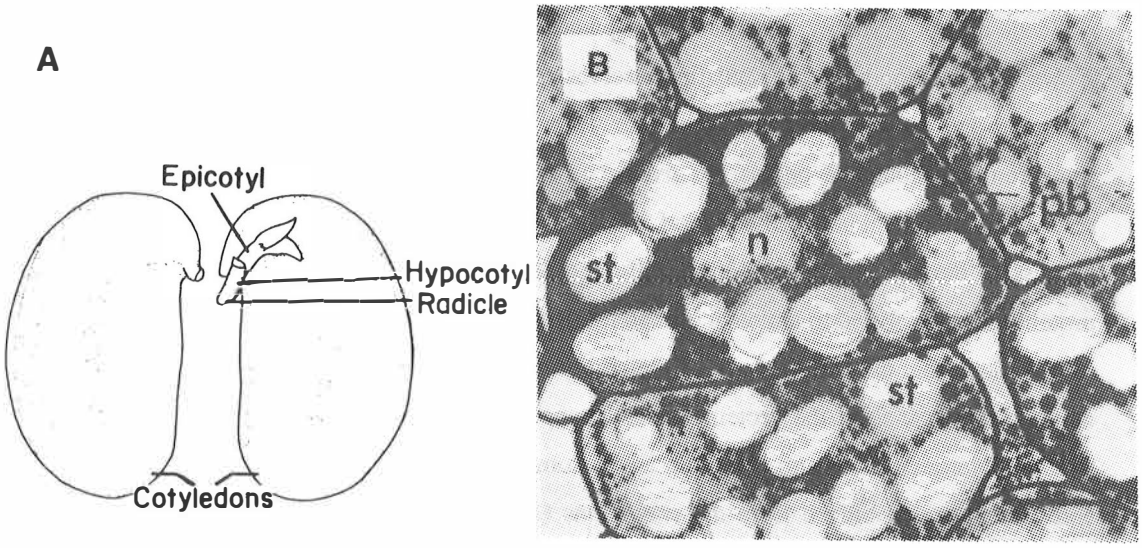
The resting legume seed is composed of two cotyledons and a small embryo, which contains a radicle and two "first leaves", the epicotyl (Fig.1A). The cells are packed full of starch grains and protein bodies (Fig. 1B). The largest cells of the kidney bean (*Phaseolus vulgaris* L.) are about 160 µm in diameter and contain starch grains up to 50 µm and protein bodies 2 to 22 µm in diameter (Öpik 1966, Pusztai et al. 1978, Mäder & Chrispeels 1984). The main reserve is starch, which is mainly used as an energy source. The protein content is about 20 % of the dry weight (Öpik 1966) and moisture accounts for approximately 10 % (Pusztai et al. 1978).

In the early stages of cotyledon development, before true protein bodies form, seed storage proteins such as legumin and vicilin accumulate in discrete areas lining the periphery of central vacuoles of the cells (Goodchild & Craig 1982). The protein bodies arise by partitioning of the central vacuole during the first 10-15 days of storage protein deposition (Öpik 1968, Craig et al. 1979 and 1980, Hara-Nishimura et al. 1987). During this period, and later in seed development, synthesized storage proteins are transported to the protein bodies by vesicles derived from the endoplasmic reticulum, the Golgi apparatus, or both (Harris 1979, Chrispeels 1983, Adler & Muntz 1983, Craig & Goodchild 1984). Thus, in resting legume seeds, the contents of the protein bodies are surrounded by a single membrane (Tully & Beevers 1976, Pusztai et al. 1977 and 1979, Pernollet 1978, Mäder & Chrispeels 1984).

Seeds do not normally germinate until plants have undergone a considerable period of growth and development, accumulating reserves and finally becoming air-dry. They are then quiescent and can be stored for months or years without harm. Air-dry seeds have a low water content, about 15% or less, but given access to water they imbibe rapidly and generally reach a water content between 30% and 50% in one or two days (Simon 1984). The opposite process to the development of the seeds, germination, can then begin and seed storage proteins are hydrolyzed to amino acids and transported to the growing seedling (Fig. 1C).

It is likely that the early growth of the embryonic axis depends substantially on the local nutrient reserves, since the protein bodies disappear first from the radicle of the pea seed (Bain & Merces 1966b) and there is a lag of some days before the main reserves of starch and protein are mobilized from the cotyledons (Bain & Merces 1966a, Basha & Beevers 1975). Digestion of reserves in cotyledons begins in the cells furthest away from the epidermis. Starch and protein bodies are digested in the storage cells more or less simultaneously (Öpik 1966).

When the cotyledonary cells return during germination to an active anabolic phase the synthesis of a new protein pattern may occur (Croissant-Sych & Bopp 1988). Newly formed hydrolases are secreted into the protein bodies of the germinating seed and storage proteins are broken down (Spencer 1984). The steps involved in protein body biogenesis are essentially reversed during seed germination. Protein bodies have been shown to fuse together and give rise to a central vacuole (Gifford et al. 1983, Spencer 1984, Alvarez & Guerra 1985, Hara-Nishimura et al. 1987).



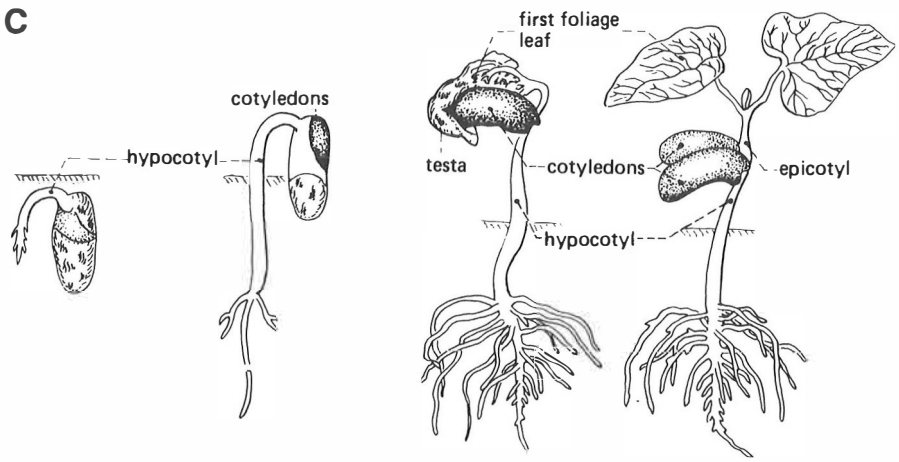


Fig.1. A: Longitudinally sectioned resting seed of kidney bean (*Phaseolus vulgaris*) with cotyledons spread out (Fahn 1985).
B: Thin (1 µm) section of embedded 24 h-imbibed mung bean cotyledons. The section has been stained with toluidine blue and shows starch grains (st), protein bodies (pb) and a nucleus (n). Marker: 10 µm; x1000 (Harries & Chrispeels 1975).
C: Seedling development of *Phaseolus vulgaris*; epigeal germination (Bewley & Black 1978).

2. Review of literature on legume storage proteins and proteases

2.1. Storage proteins of legumes

Improvement in seed protein concentration was initiated with maize, a cereal, as early as 1899 (Hopkins 1899). After 70 generations of selection for high protein, an increase from 10.9 to 25.2 % was observed (Dundley et al. 1974). However, the amount of the nutritionally poor storage protein zein was also increased. At about the same time, studies of legume proteins were initiated by identifying two globulins, legumin and vicilin (Osborne & Campbell 1898, Osborne 1924). Legumes, as the product of nitrogen fixation by symbiotic bacteria, are very important protein resources both for humans and for domestic animals.

Although legumes are rich in some essential amino acids, such as lysine, threonine, valine, isoleucine, and leucine, they have a rather poor nutritive value because of their low contents of methionine and cysteine. A greater dietary contribution could be realized by increasing the protein concentration and improving the protein quality and digestibility of legume proteins (Evans & Bandemer 1967, Ma & Bliss 1978). Thus, it is desirable to determine what scope exists for modifying the amino acid composition of legumes. Much effort has gone into characterization of the storage proteins of the seeds, study of their biosynthesis and deposition, and examination of the effects of developmental, genetic, and enviromental factors on their relative proportions (Macnicol 1983). The genetic control of development is probably the most difficult aspect of contemporary biology to investigate. The idea that certain genes are expressed only during seed development, maturation or germination is not usually doubted (Ross 1984). The expression of seed proteins is known to be regulated by both transcriptional and posttranscriptional level (Goldberg et al. 1989).

Lupin is an example of a leguminous plant with a high protein content in the seed, from 31 % to 44 % depending on the species. However, the main part of this protein is vicilins, containing only very little or no sulphur amino acids and therefore with poor nutritional value. Globulin 1, although it represents only 6 % of total globulins, has an equilibrated

amino acid composition and thus raises the overall nutritional value of the seed. According to Duranti et al. (1981) this gives indications for improving cultural practice.

Storage proteins are large multimeric molecules which are deposited in membrane-bound protein bodies and used subsequently after proteolytic beakdown as a nitrogen supply during germination (Boulter 1981). They were divided by Osborne (1924) into four groups on the basis of their solubility properties:

1. Albumins - soluble in water at neutral or slightly acidic pH

2. Globulins - insoluble in water, but soluble in salt solutions

3. Glutelins - insoluble in water or salt solutions, but soluble in strongly acidic or basic solutions

4. Prolamins - soluble in ethanol solutions, but insoluble in water

The glutelins and prolamins are the major storage proteins in the monocotyledonous plants, but are absent or present only at low levels in the dicotyledonous plants (dicots). The globulins, legumin and vicilin-type storage proteins, and occasionally also albumins, are by contrast the major storage proteins of the dicots (Ashton 1976, Wilson 1986). The classes of legume storage proteins and their properties are presented in Table 1. The relative proportions of different proteins vary widely between plant species and cultivars and furthermore environmental factors may change the subunit composition in some species (Citharel & Citharel 1986). Some legumes have a high content of lectins and proteinase inhibitors and thus these proteins, located in protein bodies, are also classified as storage proteins.

Table 1. Classes of storage proteins in seeds of legumes.

	Relative proportion (%)	Molecular weight (kDa)	Reference
Globulins			
Legumins		200 - 400 (11S)	
-Soybean (*Glycine max*) glycinin		320 - 350	Wilson 1986
-Kidney bean (*Phaseolus vulgaris*) legumin	40		Ma & Bliss 1978
-Broad bean (*Vicia faba*) legumin			Mori & Utsumi 1979
-Castor bean (*Ricinus communis*) legumin	70 - 80	219	Tully & Beevers 1976
Vicilins		110 - 190 (7S)	
-Kidney bean (*Phaseolus vulgaris*) glycoprotein II (phaseolin)		140 ± 20	Pusztai and Watt 1970 Chrispeels 1983
Albumins			
Lectins (Phytohemagglutinins)		110 -135	Wilson 1986
-Jackbean (*Canavalia ensiformis*) Concanavalin A	2 - 3		Maycox et al. 1988
-Kidney bean (*Phaseolus vulgaris*) lectins	10		Pusztai et al. 1981a, Chrispeels et al. 1984, Vitale et al. 1989
-Soybean (*Glycine max*) lectins	1 - 1.5		Liener & Pallansch 1952
Proteinase inhibitors			
-Mung bean (*Vigna radiata*)	1-6	8	Wilson 1986, Wilson & Tan-Wilson 1987
* Bowman-Birk type inhibitors		18 - 22	Shibata et al. 1986
* Kunitz type inhibitors			Mosolov et al. 1983
-Kidney bean (*Phaseolus vulgaris*) serine proteinase inhibitor	10		
Others			
-Castor bean (*Risinus communis*) albumins	10	12 (2S)	Gifford et al. 1983, Wilson 1986
-Kidney bean (*Phaseolus vulgaris*) arcelin			Osborn et al. 1988

2.1.1. Globulins (legumins and vicilins)

The relative proportions of two major classes of storage proteins, legumins (11S) and vicilins (7S), vary widely between different legume species. Typical to both proteins are high contents of glutamic acid/glutamine, aspartic acid/asparagine, and arginine (Wilson 1986). They are glycoproteins, vicilin containing about 2-6 % sugar and legumin only about 1 % or less (Pusztai & Watt 1970, Boulter 1981). Both proteins have been shown to be localized in the protein bodies (Pusztai et al. 1977, Bollini & Chrispeels 1978, Pernollet 1978).

The legumin of soybean, glycinin, has been extensively studied (Brandley et al. 1975, Kitamura et al. 1976, Staswick et al. 1981). Each glycinin molecule is composed of six acidic (Mr 37 000 to 45 000) and six basic (Mr 20 000) subunits. Each acidic chain is disulphide-bonded to a basic chain. The amounts of different acidic and basic chains vary between different cultivars.

The presence of multiple forms has also been reported in the vicilins of the legumes, including peas and beans (Boulter et al. 1979, Chen et al. 1984).These are the most intensively studied storage proteins, also called phaseolins (Sturm et al. 1987) and glycoprotein II (Pusztai & Watt 1970). The majority of the 7S globulin fraction of soybean is composed of the vicilin ß-conglycinin (Thanh & Shibasaki 1978), which can be separated by ion exchange chromatography into six distinct species.

2.1.2. Albumins

2.1.2.1. Lectins

Lectins are common in the seeds of legumes (Goldstein & Hayes 1978, Pusztai et al. 1981a and 1983). The highest content of lectins (10 %) was found in kidney bean, *Phaseolus*

vulgaris, in which five isolectins with different subunit compositions were characterized (Pusztai et al. 1983, Vitale et al. 1984 and 1989). They are encoded by a small gene family and each lectin contains two types of closely related polypeptides, E and L. These may be combined to form isolectins with subunit compositions of E_4, E_3L, E_2L_2, E_1L_3, and L_4. The subunits may also be identical as in the case of concanavalin A, a lectin of soybean seeds. Isolectins are also known as phytohemagglutinins, reflecting their ability to agglutinate erythrocytes from man and a number of animal species.

The biological function of the seed lectins is not well established (Pusztai et al. 1981a), although they have been studied rather extensively with regard to their toxicity and carbohydrate specificity. Bean lectins in the diet have been shown to cause a specific immune response (Pusztai et al. 1981b) and to bind to the luminal surface of the small intestine (Kimura et al. 1986). These phenomena can be explained by the recent observation (Vitale et al. 1989) that lectins play a role in plant defence mechanisms; e.g. arcelin (a protein similar to lectin) is toxic to an important bean pest, *Zabrotes subfasciatus*. The lectins are synthesized early in maturing seeds (Pusztai et al. 1981a) and in contrast to the 11S and 2S protein fractions they are mobilized slowly from endosperm of germinating castor bean and are present after the other proteins have been completely broken down (Gifford et al. 1983).

2.1.2.2. Proteinase inhibitors

The plant proteinase inhibitors are a diverse group of generally low molecular weight proteins. They inactivate animal and some plant proteinases by forming complexes with them (Ryan 1973) and may have a function as controllers of proteolysis or protectors from exogenous proteinases (Wilson 1986). Legume seeds have particularly high concentrations of inhibitors, which are divided into two families (1) Bowman-Birk type trypsin inhibitors and (2) Kunitz type inhibitors (Wilson 1986). A number of animal and bacterial enzymes have been observed to be inhibited by Bowman-Birk type inhibitors, including mammalian trypsin, chymotrypsin and elastase, as well as the bacterial proteinase subtilisin (Laskowski

& Kato 1980). The high content of half-cystine of the Bowman-Birk type inhibitors means that they contain a disproportionately high amount of the seed's sulphur reserves of the seed and thus are important to the legume seed as storage proteins. An exception to the division into two inhibitor classes is a specific inhibitor of the serine proteinase of microorganisms, isolated from kidney bean seeds, which does not contain cystine residues (Mosolov et al. 1983). Winged bean seeds (*Psophocarpus tetragoholobus*) contain more than ten proteinase inhbitors, seven of which are Kunitz type inhibitors with one or two reactive sites for the target enzyme, e.g. α-chymotrypsin (Shibata et al. 1986).

2.1.2.3. Low-molecular-weight albumins

In contrast to lectins the low-molecular-weight albumins are degraded rapidly during germination, mainly disappearing within 2 days of imbibition. Albumins, with sedimentation coefficients of 2S, are localized in the proteinaceous matrix of protein bodies of castor bean seeds and constitute about 40 % of the total protein of the protein bodies (Gifford et al. 1983, Wilson 1986). It has been suggested that low-molecular-weight albumins have an evolutionary relationship with Bowman-Birk proteinase inhibitors (Sharief and Li 1982) and with barley trypsin inhibitor (Odani et al. 1983).

2.2. Biosynthesis and processing of storage proteins

The biosynthesis and processing of phaseolin, the major storage protein of the common bean (*Phaseolus vulgaris*), has been examined extensively. Phaseolin is encoded by a small multigene family, with approximately seven phaseolin genes/haploid genome (Croy et al. 1980, Sturm et al. 1987). It is synthesized in the developing cotyledons on membrane-bound polysomes and sequestered in the lumen of the endoplasmic reticulum (Baumgartner et al.

1980) before its transport to the protein bodies (Bollini et al. 1982). Transport of the storage proteins through the Golgi complex to the protein bodies is mediated by small electron-dense vesicles. Late processing events occur in the protein bodies.

Signal sequences have been observed in the case of vacuolar and protein body proteins. A signal peptide is present on phaseolin polypeptides, since the two polypeptides formed by polysome run-off are slightly smaller than those formed by *in vitro* translation of isolated RNA (Bollini et al. 1982 and 1983).

Proteolytic processing resulting in the formation of smaller polypeptides occurs in the protein bodies (Chrispeels 1984). A thiol protease catalyzes the conversion of proglobulin to globulin in developing pumpkin cotyledons. The pH optimum for this vacuole-mediated conversion is 5.0 (Hara-Nishimura & Nishimura 1987), which corresponds to the pH inside protein bodies (Nishimura 1982).

Phaseolin polypeptides have one or two glycosylation steps and the size heterogeneity of mature phaseolins is due in part to the differential glycosylation of polypeptides of the same size class (Bollini et al. 1983). When cotyledonous mRNA is translated *in vitro*, two phaseolin polypeptide size classes of Mr 48000 (α) and 45000 (ß) are produced (Hall et al. 1978, Sun et al. 1978). *In vivo* the polypeptides are cotranslationally glycosylated with either one or two oligosaccharide chains on asparagine residues, resulting in A and B chains from α-polypeptide and C and D chains from ß-polypeptide. These glycosylated polypeptides are transported via tubular connections between ER and the Golgi into the Golgi apparatus, where the high mannose oligosaccharide chains (glycans) are further modified. The processing of the glycans in the Golgi apparatus, and later in protein bodies, involves enzymes such as glycosidases and glycosyltransferases (Bollini et al. 1983).

The mechanism of the biosynthesis of phaseolin and its transport into protein bodies is apparently similar to the corresponding mechanisms of other reserve proteins examined in this respect, such as lectins of kidney bean (Chrispeels 1984) and pea reserve proteins (Hurkman & Beevers 1982). Although the sugar content varies, being lowest in legumins and highest in lectins, the oligosaccharide chains of the reserve proteins seem always to be linked to asparagine residues (Vitale et al. 1984, Sturm et al. 1987).

2.3. Proteolytic enzymes of plants

Proteolytic enzymes (peptide bond hydrolases, proteases) are usually classified as endopeptidases or exopeptidases depending on whether they hydrolyze internal peptide bonds of a polypeptide or terminal peptide bonds.

The term proteinases is also used for the enzymes which have endopeptidase activity, the ability to hydrolyze internal peptide bonds in proteins with production of peptides. The exopeptidases can then simply be called peptidases. They attack the terminal peptide bonds and liberate C- or N-terminal amino acid residues from peptides and/or from proteins. The peptidases can therefore be classified as carboxypeptidases, aminopeptidases and dipeptidases.

The different groups of proteolytic enzymes from plant sources will be discussed in more detail below.

2.3.1. Proteinases

Although proteinase activities of seeds have been examined for a long time, enzymes have been purified mainly during recent years following the development of purification techniques. The formation of complexes between the enzyme and inactive proteins, or autolysis during the purification, have previously lead to the observation of multiple active peaks (Doi et al. 1980b). Occurrence and properties of the purified seed enzymes are represented in Table 2. The seed proteinases can be divided into four classes: cysteine, serine, metallo- and aspartic proteinases.

Table 2. Occurrence of different classes of proteinases in seeds.

Enzyme/Source	Molecular weight	pH optimum	Substrate	Reference
Cysteine proteinases				
-Kidney bean (*Phaseolus vulgaris*)	23 400	5.5, 6.5	azocasein	Nielsen & Liener 1984
		8.2	BAPNA	Csoma & Polgar 1984
-Mung bean (*Vigna radiata*)	23 000	5.1	vicilin (reserve protein)	Baumgartner & Chrispeels 1977
-*Vigna mungo*	33 000			Mitsuhashi & Minamikawa 1989
-Soybean (*Glycine max*)		4	soybean Kunitz inhibitor	Wilson et al. 1988
-Pumpkin (*Cucurbita moschata*)		5	pumpkin proglobulin	Hara-Nishimura & Nishimura 1987
-Vetch (*Vicia sativa*) proteinase A			vetch legumin	Shutov et al. 1984
-Rice (*Oryza sativa*)		5.7	casein	Abe et al. 1987
-Barley (*Hordeum vulgare*)	30 000	3.8	haemoglobin, hordein (reserve protein)	Poulle & Jones 1988
Serine proteinases				
-Mung bean (*Vigna radiata*) proteinase F	65 000	4.5	trypsin inhibitor	Wilson & Tan-Wilson 1987
-Soybean (*Glycine max*)	59 000	8 - 10	BAPA	Nishikata 1984
Metalloendopeptidases				
-Soybean (*Glycine max*)		8.0	reserve proteins	Bond & Bowles 1983
-Buckwheat (*Fagopyrum tartaricum*)	39 000	8.3	13S globulin	Dunaevsky et al. 1983
Aspartic proteinases				
-Soybean (*Glycine max*)		4.0	polypeptide (Mr 30 000)	Bond & Bowles 1983
-Buckwheat (*Fagopyrum tartaricum*)	28 000	3.5	13S globulin	Belozersky et al. 1984
-Hempseed (*Cannabis sativa*)		3.4	denaturated hemoglobin	St. Angelo et al. 1970
-Rice (*Oryza sativa*)	60-65 000	2.5-3.5	denaturated hemoglobin casein (+ 3.6 M urea)	Doi et al. 1980b
-Barley (*Hordeum vulgare*)				Siuro et al. 1988
-Wheat (*Triticum aestivum*)	58 000	4.5	gliadin (storage protein)	Belozersky et al. 1989

2.3.1.1. Cysteine proteinases (EC 3.4.22)

The cysteine proteinases are the most intensively studied proteolytic enzymes. These enzymes are composed of a single polypeptide chain having a molecular weight between 23 000 - 33 000. They are active in the acidic pH range and hydrolyze both proteins originating from animal tissues, such as haemoglobin and casein, and the storage proteins of resting seeds.

Vicilin peptidohydrolase acts on the 7S protein of ungerminated seeds of mung bean and is able to perform extensive hydrolysis (Baumgartner & Chrispeels 1977). It is absent from the ungerminated seeds and appears no later than on the third day of germination as a result of axis-dependent *de novo* synthesis (Chrispeels et al. 1976, Kern & Chrispeels 1978). The enzyme is synthesized on the rough endoplasmic reticulum and subsequently transported in the small dense vesicles into the protein bodies, where it takes part in the hydrolysis of the reserve proteins (Baumgartner et al. 1978, Van der Wilden et al. 1980). Recently Mitsuhashi & Minamikawa (1989) postulated that sulphhydryl-endopeptidase of germinating *Vigna mungo* seeds is first synthesized as the 45 000 precursor from which a 2 000 signal peptide is cleaved. The observed 43 000 polypeptide is further cleaved to give the 33 000 mature enzyme.

Leupeptin is a potent inhibitor of cysteine proteinases and has been shown to inhibit strongly the germination and seedling development of castor bean. Thus Alpi & Beevers (1981) suggested that the SH-proteinase is intimately involved in the mobilization of storage proteins. The same conclusion has been made in the case of cysteine proteinases of kidney bean (Nielsen & Liener 1984), vetch (Shutov et al. 1984) and soybean (Wilson et al. 1988). In addition to the hydrolysis of reserve proteins, cysteine proteinases seem to take part in the post-translational processing of proteins deposited in the protein bodies, e.g. in the processing of precursors of castor bean lectin (Harley & Lord 1985) and 11S globulin and trypsin inhibitor of developing pumpkin cotyledons (Hara-Nishimura & Nishimura 1987).

The hydrolyzed products of a pure reserve protein by a purified cysteine proteinase can in some cases be visualized in SDS-PAGE (Nielsen & Liener 1984, Wilson et al. 1988). The

first step in the hydrolysis of hordein, the main reserve protein of cereal barley, has been proposed to be catalyzed by GA_3-independent cysteine proteinase, whereas GA_3-induced cysteine proteinase is responsible for the release of polypeptides smaller than 15 000 and small peptides (Rastogi & Oaks 1986, Poulle & Jones 1988).

2.3.1.2. Serine proteinases (EC 3.4.21)

A trypsin-like serine protease has been purified from soybean seeds (Nishikata 1984). It is strongly inhibited by di-isopropylfluorophosphate (DFP) and tosyl-L-lysine-chloromethyl ketone (Tos-Lys-CH_2Cl) and probably is involved in the limited hydrolysis of certain physiological peptides during processing. The molecular weight of the enzyme is about double that of the cysteine proteinases and the enzyme is most active against BAPA between pH 8 and 10. A proteinase called proteinase F initiates degradation of the trypsin inhibitor in germinating mung bean (*Vigna radiata*) by releasing tetrapeptide Lys-Asp-Asp-Asp optimally at pH 4.5. The enzyme has a molecular weight of 65 000 and may be a serine proteinase (Wilson & Tan-Wilson 1987).

2.3.1.3. Metalloproteinases (EC 3.4.24)

A metallopeptidase of buckwheat seeds hydrolyzes the main reserve protein, 13S globulin, to high molecular weight fragments with maximum activity at pH 8.3 (Dunaevsky et al. 1983). Similarity was observed between preparations of 13S globulin isolated from seeds germinated for 3 days and the 13S globulin from dry seeds treated with the isolated proteinase. The combined action of a metallopeptidase(s) and exopeptidases could produce amino acids from reserve proteins at least in the early stage of soybean germination (Bond & Bowles 1983).

2.3.1.4. Aspartic proteinases (EC 3.4.23)

A proteinase resembling cathepsin D of the lysosomes of mammmalian tissues was first purified from hempseed (St. Angelo et al. 1970). A pepstatin-sensitive aspartic proteinase was later characterized in the seeds of rice (Doi et al. 1980b), soybean (Bond & Bowles 1983), buckweat (Belozersky et al. 1984), wheat (Belozersky et al. 1989), and barley (Siuro et al. 1988). All these enzymes have low pH optima, from 2.5 to 4.5, and they are predominant over cysteine proteinases in resting seeds. This implies that they have an endogenous function by initiating proteolysis of storage proteins in germinating wheat seeds (Belozersky et al. 1989).

2.3.2. Carboxypeptidases (EC 3.4.16)

Almost all the carboxypeptidases thus far isolated from plants liberate C-terminal amino acids of peptides and proteins optimally between pH 4 and 6. They are inhibited by di-isopropylfluorophosphate (DFP), indicating that they are serine enzymes (Mikola & Mikola 1980, Breddam et al. 1983). DFP does not affect any of the other well known plant peptidases or proteinases. Recently Alvarez (1989) reported that a lentil seed carboxypeptidase, with a molecular weight of 170 000 corresponding to barley carboxypeptidase IV (Mikola, L. 1983), may have a tryptophan residue in addition to the serine residue in its active site.

Carboxypeptidases have been studied especially in barley because of their importance in the brewing industry. Other studies have concentrated on carboxypeptidases of citrus fruit (Kubota et al. 1973 and 1980), water melon (Matoba & Doi 1974), wheat (Umetsu et al. 1981, Mikola, L. 1986), and cottonseed (Ihle & Dure 1972).

Five acid carboxypeptidases present in the starchy endosperm of germinating barley grains were separated from each other (Mikola & Mikola 1980, Mikola, L. 1983). The first group

of three enzymes liberates both proline and other amino acids from C-termini of peptides, provided that the penultimate residue is not proline. The second group of two enzymes requires a proline residue in the second position. Three of the observed enzymes (two from the first group and one of the second group) have molecular weights between 95 000 and 110 000 and the remaining two enzymes elute on gel chromatography at positions corresponding to Mr 45 000 and 170 000. Carboxypeptidase I of germinating barley consists of two peptide chains linked by disulphide bridges (Breddam et al. 1983). Both chains of the enzyme are translated from a single mRNA (Doan & Fincher 1988). The same five carboxypeptidases also occur in germinating wheat (Mikola, L. 1986).

High neutral metallocarboxypeptidase activity was detected in young seedlings of rice (Doi et al. 1980a,b). The enzyme is absent from the other tissues of rice and from barley and oat seeds and seedlings (Mikola & Saarinen 1986).

All the acid plant carboxypeptidases hitherto investigated are glycoproteins. For example, one enzyme of malted barley contains six residues of glucosamine and 8 % neutral sugar and another contains 15 % neutral sugar (Breddam et al. 1983 and 1985).

2.3.3. Aminopeptidases

The aminopeptidases are somewhat arbitrarily divided into two classes: The neutral aminopeptidases are also called arylamidases and naphthylamidases, because they rapidly hydrolyze peptide bonds between amino acids and aromatic amines (ß-naphthylamine, p-nitroaniline) optimally at neutral pH. The alkaline peptidases cleave off the N-terminal amino acid of dipeptides, oligopeptides or proteins at alkaline pH (Mikola, J. 1979 and 1983).

2.3.3.1. Neutral aminopeptidases (EC 3.4.11)

Neutral aminopeptidases have been found in all living tissues from which they have been assayed. They hydrolyze peptide bonds between amino acids and aromatic amines optimally near pH 7 and differ in their specificity towards the aminoacyl moiety. The molecular weights are approximately from 60 000 to 70 000 with the exception of an aminopeptidase from buckwheat seeds with a reported molecular weight of 37 000 (Wilson 1986, Ikeda et al. 1983). All the purified enzymes hydrolyze dipeptides and sequentially liberate N-terminal amino acid residues from longer peptides, usually at somewhat lower rates than from the corresponding aromatic amines (Mikola & Mikola 1986).

The wealth of data available suggests that the total number of naphthylamidases in most plants is probably five (Mikola & Mikola 1986). In addition to these enzymes, an iminopeptidase characterized by high molecular weight (220 000) and the ability to hydrolyze Pro-ß-NA and Hyp-ß-NA as well as peptides with an N-terminal proline residue has been characterized (Ninomiya et al. 1982 and 1983). The first neutral aminopeptidase of plants was purified from barley grains using phenylalanyl-ß-naphthylamide (Phe-ß-NA) as a marker substrate (Kolehmainen & Mikola 1971). Naphthylamidases have been found in all dicot seeds that have been examined for their presence, including pea (Elleman 1974), peanut (Mikola 1976), mung bean (Van der Wilden et al. 1980), common bean (Pusztai & Duncan 1971, Blätter & Feller 1988), vetch (Shutov & Polo 1983), pumpkin (Hara & Matsubara 1980), buckwheat (Ikeda et al.1983), cowpea (Wynn & Murray 1985) and castor bean (Tully & Beevers 1978).

The naphthylamidases generally appear to fall into three groups on the basis of their substrate specificities, i.e. those hydrolyzing the arylamides of (1) arginine, (2) proline, and (3) neutral and aromatic amino acids such as leucine, alanine, phenylalanine, etc. (Wilson 1986). Multiple forms within a group may be present in any particular dicot species, especially in groups (1) and (3) above. At least four aminopeptidase forms have been found in kidney bean seeds (Blätter & Feller 1988) and three in sweet bean and broad bean (Murray & Waters 1985). The naphthylamidases appear to be sulphhydryl-dependent, being inhibited

by NEM and pCMB and also to varying extents by 1,10-phenantroline. A number of the arginine-specific enzymes are inhibited by DFP and/or PMSF (Wilson 1986). In dicotyledons, naphthylamidases may assist in the mobilization of reserve proteins following seed germination (Collier & Murray 1977, Crump & Murray 1979), but generally they display highest activities in tissues with rapid rates of growth and protein turnover (Murray & Waters 1985).

2.3.3.2. Alkaline peptidases

At least one aminopeptidase (EC 3.4.11) and one dipeptidase (EC 3.4.13) can be classified into a single group, alkaline peptidases, on the basis of alkaline pH optima. These enzymes have not been as intensively studied as the neutral aminopeptidases.

In addition to their alkaline pH optima, the alkaline aminopeptidases differ from neutral aminopeptidases on the basis of molecular weight and substrate specificity. An alkaline aminopeptidase, purified almost to homogeneity from germinating barley (Sopanen & Mikola 1975), has a molecular weight of about 260 000 and a high activity against leucine amide and di- and tripeptides with N-terminal leucine or methionine. Leucine-ß-naphthylamide , on the contrary, is hydrolyzed only very slowly. The highest reaction rates are obtained at pH 8.5 to 10.5. The same enzyme is also abundant in germinating seeds of Scots pine (Salmia & Mikola 1975), peanut (Mikola 1976), and squash (Ashton & Dahmen 1967).

The properties of barley leucine aminopeptidase are rather similar to those of the well known leucine aminopeptidases of swine kidney (Spackman et al. 1955) and *Escherichia coli* (Vogt 1970). The barley aminopeptidase is inactivated in the absence of dithiothreitol or 2-mercaptoethanol, but it cannot be a "true" sulphhydryl enzyme with a reactive SH-group at the active centre, because it is not inactivated by p-hydroxymercuribenzoate (Sopanen & Mikola 1975). The squash aminopeptidases appear to be metallo-enzymes, being inhibited by

EDTA and activated by Mg^{2+} (Ashton & Dahmen 1967). A purified N-terminal exopeptidase (Mr 300 000) from *Streptococcus durans* has similar properties to thiol compounds (Machuga 1984). The enzyme is a hexameric protein; one subunit has a molecular weight of 49 400.

Another alkaline peptidase occurring in barley has also been purified to homogeneity (Sopanen 1976). This enzyme hydrolyzes dipeptides such as Ala-Gly and Ala-Leu rapidly whereas the hydrolysis of tri- and oligopeptides is not observed. The molecular weight of this dipeptidase is about 150 000 and its pH optimum is 8.8. It is composed of at least two subunits and strongly inhibited by metal chelators and suphhydryl reagents. Another plant dipeptidase was purified from cotyledons of *Cucurbita maxima* and found to be most active at about pH 8 on Leu-Gly (Ashton & Dahmen 1967). However, data on inhibitor effects and molecular properties are not available.

2.4. Functions of different groups of proteolytic enzymes in germinating legumes

Proteolysis of storage proteins, supplying the embryonic axis with nitrogen compounds during the early stages of germination, is an essential process of seed germination. According to a number of studies, hydrolysis of storage proteins may be a complex process consisting of several individual steps (Baumgartner & Chrispeels 1977, Mikola 1981, Shutov & Vaintraub 1987). An important set of questions is which proteolytic enzymes carry out the proteolysis, what is the sequence of their action, and how is this proteolysis regulated (Dunaevsky & Belozersky 1989).

In the physiological context, an inevitable consequence of the specificities of the endopeptidases and exopeptidases is that the former are responsible for the early stages of protein breakdown and the latter enzymes take over at an intermediate stage and complete the

generation of free amino acids (Barrett 1986). As is generally found with biochemical pathways, the endopeptidase-catalyzed initial stages are typically the rate-limiting ones. Once the degradation of a protein molecule has started it proceeds rapidly, with little or no accumulation of partial degradation products. Most of the known examples of limited proteolysis, such as post-translational processing of proteins and activation of enzyme precursors, are attributable to endopeptidases, but there is no doubt that exopeptidases also participate in these important aspects of biological control (Barrett 1986).

Cysteine proteinases have been shown to hydrolyze the storage proteins of resting seeds. When leupeptin, a potent inhibitor of cysteine proteinases, was suitably introduced into the endosperm of castor bean it strongly inhibited germination and seedling development (Alpi & Beevers 1981). These results suggest that the mobilization of storage proteins carried out by cysteine proteinases is involved in early seedling growth. Two distinct cysteine proteinases were identified that attack the native Kunitz soybean trypsin inhibitor: protease K1 cleaves five carboxyl-terminal amino acid residues from the native protein and peaks earlier in seedling growth than protease K2, which produces no electrophoretically recognizable products. These proteinases acting at pH 4 are partially sensitive to leupeptin and are presumably localized in the degradative vacuoles derived from fusion of the protein bodies. Protease K3, active *in vitro* at pH 8, is also involved in the degradation of Kunitz soybean trypsin inhibitor *in vivo*. A portion of the inhibitor appears to be cytosolic (Horisberger & Tacchini-Vonlanthen 1983) and thus may be subjected to attack by the enzyme (Wilson et al. 1988). A different cysteine proteinase, G1, appears to initiate degradation of glycinin, the major storage protein of soybean by hydrolyzing only an acidic chain of 15 000 (Wilson et al. 1988). A similar observation has been reported in the mung bean, *Vigna radiata*, in which degradation of the major storage globulin, vicilin, is initiated by vicilin peptidohydrolase (Baumgartner & Chrispeels 1977), whereas degradation of the Bowman-Birk-type trypsin inhibitor is initiated by another distinct protease, proteinase F (Wilson & Tan-Wilson 1987).

Recently, a cathepsin D-like proteinase from wheat seeds has been suggested to initiate the proteolysis of storage proteins in germination (Belozersky et al. 1989). The same pepstatin-sensitive activity has been observed to be dominant in the resting seeds of barley but only a

minor increase in activity takes place during germination (Mikola, L., unpublished results). Further investigations are necessary to clarify the role of the aspartic proteinase in germinating seeds.

The first step, a limited proteolysis of storage proteins in germinating seeds, appears to take place inside the protein bodies and later in the vacuoles that are formed by the fusion of protein bodies (Yatsu & Jacks 1968, Shutov & Vaintraub 1987, Wilson et al. 1988, Dunaevsky & Belozersky 1989). As germination proceeds, the water-insoluble globulin is converted into a water-soluble form as has been observed in pumpkin seeds (Hara & Matsubara 1980). It seems that after the first steps of hydrolysis effected by acid proteinases, high molar concentrations of peptides are produced. These peptides are apparently the main substrates of carboxypeptidases (Mikola 1981, Shutov & Vaintraub 1987). The end products of the activities of these acid proteases inside the protein bodies are obviously a variety of small peptides and free amino acids. Where and how these peptides are broken down into amino acids in leguminous seeds, was the main subject of the present work. The naphthylamidases are located in the cytoplasm and these enzymes have been observed to hydrolyze the derivatives of most amino acids (Shutov & Vaintraub 1987). Also high alkaline peptidase activities are present in all the seeds studied with respect to these enzymes (Mikola & Mikola 1986). Amino acids and peptides produced in the starchy endosperm of the barley grain are taken up by the scutellum, where the peptides are hydrolyzed apparently by the neutral and/or alkaline amino- and dipeptidases to amino acids (Mikola 1987).

It is not surprising that the seed and seedling contain several proteases, each specifically initiating the degradation of one or more of the seed reserve proteins. The timing and extent of reserve mobilization can be fine-tuned by regulating the appearance and quantity of these initiating proteases (Wilson et al. 1988).

It has been observed that different reserve proteins are not degraded simultaneously. The two major vicilins of lupin seeds are degraded first and at different rates, indicating a structural diversity of the two proteins (Duranti et al. 1984). Conglutin τ, a lectin, is resistant to proteolysis over the period during which the two major vicilins are hydrolysed. This

property may be a result of the higher content of bound sugar and the higher surface hydrophobicity of this protein (Duranti et al. 1981 and 1984). The lectins of castor beans, located within the protein bodies, are mobilized slowly and are present after the other protein fractions have been completely broken down (Gifford et al. 1983).The albumins, M_r 43 000 and 32 000, are on the contrary initially degraded in the cotyledons of cowpeas (Vidovic & Murray 1984).

The regulation mechanism of the protein degradation process in germinating seeds is almost unknown. Experimental evidence suggests that inhibitors do not play an important role in the prevention of germination (Morris et al. 1984). The dry mung bean seed contains an inhibitor of the major endopeptidase of the germinating seed, vicilin peptidohydrolase. Baumgartner & Chrispeels (1976) suggested that the inhibitor is not involved in regulation of the levels of vicilin peptidohydrolase because (1) the inhibitor decreases faster during germination than proteinase activity increases, and (2) the inhibitor is cytosolic in localization, whereas the proteinase is found in protein bodies. The authors hypothesized that the function of the inhibitor is protection of the cytoplasm from the proteinase in the event of accidental rupture of the protein bodies/vacuoles during degradation of storage protein. Lectins, such as concanavalin A, accumulating in protein bodies of developing jackbean, evidently also function in developing rather than in germinating seeds (Maycox et al. 1988).

3. Aims of the present study

Legumes constitute a valuable source of protein for appreciable segments of the world`s population. As the product of nitrogen fixation by symbiotic bacteria, legume proteins are available at low costs. However, these mainly have poor nutritional value and some proteins are toxic unless subjected to heat treatment. Extensive breakdown of the storage proteins is known to occur during germination. The study of how this occurs provides knowledge concerning both the mechanism of the proteolysis and the enzymes involved. It is a contrasting process to the mobilization of proteins from senescing leaves and the transport of amino-nitrogen to the developing seed. Attempts to improve the nutritional quality and agricultural utilization of legumes clearly need more information about protein mobilization. The aim of this study was to clarify the mechanism of the proteolysis, especially those steps which are not characterized or characterized poorly. In this work the occurrence and properties of alkaline peptidases in germinating kidney bean were studied extensively to determine their role in the hydrolysis of peptides produced by acid proteinases and carboxypeptidases.

The specific aims of this study are described below:

1) To find out whether alkaline peptidases, which are abundant in barley grains, also occur in kidney bean, a legume; and to characterize the partially purified enzymes (I).

2) To study the subcellular localization of the alkaline, neutral, and carboxypeptidases of resting kidney bean, and so to clarify the mechanism responsible for the hydrolysis of storage proteins (II).

3) To determine the importance of the different proteolytic enzymes in the hydrolysis of storage proteins in germinating seeds. Activities of the peptidases and proteinases were examined during germination of kidney bean (III).

4) The role of leucine aminopeptidase, which has been examined intensively in mammalian tissues, is almost unknown in plants. In one study the leucine aminopeptidase of kidney bean was purified and its molecular and chemical properties were examined (IV).

4. Materials and methods

Details of the materials and methods are given in the original publications I-IV and only a brief summary is presented here.

4.1. Plant material

Kidney beans, *Phaseolus vulgaris* L. cv. Prosessor, were obtained from Hurst Gunson Cooper and Taber Ltd., Witham, Essex, England.

The testa and embryonic axes of the resting beans were removed manually and the cotyledons were ground to a fine powder in a Moulinex electric coffee grinder (I,II). Lipids were removed with acetone at -20°C and the dried powder was used as starting material for the purification of leucine aminopeptidase (IV).

For the enzyme activity measurements of germinated seeds, the cotyledons and the axial tissues were separated and used immediately for preparation of the homogenates.

4.2. Germination

Dry beans were surface-sterilized with 1 % sodium hypochlorite and germinated in sterilized moist vermiculite at 20°C in the dark as described in (III).

4.3. Extracts and homogenates

Ground cotyledons (I) or ground cotyledons extracted with acetone (IV) were suspended in

cold water containing Mg^{2+}, DTT and glycerol (I,IV) and benzamidine (IV) to preserve the peptidase activities. The majority of the water-soluble seed proteins were precipitated by adjusting the pH of the suspension to 4.9-5.0 with acetic acid. Immediately after centrifugation the pH of the extracts was increased to 7.5 by adding 1 M Tris base. The small amounts of precipitates formed were removed by additional centrifugation.

Cotyledons and axial tissues were homogenized separately in extraction buffer at pH 7.0 for measurements of alkaline and naphthylamidase activities and at pH 5.5 for carboxypeptidase and proteinase activities. Extraction was facilitated by adding sea sand during grinding in a mortar; the separated supernatants were used immediately for the activity measurements (III).

The dry powder of cotyledons was homogenized in anhydrous glycerol containing 1 mM DTT in a Potter-Elvehjem homogenizer equipped with a suitable plunger to break cells but preserve organells (II). The homogenate was centrifuged to remove large tissue particles and used for sedimentation runs in KI/glycerol density gradients. The homogenates in dense KI/glycerol were used for flotation runs and those in buffered 65% sucrose for short sedimentation runs.

4.4. Assays of enzyme activities

The assays of leucine aminopeptidase activity (hydrolysis of 5 mM Leu-Gly-Gly and Leu-Tyr at pH 9.2) and of dipeptidase activity (hydrolysis of 10 mM Ala-Gly at pH 8.5) were performed as described earlier (Sopanen & Mikola 1975, Sopanen 1976). The enzymatic reactions were carried out at 30°C, the residual substrate was blocked by cupric ions and the liberated amino acids were estimated with TNBS-reagent. Carboxypeptidase activity was determined using 1.82 mM Z-Phe-Ala as substrate at pH 5.9 as described above but without cupric ions (Mikola & Kolehmainen 1972). Naphthylamidase activity was assayed using 0.3 mM Leu-ß-NA as substrate (Kolehmainen & Mikola 1971) at pH 6.4. Proteinase activities

(hydrolysis of haemoglobin at pH 3.7 and of casein at pH 5.4 and 7.0) were measured as described by Salmia et al. (1978), except that the soluble peptides after precipitation with 3 % TCA were assayed as described by Peterson (1977). The marker substrates and in most cases their concentrations, buffer conditions, pH optima and incubation times were optimised for each enzyme reaction.

4.5 Chromatography

Ion exchange chromatography on DEAE-Sephacel and gel permeation chromatography on Sephacryl S-300 were used to fractionate the alkaline aminopeptidase, dipeptidase and naphthylamidase activities (I).

The runs were carried out in the cold using thermostated water-jacketed columns. In addition to these chromatography steps, HPLC runs on Mono Q and Superose columns were used as purification steps for the leucine aminopeptidase (IV).

5. Results and discussion

5.1. Nitrogen content of kidney bean and its mobilization during germination (III)

The content of nitrogen in resting kidney bean was 3.7 % (measured by the Kjeldahl method), corresponding to about 23 % of protein. The beans were germinated in the dark at 20℃ in moist vermiculite. In these conditions the hydrolysis and the mobilization of storage proteins began after three days and was rapid from the 5th day. After 11 days, half of the nitrogen content had been transferred from the cotyledons to the axial tissues. At the end of the germination (after 18 days) only 10% of the original total nitrogen and 14 % of the original dry weight remained in the cotyledons (Fig.2).

5.2. Activities of different proteolytic enzymes in resting and germinating kidney bean (I,III)

Aqueous extracts prepared from cotyledons of resting kidney beans showed high activities in the hydrolysis of Leu-Tyr and Ala-Gly (Table 3), whereas the activities towards standard substrates of plant naphthylamidases and carboxypeptidases (Mikola & Mikola 1986) were lower by one or two orders of magnitude, respectively. The details of activity changes per seed during the course of germination are presented in paper III. In Table 3 the activities are presented per mg of protein of the cotyledons of resting and 11 day-germinated beans.

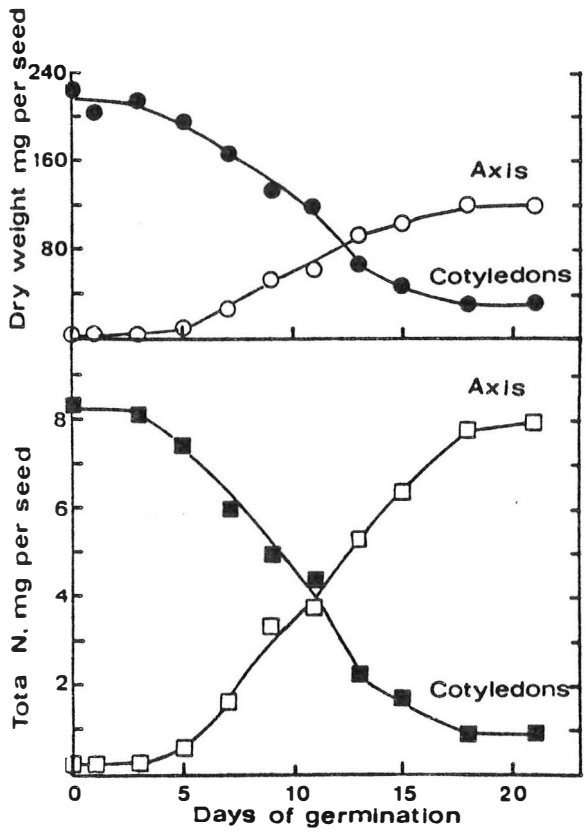


Fig. 2. Changes in dry weight and total nitrogen of kidney bean seeds germinated in the dark at 20°C.

Table 3. The activities of proteinases and peptidases in the cotyledons of resting and 11 day-germinated kidney beans against different substrates. One unit of proteinase activity liberated TCA-soluble reaction products equivalent to 1 μg of BSA in 1 min at 30°C. Peptidase activities were expressed as μmol substrate hydrolyzed per minute. All activities are presented per mg of protein.

Enzyme/substrate		Activity (units/mg protein)	
		resting seed	11 day-germinated seed
Proteinases			
haemoglobin,	pH 3.7	1.9	9.9
casein,	pH 5.4	0.52	28
casein,	pH 7.0	0.29	8.5
		Activities (units/mg protein) $x10^{-3}$	
		resting seed	11 day-germinated seed
Peptidases			
Carboxypeptidases			
Z-Phe-Ala,	pH 5.9	1.0	43
Naphthylamidases			
Leu-ß-NA,	pH 6.4	12	5.0
Leucine aminopeptidase			
Leu-Tyr,	pH 9.2	43	31
Dipeptidase			
Ala-Gly,	pH 8.5	130	65

5.2.1. Proteinases (III)

The proteinase activities were low in the resting kidney bean cotyledons, but increased rapidly during germination. The highest activity in resting seeds was assayed with haemoglobin at pH 3.7. This activity was inhibited completely by 2 μM pepstatin A, which is an inhibitor of carboxyl endopeptidases (such as pepsin and cathepsin D). Recently, acid aspartic proteinases specifically inhibited by pepstatin were purified from resting seeds of wheat (Belozersky et al. 1989) and barley (Mikola, L., unpublished results). Like in barley, the amount of the pepstatin-sensitive proteinase activity of kidney bean remained almost the same throughout the germination; the increase observed in haemoglobin hydrolysis at pH 3.7 was insensitive to pepstatin and thus may have been caused by a newly synthesized proteinase(s).

The highest proteinase activity measured in germinating beans was the caseolytic activity at pH 5.4. This activity was not inhibited by pepstatin but a partial sensitivity to leupeptin (an inhibior of thiol-proteinases and endopeptidases, Alpi & Beevers 1981) was observed as has also been reported for the activity against azocasein at pH 5.5 (Nielsen & Liener 1984).

These results support the view that at least two acid and one near-neutral proteinases are present in the cotyledons of germinating kidney bean.

5.2.2. Carboxypeptidases (I,III)

Like the proteinase activities, carboxypeptidase activity was low in resting bean and the maximum level was observed when the mobilization of nitrogen was highest. Z-Phe-Ala at pH 5.9 was used as a marker substrate (I-III). Carboxypeptidase I of germinating barley and wheat hydrolyses this substrate at a high rate (Mikola, L. 1983 and 1986) and the proportion of this enzyme increases during germination both in the above mentioned cereals and in the cow bean (*Vigna unguiculata*), a legume (Mikola, L., unpublished results). The hydrolysis of Z-Phe-Phe was also detected in this investigation (unpublished results). Obviously this is

caused by carboxypeptidase III hydrolyzing Z-Phe-Phe and Z-Ala-Phe (Mikola & Saarinen 1986). Carboxypeptidase III has a high activity in resting cowpeas, which in contrast to the other measured carboxypeptidase activities decreases during the course of germination (Mikola, L, unpublished results). The measured hydrolysis of Z-Phe-Glu, Z-Gly-Pro-Ala and Z-Pro-Ala confirms further the results of L. Mikola that the same carboxypeptidase family as in cereals also occurs in legumes but with some differences in expression.

5.2.3. Naphthylamidases (I,III)

The naphthylamidase activity (hydrolysis of Leu-ß-naphthylamide at pH 6.4) decreased during germination and was the lowest of the measured peptidase activities when the mobilization of nitrogen was highest (Table 3). The results of other research groups (Chrispeels & Boulter 1975, Crump & Murray 1979) have shown the same tendency.

There are at least three (Wynn & Murray 1985, Murray & Water 1985) or four different naphthylamidases (Blätter & Feller 1988) in leguminous seeds. The activities acting on Phe-ß-NA at pH 6.4 and on Arg-ß-NA at pH 7.1, reported as representing other naphthylamidases (Wynn & Murray 1985), were observed to be high in the resting kidney bean (unpublished results).

In DEAE-Sephacel ion exchange chromatography the bulk of the measured activities of naphthylamidases eluted between the leucine aminopeptidase and dipeptidase activities. The activity hydrolyzing Arg-ß-NA peaked slightly earlier than the two other naphthylamidase activities. In gel filtration the naphthylamidase activities eluted after the alkaline peptidases and the activity against Phe-ß-NA peaked slightly later than the two other naphthylamidase activities. The peptidase inhibitors bestatin and amastatin had different influences on the three activities: The activity against Phe-ß-NA was not inhibited, but the activity against Arg-ß-NA was inhibited totally by 10 μM amastatin and partially by 10 μM bestatin. These inhibitors decreased the activity hydrolyzing Leu-ß-NA by approximately 30 % (unpublished results).

5.2.4. Dipeptidase (I,III)

The high dipeptidase activity (hydrolysis of Ala-Gly at pH 8.5) of resting bean decreased dramatically during the course of germination. However, it was still the highest activity measured during the peak of nitrogen mobilization (Table 3).

The activity hydrolyzing Ala-Gly at pH 8.5 was separated as a single peak by ion exchange and gel chromatography (Fig. 1 and 2,I). This enzyme preparation hydrolyzed Ala-Leu more rapidly than Ala-Gly. However, Ala-Gly was used as a marker substrate for the dipeptidase activity because the alkaline aminopeptidase also hydrolyzed Ala-Leu. The molecular weight of 105 000 determined by gel fitration, is close to the 100 000 reported for a bacterial dipeptidase (Hwang et al. 1981).

The dipeptidase, partially purified by ion-exchange or gel permeation chromatography, was completely inhibited even by 1 μM bestatin, as is the bacterial enzyme mentioned above. Recently it was reported that bestatin competitively inhibited a mouse ascites tumor dipeptidase with a K_i-value of 2.7 nM (Patterson 1989). However, the maximum inhibition obtained with fresh or stored (6-48 h at 0-5℃) extracts of kidney bean varied between 20 and 50 %. A number of experiments were carried out to determine whether the unfractionated extracts contained two enzymes hydrolyzing Ala-Gly and Ala-Leu, one inhibited and the other not inhibited by bestatin. No evidence for the presence of two enzymes was obtained. The partially purified dipeptidase was inhibited completely by 10 μM p-hydroxymercuribenzoate, showing that it is an SH-enzyme. The partial inhibition by o-phenantroline may suggest a requirement for some divalent cation.

5.2.5. Leucine aminopeptidase (I,III,IV)

An alkaline peptidase activity, acting on tripeptides and some dipeptides optimally near pH 9, decreased during germination (Table 3).

This aminopeptidase was purified 317-fold from the cotyledons of resting kidney beans with a yield of 9.2 %. The enzyme rapidly liberated the N-terminal leucine from Leu-Gly-Gly but did not split the second peptide bond between the two glycines. Two other non-polar amino acids, alanine and methionine, were also liberated rather rapidly. The enzyme hydrolyzed the peptide bonds between two polar amino acid residues in Tyr-Gly and Gln-Gln. It liberated also basic amino acids, such as histidine and arginine. Although the purified aminopeptidase appeared to have a rather broad specificity with different peptides, there were at least two examples, glycine at N-termini and proline in the second position, which prevented the hydrolysis.

The purified enzyme had a large molecular size (mol. wt 360 000), which distinguishes it from all the other peptidases known in legume seeds. Studies of the molecular structure suggest that the enzyme is composed of three pairs of subunits (Mr 58 000 and 60 000), which are not linked by disulphide bridges (IV).

Similar aminopeptidase activity has been observed in other plants, such as peanuts (Mikola 1976) and Scots pine seeds (Salmia & Mikola 1975). However, the enzyme has been purified only from barley (Sopanen & Mikola 1975). The enzymatic properties of the barley enzyme have been studied extensively but its subunit composition has not been reported. However, the aminopeptidases from barley and kidney bean seem to belong to the universal group of aminopeptidases, called leucine aminopeptidases. These enzymes apparently have no significant relationship with other proteins in terms of their amino acid sequences (Cuypers et al. 1982).

Mg^{2+} ions activated the aminopeptidase of kidney beans about threefold, as has been observed for the barley enzyme (Sopanen & Mikola 1975). When the aminopeptidase was incubated with zinc ions, the activity was lost completely as was the case with the leucine aminopeptidase of hog lens (Oettegen & Taylor 1985). The crystalline enzyme of bovine lens contains two zinc ions per subunit, one of which can easily be replaced by a manganese or magnesium ion, resulting in increased peptidase activity (Carpenter & Vahl 1973, Thompson & Carpenter 1976). At least one of the zinc ions is thought to bind at or near the active site of

the enzyme (Himmelhoch 1970). Thus, the purified enzyme seems to belong to a class of two-metal peptidases, previously described for mammalian tissues (Cuypers et al. 1982).

Bestatin, which is an inhibitor of some mammalian aminopeptidases such as the leucine aminopeptidase of swine kidney (Suda et al. 1976), caused complete inhibition of the partially purified enzyme of kidney bean at a concentration of 10 μM (I). The purified aminopeptidase was inhibited competitively and the K_i value of 1.5×10^{-9} M indicated high affinity of the inhibitor to the enzyme.

5.3. Subcellular localization of the peptidases (II)

The abundance of the leucine aminopeptidase and dipeptidase suggests that they may have a major role in the degradation of storage proteins during the initial stage of germination. On the other hand their alkaline pH optima raise the question of the cellular compartment in which they function. It seems unlikely that they act inside the protein bodies, the contents of which are acidic. In order to answer this question, the localization of these enzymes was studied by subcellular fractionation in aqueous sucrose and non-aqueous glycerol media and in non-aqueous potassium iodide gradients in glycerol.

In aqueous media the organelles of dry seeds, especially protein bodies, are very fragile. Protein bodies may contain about 80 % of the legume seed proteins recovered in fractionation of cell homogenate in non-aqueous glycerol media. However, when finely ground cotyledons were homogenized in 65 % sucrose solution at pH 6.1 to minimize the rupture of protein bodies, and centrifuged rapidly through 70 % sucrose, only about 10 % of the total protein was recovered in the pellet. These results are in agreement with the observations of Pusztai et al. (1977 and 1978). The pellet probably represented partially fragmented or depleted protein bodies, whereas the bulk of the "soluble protein" was derived from the ruptured protein bodies. α-Mannosidase (EC 3.2.1.24) was used as marker enzyme. This

enzyme has been reported to be mainly localized in protein bodies and partially (15 % of the total activity) in the cell walls (McGee & Murray 1985, Van der Wilden & Chrispeels 1983). In this study the enzyme was completely located in the protein bodies and the cell wall zone when cell fractionation was accomplished using a density gradient in glycerol containing an increasing concentration of potassiun iodide (Fig. 1,II). However, only about 20 % of the α-mannosidase activity was recovered in the "protein body" pellet by sedimentation in aqueous 65/70 % sucrose solution (Table 2,II) showing that cell fractionation of resting seeds is unsuccessful in aqueous media.

The first differential centrifugation in glycerol was carried out by Yatsu & Jacks (1968), who observed that the protein bodies of resting cotton seeds contained acid proteinase activity. Protein bodies isolated from resting mung bean with 80 % glycerol contain all, or nearly all, of the carboxypeptidase and acid caseolytic activities. The cytoplasm contains all, or most, of the neutral aminopeptidase activity (naphthylamidase) and the trypsin-like activity determined using benzoyl arginine-p-nitroanilide as substrate (Harries & Chrispeels 1975).

Although the subcellular fractionation in KI/glycerol gradients carried out by Begbie (1979) were successful, the recoveries of dipeptidase and aminopeptidase activities were only 50 and 17 %, respectively. Microscopic examination of the protein body zone showed that there were a number of large cellular fragments among the protein bodies. The proportion of cytosolic enzymes occupying the interstitial space in undisrupted fragments might be stabilized relative to that released into glycerol solution. Thus, it may be speculated that the activities associated with the protein body zone might be disproportionately preserved because of the protective effect of the residual cellular structure (Fig.1,II).

The results of the three types of cell fractionation experiments (Fig. 1, Tables 1 and 2,II) showed that the two alkaline peptidases, dipeptidase and aminopeptidase, are not located in the protein bodies and are not associated with any other cellular organelles. Waters et al. (1982) studied the localization of alkaline peptidases hydrolyzing Leu-Tyr and Ala-Gly in mesophyll cells of wheat leaves and found that the two activities were localized mainly in the cytosol, with only small amounts in the chloroplasts. The cytosolic localization of the

alkaline peptidases is in contrast to the distribution of the carboxypeptidase, which is a particle-bound enzyme like α-mannosidase both in glycerol and in high-density sucrose media. When essentially pure cell walls were isolated by differential and discontinuos gradient centrifugation in glycerol (Table 3 and Fig. 2,II), most of the recovered carboxypeptidase activity was associated with protein bodies (70%) and a minor, but significant, proportion (30%) with the cell wall compartment. A possible function of carboxypeptidases in cell walls remains to be clarified. A similar distribution was found for α-mannosidase, of which 15 % of the total activity was cell wall-associated. This agrees well with the data presented by Van der Wilden & Chrispeels (1983) for the mature cotyledons of *Phaseolus vulgaris*.

5.4. Possible functions of alkaline peptidases in germinating legumes

High alkaline aminopeptidase and dipeptidase activities in the cytoplasm of the resting seed suggests their important role in the hydrolysis of reserve proteins. However, their occurrence and properties are not well known and therefore most speculations concerning the mechanism of the hydrolysis do not take their possible participation into account.

Bestatin was a potent inhibitor of the alkaline peptidases of kidney bean. It has also been shown to induce the accumulation of acid soluble peptides in rat liver *in vivo* (Takahashi et al. 1987). This can be explained if bestatin inhibits leucine aminopeptidase and arylamidase hydrolyzing the peptides. Thus, these cytosolic enzymes have an important role in the intracellular proteolytic system in animals (Takahashi et al. 1987). In cases when plant peptidases have been examined more thoroughly, they have been found to be very similar in different plants (Mikola & Mikola 1986). The leucine aminopeptidase purified in this work showed uniform properties with the mammalian enzymes. Thus, it seems likely that at least some of the proteolytic enzymes are very similar in plants and in animals. It is now possible

to suggest that peptidases are necessary in germinating seeds in order to liberate free amino acids from peptides. The results with barley grains support this conclusion (Mikola & Kolehmainen 1972, Sopanen 1979). The starchy endosperm of germinating barley grain contains the bulk of the reserve proteins, which are hydrolyzed by acid proteinases and carboxypeptidases into peptides and free amino acids taken up by the scutellum. The neutral and alkaline peptidases are absent from the starchy endosperm but have high activities in the scutellum, where they may hydrolyze the peptides to free amino acids.

Collier & Murray (1977) found that the maximum activity of Leu-ß-naphthylamidase in germinating pea cotyledons was only half that found in the developing cotyledons. Thus, it is possible that this enzyme catalyzes the protein turnover needed for the high rate of protein biosynthesis in developing seeds, rather than ensuring a high net rate of protein breakdown during germination. Because there are no observations of the alkaline peptidase activities in developing seeds, no conclusions concerning their role during seed development can be reached. The high activities in resting beans may, however, reflect their importance in developing seeds. It seems likely that the alkaline peptidases are not synthesized in the germinating seed *de novo* and that the observed decrease in enzyme activities is the result of hydrolysis of the cell content including these enzyme proteins.

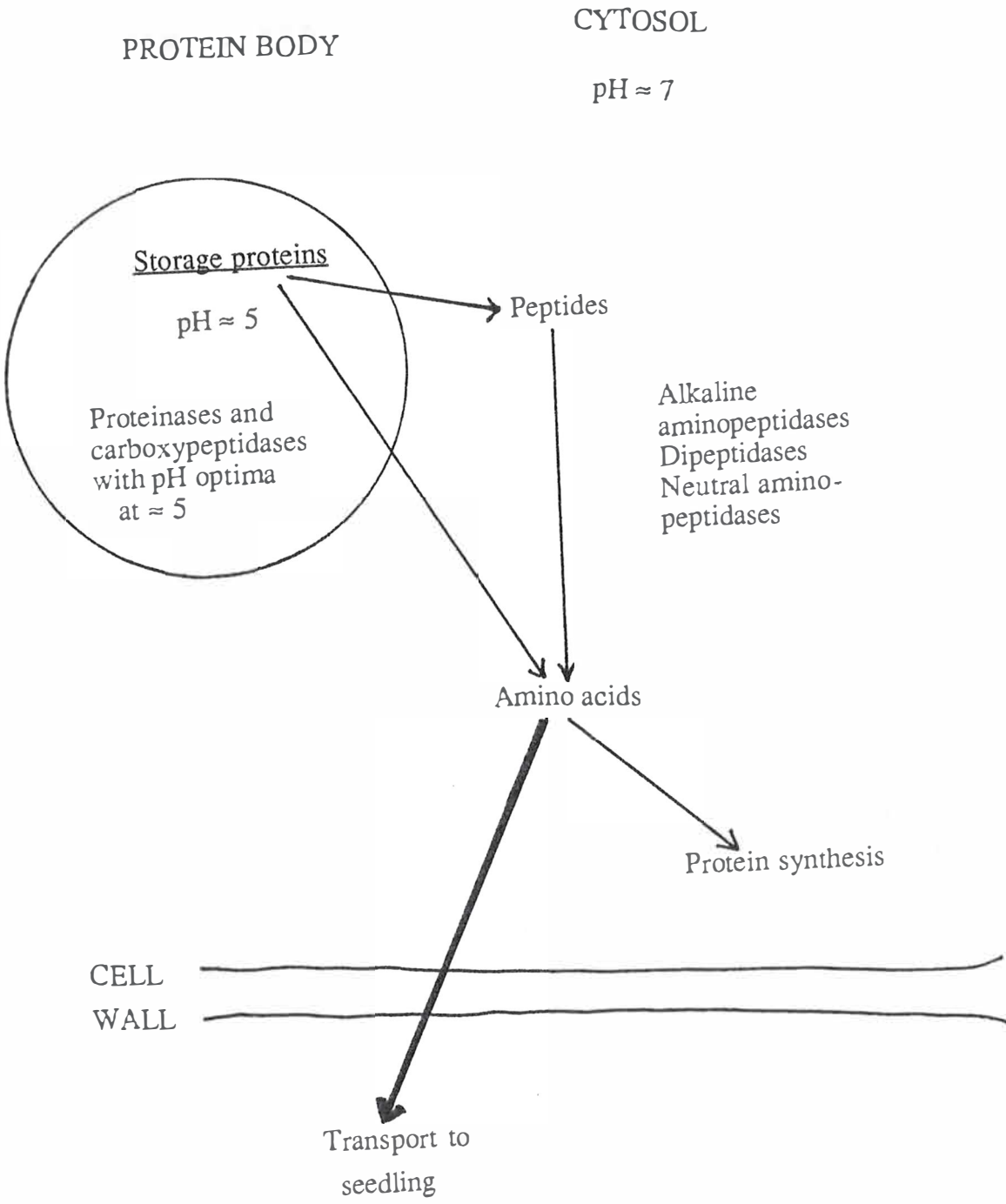
6. Concluding remarks

In the resting seed of kidney bean the protein bodies contain storage proteins that are degraded during germination. The protein bodies fuse together and form a central vacuole during the hydrolysis of proteins. Hydrolysis of the storage molecules supplies amino acids and their derivatives for the metabolism and energy needs of the developing plant.

The acid proteinases, endopeptidases and carboxypeptidases, may initiate the hydrolysis inside the protein bodies and related vacuoles. The first step is a limited specific hydrolysis catalysed most probably by cysteine proteinases, since two different cysteine proteinases have been observed to be involved in initiating the proteolysis of two distinct proteins in soybean (Wilson et al. 1988). Carboxypeptidases may act on large peptides, produced by the acid proteinases, probably resulting in small oligopeptides and free amino acids. Because the proteinase and carboxypeptidase activities are low in resting seeds, additional enzyme protein is probably synthesized in the cytoplasm and transported into the protein bodies (Van der Wilden et al. 1980).

The high alkaline aminopeptidase and dipeptidase activities suggest their important roles in the mobilization of seed reserve proteins during germination. Moreover, their localization in the cytosol is in accordance with the view that the oligopeptides arising from the proteolysis within the vacuoles may pass to the cytosol, where their hydrolysis to amino acids is completed by aminopeptidases and dipeptidases. The free amino acids are then transported to the growing seedling.

As a conclusion of this work and litterature the role of different proteolytic enzymes in mobilization of storage proteins in germinating legumes may be presented in a generalized model:


PROTEIN BODY
CYTOSOL
pH ≈ 7
Storage proteins
pH ≈ 5
Proteinases and carboxypeptidases with pH optima at ≈ 5
Peptides
Alkaline aminopeptidases
Dipeptidases
Neutral amino-peptidases
Amino acids
Protein synthesis
CELL WALL
Transport to seedling

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Selostus: Itävien palkokasvien siemenissä esiintyvät proteolyyttiset entsyymit ja niiden ominaisuudet.

Itävien palkokasvien siementen varastoproteiinit sijaitsevat lähes täysin sirkkalehtisolujen proteiinijyväsissä. Itämisen aikana varastoproteiinit hydrolysoidaan vapaiksi aminohapoiksi, jotka kuljetetaan kasvavaan taimeen ja käytetään siellä muodostuvien proteiinien biosynteesiin. Proteolyyttiset entsyymit, proteinaasit ja peptidaasit, katalysoivat varasto-proteiinien hydrolyysiä.

Lepäävän pensaspavun (*Phaseolus vulgaris*) sirkkalehdissä on alhaiset aktiivisuudet neutraaleja aminopeptidaaseja (Leu-β-NA markkerisubstraattina) ja karboksipeptidaaseja (Z-Phe-Ala markkerisubstraattina), mutta alkaalisten aminopeptidaasien aktiivisuudet ovat korkeat. Toinen alkaalisista peptidaaseista on aminopeptidaasi, joka hydrolysoi nopeimmin leusiini- ja alaniinitähteet di- ja oligopeptidien aminopäästä pH-optimin ollessa noin yhdeksän. Toinen peptidaaseista on dipeptidaasi, joka pilkkoo dipeptidejä, esim. Ala-Gly:tä aminohapoiksi pH-optimin ollessa 8,5. Molemmat alkaaliset peptidaasit voitiin erottaa neutraaleista aminopeptidaaseista sekä ioninvaihto- että geelisuodatus-kromatografialla. Puhdistetun aminopeptidaasin suuri molekyylikoko (360 000) erottaa sen muista tunnetuista palkokasvien peptidaaseista. Ominaisuuksiensa perusteella se on leusiiniaminopeptidaasi, joka tunnetaan erittäin hyvin esimerkiksi eläinsoluista.

Alkaalisten peptidaasien todettin olevan sytosolisia entsyymejä kokeissa, joissa soluorganellien fraktiointi suoritettiin sekä vedettömissä että vesipitoisissa liuoksissa. Suurin osa karboksipeptidaasiaktiivisuudesta (70 %) oli proteiinijyväsissä, mutta merkittävä osuus (30 %) myös soluseinissä.

Neutraalit ja alkaaliset peptidaasiaktiivisuudet alenivat itämisen kuluessa. Kun taas karboksipeptidaasi- ja endoproteinaasiaktiivisuudet kasvoivat ja saavuttivat huippuarvot samanaikaisesti, kun typen kuljetus siemenestä kasvavaan taimeen oli nopeimmillaan. Kuitenkaan itämisen missään vaiheessa karboksipeptidaasiaktiivisuus ei ollut alkaalisia peptidaasiaktiivisuuksia korkeampi. Näinollen näyttää todennäköiseltä, että alkaalisilla peptidaaseilla on merkittävä tehtävä varastoproteiinien hydrolyysissä.

Happamat proteinaasit, endopeptidaasit ja karboksipeptidaasit, aloittavat varastoproteiinien hydrolyysin proteiinijyvästen sisällä, minkä seurauksena muodostuu vapaita oligopeptidejä ja aminohappoja. Nämä hydrolyysituotteet siirtyvät sytosoliin, jossa peptidit pilkottaneen edelleen aminopeptidaasien ja dipeptidaasien katalysoimissa reaktioissa aminohapoiksi. Muodostuneet aminohapot kuljetetaan kasvavaan taimeen.

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