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Cysteine-containing peptides are produced by sequential clipping, but not released, from lupin 11S storage globulin during early germination

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Abstract: The digestion of the seed storage proteins is a finely regulated process operated by several proteases whose action is influenced by the exposure of specific regions, which became progressively available upon their action. We focused our study on the initial stages of germination, where more subtle modifications to the storage proteins are expected. Small-size peptides containing cysteine residues and other possible metal-binding regions are *de facto* produced but are not released from the "parental" protein since they remain bound trough disulphide bridges. The meaning of these findings is discussed.

Keywords: Seed storage proteins; proteolysis; germination.

1 Introduction

In mature legume seeds the majority of proteins belong to the so-called "Storage Proteins" (SP). They have no enzyme activities and act as amino acid reserves destined to support the nascent seedling. In legume seed SPs are globulins usually classified according to their sedimentation coefficients (S) as 7S (also named as vicilins) and 11S (known with the trivial name of legumins). The 11S globulins are hexameric proteins of about 300 kDa. Each monomer is made by two subunits, which origin by a single precursor [1]. As all SPs, they are synthesized during seed developing and deposited inside the cells in specific membrane-bound organelles, called Protein Bodies (PBs), in which a variety of proteins and enzyme are also stored [1,2].

Lupin 11S globulin accounts up to 35% of the total SPs and is formed by polypeptides that originated from Post-Translational Proteolysis (PTP) at a single cleavage site of few precursors polypeptides of about 60 kDa, occurring during seed development [3]. This PTP thus forms two polypeptide chains, also referred to as acidic (α) and basic (β) chains, linked by disulphide bonds, which represent the legumin monomer. In mature *Lupinus albus* seeds are present α-polypeptides with M*r*s ranging from 42–52 kDa and β polypeptides of 20–22 kDa [4]. Such heterogeneity arises from the action of proteases acting during seed filling. The extent of this processing has no observed parallel in other plant species, including pea and soybean, where the endogenous cleavage of legumins during seed developing has been described previously [5].

Seed germination is a complex physiological process where many biochemical processes are initiated or resumed following water imbibition [6]. SPs are hydrolysed up to free amino acids to support the seedling growth. The complete degradation of storage proteins takes place inside the storage organelle. This process is usually triggered by pre-existing and by newly formed proteases [3,7]. Limited proteolysis that triggers unlimited degradation of storage globulins are catalysed by low specificity endopeptidases [3]. The final products of the action of the proteases are free amino acids and small peptides, that in dicots, are transported outside of the PBs into the cytosol, where the peptides will be degraded further to amino acids by the action of aminopeptidases [1]. Structural features of SPs prevent cleavage by proteinases that are simultaneously present in the same compartment. It is generally accepted that the structural compactness of SPs is an important feature for their controlled proteolysis. Disulphide bridges, which are formed between the domains of legumin precursor, stabilize the conformation of mature legumin subunits [1,3]. Cleavage of susceptible

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sites causes the destabilization of the tertiary structure of storage globulins and bestows susceptibility to unlimited proteolysis [2].

Here we show the results about the effects of the early proteolytic events during germination occurring to lupin legumin, that lead to the production of peptides involved in disulphide bridges. Consequences on the metal-binding capacities of the protein are also discussed.

2 Material and Methods

L. albus. seeds (cv. Multitalia) were generously supplied by Dr. A. Conocchiari (Agroservice S.p.A., Italy). All chemicals were from Sigma-Aldrich (Milano, Italy) unless otherwise indicated. Protein concentrations were determined according to the Bradford assay [8], using bovine serum albumin as a standard. Before germination, seeds were surface-sterilized with 0.2% sodium hypochloride for 20 min, rinsed thoroughly with distilled water, vernalized overnight at 4°C, and then germinated at 22°C for 48, 72 and 96 hours. At the end of the incubation time, the seeds were immediately frozen with liquid nitrogen and kept at -80°C.

Purification of lupin 11S globulin (legumin) from mature and germinated seeds was performed using a previously described procedure based on anion exchange chromatography separation [7]. To prevent hydrolysis during the procedure, proteins were extracted in the presence of a protease inhibitors cocktail. At the end of the purification, the protein was extensively dialyzed against water, lyophilized and kept at -20°C in a sealed jar until used.

Reactive cysteine thiols were measured essentially according to Iametti et al. [9]. Protein samples were dissolved (1 mg mL^{-1}) in solution containing 8 M urea and, when indicated, 10 mM DTT. After 10 min incubation at room temperature, excess DTT was removed by rapid gel filtration on syringe-type Sephadex G-25 columns. DTNB (0.9 mL, 2 mM in 50 mM sodium phosphate buffer, pH 7.5) was added to 0.1 mL of the eluted protein. After 15 min incubation at room temperature, solutions were centrifuged for 5 min at 12000 × *g*, and the absorbance of the supernatants was red at 412 nm. Each sample has been analysed in triplicate.

SDS-PAGE was carried out on 12% or 16% polyacrylamide gels according to Laemmli [10], under reducing conditions, using a MiniProtean III electrophoresis apparatus (Bio-Rad). Runs were carried out at constant 16 mA for each gel. Coomassie Brilliant Blue or silver staining [11] was used for gel staining as indicated. Molecular weight markers were LMW-SDS Marker Kit and Low-range Amersham Rainbow Marker from GE Healthcare (Milan, Italy).

Analysis of the oligomeric structures of legumin were carried out by size exclusion chromatography using a Waters HPLC (model 625, Milford, MA) and a Superose 12 HR 10/30 column (GE Healthcare) equilibrated in 50 mM sodium phosphate buffer, pH 7.5, containing 0.1 M NaCl. The calibration curve for M*r* assessment was set using thyroglobulin (670 kDa), amylase (200 kDa), glucose oxidase (180 kDa), alcohol dehydrogenase (150 kDa), transferrin (76 kDa), bovine serum albumin (67 kDa) and egg albumin (45 kDa) as standard protein markers. Protein elution was monitored at 280 nm.

3 Results

Lupin legumin was purified from germinating seeds at 48, 72 and 96 hrs after the onset of germination. Dry seeds were used as control. The SDS-PAGE patterns are shown in Figure 1A. Their most striking was the appearance of a band of about 60 kDa, that rapidly appeared and started to fade between 72 and 96 hrs. This polypeptide is most likely the legumin precursor, not yet processed, which origin from the translation of stored mRNA, namely pre-existing messenger trapped in the cotyledonary tissue during the desiccation of the mature seed, as previously reported for lupin [5]. The group of polypeptides with M*r* between 40 and 50 kDa, namely the legumin α chains, varied quantitatively, indicating that proteolysis took place. The appearing of apparently new polypeptides in this M*r* range represent intermediate products rather than newly expressed protein [1-3]. The β subunit of about 20 kDa remained unvaried in all samples.

Analysis of the oligomeric structures of legumin was performed by gel filtration chromatography under native conditions. The results are shown in Figure 1B. The global native arrangement of the legumin monomers was essentially maintained during the time course at the early stages of germination, since no shifts of M*r* could be detected. Only the sample collected at 96 hrs showed a modest decrease in size, indicating that a significant proteolytic degradation occurred and that some regions close to the protein surface or otherwise on it began to be trimmed off.

To assess any change of the disulphide bonds pattern during germination, we measured the reacting cysteine thiols by using the Elmann reagent [12]. The results are summarized in Table 1. Analysis of 8M-denatured, non-reduced proteins indicated the absence of free cysteines.

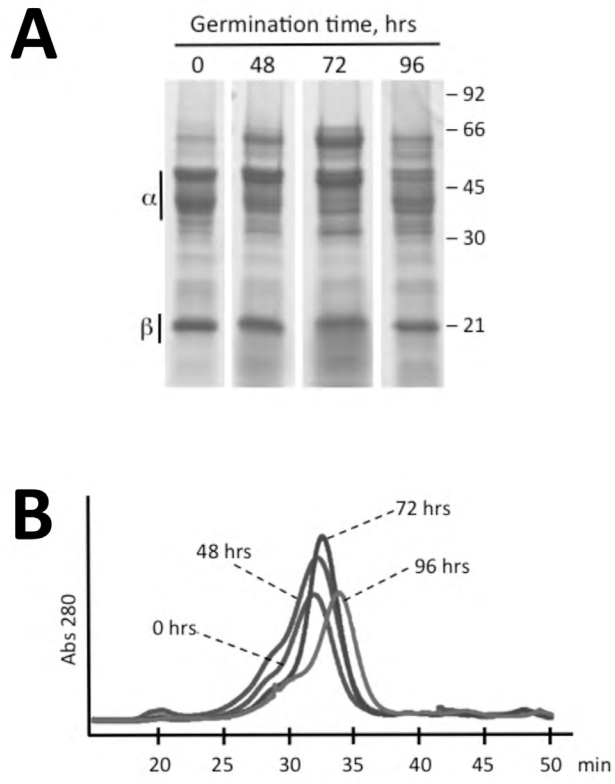

Fig. 1. Lupin 11S globulin analysis during germination time course. **A:** SDS-PAGE under reducing condition. α indicates the polypeptide family forming the major subunit. β indicate the position of the small subunit. Weight molecular markers are reported as kDa. **B:** Gel-filtration under native conditions.

Thus, all –SH groups are involved in disulphides bonds even at a late stage off germination, when hydrolysis operated by endogenous proteases is evident. Instead, when denaturation was carried out in the presence of the reducing agent, some differences among the sample became evident. Although experimental data are impaired by a non negligible standard error, it appears fairly clear that thiol groups are determined in even number in all cases.

Table 1. Number of cysteine residues in legumin protein during germination time course, assesssed under not-reducing and reducing conditions.

Germination time (hrs)	# Cys	
	- DTT	+ DTT
0	0,5 ± 0.2	6.3 ± 0.6
48	0.3 ± 0.2	4.1 ± 0.3
72	0.3 ± 0.1	3.7 ± 0.2
96	0.6 ± 0.3	2.2 ± 0.3

Figure 2 reports the electrophoretic patterns of the fractions retained by the gel-filtration spun columns. The left gel concerns the unreduced proteins, whereas the right gel shows the separation of the reduced samples. In this latter case some small-sized polypeptides are also appearing, indicating that cysteine-containing peptide may be released from 11S globulin only after reduction.

4 Discussion

Dramatic changes occur within the seed's tissues during germination, when anabolic and catabolic events inherently overlap and interplay. The digestion of the seed storage proteins is a key process aimed to provide free amino acids essential to support the seedlings growth. This complex process is operated by pre-existing and *de novo* synthesized proteases [1-3]. We focused our study on the initial stages of germination, where more subtle modifications to the storage proteins are expected, to contribute in shedding some light on the possible structural repercussions occurring to legumin during proteolysis. As a matter of facts, small structural modifications are at the basis of the controlled degradation of the storage reserves of the germinating seed. It has been previously shown that the first step of the lupin legumin degradation is the selective removal of a peptide of about 3 kDa located at the surface of the protein, due to the action of a highly specific protease cutting at a double arginine sequence. The core of this peptide is formed by two consecutive stretches, the first made of six histidine residues and the second of nine glutamic acids [7]. The loss of this peptide apparently did not cause major structural variations in the compactness of the legumin, as evidenced by the chromatographic determination shown in Figure 1B. Due to its peculiar amino acid composition, this peptide is likely located at the surface of the native protein, being charged negatively at physiological pH. It has been postulated that it may be involved in metal binding, acting as a carrier for divalent ions [7], once it is released from the "parental" protein.

In the present work we followed the fate of the peptides originating by the proteolytic processing located around the cysteine residue, all involved in disulphide bridges in the native legumin. According to the deduced primary structure of Lupin legumin (Figure 3), all cysteine of the β polypeptide must be involved in inter-chain bonds. Thus, intra-chain bonds may be found only in the major subunits of the monomers. It has been previously showed, and here we confirm (Figure 1A), that the β subunit is hydrolysed only in the final stages of germination [2,4]. It follows that

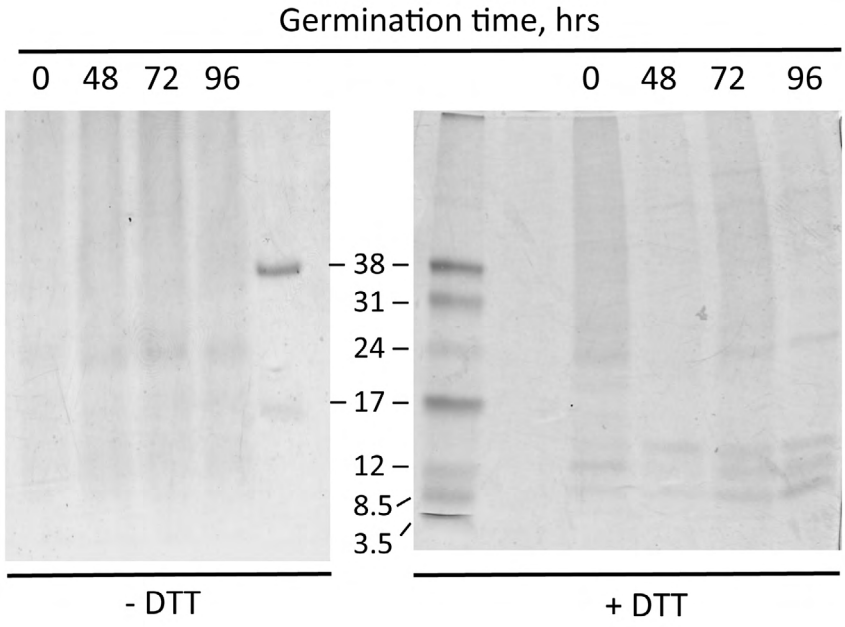

Fig. 2. SDS-PAGE analysis of the retained fraction obtained by gel filtration from the purified 11S globulin during germination time course. On the left gel the proteins were denatured with urea before the chromatography, whereas the proteins on the second gel were treated with urea and DTT.

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STFRQQPQENECQFQRLNALEPDNTVQSEAGTIETWNPKNDELRCAGVALSRCTIQRNGL   60
RRPFYTNAPQEIYIQQGRGIFGMIFPGCGETYEEPQESEKGQGPRPQDRHQKVEHFKEGD  120
IIAVPTGIPFWMYNDGQTPVVAITLIDTTNLDNQLDQIPRRFYLSGNQEQEFLQYQEKEG  180
GQGQQQEGGNVLSGFDDEFLEEALSVNKEIVRNIKGKNDDREGGIVEVKGGLKVIIPPTM  240
RPRHGREEEEEEEEEDERRGDRRRRHPHHHHHEEEEEEEEEWSHQVRRVRRPHRHHHHRK  300
DRNGLEETLCTMKLRHNIGESTSPDAYNPQAGRFKTLTSIDFPILGWLGLAAEHGSIYKN  360
ALFVPYYNVNANSILYVLNGSAWFQVVDCSGNAVFNGELNEGQVLTIPQNYAAAIKSLSD  420
NFRYVAFKTNDIPQIATLAGANSEISALPLEVVAHAFNLNRDQARQLKNNNPYKFLVPPP  480
QSQLRAVA                                                       488
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Fig. 3. Deduced amino acid sequence of lupin 11S globulin precursor (UniProtKB: Q53I54). The signal peptide has been removed. Black blocks indicate the cysteine residues. Residues in bold (at positions 110, 112 and 118) indicate a possible metal-binding active site typical of cupin proteins. The cleavage site originating acidic and basic subunits of the 11S globulin precursor is underlined. The basic subunit is reported in italics.

the peptides involved in disulphide bonds that we have shown to be trimmed derive from the α subunit.

Measurement of reactive thiols (Table 1 and Figure 2) indicated that no release of peptides from the parental protein occurs, since they remain bound to the remainder of the protein through disulphide bridges. It has been shown that in several cases reduction of seed proteins during germination is conducted by *ex novo* synthesized thioredoxin h [13]. It is likely that in our case a possible action of thioredoxin h became evident at later times. The reduction of disulphide groups of wheat and rice SPs has been shown to increase progressively during seed germination [14]. Thus, reduction of the disulphide bridges may also represent a key event in the regulation of the legumin breakdown. Our results indicate that cysteine-containing peptides are produced sequentially. The first peptide appears within 48 hrs from the onset of germination, and the second is appearing later, typically between 72 and 96 hrs. It remain to be determined if the first event is necessary to trigger the production of the second peptide or whether the two have no mutual relationship and thus other proteolytic events must take place in due time.

In homologous legumin proteins from different origin, the intra-chain bonds are positioned in slightly

buried regions compared with the inter-chain bond. In addition, the inter-chain disulphide bonds are located on the interface between protomers. These bonds could contribute to structural stability and folding of the protein.

Legumins belong to the cupin superfamily, which present a typical conserved β-barrel fold. Studies based on sequence alignment reveal that most plant cupins contain a single metal-binding site, characterized by two conserved histidines and a glutamic acid, able to bind manganese ions [15]. Although legumins are not the most important proteins with regard to metal-binding capacity, the lupin one presents some molecular features that indicate potential metal-binding sites other that the mentioned histidine/glutamate-rich peptide [7]. Lupin legumin (Figure 3) shows a conserved cupin metal-binding site located close to one cysteine in the major subunit, in which the second hystidine is substitute by a lysine. Such sequence variation is also present in few other legumin sequences found in peanut, soybean and fababean [16] and could alter the binding features of the site.

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Contributions: Conceived and designed the experiments: JC, AS. Performed the experiments: AS, JC, EG, MM. Analysed data: JC, AS, EG, MD. Contributed reagents/materials/analysis tools: AS, MD. Wrote the paper: AS, JC.

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