JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Metal Ions Restore the Proteolytic Resistance of Denatured Conglutin γ, a Lupin Seed Glycoprotein, by Promoting Its Refolding

MARCELLO DURANTI,*,† ANNALISA DI CATALDO,† FABIO SESSA,† ALESSIO SCARAFONI,† AND FABRIZIO CECILIANI‡

Dipartimento di Scienze Molecolari Agroalimentari and Istituto di Fisiologia Veterinaria e Biochimica, Università degli Studi di Milano, Milano, Italy

The susceptibility to trypsin of conglutin γ, a lupin seed glycoprotein affected by this enzyme only when in a non-native conformation, was used to study the effect of Zn^{2+} and other metal ions on the structural dynamics of the protein. When acid-treated trypsin-susceptible conglutin γ was incubated at neutral pH in the presence of Zn^{2+}, it became resistant to tryptic attack, contrary to the protein treated in the absence of Zn^{2+}. The time course of this refolding event has been quantitatively evaluated by SDS-PAGE. Amino acid sequencing of the major polypeptide fragments, produced by trypsin before completion of the refolding process, indicated that only a few cleavable bonds were accessible to the enzyme. This suggested that the presence of metal ions affected the pathway of degradation of the protein, by inducing its folding. Among the other metal ions tested, Ni^{2+} also promoted the adoption of a trypsin-resistant conformation of conglutin γ, whereas Mn^{2+} and Ca^{2+} had only much lower effects. The relevance of these findings for a deeper understanding of the in vivo degradation of plant food proteins and how it is affected by metal ions are discussed.

KEYWORDS: **Conglutin γ; *Lupinus albus*; Ni^{2+}; renaturation; trypsin; Zn^{2+}**

INTRODUCTION

The molecular understanding of plant protein structural dynamics in relation to their susceptibilities to proteolytic enzymes and the elucidation of the factors affecting these processes are consistent with the oriented utilization of plant proteins for human and animal nutrition. The degradability of food proteins, by affecting amino acid bioavailability, is a relevant issue, especially for the seed globulins, known for being more recalcitrant to proteolysis than the animal proteins (*1*). On the other hand, limited proteolysis of plant proteins may represent a tool for their nutritional improvement (*2*), their functionalization (*3*), or the liberation of bioactive peptide fragments, both with direct effects on cell metabolism or as binders of metals, hormones, and other small active molecules.

A relevant example of seed protein is lupin conglutin γ, a glycoprotein (*4*) of 46 kDa molecular mass, made up of two −S−S− bound subunits of 29 and 17 kDa, respectively (*5*). Indeed, in addition to its relative abundance in lupin cotyledons, 5−6% of the total proteins, this protein shows several features that make it an extremely interesting seed protein. Its deduced amino acid sequence (*6*) markedly differs from that of the canonical legume storage proteins with many fewer acidic amino acid residues and more lysine, cysteine, tryptophan, and hydroxylated amino acid residues (*4*). Conglutin γ is not degraded during seed germination (*7*, *8*) and is also resistant to trypsin in vitro when purified under native conditions (*9*). Additionally, although conglutin γ is primarily deposited in the protein storage vacuoles of the developing lupin seeds (*10*), it was also located in the cytoplasmic spaces between the protein bodies (*11*), within the epidermal cotyledonary cells (*10*), and in the intercellular spaces of the germinating cotyledons (*12*). A number of data (*10*, *13*; F. Sessa, A. Di Cataldo, A. Scarafoni, and M. Duranti, unpublished results) have shown a spread of the presence of conglutin γ-like proteins among several leguminous species.

In a previous work (*14*), the resistance of native conglutin γ to trypsin, while the unfolded protein is fully degraded, was used as an effective molecular probe to show that the folding of the acid-denatured protein to a stable conformation is favorably affected by the presence of the N-linked saccharide side chain. More recently, the interaction of conglutin γ with a number of metal ions and its binding to both Zn^{2+} and Ni^{2+} in metal affinity chromatography have been described. Other metals, such as Mn^{2+}, Mg^{2+}, and Ca^{2+}, were found to be ineffective (*15*). However, the specificity and the functional reasons of the metal binding activity remain obscure.

The aim of this work was to study the effect of the interaction of Zn^{2+} and other metal ions with denatured conglutin γ on the

* Address correspondence to this author at the Dipartimento di Scienze Molecolari Agroalimentari, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy (telephone +39-02-2663662; fax +39-02-70633062; e-mail marcello.duranti@unimi.it).

† Dipartimento di Scienze Molecolari Agroalimentari.

‡ Istituto di Fisiologia Veterinaria e Biochimica.

10.1021/jf010512c CCC: $22.00
Published on Web 02/26/2002

adoption of a trypsin-resistant conformation and to get insight into the degradation dynamics of this nutritionally/functionally relevant metal binding plant protein. To these purposes, the modalities and the kinetics of the metal-induced restoration of the resistance against trypsin of denatured conglutin γ and the identification of the major polypeptide fragments liberated by this enzyme are presented.

MATERIALS AND METHODS

Plant Material. *Lupinus albus* L. seeds of the sweet Multitalia variety were kindly provided by L. Postiglione and M. Fagnano (University of Naples, Italy).

Purification of Conglutin γ from Mature Lupin Seeds. Conglutin γ was purified according to the method of Duranti et al. (*16*) under non-denaturing conditions by using a combination of anion and cation exchange chromatography. This form of the protein was proved to be fully resistant to trypsin. A further purification step was accomplished by reversed phase (RP) HPLC on a Symmetry 300 C_{18} column (0.46 cm $\times$ 25 cm) (Waters) equilibrated with 0.1% TFA in Milli-Q water, pH 2.2, in conditions that cause the protein to be cleaved by trypsin (*14*). Elution of the protein was obtained with a continuous gradient from 0 to 75% acetonitrile in 75 min, at a flow rate of 0.8 mL/min. The purified protein was dried in a Speed Vac (Savant) and resuspended in the appropriate buffer for the renaturation trials performed in this work (see below). Whenever the native conglutin γ was required for control experiments, the last purification step was omitted.

Conglutin γ concentrations were determined by measuring the absorbances at 280 nm of the solutions and assuming an absorption coefficient of 1 for a 1 mg/mL solution (*17*). A molecular weight of 46 kDa was used to calculate molar concentrations of the protein. The homogeneity of conglutin γ preparations was checked by SDS-PAGE.

Use of Trypsin as a Refolding Probe of Conglutin γ. Acid treatment of conglutin γ was previously shown to make the protein sensitive to trypsin (*14*). On this basis, RP-HPLC-purified conglutin γ (10.6 nmol) was dissolved in 1 mL of 0.1% TFA in Milli-Q water containing $ZnCl_2$ at the desired concentration and trypsin (Sigma, catalog no. T1426) at a final concentration of 0.82 μM. A control without Zn^{2+} was also set up. The pH of the solutions was then brought to 7.2 with 3 M Tris base, and identical aliquots were withdrawn after 1.5 h of incubation with the enzyme at 37 °C.

To allow the refolding of acid-treated conglutin γ to take place, solutions of the protein in the presence or absence of Zn^{2+}, after the adjustment to neutrality, were incubated at 20 °C prior to trypsin treatment. After fixed preincubation times, trypsin in the same ratios as above was added and allowed to act for 1.5 h. SDS-PAGE was used to determine the relative amounts of uncleaved conglutin γ at various refolding times, thus allowing estimates of the refolding kinetics of the protein in the presence and absence of Zn^{2+}.

Electrophoretic and Sequencing Techniques. SDS-PAGE was carried out essentially as described by Laemmli (*18*) on 12.5 or 15% polyacrylamide gels. Electrophoretic separations were performed by using either a Minigel Bio-Rad system or a Hoefer electrophoresis unit (16 $\times$ 18 cm gel) in order to get a more accurate estimation of the polypeptide molecular weights. Gels were stained with Coomassie Brilliant Blue R250. The relative molecular masses (M_r) of the polypeptide bands were determined by comparison with a standard protein solution (Pharmacia) containing phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), egg albumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa). In this work the molecular mass of the uncleaved unreduced conglutin γ polypeptide is that deduced by its amino acid sequence (*6*), which may not coincide with the apparent M_r observed in SDS-PAGE because the protein was also glycosylated (*5*).

Gels were digitalized by a Studio Scan II scanner (Agfa) with PhotoLook 32 (Agfa) and quantitatively processed with Cream (Kem-En-Tec, Copenhagen, Denmark) software.

The blotting of the polypeptides onto nitrocellulose membrane was carried out essentially according to the method of Towbin et al. (*19*) in a Sartorius semidry blotting apparatus at a constant current of 50 mA for 1 h at room temperature. The presence of mannose/glucose/*N*-acetylglucosamine-type carbohydrate covalently linked to the blotted polypeptides was estimated by reaction with concanavalin A and visualization by peroxidase according to the method of Hawkes (*20*).

For polypeptide sequencing, blotting onto PVDF membranes was performed according to the method of Matsudaira (*21*) in a tank apparatus Bio-Rad Trans Blot Cell at a constant current of 100 mA for 1.5 h at 4 °C. The amino-terminal amino acid sequence was determined by Edman degradation using a Procise automated liquid phase sequencer (Perkin-Elmer).

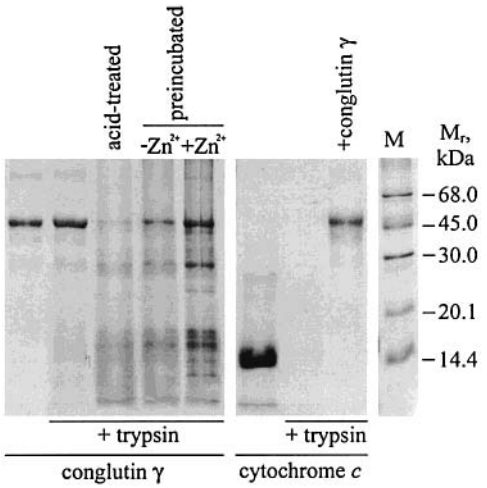


Figure 1. Effect of Zn^{2+} on the adoption of a trypsin-resistant conformation of denatured conglutin γ. Acid-treated conglutin γ (0.5 mg/mL) was incubated with trypsin in the ratio 25:1 (w/w) in a 10 mM Tris-HCl buffer, pH 7.5, for 24 h at 37 °C before and after preincubation for 4 h in the same buffer in the absence or presence of 2 mM Zn^{2+}. The same volumes of the samples with identical initial protein concentration were loaded on the SDS-PAGE gel. The gel was run under nonreducing conditions. For comparison, the patterns of untreated conglutin γ incubated with and without trypsin, in the same conditions as above, are also shown. When Zn^{2+} was absent, a more extensive degradation of the polypeptides occurred, as denoted by the clearer pattern in the corresponding lane. The action of trypsin, in the presence of 2 mM Zn^{2+}, on cytochrome *c* with and without conglutin γ added, was also checked. See Materials and Methods for details. M: marker proteins.

RESULTS

Susceptibility of Conglutin γ to Trypsin. The susceptibilities to trypsin of native and acid-treated conglutin γ and of the denatured protein preincubated at neutral pH in the absence or presence of Zn^{2+} were monitored by SDS-PAGE (**Figure 1**). Although no change of the native protein electrophoretic pattern with respect to the control without trypsin was observed, disruption of the non-covalent bonds by extremely acidic pH allowed trypsin to access the numerous arginine and lysine residues of conglutin γ. As a consequence, an almost complete degradation of the polypeptide backbone, giving rise to small peptide fragments, many of which were not retained in the gel, took place. Conversely, when acid-treated conglutin γ was preincubated in the renaturing buffer in the presence of Zn^{2+} for 4 h, most of the protein molecules became again resistant to trypsin, like the native ones, as deduced from the intensity of the intact conglutin γ band. Some polypeptide fragments were also present. The major ones have been analyzed, as described in a following section. If Zn^{2+} was not present, only a limited amount of protein was unaffected by trypsin, whereas most of it was degraded to smaller fragments, the majority of which seemingly escaped the gel, as happened with the unfolded conglutin γ.

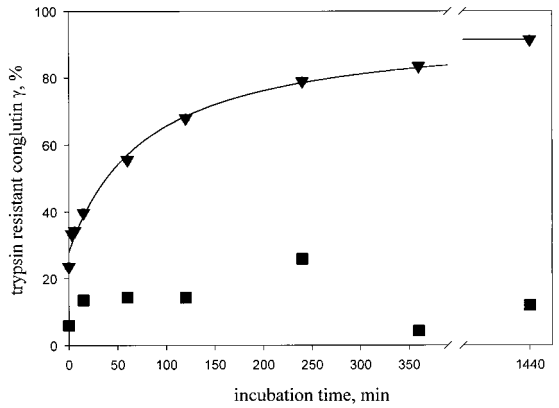


Figure 2. Time course of the renaturation of acid-treated conglutin γ. Samples of 10.6 μM conglutin γ were incubated at neutral pH for various times with a 200-fold molar excess (▼) or in the absence (■) of Zn^{2+} and subsequently treated with trypsin for 1.5 h at 37 °C as detailed under Materials and Methods. Points represent the averaged (duplicate) percentages of uncleaved conglutin γ 46 kDa polypeptide after SDS-PAGE analysis. The continuous line denotes the hyperbolic calculated fit.

To verify whether conglutin γ itself affected trypsin activity, the same molar concentrations of bovine hearth cytochrome *c* as conglutin γ were added to the incubation mixture with trypsin in the same ratio as in the refolding experiments. Complete degradation of cytochrome *c* took place, as if it was incubated alone with trypsin (**Figure 1**), thus indicating that conglutin γ itself had no trypsin inhibitory activity.

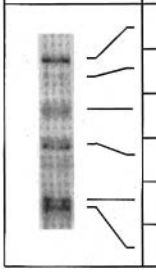
Time Course of Conglutin γ Refolding Patterns in the Presence and Absence of Zn^{2+}. To compare the patterns of adoption of the trypsin-resistant conformation by conglutin γ in the presence and absence of Zn^{2+}, the time course of these events was studied by plotting the relative amounts of trypsin-resistant conglutin γ versus the refolding time (**Figure 2**). A hyperbolic curve, with a correlation coefficient of 0.996, was obtained with conglutin γ refolded in the presence of a molar excess of Zn^{2+}, whereas with an equimolar Zn^{2+} concentration to the protein (not shown) and in the control without Zn^{2+}, respectively, a series of scattered data, roughly parallel to the abscissa and close to zero, were obtained (**Figure 2**). Despite the scarce reliability of the latter scanning measurements at these low uncleaved polypeptide concentrations, the results indicated that only a much lower amount of conglutin γ was resistant to trypsin if Zn^{2+} was not present, as qualitatively shown in **Figure 1**.

At zero refolding time, a certain amount of conglutin γ, varying from 6 to 24% in the different samples, appeared to be resistant to trypsin action, as already noted in a previous work (*14*), seemingly due to the incomplete protein unfolding under the denaturing, but nonreducing, conditions used.

Identification of the Major Tryptic Polypeptide Fragments. The most prominent polypeptide fragments liberated by trypsin at the middle point of the renaturation process were separated by SDS-PAGE under reducing conditions, blotted to PVDF membrane, and sequenced. This approach allowed us to identify these fragments, as detailed in **Table 1**.

In the reduced SDS-PAGE pattern shown, two polypeptide bands, namely, those with molecular mass of 31200 and 17700 Da, were the uncleaved conglutin γ heavy and light subunits, respectively, as confirmed by their N-terminal sequences. The other polypeptides corresponded to fragments of the heavy subunit cleaved at two K and one R residue, respectively, located

Table 1. Identification of the Major Tryptic Polypeptide Fragments Liberated during Conglutin γ Refolding in the Presence of Zn^{2+} [a]

SDS-PAGE pattern under reducing conditions	Determined Mr, Da	N-terminal sequencing (sequence position [&])	Calculated molecular mass	Glycosylation[•] ($NN_{(99)}T$)
	31,200	$Y_{(1)}$HNSQP	29,007	+
	28,200	$T_{(36)}$PLMQV	25,141	+
	22,600	$A_{(78)}$NTHQ	20,326	+
	17,700	*$Y_{(268)}$HESSE*	*16,352*	-
	12,500	$G_{(153)}$LPNNV	12,093	-
	11,900	*$Y_{(268)}$HESSE*	*n.d.*	-

[a] Italic type relates to conglutin γ light subunit. [&] Except for the N termini of the heavy and light conglutin γ subunits (residues 1 and 268, respectively), all sequences shown follow a K or and R residue. [•] Determined by concanavalin A Western blotting (see Materials and Methods). n.d., not detectable.

in the first half of the molecule, and one fragment derived from the C-terminal truncated light subunit, where several K and R residues are also present. Their masses calculated from the deduced amino acid sequence fitted with the M_r values determined by SDS-PAGE and also the presence of a glycosyl moiety were consistent with its position in the heavy chain sequence, as also shown in **Table 1**. Because many other cleavable K and R residues are present in the conglutin γ sequence, although they appeared not to be cleaved by trypsin or at least they do not give rise to relevant fragments, these uncleaved positions likely represent inaccessible regions of the fully or partly folded protein structure.

Effects of Other Metal Ions. On the basis of our previous results (*15*), showing the interaction of various divalent metal ions with conglutin γ, the influence of other metals on the refolding pattern of conglutin γ was studied. The metal ions were selected on the basis of their observed greatest (Zn^{2+} and Ni^{2+}) and lowest (Mn^{2+} and Ca^{2+}) extent of interaction with conglutin γ, as described in a previous paper (*15*). In this experiment, the SDS-PAGE patterns of identical volumes of treated conglutin γ solutions were used to compare the effect of the various metal ions on the structural dynamics of the protein (**Figure 3**). Ni^{2+} was shown to be more effective than Zn^{2+} in promoting conglutin γ adoption of the trypsin-resistant conformation at short refolding time, whereas the percentages of refolded protein with Mn^{2+} and Ca^{2+} were only 42 and 19% of the Ni^{2+}-treated sample, respectively. These results were obtained by considering only the relative intensity of the intact conglutin γ band as the data reported in **Figure 2**. Major fragments around 30 and 17 kDa were detected for Ni^{2+}- and Zn^{2+}-treated samples, whereas most of the peptides were not retained in the gel with both Mn^{2+} and Ca^{2+}.

DISCUSSION

In this work, the susceptibility to trypsin of a metal binding plant glycoprotein (*15*) was used to monitor the effect of various metal ions on the structural dynamics of the protein, in order to get a deeper insight into the extent of its proteolysis as affected by the presence of metal ions. Other experimental approaches, such as activity measurements or direct physical measurements, such as far-UV circular dichroism, could not provide evidence of conglutin γ folding/unfolding processes because this protein lacks any detectable biological activity and

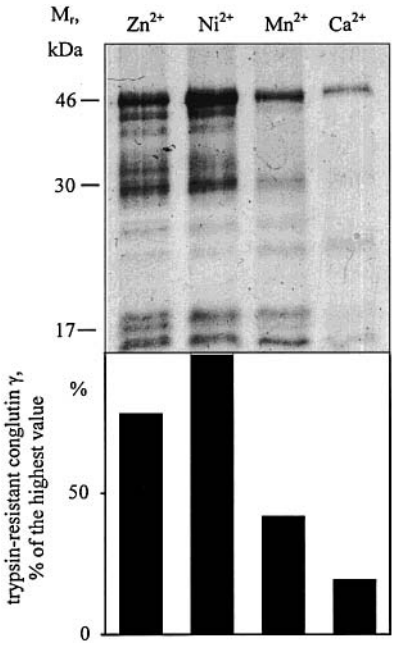


Figure 3. Effect of divalent metal ions on the refolding pattern of conglutin γ. Conglutin γ was incubated in the presence of 2 mM concentrations of the metal ions indicated in the figure for 40 min and then treated with trypsin, as already described. Identical volumes of each sample were withdrawn, denatured, and analyzed by SDS-PAGE. The histogram shows the relative amounts of uncleaved conglutin γ polypeptide as a percent of the corresponding area in the Ni^{2+}-treated sample.

is predominantly structured in the β conformation (*14*, *22*). However, the sensitivity to proteolytic attack has already been shown to represent an effective probe of protein structural changes (*23*, *24*). From the structural viewpoint, further interest in this study arises from the fact that, 17 lysine and 7 arginine residues being present in the sequence of conglutin γ, the complete trypsin resistance of such a large-sized, potentially highly cleavable protein in its native conformation is quite unusual.

Our results have shown that the interaction of the denatured and trypsin-susceptible conglutin γ with Zn^{2+} at neutral pH values triggers the adoption of a proteolysis-resistant conformation, as observed by the increasing presence of the intact 46 kDa conglutin γ band. Our experimental approach was based on the analysis of the same volumes of protein solutions with identical initial protein concentrations, and the effect of the metal was monitored by comparing the intensity of the Coomassie blue stained conglutin γ intact band.

If metal ions were absent during the refolding step, degradation gave rise to very small peptides undetectable in SDS-PAGE, whereas proteolysis, giving rise to major polypeptides, which have been identified by N-terminal sequencing, in the presence of the metal was only limited.

The comparative effects of the four metal ions tested on the refolding pattern of conglutin γ (**Figure 3**) are consistent with our previous findings on the interaction of divalent cations with conglutin γ (*15*) and suggest a preferential interaction of this protein with transition metal ions. Despite Ni^{2+} having shown a significant structurizing capacity and its status as an essential nutrient (*25*), in this work we have focused on Zn^{2+} for its greater physiological relevance, its deficiency in humans being known (*25*).

The presence of major polypeptide fragments liberated by trypsin action before completion of the refolding process (**Table 1**) suggests that some cleavage sites belong to exposed regions of the folding protein, whereas other cleavable bonds are hindered in the protein folding core and the mentioned metal ions play a major role in these structural dynamics. Incidentally, the liberation of polypeptide fragments of similar size from conglutin γ refolded in the presence of different metal ions suggests a unique or a predominant refolding pathway followed by the protein, according to the widely accepted models of protein folding (*26*, *27*).

These results, in addition to the molecular insight provided, might be relevant to a deeper understanding of the in vivo stepwise degradation of food proteins and how these processes are affected by the presence of other food components, such as the metal ions. This is of special interest if a given protein, such as conglutin γ, is being isolated and processed in view of its utilization as a food ingredient (*28*). In this respect, the metal binding capacity of conglutin γ and its fragments may be of interest in influencing the supply and bioavailability of minerals in the human diet.

On the other hand, it has been shown that specific polypeptide fragments of legume storage proteins may develop biological activities, which can have either beneficial or adverse effects on human and animal health, such as a lectin-like activity arising from the proteolysis of lupin 7S globulins (*29*), the soybean hypocholesterolemic activity of peptides from the conglycinin fraction (*30*), and the secretagogue activity of phaseolin fragments (*31*). In this respect, the oriented modulation of plant protein limited proteolysis may open new perspectives in the biological utilization of these nutrients and certainly deserves further studies at molecular and physiological levels.

LITERATURE CITED

(1) Carbonaro, M.; Grant, G.; Cappelloni, M.; Pusztai, A. Perspectives into factors limiting in vivo digestion of legume proteins: antinutritional compounds or storage proteins. *J. Agric. Food Chem.* **2000**, *48*, 742−749.

(2) Hajos, G.; Gelencser, E.; Grant, G.; Bardocz, S.; Sakhri, M.; Duguid, T. J.; Newman, A. M.; Pusztai, A. Effect of proteolytic modification and methionine enrichment on the nutritional value of soya albumins for rats. *J. Nutr. Biochem.* **1996**, *7*, 481−487.

(3) Schwenke, K. D. Enzyme and Chemical Modification of Proteins. In *Food Proteins and their Applications*; Damodaran, S., Paraf, A., Eds.; Dekker: New York, 1997; pp 393−423.

(4) Duranti, M.; Restani, P.; Poniatowska, M.; Cerletti, P. The seed globulins of *Lupinus albus*. *Phytochemistry* **1981**, *20*, 2071−2075.

(5) Restani, P.; Duranti, M.; Cerletti, P.; Simonetti, P. Subunit composition of the seed globulins of *Lupinus albus*. *Phytochemistry* **1981**, *20*, 2077−2083.

(6) Scarafoni, A.; Di Cataldo, A.; Vassilevskaia, T. D.; Bekman, E. P.; Rodrigues-Pousada, C.; Ceciliani, F.; Duranti, M. Cloning, sequencing and expression in the seeds and radicles of two *Lupinus albus* conglutin γ genes. *Biochim. Biophys. Acta* **2001**, *1519*, 147−151.

(7) Duranti, M.; Cucchetti, E.; Cerletti, P. Changes in composition and subunits in the storage proteins of germinating lupin seeds. *J. Agric. Food Chem.* **1984**, *32*, 490−493.

(8) Citharel, J.; Delamarre, D. The γ-conglutin of *L. albus* L. var. Lutop (Leguminoseae). Synthesis during seed maturation. *Plant Physiol. Biochem.* **1989**, *27*, 211−218.

(9) Duranti, M. Enzymatic subunit splitting of lupin storage proteins. *Nahrung* **1986**, *30*, 271−274.

(10) Duranti, M.; Faoro, F.; Harris, N. Immunocytochemical localisation of conglutin γ and legumin-like globulin in developing and mature seeds of *Lupinus albus*. *Protoplasma* **1991**, *161*, 104−110.

(11) Esnault, M. A.; Citharel, J.; Thomas, D.; Guegan, P.; Cavalier, A. Behaviour of conglutin γ, a major lupin seed protein during germination. *Plant Physiol. Biochem*. **1996**, *34*, 101−109.
(12) Duranti, M.; Faoro, F.; Harris, N. The unusual extracellular localisation of conglutin γ in germinating *Lupinus albus* seeds rules out its role as a storage protein. *J. Plant Physiol*. **1994**, *143*, 711−716.
(13) Hirano, H.; Kagawa, H.; Okubo, K. Characterization of proteins released from legume seeds in hot water. *Phytochemistry* **1992**, *31*, 731−735.
(14) Duranti, M.; Gius, C.; Sessa, F.; Vecchio, G. The saccharide chain of lupin seed conglutin γ is not responsible for the protection of the native protein from degradation by trypsin, but facilitates the refolding of the acid-treated protein to the resistant conformation. *Eur. J. Biochem*. **1995**, *230*, 886−891.
(15) Duranti, M.; Scarafoni, A.; Di Cataldo, A.; Sessa, F. Interaction of metal ions with lupin seed conglutin γ. *Phytochemistry* **2001**, *56*, 529−533.
(16) Duranti, M.; Scarafoni, A.; Gius, C.; Faoro, F.; Negri, A. Heat-induced synthesis and tunicamycin-sensitive secretion of the putative storage glycoprotein conglutin γ from mature lupin seeds. *Eur. J. Biochem*. **1994**, *222*, 387−393.
(17) Duranti, M.; Sessa, F.; Scarafoni, A.; Bellini, T.; Dallocchio, F. Thermal stabilities of lupin seed conglutin γ protomers and tetramers. *J. Agric. Food Chem*. **2000**, *48*, 1118−1123.
(18) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(19) Towbin, M.; Stahelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4350−4354.
(20) Hawkes, R. Identification of concanavalin A-binding proteins after sodium dodecyl sulphate-gel electrophoresis and protein blotting. *Anal. Biochem*. **1982**, *123*, 143−146.
(21) Matsudaira, P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem*. **1987**, *262*, 10035−10037.
(22) Blagrove, R. J.; Gillespie, J. M. Isolation, purification and characterization of the seed globulins of *Lupinus albus*. *Aust. J. Plant Physiol*. **1975**, *2*, 13−27.
(23) Fontana, A.; Zambonin, M.; Polverino De Laureto, P.; De Filippis, V.; Clementi, A.; Scaramella, E. Probing the conformational state of apomyoglobin by limited proteolysis. *J. Mol. Biol*. **1997**, *266*, 223−230.
(24) Fontana, A.; Polverino De Laureto, P.; De Filippis, V.; Scaramella, E.; Zambonin, M. Probing the partly folded states of proteins by limited proteolysis. *Folding Des*. **1997**, *2*, R17−R26.
(25) Seiler, H. G.; Sigel, A.; Sigel, H. *Handbook on Metals in Clinical and Analytical Chemistry*; Dekker: New York, 1994.
(26) Karplus, M.; Weaver, D. L. Protein folding dynamics. *Nature* **1976**, *260*, 404−406.
(27) Jaenicke, R. Folding and association of proteins. *Biophys. Struct. Mech*. **1982**, *8*, 231−256.
(28) Holley, W.; Luck, T.; Müller, K.; Schäfer, C.; Wäshe, A. Properties, functions and applications of lupin protein isolates and joint-products. *Proceedings of the 4th European Conference on Grain Legumes*, Crakow, Poland; AEP: Paris, 2001; pp 402−403.
(29) Ramos, P. C.; Rodrigues, D.; Ferreira, R.; De Seixas Boavida, M.; Franco, E.; Teixeira, A. R. N. Accumulation of a lectin-like breakdown product of β-conglutin catabolism in cotyledons of germinating *Lupinus albus* L. seeds. *Planta* **1997**, *203*, 26−34.
(30) Manzoni, C.; Lovati, M. R.; Gianazza, E.; Morita, Y.; Sirtori, C. Soybean protein products as regulators of liver low-density lipoprotein receptors: II. α-α′ rich commercial soy concentrate and α′ deficient mutant differently affect low-density lipoprotein receptor activation. *J. Agric. Food Chem*. **1998**, *46*, 2481−2484.
(31) Santoro, L.; Grant, G.; Pusztai, A. *In vivo* degradation and stimulating effect of phaseolin on nitrogen secretion in rats. *Plant Foods Hum. Nutr*. **1999**, *53*, 223−236.

Received for review April 19, 2001. Revised manuscript received December 4, 2001. Accepted December 18, 2001. This work has been funded by MURST 60%-2000 and EU-INTAS Contract 2000-0256.

JF010512C