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ARTICLE

Susceptibility of Lupin γ-Conglutin, the Plasma Glucose-Lowering Protein of Lupin Seeds, to Proteolytic Enzymes

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Lupin seed γ-conglutin, orally administered to animal models, has been shown to display glucose-controlling properties. Therefore, we have addressed the study of γ-conglutin susceptibility to proteolytic enzymes *in vitro* as the basis to unveil its metabolic fate in the body. Pepsin treatment at pH 2.0 and 3.0 caused extensive proteolytic breakdown, while at pH 4.0, where pepsin is minimally active, γ-conglutin was unaffected. Aliquots of the pepsin-treated protein were further incubated with pancreatin at neutral pH. If the protein backbone was already cleaved by pepsin action, then the breakdown by pancreatin was almost complete; alternatively, pancreatin did not affect at all γ-conglutin polypeptide chain. This was not due to an inhibitory activity of γ-conglutin, because co-incubation with casein showed complete breakdown of the milk protein. Furthermore, γ-conglutin was incubated with bromelain, a proteinase effective between pH 4.0 and 7.0. A sharp transition from the uncleavable to the fully cleavable form of γ-conglutin was observed below pH 4.25. Therefore, it was concluded that (i) γ-conglutin is resistant to proteolysis at pH greater than 4.0, likely because of a compact native conformation, (ii) an acidic pH renders the protein susceptible to proteases, suggesting the occurrence of a trans conformation, which has also been observed by circular dichroism spectral analysis, and (iii) the protein undergoes an "all or none" degradation pathway, regardless of the enzyme used.

KEYWORDS: Conglutin; *Lupinus albus*; protease susceptibility; pH

INTRODUCTION

White lupin seeds contain relatively large amounts of γ-conglutin, 3−5% of total globulins, predominantly but not exclusively stored in the storage vacuoles of the mature seeds (*1*). γ-Conglutin is a monoglycosylated protein consisting of two disulfide-linked 30 and 17 kDa subunits (*2*). Two γ-conglutin genes have been identified, but only one gene product has been detected in the mature seed (*3*). The protein is expressed as a pre-pro-protein, which, upon removal of the signal peptide, gives rise to a single pro-protein polypeptide chain. Subsequent proteolytic cleavage in a serine-rich region of the pro-protein leads to the two disulfide-linked subunits (*4*).

Despite being deposited into the storage vacuoles as all seed storage proteins, γ-conglutin does not play a reserve role, because it is unaffected by the proteolytic enzymes of the germinating lupin seeds (*5*).

In a relatively recent work, γ-conglutin was found to be capable of interacting *in vitro* with the mammalian protein hormone insulin and lowering plasma glucose concentrations of glucose-overloaded rats upon oral administration of the protein prior to glucose load (*6*). Bioactivity of a dietary protein is rather peculiar, because the expected exhaustive gastro-intestinal degradation should prevent any biological activity to be manifest. Nonetheless, the number of dietary proteins found to survive proteolysis during gastro-intestinal digestion is currently rising. Some of them are intrinsically resistant, such as the hydrolytic enzyme inhibitors, because of their compact disulfide-stabilized structure (*7*). Other proteins, including gluten (*8*), lectins (*9*), and plant protein allergens (*10*), display peculiar amino acid sequences and/or particularly stable and compact structures, which prevent the protease attack or decrease its extent.

Therefore, the study of protein susceptibility to proteolytic enzymes, especially if these proteins are involved in relevant biological responses, such as white lupin γ-conglutin, is crucial.

Actually, γ-conglutin was already found to be resistant to trypsin attack under *in vitro* conditions (*11*). In that work, it was also shown that a preliminary acidic treatment of the protein made the protein more proteolysis-prone, provided that γ-conglutin was immediately submitted to trypsin action prior to protein renaturation at the neutral pH value needed for trypsin action. These preliminary findings suggested that γ-conglutin could be cleaved by trypsin only if its native conformation was lost.

In the present work, we investigated γ-conglutin susceptibility to *in vitro* proteolysis with various proteases and under different experimental conditions. Our aim was not mimicking the digestion physiological process but rather to focus on the structural constrains that hinder γ-conglutin breakdown and to envisage the molecular bases of γ-conglutin peculiar behavior. To this

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purpose, various enzymes and pH conditions were assayed. A sharp pH dependence of γ-conglutin susceptibility was evidenced, and this may at least in part explain the behavior of the lupin protein observed *in vivo*.

MATERIALS AND METHODS

Materials. White lupin seeds (*Lupinus albus*, var. Multitalia) were a kind gift of AGROSERVICE SpA, Rocchetta, San Severino Marche, Italy. Porcine pepsin and pancreatin and pineapple steam bromelain were from Sigma Aldrich (Milan, Italy).

γ-Conglutin Purification. γ-Conglutin was purified as described in a previous work (*12*) by using a combination of anion- and cation-exchange chromatography. The dialyzed, purified protein was freeze-dried and resuspended in the appropriate buffers before use. The protein concentration was assessed by 280 nm optical absorbance using a Lambda II spectrophotometer (Perkin-Elmer). The extinction coefficient for a solution of 1 mg/mL was 0.733, as calculated according to the protein amino acid sequence (*13*).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was carried out according to Laemmli (*14*) on 12% polyacrylamide precast gel (BioRad, Milan, Italy). For the runs under reducing conditions, 2% 2-mercaptoethanol was added to the sample-denaturing buffer. Polypeptide bands were stained by Bio-Safe Coomassie G-250 (BioRad, Milan, Italy), and the relative molecular mass of the polypeptides was determined by a comparison to standard protein solutions (BioRad, Milan, Italy).

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). The RP-HPLC analysis of γ-conglutin, treated with pepsin and pancreatin, was carried out on a Simmetry300 C18 column (4.6 × 250 mm) (Waters), equilibrated with 0.1% trifluoroacetic acid in water at pH 2.2. The elution of the peptide fractions, at a flow rate of 0.8 mL/min, was carried out by a continuous gradient of 0−75% acetonitrile for 75 min.

Enzymatic Treatments on γ-Conglutin. For the pepsin treatment, freeze-dried γ-conglutin was resuspended in a solution 0.12 M NaCl, adjusted to the desired pH value with 1 M HCl. Then, the pepsin solution (1 mg/mL in water) was added to the samples in the ratio of 1:30 enzyme/γ-conglutin (w/w). The samples were incubated for 1 h at 37 °C under gentle stirring, and the reaction was stopped with a cocktail of protein inhibitors (fast protease inhibitor, Sigma Aldrich, Milan, Italy). To the samples incubated with pancreatin after pepsin treatment, the inhibitors were not added and the pH of the solutions was adjusted to 7.5 by adding an equal volume of 0.1 M Tris-HCl at pH 7.5.

For the pancreatin treatment, the enzyme solution (1 mg/mL in water) was added to the samples in the ratio of 1:30 enzyme/γ-conglutin (w/w). The samples were incubated for 1 h at 37 °C under gentle stirring, and the reaction was stopped with a cocktail of protein inhibitors (fast protease inhibitor, Sigma Aldrich, Milan, Italy). In the samples containing both γ-conglutin and casein, the two proteins were in the ratio 1:1 (w/w).

For the bromelain treatment, freeze-dried γ-conglutin was resuspended in a 0.1 M Na-acetate, 0.3 M NaCl, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) buffer. The solution was adjusted at the desired pH value with 1 M HCl. The enzyme (1 mg/mL in water) was added in the ratio of 1:33 enzyme/γ-conglutin (w/w) to the protein substrate, and the samples were incubated at 37 °C under gentle stirring for different times. The reaction was stopped with a cocktail of protein inhibitors (fast protease inhibitor, Sigma Aldrich, Milan, Italy).

Circular Dichroism (CD) Study. CD spectra were recorded at 20 °C between 240 and 360 nm using a J-810 Jasco spectropolarimeter and analyzed by means of the dedicated Jasco software. The cell path was 1 cm; the scanning speed was 10 nm/min; and the brand width was 2 nm. The protein solutions (2 mg/mL) were prepared by dissolving γ-conglutin in different buffer systems, namely, 50 mM sodium phosphate at pH 2.0 and 3.0, 50 mM sodium acetate at pH 4.0, and 50 mM Tris-HCl at pH 7.5. All of the solutions contained 0.1 M NaCl. CD measurements were corrected by subtracting the buffer spectra.

RESULTS

The typical electrophoretic pattern of reduced γ-conglutin with two main bands of 30 and 17 kDa, respectively, and a weaker band of 50 kDa, corresponding to the unprocessed γ-conglutin precursor (Duranti, unpublished data), is shown in lane R of **Figure 1**. When γ-conglutin was incubated with pepsin at different pH values for 60 min, the pattern of the sample incubated at pH 2.0 appeared significantly affected by the enzyme, with the complete disappearance of the 30 and 17 kDa bands and the formation of some weakly stained low M_r polypeptides (about 10 kDa and less than 5 kDa). At pH 3.0, the pattern showed minor changes with the formation of a few polypeptides with M_r between 10 and 5 kDa. At a greater pH value, i.e., 4.0, the two main bands were not affected at all. Indeed, peptic activity is extremely low at these pH values (*15*). When pepsin-treated samples were further incubated with pancreatin at pH 7.5, the extent of degradation was complete for the sample previously attacked by pepsin at pH 2.0, as assessed by the disappearance of any Coomassie Blue reacting band in the corresponding lane. On the other hand, the γ-conglutin samples, which were not preliminarily attacked by pepsin, did keep full covalent integrity of the two main subunits, because it occurred with the protein directly incubated with pancreatin (lane D of **Figure 1**). This finding suggests that γ-conglutin is totally resistant to the attack of pancreatin enzyme pool at neutral pH, unless previously and extensively degraded by pepsin.

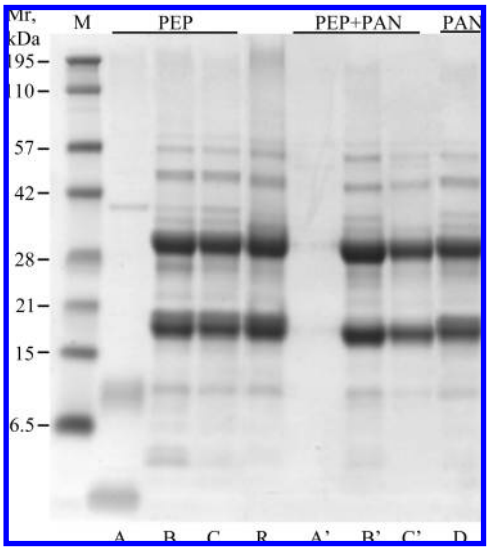

Figure 1. SDS−PAGE patterns under reducing conditions of γ-conglutin incubated with pepsin alone (PEP), pepsin followed by pancreatin (PEP + PAN), and pancreatin alone (PAN). γ-Conglutin was incubated with pepsin [30:1 (w/w), 1 h at 37 °C] at the pH values of 2.0, 3.0, and 4.0 for the samples marked with A, B and C, respectively. The corresponding samples marked with a prime (A′, B′, and C′) were adjusted to pH 7.5 and further incubated with pancreatin [30:1 (w/w), 1 h at 37 °C]. The sample in lane D consists of γ-conglutin directly incubated with pancreatin as above. M, marker proteins; R, reference γ-conglutin.

Similar results were obtained by RP-HPLC analysis of γ-conglutin samples treated with pepsin at pH 2.0, 3.0, and 4.0 (panels **A**, **B**, and **C** of **Figure 2**). The elution profiles at the tested pH values differed significantly. At pH 4.0, only one peak, corresponding to the intact γ-conglutin, was visible in the chromatogram at about 55 min elution time. The unprocessed γ-conglutin was still visible at pH 3.0, along with some γ-conglutin proteolytic fragments. The most dramatic changes were observed at pH 2.0, where γ-conglutin was extensively degraded. Most of the generated peptides were not visible in the SDS−PAGE, likely being too small to be retained by the polyacrylamide gel network. A similar pattern was observed

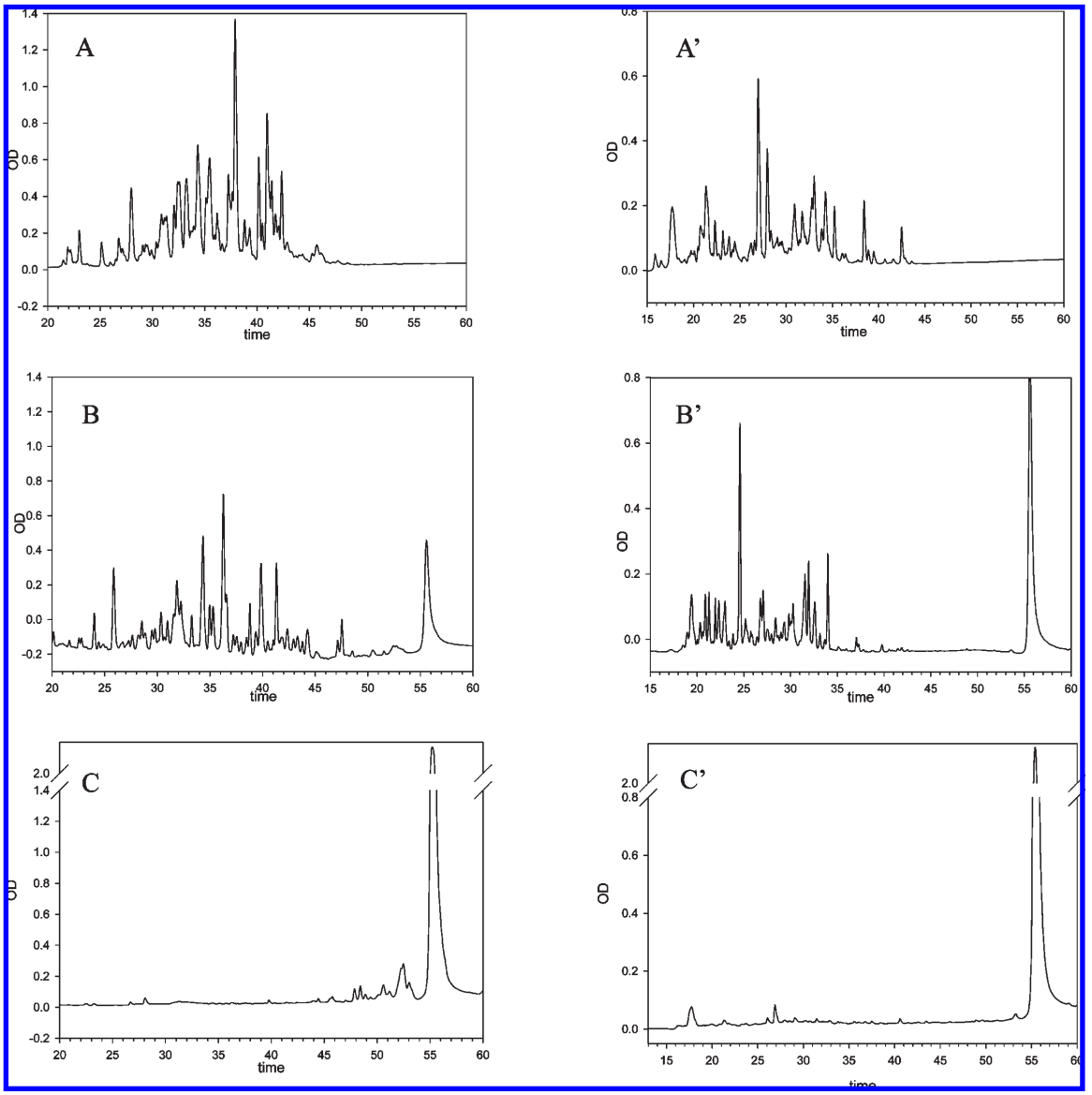

Figure 2. RP-HPLC chromatograms of γ-conglutin incubated with pepsin alone (panels A, B, and C) and pepsin followed by pancreatin (panels A′, B′, and C′). γ-Conglutin was incubated with pepsin [30:1 (w/w), 1 h at 37 °C] at pH (A) 2.0, (B) 3.0, and (C) 4.0, respectively, and subsequently with pancreatin [30:1 (w/w), 1 h at 37 °C], upon adjustement to pH 7.5 (panels A′, B′, and C′). The optical density was measured at 220 nm.

when pancreatin incubation at pH 7.5 followed pepsin treatment at the three pH values considered (panels **A′**, **B′**, and **C′** of **Figure 2**). Once again, if pepsin attack was not effective on γ-conglutin, as it occurred at pH 4.0 and partially at pH 3.0, pancreatin did not cause any cleavage of γ-conglutin covalent continuity, as previously shown in the SDS−PAGE patterns of **Figure 1**.

To rule out the possibility that γ-conglutin played a role as an inhibitor of pancreatic enzymes, the same concentrations of γ-conglutin and casein were co-incubated with pancreatin at pH 7.5. **Figure 3** shows the time course of incubation with pancreatin as assessed by SDS−PAGE under nonreducing conditions. γ-Conglutin, when added, was visible in this gel as a single main band of about 50 kDa, corresponding to the unreduced protein. As it can clearly be seen, the three characteristic main bands of casein completely disappeared already after 30 min of pancreatin incubation. This showed that the activity of pancreatin enzymes was totally unaffected by the presence of γ-conglutin. Therefore, the intrinsic resistance of γ-conglutin to pancreatin is not due to any inhibitory activity of the lupin protein, and it is likely related to the compact conformation of the protein at neutral pH values.

To obtain greater insight into γ-conglutin susceptibility in a critical pH range where pepsin is inactive, the protein was incubated with bromelain, a low-specificity proteolytic enzyme most effective between pH 4.0 and 7.0 (*16*). The time course of γ-conglutin incubation with bromelain, as assessed by SDS−PAGE, is shown in **Figure 4**. As it can be seen, a progressive degradation of γ-conglutin bands, especially of the 30 kDa subunit, with the accumulation of a polypeptide fragment around 10 kDa, was monitored at pH 4.0. The extent of proteolysis significantly decreased already at pH 4.25, and any breakdown was totally abolished at pH 4.5 and 5.0. This finding provides evidence that incubation at a pH lower than 4.25 makes γ-conglutin susceptible to proteolytic degradation. Thus, the transition between a noncleavable to a fully cleavable form of γ-conglutin takes place in a sharp range of acidic pH values.

To confirm the effect of pH changes on γ-conglutin structural properties, a near-UV CD study was undertaken. The spectra taken at critical pH values, that is, 2.0, 3.0, 4.0, and 7.5, as a

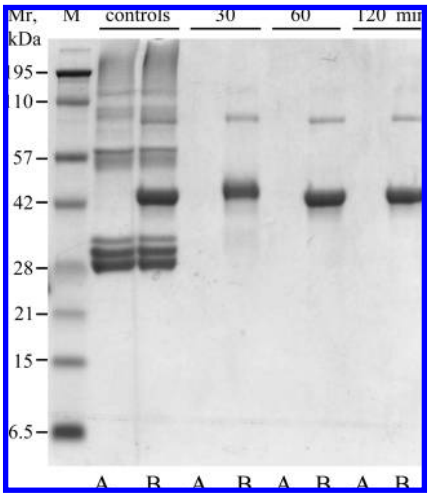

Figure 3. Time course of γ-conglutin and casein incubation with pancreatin, as monitored by SDS−PAGE under nonreducing conditions. γ-Conglutin and casein were digested with pancreatin at pH 7.5 and 37 °C for the times indicated. A, casein; B, 1:1 γ-conglutin/casein (w/w) mixture; controls, untreated proteins; M, marker proteins.

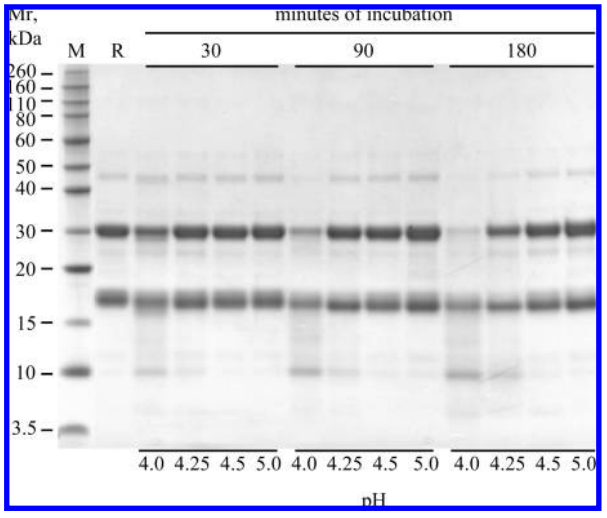

Figure 4. Time course of γ-conglutin incubation with bromelain, as monitored by SDS−PAGE under reducing conditions. γ-Conglutin was incubated with bromelain in the ratio of 1:33 (w/w) at the pH values indicated and 37 °C for the times indicated. M, marker proteins; R, reference γ-conglutin.

control, are reported in **Figure 5**. The aromatic chromophores (Trp, Tyr, and Phe) responsible for dichroic signals in the near-UV region (*17*) are well-represented in the amino acid sequence of γ-conglutin, summing to about 8% of the total amino acids (UniProtKB/TrEMBL database, accession number Q9FSH9). Therefore, spectral changes arising from the pH-dependent perturbation of the aromatic amino acid environment could reasonably be expected. As a matter of fact, a remarkable overall variation in the shape of the spectra taken at pH 2.0, 3.0, 4.0, and 7.5, respectively, is visible in **Figure 5**. In particular, the positive peaks in the region between 270 and 290 nm, typical of the aromatic amino acids, almost disappeared in the spectra of the protein at strongly acidic pH values. This result strongly supports the conclusion that a dramatic change in γ-conglutin native conformation takes place between pH 4.0 and 3.0, in excellent agreement with the findings obtained using the proteolytic enzymes.

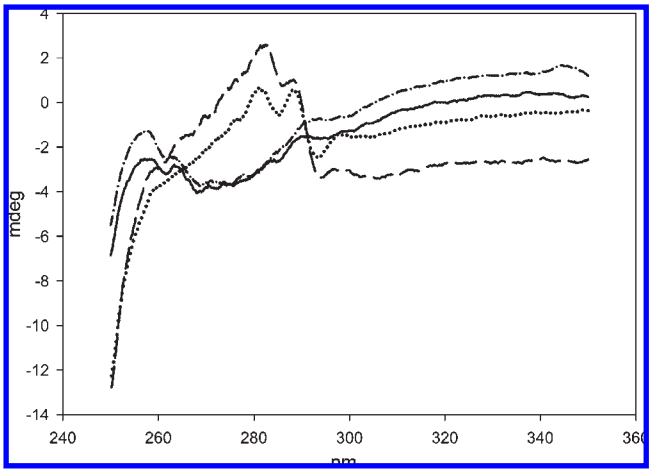

Figure 5. Near-UV CD spectra of γ-conglutin at various pH values. (- · -) pH 2.0, (—) pH 3.0, (- - -) pH 4.0, and (· · ·) pH 7.5. Experimental details are given in the Materials and Methods.

DISCUSSION

This work provides evidence of the resistance of lupin γ-conglutin to various proteolytic enzymes at pH values greater than 4.0 and extends previous results obtained by treating γ-conglutin with trypsin (*11*). Our present approach with different enzymes and in a wide range of pH values focused the sharp correlation between the observed resistance and the conformational accessibility of the lupin protein, as it is affected by pH. As a matter of fact, γ-conglutin is almost completely degraded by pepsin at pH 2.0 and totally cleaved down to small peptides and amino acids if pancreatin is subsequently applied. However, if pepsin is not effective, as it occurs at greater pH values, then pancreatin has no effect at all on γ-conglutin (**Figures 1** and **2**). This finding was not attributed to any inhibitory activity of γ-conglutin, because casein co-incubated with the lupin protein appeared completely degraded by pancreatin (**Figure 3**). The strong pH dependence of γ-conglutin susceptibility to proteolysis was shown by its incubation with bromelain; in the sharp transition between pH 4.0 and 4.25, γ-conglutin becomes almost completely resistant to enzyme attack. This set of data strongly supports the hypothesis of an intrinsic resistance of γ-conglutin to proteolysis unless its native conformation is altered by the pH. This is an intriguing finding, if one considers that the amino acid residues primarily contributing to the trypsin- and chymotrypsin-cleavable peptide bonds, i.e., arginine, lysine, leucine, and the aromatic residues, account for about 25% of the total amino acids in the γ-conglutin sequence (UniProtKB/TrEMBL database, accession number Q9FSH9). Therefore, all of these amino acid residues must be scarcely accessible to pancreatic enzymes when γ-conglutin is in its native conformation. Moreover, the resistance to bromelain, a broad spectrum protease, at pH greater than 4.25 suggests that many other amino acid residues are inaccessible in the protein native conformation. Therefore, although the γ-conglutin 3D structure is not available, it can be argued that its native conformation must be extremely compact, as compared to the canonical seed proteins. Such conformational stability is supported by the γ-conglutin six disulfide bridges, as it occurs for other plant proteins, including most plant hydrolase inhibitors (*7*) and various seed allergens (*10*). However, disulfide bridges are not the first cause of γ-conglutin resistance to proteases, because −S−S− bonds are not known to be affected by the acidic pH values, where the protein becomes susceptible to proteases. Rather, the sharp transition at pH lower than 4.25 speaks in favor of the breakdown of some critical ionic bonds, which glutamate residues

(pK_{a2} = 4.25) do contribute to. Interestingly, a similar transition around pH 4.25 has been observed for the *in vitro* insulin binding capacity of γ-conglutin (*6*).

The near-UV CD spectral analysis, by providing a valuable fingerprint of the tertiary structure of a protein (*18*), turned out to be effective in detecting γ-conglutin structural changes occurring in a pH range, which matched that detected with the proteases. Indeed, the finding that proteolysis data may well correlate with the extent of conformational transitions inferred from CD spectra has already been emphasized (*19*).

In a previous work, the reversible γ-conglutin association/dissociation equilibrium as a function of pH was described (*20*). In particular, it was shown that dissociation of the tetrameric form of this protein to the monomer occurs at pH around 5.5 and it is almost complete at pH 5.0. Therefore, protein quaternary structure modification has no apparent effect on the degradability of the protein, because the transition to a fully cleavable form of γ-conglutin takes place below pH 4.5, where the protein is predominantly in the monomeric form.

The findings presented in this work represent a first attempt to explain γ-conglutin biological activity *in vivo*, which consists of a decrease of the plasma glucose concentration in glucose-overloaded rats upon oral treatment with the protein (*6*). Indeed, provided that not all γ-conglutin molecules are affected by the acidic conditions of the stomach, then their backbones and likely conformations too may not be altered during the transit in the duodenal tract at a neutral pH. The mechanism underlying the biological activity of γ-conglutin has not been unveiled yet; thus, it is not possible to argue whether the integrity of this lupin protein is a prerequisite for its biological effect or smaller peptides thereof display bioactivity too. However, the full length protein in its native conformation has recently been shown to activate the insulin-signaling pathway in the miocyte model (*21*). The hypothesis of an at least partial preservation of γ-conglutin structure and activity into the gastro-intestinal tract is also consistent with the observed γ-conglutin immunogenic activity as measured in orally treated mice (*22*). Further *in vivo* studies aimed at confirming the transit of unaltered γ-conglutin in the gastro-intestinal tract; its absorption by intestinal cells and presence in the sera of treated animals have recently been undertaken.

In conclusion, in this work, we have shown that (i) the compact native conformation of γ-conglutin is responsible for its resistance to proteolytic attack at neutral pH values, (ii) a transition in the native resistant conformation to make it fully susceptible to the proteases is triggered by a sharp pH variation, and (iii) the protein undergoes an "all or none" degradation pathway, regardless of the enzyme used.

This work, by showing the behavior of γ-conglutin with respect to proteolytic attack, opens the way to further studies aimed at understanding the modalities of action of this lupin protein as a candidate natural drug for the oral treatment of insulin-resistance-related diseases.

LITERATURE CITED

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

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

(3) 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. Biophis. Acta* **2001**, *1519*, 147–151.

(4) Kolivas, S.; Gayler, K. R. Structure of the cDNA coding for conglutin γ, a sulphur-rich protein from *Lupinus angustifolius*. *Plant Mol. Biol.* **1993**, *21*, 397–401.

(5) Duranti, M.; Faoro, F.; Harris, N. The unusual extracellular localization of conglutin γ in germinating *Lupinus albus* seeds rules out its role as a storage protein. *J. Plant Physiol.* **1994**, *143*, 711–716.

(6) Magni, C.; Sessa, F.; Accardo, E.; Vanoni, M.; Morazzoni, P.; Scarafoni, A.; Duranti, M. Conglutin γ, a lupin seed protein, binds insulin in vitro and reduces plasma glucose levels of hyperglycemic rats. *J. Nutr. Biochem.* **2004**, *15*, 646–650.

(7) Clemente, A.; Vioque, J.; Sanchez-Vioque, R.; Bautista, J.; Millan, F. Factors affecting the *in vitro* digestibility of chickpea albumins. *J. Sci. Food Agric.* **2000**, *80*, 79–84.

(8) Hausch, F.; Shan, L.; Santiago, N. A.; Gray, G. M.; Khosla, C. Intestinal digestive resistance of immunodominant gliadin peptides. *Am. J. Physiol.* **2002**, *283*, G996–G1003.

(9) Rhodes, J. M.; Campbell, B. J.; Yu, L.-G. Lectin−epithelial interactions in the human colon. *Biochem. Soc. Trans.* **2008**, *36*, 1482–1486.

(10) Moreno, J.; Clemente, A. 2S Albumin storage proteins: What makes them food allergens? *Open Biochem. J.* **2008**, *2*, 16–28.

(11) Duranti, M.; Gius, C.; Sessa, F.; Vecchio, G. The saccharide chain of lupin seed conglutin gamma 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.

(12) Duranti, M.; Scarafoni, A.; Gius, C.; Faoro, F.; Negri, A. Heat induced synthesis and tunicamycin-secretion of the putative storage glycoprotein conglutin γ from mature lupin seeds. *Eur. J. Biochem.* **1994**, *222*, 387–393.

(13) Gasteiger, E.; Hoogland, C.; Gattiker, A., Duvaud, S.; Wilkins, M. R.; Appel, M. R.; Bairoch, A. Protein identification and analysis tools on the ExPASy server. In *The Proteomics Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totova, NJ, 2005; pp 571−608.

(14) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(15) Schlamowitz, M.; Peterson, L. U. Studies on the optimum pH for the action of pepsin on "native" and denatured bovine serum albumin and bovine hemoglobin. *J. Biol. Chem.* **1959**, *234*, 3137–3145.

(16) Harrach, T.; Eckert, K.; Maurer, H. R.; Machleidt, I.; Machleidt, W.; Nuck, R. Isolation and characterization of two forms of an acidic bromelain stem proteinase. *J. Protein Chem.* **1998**, *17*, 351–361.

(17) Strickland, E. H. Aromatic contribution to circular dichroism spectra of proteins. *CRC Crit. Rev. Biochem.* **1974**, *5*, 113–175.

(18) Kelly, S. M.; Jess, T. J.; Price, N. C. How to study proteins by circular dichroism. *Biochim. Biophys. Acta* **2005**, *1751*, 119–139.

(19) Polverino De Laureto, P.; Frare, E.; Gottardo, R.; Van Dael, H.; Fontana, A. Partly folded states of members of the lysozyme/lactalbumin superfamily: A comparative study by circular dichroism spectroscopy and limited proteolysis. *Protein Sci.* **2002**, *11*, 2932–2946.

(20) 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.

(21) Terruzzi, I.; Senesi, P.; Magni, C.; Montesano, A.; Scarafoni, A.; Luzi, A.; Duranti, M. Insulin-mimetic action of conglutin-γ, a lupin seed protein, in mouse myoblasts. *Nutr., Metab. Cardiovasc. Dis.* **2009**, manuscript submitted.

(22) Foss, N.; Duranti, M.; Magni, C.; Frøkiær, H. Assessment of lupin allergenicity in the cholera toxin model: Induction of IgE response depends on the intrinsic properties of the conglutins and matrix effects. *Int. Arch. Allergy Immunol.* **2006**, *141*, 141–150.

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