Seed Proteins from *Quercus suber*

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A study on the extraction and characterization of the cotyledonary proteins from cork oak (*Quercus suber* L.) seeds is reported. The seed proteins, which constitute approximately 5% of the seed weight, are glutelins on the basis of solubility criteria. They are readily solubilized in sodium borate buffer, pH 10, 50 mM, containing 1% (v/v) β-mercaptoethanol and 1% (w/v) sodium dodecyl sulfate. This fraction is made up of a large number of polypeptides with molecular masses ranging from 10 to above 100 kDa and occurs in the form of large aggregates. The level of glycosylation is low, with two main glycopolypeptides present (43 and 65 kDa). However, the major polypeptides are not glycosylated. *In vitro* digestibility studies indicated that trypsin and α-chymotrypsin produce a partial proteolysis of the cotyledonary proteins, particularly among the larger molecular mass polypeptides. The partial proteolysis seen in trypsin digests is not due to the presence of enzyme inhibitors in the seeds. On the other hand, pepsin almost completely digests the seed proteins. Taken together, these results may be considered as a good indication of the potential nutritional quality of the proteins from *Q. suber* seeds.

Keywords: *Cork oak; glutelin; in vitro proteolysis; protein; Quercus suber; seed*

INTRODUCTION

The economic interest of cork oak (*Quercus suber* L.) in the west Mediterranean region is not restricted to the production of cork or wood. The acorns, especially from some sweet varieties, are widely used to feed swine and other animals. However, very little information exists concerning the protein content, composition, and quality of *Q. suber* seeds.

Although the vast majority of individual proteins present in mature seeds fulfills metabolic or structural roles, the seeds also contain a few protein species that are present in high amounts and provide a store of amino acids for use during germination and seedling growth. These storage proteins are of particular importance not only because they comprise nearly the total protein content of the seed but also because they determine its quality for various uses (Shewry et al., 1995).

Seed proteins have been classified in many different ways. Osborne's classification, based on solubility, dates from the turn of the century and is still one of the most useful methods. By solubility criteria, proteins are classified into groups on the basis of their extraction and solubility in water (albumins), dilute saline solutions (globulins), alcohol/water mixtures (prolamins), and dilute acid or alkali solutions (glutelins) (Ashton, 1976). However, the divisions between these groups of proteins are not always clearly defined. For instance, the extraction depends on the sequence in which the solvents are used, on the vigor of the extraction, and on the conditions of the starting material (Padhye and Salunkhe, 1979; Shewry et al., 1980). On the other hand, the globulins from different plant sources usually require salt solutions of different ionic strengths to solubilize completely (Danielson, 1955).

Extraction of glutelins in alkali solutions leads to degradation of cystine residues in corn proteins, when pH values above 10 were used (Tecson et al., 1971). A comparison of various extraction procedures for the preparation of glutelins from milled rice showed that a solution containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol, in sodium borate buffer, pH 10, is an effective extractant (Tecson et al., 1971).

Resistance of some storage proteins to proteolysis is generally considered as a major cause for the poor nutritional value of seeds. On the other hand, seeds often possess inhibitors of proteases that may be responsible for a decreased digestibility of the proteins. Koshiyama et al. (1981a,b) detected some trypsin and chymotrypsin inhibitory activities in fractions of the 2S globulins from soybean seeds and found that two of these fractions were identical with the Kunitz trypsin inhibitor. Trypsin and chymotrypsin inhibitory activities were also observed in studies of *in vitro* digestibility of dry bean proteins (Deshpande and Nielsen, 1987a,b). Factors such as structural constraints, the compact structure of proteins, and the stability imposed on their three-dimensional structure by carbohydrate residues were proposed to explain the resistance of phaseolin, the major storage protein from dry bean, to trypsin and chymotrypsin (Deshpande and Nielsen, 1987b; Deshpande and Damodaran, 1989). Other factors that may be responsible for the low *in vitro* digestibility of legume proteins include starch complexed with proteins, product inhibition during digestion, and the presence of phytic acid and polyphenols (Deshpande and Nielsen, 1987a).

The purpose of our study was to purify the major proteins from *Q. suber* seeds and to characterize them with respect to solubility, polypeptide composition, structure, presence of carbohydrate residues, and susceptibility to *in vitro* proteolytic attack.

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MATERIALS AND METHODS

Plant Materials. Mature *Q. suber* L. (cork oak) seeds were obtained from a grove near Nisa, Alentejo, Portugal, in November 1994 and stored at −70 °C. Seeds were defrosted at room temperature before their study.

Autotrophic *Lemna minor* L. cultures were maintained in a complete sterile medium as described by Ferreira and Davies (1986), under a photoperiod of 14 h of light, at 25 °C, and 10 h of dark, at 20 °C.

Protein Fractionation Based on Solubility Criteria. Albumins, globulins, prolamins, and glutelins from *Q. suber* cotyledons were sequentially purified using appropriate extraction solutions. The cotyledons of 20 seeds were separated from teguments and embryos and ground in Tris-HCl buffer, pH 7.5, 10 mM, containing 14 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 10 mM $CaCl_2$ (5 mL/g of fresh weight), and the resulting extract was filtered through four layers of gauze. Albumins were extracted by stirring the sample for 2 h at 4 °C. The insoluble proteins were removed by centrifugation at 27000*g* for 15 min. For globulin extraction, the pellet was resuspended in Tris-HCl buffer, pH 7.5, 10 mM, containing 1 M NaCl, 14 mM β-mercaptoethanol, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (5 mL/g of fresh weight) and stirred for 2 h at 4 °C. The presence of $MgCl_2$ and $CaCl_2$, during the extraction of albumins, and of EDTA and EGTA, during the extraction of globulins, increases the extraction efficiency of these proteins and avoids cross-contamination, as shown by Franco (1996) for several legume species. The procedure was sequentially repeated to obtain prolamin and glutelin fractions from the pellet containing the insoluble material. The following extraction solutions were used: 75% (v/v) ethanol for prolamins; sodium borate buffer, pH 10, 50 mM, containing 1% (v/v) β-mercaptoethanol and 1% (w/v) SDS for glutelins [as suggested by Shewry et al. (1980) for extraction of glutelins from barley seeds]. Extraction of glutelins was performed at room temperature to keep SDS in soluble form.

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Protein samples were mixed with 4 volumes of cold acetone, incubated at −20 °C for 1 h, and then pelleted by centrifugation at 15800*g* for 15 min. The resulting pellet was resuspended in Tris-HCl buffer, pH 6.8, 80 mM, containing 2% (w/v) of SDS and 0.1 M β-mercaptoethanol, boiled for 3 min, and analyzed by SDS−PAGE in 12.5% (w/v) acrylamide gels as described by Ferreira and Davies (1986), except that *m*-cresol purple was used as tracking dye and 0.1 M sodium acetate was included in the anode buffer to increase the resolution of polypeptides with molecular masses ranging from 2.5 to >200 kDa (Christy et al., 1989). The polypeptides in gels were fixed with 10% (w/v) trichloroacetic acid and stained with Coomassie Brilliant Blue R-250.

The control samples utilized in all electrophoretic runs, containing total cotyledonary proteins, were prepared in the following way: cotyledons were ground in Tris-HCl buffer, pH 7.5, 100 mM, containing 14 mM β-mercaptoethanol (3 mL/g of fresh weight) and filtered through four layers of gauze, and the proteins contained in 150 μL of extract were precipitated with acetone and treated as described above.

The large and small subunits of ribulose bisphosphate carboxylase were identified in SDS−PAGE gels as described before (Ferreira and Davies, 1986).

Molecular mass markers were obtained from Sigma Chemical Co. (MW-SDS-70L mixture): albumin (bovine plasma), 66 kDa; albumin (egg), 45 kDa; glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), 36 kDa; carbonic anhydrase (bovine), 29 kDa; trypsinogen (bovine pancreas), 24 kDa; trypsin inhibitor (soybean), 20 kDa; α-lactalbumin (bovine milk), 14.2 kDa.

Glycoprotein Detection. After SDS−PAGE of protein samples, the polypeptides were transferred from the gel to a nitrocellulose membrane in a semidry electrophoretic transfer cell Trans-Blot SD from Bio-Rad Laboratories, using Tris−glycine buffer, pH 9.2, 87 mM, containing 20% (v/v) methanol and 1.3 mM SDS as transfer solution.

After the polypeptide transfer, the glycosylated polypeptides present on the membrane were specifically detected by affinoblotting using concanavalin A−peroxidase, as described by Faye and Chrispeels (1985), except that polyoxyethylenesorbitan monolaurate was used to reduce the background, instead of the blocking agent gelatin.

Affinoblotting with concanavalin A−peroxidase is a high-resolution technique to characterize the degree of protein glycosylation (Faye and Chrispeels, 1985). This procedure is much more sensitive, simpler, and faster than using conventional staining such as periodic acid or Schiff reagent, allowing the detection of bands containing as little as nanogram quantities of glycopolypeptides. At the same time, the sensitivity of this method appears to be at least similar to that of techniques involving ^{125}I-labeled concanavalin A or immunological detection (Clegg, 1982; Faye and Chrispeels, 1985). However, it must be emphasized that concanavalin A specifically recognizes molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related residues (Goldstein et al., 1965). In other words, glycoproteins exist that are not detected by this method. Concanavalin A has, nevertheless, a rather broad binding specificity, being able to bind to most glycoproteins. On the other hand, previous studies have shown that, at least for legume seeds, mannose residues appear to be present in all glycosylated protein subunits (Eaton-Mordas and Moore, 1978, 1979; Duranti et al., 1981).

Gradient Ultracentrifugation. The samples analyzed by ultracentrifugation through sucrose and Percoll gradients were obtained as described above for the control samples utilized in the electrophoretic runs (see SDS−PAGE). One milliliter of sample was layered on the top of each gradient.

Linear sucrose gradients, from 52% to 62% (w/v) of sucrose, were prepared on a 3 mL 65% (w/v) sucrose bed (Yamagata et al., 1982) according to the method described by Luthe (1983), using sucrose solutions prepared in Tris-HCl buffer, pH 7.5, 100 mM. Samples were ultracentrifuged at 61500*g* for 5 h at 4 °C, in a Beckman XL-90 ultracentrifuge using a swinging bucket SW28 rotor (Beckman).

Percoll gradients were preformed by centrifugation, at 14500*g* during 100 min, of 27 mL of Tris-HCl buffer, pH 7.5, 100 mM, containing 0.25 M sucrose and 40% (v/v) Percoll (Pharmacia Fine Chemicals). After the sample application, gradients were centrifuged at 5000*g* for 20 min in a swinging bucket rotor (SW28, Beckman).

Immediately after centrifugation, the gradients were fractionated in 1 mL samples using a peristaltic pump and analyzed for $A_{280}-A_{340}$, for protein detection. The value of A_{340} rectifies the refraction effect due to the sucrose concentration. The pellet was resuspended in 1 mL of Tris-HCl buffer, pH 7.5, 100 mM. The protein content of each fraction was analyzed by SDS−PAGE.

Protein Digestibility Analysis. *In vitro* proteolysis was performed using three different proteases: trypsin (EC 3.4.21.4, type III, from bovine pancreas), α-chymotrypsin (EC 3.4.21.1, type II, from bovine pancreas), and pepsin A (EC 3.4.23.1, from porcine stomach mucosa), all purchased from Sigma.

In the case of trypsin and α-chymotrypsin, *Q. suber* cotyledons were triturated in Tris-HCl buffer, pH 8.0, 100 mM (5 mL/g of fresh weight) and filtered through four layers of gauze, and 200 μL of the extract was incubated for 15 min at 30 °C in the presence of 50 μg/mL of protease. In the case of pepsin, the same procedure was applied, except that the buffer used was formate, pH 4.0, 100 mM. After the incubation period, the proteolytic reaction was stopped by adding 4 volumes of cold acetone (−20 °C), and the remaining polypeptides were subsequently analyzed by SDS−PAGE.

When appropriate, *L. minor* proteins were extracted and subjected to *in vitro* proteolysis in the presence of trypsin. In this case the plants were frozen in liquid nitrogen and ground to a fine powder, and the proteins were extracted in 3 mL/g of Tris-HCl buffer, pH 8.0, 100 mM. The homogenate was filtered through four layers of gauze and clarified by centrifugation at 30000*g* for 30 min.

Other Assays. The total nitrogen content of *Q. suber* seeds was determined according to the method of Kjeldahl, using a Kjeltec System 1030 (Tecator, Sweden), following the instruc-

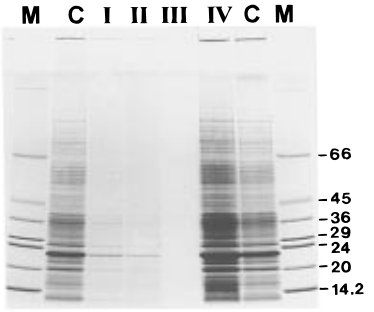


Figure 1. Polypeptide patterns of albumin (lane I), globulin (lane II), prolamin (lane III), and glutelin (lane IV) fractions from *Q. suber* cotyledons. The protein fractions were sequentially extracted as described under Materials and Methods and the resulting samples (250 μL) analyzed by SDS−PAGE. Lanes C, control samples; lanes M, molecular mass markers (kDa).

tions of the manufacturer. Total protein content was estimated by multiplying the total nitrogen by the factor 6.25, a procedure that overestimates the protein values due to the presence of variable amounts of non-protein nitrogenous compounds in seeds.

Carbohydrate concentrations were measured according to the phenol−sulfuric acid method described by Dubois et al. (1956).

RESULTS AND DISCUSSION

Our first approach to the study of the composition of *Q. suber* seeds was to determine their content in water, proteins, and carbohydrates. We obtained values of 40.86 ± 1.62% (w/w) for water, 5.03 ± 0.76% (w/w) for proteins, and 53.34 ± 4.20% (w/w) for carbohydrates. These values are in good agreement with those of Natividade (1950), who reported concentrations of 40.58% (w/w), 4.03% (w/w), and 49.12% (w/w), respectively.

Classification of proteins based on solubility is an ambiguous system. However, fractionation based on solubility criteria is a convenient system to begin the characterization of the seed storage proteins from a species that has not been studied before. We have therefore subjected the cotyledons of *Q. suber* seeds to a sequential extraction procedure, to analyze, by SDS−PAGE, the polypeptide composition of the albumin, globulin, prolamin, and glutelin fractions. The results are illustrated in Figure 1 and show that the proteins are predominantly soluble in sodium borate buffer, pH 10, 50 mM, containing 1% (v/v) β-mercaptoethanol and 1% (w/v) SDS. There are small amounts of proteins in the albumin (Figure 1, lane I) and globulin (Figure 1, lane II) fractions. However, the corresponding polypeptide patterns are identical to the one obtained for glutelins, suggesting that the presence of proteins in the albumin and globulin fractions is due to a glutelin contamination. No proteins were detected in the pellet obtained after glutelin extraction. In conclusion, the results presented in Figure 1 indicate that the storage proteins present in *Q. suber* cotyledons are predominantly glutelins.

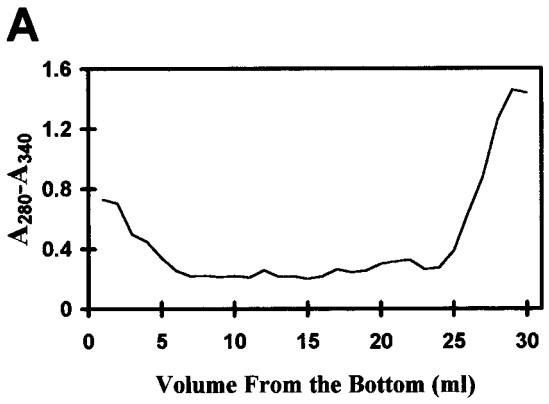
Although the most common seed storage proteins are albumins, globulins, and prolamins (Shewry et al., 1995), there are seeds that have been reported to contain glutelins as the major storage proteins. It is the case, for example, of rice, where glutelins constitute over 70−80% of the total seed protein (Padhye and Salunkhe, 1979; Yamagata et al., 1982).

Knowledge on the seed storage proteins from tree species lags far behind that of annual plants. The work that has been published indicates that there is considerable heterogeneity in the seed storage proteins when different trees are considered. Thus, for example, Ampe et al. (1986) have purified and characterized a 2S albumin, the major storage protein from the seeds of *Bertholletia excelsa*. Katsuta (1961) reported that the seeds of *Pinus thunbergii* contain albumin, globulin, and glutelin, and Allona et al. (1994) studied a low molecular mass globulin from the seeds of *Pinus pinaster* that shows some homology with the 2S proteins of angiosperms. Blagrove et al. (1984) reported a study on macrozin, the major storage globulin from seeds of *Macrozamia communis*. Macrozin was not identified as a vicilin-like or legumin-like protein. However, Leal and Misra (1993) published a work on the molecular cloning and characterization of a legumin-like storage protein from Douglas fir seeds, and Jensen and Berthold (1989) reported that the major storage protein in the seeds of the gymnosperm *Ginkgo biloba* is a legumin-like globulin. On the other hand, Newton et al. (1992) published a study on vicilin-like seed storage proteins in the gymnosperm *Picea glauca/engelmannii*.

Preliminary experiments suggested the existence of large aggregates of proteins in *Q. suber* cotyledons. Therefore, we have analyzed these proteins by sucrose density gradient ultracentrifugation following the method described by Yamagata et al. (1982) for the separation of two types of protein bodies from rice seeds. The results obtained, presented in Figure 2, show that it was not possible to obtain purified aggregates. After the ultracentrifugation, most proteins were pelleted and there was a small amount of proteins at the top of the gradient. A polypeptide pattern, similar to the one from control samples (which should contain all of the cotyledon proteins), was obtained for the pellet and for the proteins at the top of the gradient. These results suggest that the proteins from *Q. suber* cotyledons are in the form of large aggregates that are found at the bottom of the gradient after ultracentrifugation. The proteins at the top of the gradient were identified with those extracted as albumins and globulins. Similar results were obtained when *Q. suber* seed proteins were analyzed by ultracentrifugation on Percoll gradients or by gel filtration on a Sepharose CL-4B (Pharmacia Fine Chemicals) column (data not shown).

Many seed storage proteins from different plants are glycosylated. This is certainly true for legume seeds (Derbyshire et al., 1976). In an attempt to determine the extent of glycosylation of *Q. suber* seed proteins, we have probed the total polypeptide pattern with concanavalin A−peroxidase. The presence of glycosyl residues (α-D-glucopyranosyl, α-D-mannopyranosyl, and other related residues), bound by *N*-glycosidic linkages to proteins, was recognized by concanavalin A (Faye and Chispeels, 1985). The results obtained, presented in Figure 3, show that several polypeptides are glycosylated. However, a comparison between the pattern of total polypeptides (Figure 3A, lane C) and the pattern of glycosylated polypeptides (Figure 3B, lane C) reveals that the major or most representative polypeptides from *Q. suber* seeds are not glycosylated. Unlike many other species, the seeds from *Q. suber* seem to be particularly poor in glycoproteins.

The importance of characterizing the seed proteins from a species that has not been studied before depends largely on its potential use for animal and/or human consumption. Equally important is to determine the nutritive value of the seed proteins. The resistance of proteins to digestion could be a determining factor to



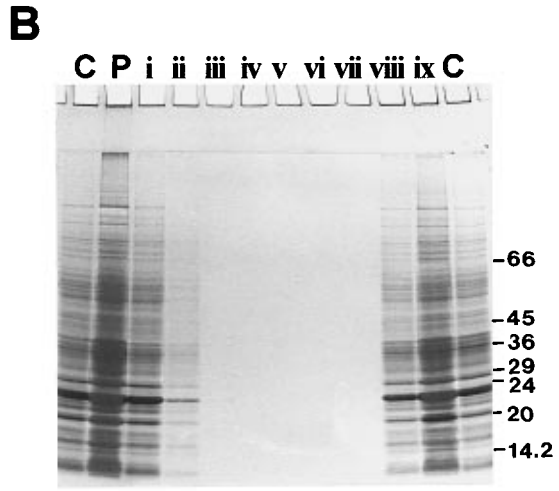


Figure 2. Sucrose density gradient analysis of total cotyledonary proteins from *Q. suber*. Total protein was extracted and subjected to ultracentrifugation through linear sucrose gradients as described under Materials and Methods. The sucrose gradients (52−62% w/v sucrose) were prepared on a 3 mL 65% (w/v) sucrose bed. The gradient was fractionated and each 1 mL fraction assayed for $A_{280}-A_{340}$ (A) and, after acetone precipitation, analyzed by SDS−PAGE (B). Only the fractions containing protein were analyzed by SDS−PAGE: the five fractions at the bottom of the gradient (i, ii, iii, iv, v; 0−5 mL) and the four fractions at the top of the gradient (vi, vii, viii, ix; 26−30 mL). Lane P, pellet; lanes i, ii, iii, iv, v, samples corresponding to the first five fractions from the bottom of the gradient; lanes vi, vii, viii, ix, samples corresponding to the last four fractions at the top of the gradient. Intermediary fractions, containing no proteins, are not represented. Lanes C, control samples. Molecular masses are indicated in kDa.

their nutritional quality. In this respect, we have performed *in vitro* digestibility studies of *Q. suber* seed proteins to evaluate their resistance to proteolytic attack. To this end, total cotyledonary protein was extracted and incubated in the presence of commercial proteolytic enzymes. The results obtained, represented in Figure 4A for trypsin (lane T), α-chymotrypsin (lane Ch), and pepsin (lane P), indicate that intense proteolysis occurs when the total cotyledonary protein is incubated, *in vitro*, in the presence of proteases, particularly in the case of pepsin. Trypsin and α-chymotrypsin seem to degrade the larger molecular mass polypeptides. Particularly resistant to attack by these enzymes is a major 23 kDa polypeptide. The effect of pepsin is much more extensive, with very few polypeptides remaining after the incubation with this enzyme. This result was not due to exposure to a low pH value (data not shown). The increased stained material at the bottom of lanes

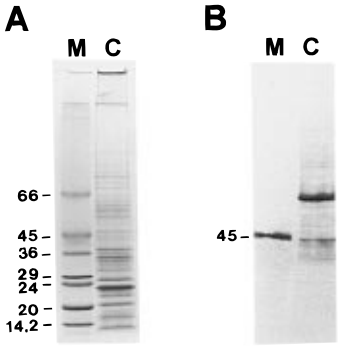


Figure 3. Glycoprotein analysis. Control samples (C), containing the total cotyledonary proteins from *Q. suber* seeds, and molecular mass markers (M) were analyzed by SDS−PAGE and the polypeptides stained with Coomassie Brilliant Blue R-250 (A) or transferred to a nitrocellulose membrane and probed with concanavalin A−peroxidase for glycoprotein detection (B). Among the molecular mass markers used, only ovalbumin (45 kDa) is glycosylated. Molecular masses of markers are indicated in kDa.

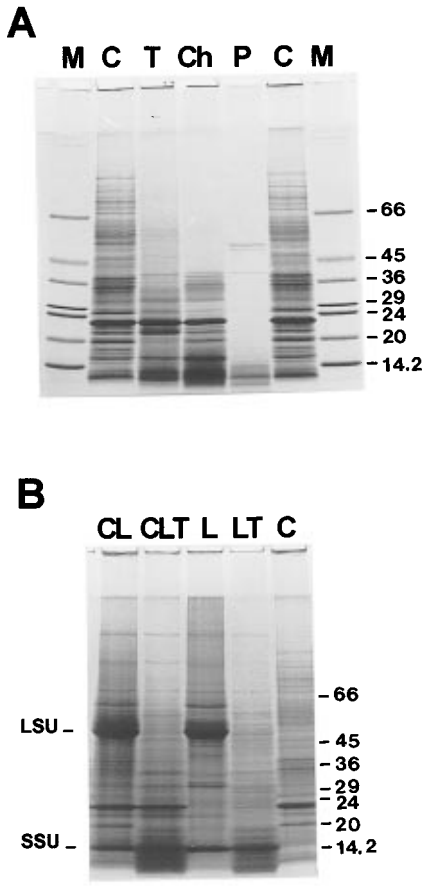


Figure 4. *In vitro* proteolysis of proteins from *Q. suber* cotyledons: (A) total cotyledonary protein was extracted and incubated in the presence of trypsin (lane T), α-chymotrypsin (lane Ch), and pepsin (lane P) as described under Materials and Methods; (B) for testing trypsin inhibition by seed components, a mixture (lane CL) of control sample (lane C) and *L. minor* soluble proteins (lane L) was incubated in the presence of trypsin during 15 min at 30 °C (lane CLT). Ribulose bisphosphate carboxylase subunits are completely digested when a *L. minor* extract is incubated in the presence of trypsin (LT). LSU and SSU, large and small subunits of ribulose bisphosphate carboxylase. Lanes M, molecular mass markers (kDa).

T, Ch, and P (Figure 4A) is indicative of the presence of proteolytic products of the digestion. Taken together, the actions of the three proteolytic enzymes on *Q. suber* seed proteins lead to complete digestion, which may be

envisaged as a good indication of the nutritional quality of these seeds.

The result presented in Figure 4A, lane T, indicates that the proteolytic activity of trypsin on *Q. suber* seed proteins is far from leading to a complete digestion. Furthermore, the digestion with trypsin was not enhanced by a longer period of incubation (data not shown). The incomplete proteolytic digestion caused by trypsin could be due to the presence of trypsin inhibitor(s) in the seeds, which is commonly reported for seeds from other species. To test this hypothesis, we have analyzed the effect of trypsin on the subunits of ribulose bisphosphate carboxylase from *L. minor*. Trypsin leads to complete degradation of the subunits (Figure 4B, lane LT). The same result was observed when we studied the effect of trypsin on the total cotyledonary protein in the presence of ribulose bisphosphate carboxylase (Figure 4B, lane CLT). Furthermore, a fate identical to that of ribulose bisphosphate carboxylase subunits occurs when more of this enzyme is added at the end of the incubation period and the mixture is incubated for an extra 15 min (data not shown). These results led us to conclude that *Q. suber* seed polypeptides remaining after incubation with trypsin were not due to enzymatic inhibition but could be related with other factors such as the structure of the proteins in the seeds.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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