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Protein Cryoprotective Activity of a Cytosolic Small Heat Shock Protein That Accumulates Constitutively in Chestnut Stems and Is Up-Regulated by Low and High Temperatures¹

Maria-Angeles Lopez-Matas², Paulina Nuñez³, Alvaro Soto⁴, Isabel Allona, Rosa Casado, Carmen Collada, Maria-Angeles Guevara⁴, Cipriano Aragoncillo, and Luis Gomez*

Departamento de Biotecnología, Escuela Técnica Superior de Ingenieros de Montes, Universidad Politécnica de Madrid, E-28040 Madrid, Spain

Heat shock, and other stresses that cause protein misfolding and aggregation, trigger the accumulation of heat shock proteins (HSPs) in virtually all organisms. Among the HSPs of higher plants, those belonging to the small HSP (sHSP) family remain the least characterized in functional terms. We analyzed the occurrence of sHSPs in vegetative organs of *Castanea sativa* (sweet chestnut), a temperate woody species that exhibits remarkable freezing tolerance. A constitutive sHSP subject to seasonal periodic changes of abundance was immunodetected in stems. This protein was identified by matrix-assisted laser-desorption ionization time of flight mass spectrometry and internal peptide sequencing as CsHSP17.5, a cytosolic class I sHSP previously described in cotyledons. Expression of the corresponding gene in stems was confirmed through cDNA cloning and reverse transcription-PCR. Stem protein and mRNA profiles indicated that CsHSP17.5 is significantly up-regulated in spring and fall, reaching maximal levels in late summer and, especially, in winter. In addition, cold exposure was found to quickly activate *shsp* gene expression in both stems and roots of chestnut seedlings kept in growth chambers. Our main finding is that purified CsHSP17.5 is very effective in protecting the cold-labile enzyme lactate dehydrogenase from freeze-induced inactivation (on a molar basis, CsHSP17.5 is about 400 times more effective as cryoprotectant than hen egg-white lysozyme). Consistent with these observations, repeated freezing/thawing did not affect appreciably the chaperone activity of diluted CsHSP17.5 nor its ability to form dodecameric complexes in vitro. Taken together, these results substantiate the hypothesis that sHSPs can play relevant roles in the acquisition of freezing tolerance.

Cold acclimation is a complex process by which the freezing tolerance of certain plants increases after a period of exposure to low nonfreezing temperatures. Because of the enormous agricultural impact of freezing injury, especially in temperate regions, the molecular mechanisms associated with cold acclimation have been the subject of intensive research over the past decades. Studies with *Arabidopsis* and cold-hardy herbaceous plants, such as winter cereals (*Triticum aestivum*, *Hordeum vulgare*), spinach (*Spinacia*

oleracea), oilseed rape (*Brassica napus*), or cabbage (*Brassica oleracea*) have led to the identification of numerous genes potentially involved in freezing tolerance (for recent reviews, see Thomashow, 1999; Smallwood and Bowles, 2002). Many of these genes encode proteins with known activities, like enzymes for the synthesis of compatible solutes or for the modification of membrane lipids. In other instances, however, the function of the gene products remains unknown. In a few cases the encoded proteins have been shown to contribute functionally to freezing tolerance, such as the stromal polypeptides COR15a (Artus et al., 1996; Steponkus et al., 1998) and WCS19 (NDong et al., 2002). The signal transduction networks involved in cold-regulated gene expression have also been studied in a number of herbaceous species, including *Arabidopsis* and several crops (Shinozaki et al., 2003).

The molecular aspects of cold acclimation remain largely unexplored in long-lived woody plants. However, it is well established that the capacity of temperate zone woody perennials to cold acclimate is much higher than that of herbaceous species (Weiser, 1970). This is probably a consequence of having longer life cycles and generation times, and also of the more extreme conditions endured by their aerial parts in winter. Woody plants seem to attain freezing tolerance

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² Present address: Facultad de Ciencias, Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, E-28049 Madrid, Spain.

³ Present address: Departamento de Silvicultura, Facultad de Ciencias Forestales, Universidad de Chile, Santiago, Chile.

⁴ Present address: Centro de Investigación Forestal-CIFOR, Instituto Nacional de Investigaciones Agrarias, Ministerio de Agricultura, Pesca y Alimentación, E-28040 Madrid, Spain.

* Corresponding author; e-mail lgomez@montes.upm.es; fax 34-91-3366387.

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through various stages, which are sequentially activated by daylength shortening and increasingly lower temperatures (Weiser, 1970; Sakai and Larcher, 1987). Very recently, evidence has been obtained in hybrid poplar (*Populus tremula* × *P. tremuloides*) that these environmental cues trigger cold acclimation through distinct pathways (Welling et al., 2002). In herbaceous plants, low temperature is the primary signal responsible for inducing this process (Thomashow, 1999).

Among the proteins induced or up-regulated in plants by low temperatures there are heat shock proteins (HSPs). These include members of the HSP70 family in spinach (Neven et al., 1992; Anderson et al., 1994) and soybean (*Glycine max* L. Merr.; Cabané et al., 1993), and also HSP90 isoforms in rice (*Oryza sativa*; Pareek et al., 1995) and oilseed rape (Krishna et al., 1995). Homologous cold-responsive HSPs have been described in other organisms, such as *Drosophila melanogaster* (Burton et al., 1988) or mice (*Mus musculus*; Matz et al., 1995), conveying the generality of this response. Since HSP70 and HSP90 exhibit molecular chaperone activity, a protective role against freeze-induced protein denaturation has been hypothesized (e.g. Guy et al., 1998).

In only a few instances low temperatures have been shown to stimulate the accumulation of small HSPs (sHSPs), which are the most diverse and abundant HSPs synthesized by plants (Vierling, 1991; Waters et al., 1996; van Montfort et al., 2001). This was first described by van Berkel et al. (1994) in cold-stored potato (*Solanum tuberosum*) tubers. Chilling-induced sHSP synthesis has also been observed in tomato (*Lycopersicon esculentum* L. cv Daniella) fruits, but only following heat treatment (Sabehat et al., 1998). More recently, Ukaji et al. (1999) reported that accumulation of WAP20, an endoplasmic reticulum-localized sHSP, is associated with cold acclimation in *Morus bombycis* (mulberry tree). Winter-specific accumulation of sHSPs has also been observed in *Acer platanoides*, *Sambucus nigra*, and *Aristolochia macrophylla* (Lubaretz and zur Nieden, 2002). The possible relationship of these sHSPs with protective mechanisms against low temperature stress is still a matter of speculation. Many experiments have shown that sHSPs have molecular chaperone activity (van Montfort et al., 2001). Besides, members of this protein family can enhance stress tolerance in a variety of cell systems (e.g. Lavoie et al., 1993; Yeh et al., 1997; Soto et al., 1999). Direct evidence for a chaperone function in cellular thermotolerance has been recently obtained for a cyanobacterial sHSP (Giese and Vierling, 2002). Other studies have reported that sHSPs have stabilizing effects on model membranes formed of synthetic and cyanobacterial lipids, suggesting a role for these proteins in preserving membrane integrity during thermal fluctuations (Török et al., 2001; Tsvetkova et al., 2002). While these properties might contribute to cell survival under freezing stress, more studies are obviously needed to understand

the hypothetical role of sHSPs in relation to cold acclimation.

Here we analyze the accumulation patterns of sHSPs in vegetative tissues of both adult chestnuts (*Castanea sativa*; field conditions) and seedlings kept under controlled conditions. A major constitutive sHSP subject to seasonal periodic changes of abundance was immunodetected in stems. This protein was identified by mass fingerprinting and internal amino acid sequencing as CsHSP17.5, a cytosolic class I sHSP isolated previously from mature chestnut cotyledons (Collada et al., 1997). The main finding is that this protein, the expression of which is maximal in winter and quickly responds to cold exposure (4°C), shows significant protein cryoprotective activity in vitro. Besides suggesting a novel function for this protein family, our results substantiate the notion that sHSPs may play relevant roles in the acquisition of freezing tolerance. A similar function has been postulated for high-molecular mass chaperones of the HSP70 family (Guy et al., 1998; Sung et al., 2001).

RESULTS

Detection of sHSPs in Vegetative Organs

To investigate the occurrence of sHSPs in chestnut vegetative organs, 16- to 46-week-old seedlings kept under normal growth conditions were subjected to heat stress (38°C for 4 h), and total proteins were isolated from leaves, stems, and roots. Samples corresponding to equal quantities of protein from both stressed and nonstressed plants were then fractionated by SDS-PAGE and immunoblotted with purified polyclonal antibodies against seed CsHSP17.5 (Collada et al., 1997). While all organs analyzed contained cross-reactive polypeptides following the heat treatment, these were undetectable in leaves and roots

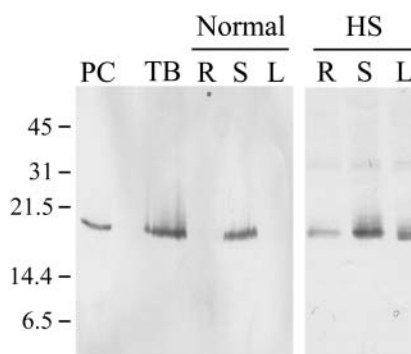


Figure 1. Chestnut proteins recognized by the CsHSP17.5 antiserum. Crude protein extracts were obtained from leaves (L), stems (S), and roots (R) of seedlings kept under normal growing conditions or heat-stressed (HS) for 4 h at 38°C. Proteins were also extracted from tree branches (TB) harvested under field conditions (a sample from January is shown). Equal quantities of total protein (25 µg) were fractionated by SDS-PAGE and subjected to immunoblot analysis using purified polyclonal antibodies to CsHSP17.5. Purified seed CsHSP17.5 (0.5 µg) was routinely included in the gels as positive control (PC). Migration of molecular mass standards are given in kilodaltons on the left.

of nonstressed plants (Fig. 1 shows a representative blot). However, a prominent band was consistently observed in stem samples of control seedlings. In all cases, the apparent size of the reactive polypeptides was very similar to that of purified seed CsHSP17.5, included as a positive control. No signals were detected when equivalent blots were reacted with preimmune serum (data not shown). These results suggest that some member(s) of the sHSP family might accumulate constitutively in chestnut stems. Since sHSPs are generally undetectable in nonstressed vegetative organs (Waters et al., 1996), we decided to characterize further the stem proteins. Interestingly, the antibodies also recognized a single 20-kD band in stem samples from adult trees, namely, second-year branch internodes (an example is presented in Fig. 1, lane TB).

Characterization and cDNA Cloning of the Major Stem sHSPs

Two-dimensional western analysis revealed that the 20-kD band detected in stems contained two major cross-reactive components in both seedlings and adult trees. These polypeptides, labeled as St1 and St2 in Figure 2A, had similar pIs (approximately 6) and overlapped when extracts from seedlings and trees were coelectrophoresed (data not shown; Fig. 2, B and C). Since their N-terminal residues appeared to be blocked (direct sequencing of polyvinylidene difluoride [PVDF]-adsorbed samples), a preparation highly enriched in St1 and St2 was obtained from whole branch slices by selective extraction and size-exclusion HPLC (see "Materials and Methods"). Due to the unusual abundance of condensed tannins and other phenolic compounds in chestnut bark, proteins were extracted under strongly reducing conditions and washed extensively with acetone. Moreover, alkylation was necessary to prevent significant aggregation and to obtain reproducible chromatograms. Both St1 and St2 eluted in a single HPLC peak along with a few minor proteins (Fig. 3A). To gain information on their primary structure, preparative two-dimensional electrophoresis was carried out followed by endoproteinase Asp-N cleavage of the eluted proteins. The resulting peptidic fragments were then fractionated by reverse-phase HPLC on Nucleosil C-4, with virtually identical chromatograms being obtained for both proteins (Fig. 3C). As shown in Figure 3D, St1 and St2 had matching internal sequences and, furthermore, these agreed at every residue with an internal region of seed CsHSP17.5 spanning amino acids 50 to 77. Such region corresponds to the N-terminal portion of the conserved α -crystallin domain present in all sHSPs.

Two different approaches were then followed to further characterize St1 and St2. On one hand, both polypeptides were subjected to matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) analysis following two-dimensional electrophoresis and trypsin digestion. The resulting peptide-mass

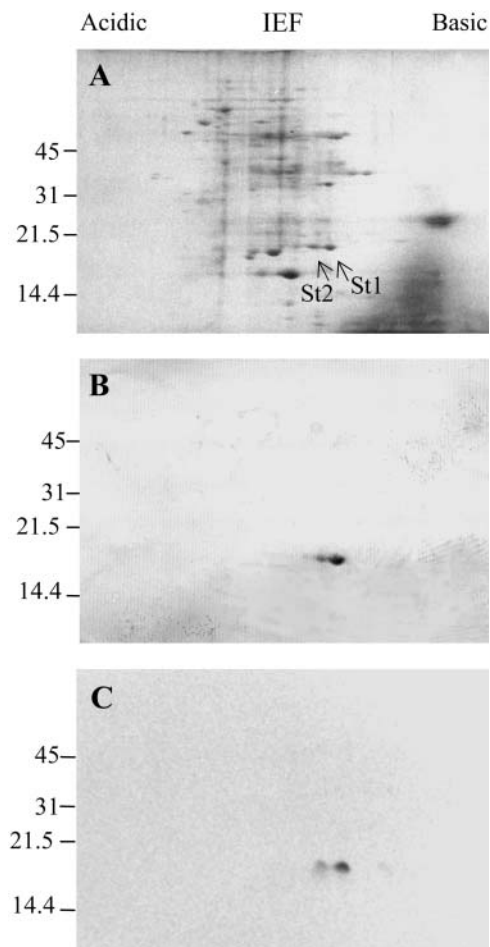


Figure 2. Identification of individual cross-reactive polypeptides in stem tissues of 24-week-old chestnut seedlings. Total proteins (30 μ g) were separated by two-dimensional electrophoresis (IEF, pH 3–10 \times SDS-PAGE) and then silver-stained (A) or immunoblotted with the CsHSP17.5 antiserum (B). The two major cross-reactive polypeptides detected in stems were designated as St1 and St2. For comparison purposes, an equivalent extract from adult trees (second-year branches) was also reacted with the CsHSP17.5 antiserum (C). Approximate molecular masses, in kilodaltons, are indicated.

fingerprints were essentially indistinguishable in the range of 0.9 to 3.1 kD, lending further support to the notion that St1 and St2 are modified forms of a single amino acid chain (Table I). The nature of such modification remains to be determined. Moreover, the sharp correspondence between the observed peptide masses and the predicted fingerprint for CsHSP17.5 strongly supports the hypothesis that St1/St2 and the seed sHSP (theoretical pI, 5.95) have identical primary structures, a notion anticipated by internal amino acid sequencing (Fig. 3D). Interestingly, the N-terminal peptides of St1 and St2 were predicted to be acetylated in Ala-2 (see Table I), which would explain the lack of substantial signals upon automatic sequencing. The second approach involved the construction of a chestnut stem cDNA library (4.5×10^7 pfu mL^{-1}) and its screening at moderate stringency

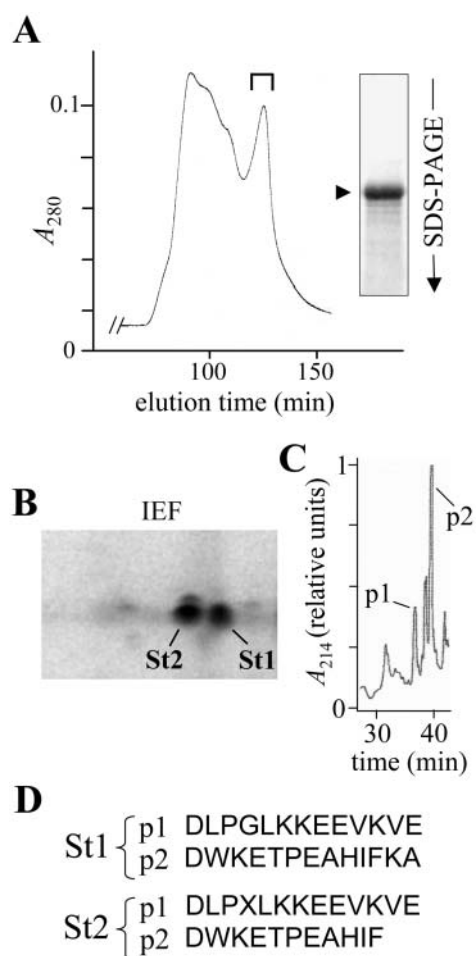


Figure 3. Purification and partial characterization of polypeptides St1 and St2. **A**, Size-exclusion HPLC of carboxymethylated stem proteins from adult trees (see "Materials and Methods"). The column (Spherogel TSK-G) was eluted with 50 mM ammonium acetate, pH 5.5, 0.1% SDS. The single peak containing St1 and St2 is indicated by a horizontal bar (only the relevant portion of the chromatogram is shown). SDS-PAGE analysis of this peak (inset) revealed the presence of several minor polypeptides in addition to St1 and St2 (the position of which is indicated by an arrowhead). **B**, To further characterize St1 and St2, the polypeptides present in the HPLC peak were separated by two-dimensional electrophoresis and then electrotransferred to PVDF (the appropriate portion of a membrane is shown). Both St1 and St2 appeared to be N-terminally blocked upon direct sequencing of PVDF-adsorbed samples. **C**, Reverse-phase HPLC separation of the peptidic fragments obtained after *in situ* Asp-N cleavage of PVDF-adsorbed St1. Essentially the same chromatogram was obtained for St2. **D**, Amino acid sequence of peptides p1 and p2, obtained after Asp-N digestion of both St1 and St2 (their position in the HPLC chromatogram is indicated in **C**).

with the cDNA for seed CsHSP17.5. Eight independent positive clones were randomly selected and their inserts sequenced. All inserts corresponded to a single nucleotide sequence that showed no mismatches with the *Cshsp17.5* seed cDNA, except for the presence of six additional nucleotides at the 5'-untranslated end (accession no. AJ582679). We conclude from these experiments and the reverse transcription (RT)-PCR

experiments reported below that CsHSP17.5 is the predominant sHSP in chestnut stems.

Seasonal Changes of Protein and mRNA Abundance

To investigate sHSP seasonal accumulation patterns, branch segments were collected from adult trees at monthly intervals and their proteins subjected to western-blot analysis with anti-CsHSP17.5 antibodies. In the representative immunoblot shown in Figure 4A, a cross-reactive 20-kD band is observed in all samples analyzed (January through December 1999). These experiments, along with western-blot analyses of nonstressed seedlings (see Fig. 1A), strongly suggest that CsHSP17.5 is constitutively expressed in chestnut stems. However, its relative abundance appears to vary on a seasonal basis, with the highest levels occurring in winter and late summer (there is approximately a 4-fold difference in signal intensity between January and April). Essentially the same accumulation patterns were observed for 1999 and 2000, supporting the generality of these findings. To test whether the observed changes in protein abundance truly reflected seasonal fluctuations of gene activity, total RNA was isolated from equivalent samples and hybridized at high-stringency with the full-length cDNA for CsHSP17.5 (Fig. 4B). The RNA blots revealed annual oscillations in the amount of hybridizing transcripts, which were basically concurrent with protein data. In fact, after a gradual increase

Table 1. Peptide mass fingerprints for St1/St2

Position in CsHSP17.5	Observed Mass ^a	Predicted Mass ^b	Difference
2–9	862.4790	820.4670	42.012 ^c
10–38	3181.5242	3181.5265	–0.023
39–60	2562.2914	2562.2986	–0.072
48–60	1613.8369	1613.8322	0.047
48–67	2308.2282	2308.2335	–0.053
48–68	2436.3298	2436.3285	0.013
61–87	2951.5715	2951.5683	0.032
68–87	2257.1698	2257.1670	0.028
69–87	2129.0785	2129.0720	–0.065
73–87	1643.8192	1643.8235	–0.043
95–103	1239.6377	1239.6341	0.036
95–107	1614.7932	1614.7917	0.015
111–119	1130.6366	1130.6429	–0.063
111–124	1713.9785	1713.9758	–0.027
112–119	974.5425	974.5418	0.007
112–124	1557.8674	1557.8747	–0.073
120–138	2068.1489	2068.1470	0.019
125–142	1999.0485	1999.0528	–0.043

After fractionation of stem proteins by IEF × SDS-PAGE, silver-stained St1 and St2 were subjected to trypsin digestion and MALDI-TOF mass spectrometry analysis. Essentially identical mass fingerprints were obtained for St1 (shown here) and St2. ^aObserved molecular mass inferred by MALDI-TOF mass spectrometry. ^bPredicted molecular mass for the indicated tryptic peptides of CsHSP17.5. All peptides shown had close agreement between predicted and observed masses. ^cPredicted to be acetylated in Ala-2 by FindMod (Wilkins et al., 1999).

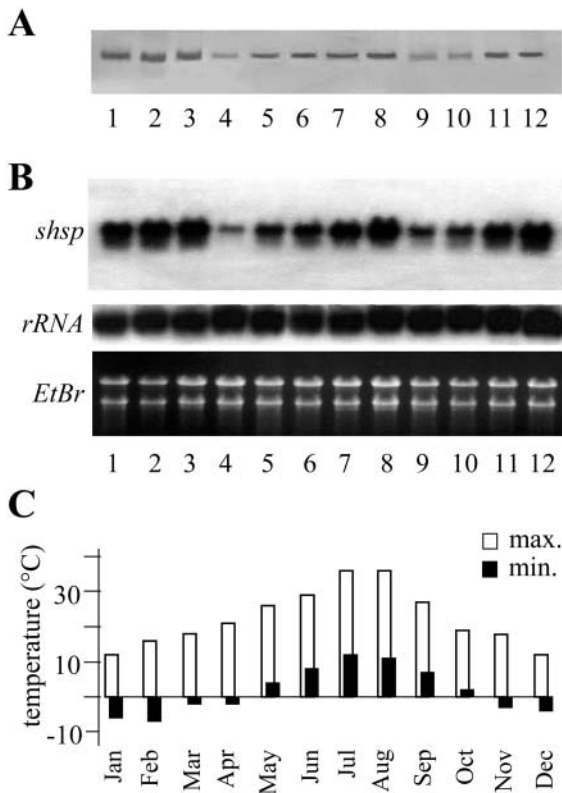


Figure 4. Seasonal changes in protein and RNA profiles. Second-year branch internodes were harvested from adult chestnuts at monthly intervals during two complete years (only 1999 is shown). A, Immunoblot analysis of SDS-PAGE-separated stem proteins using purified polyclonal antibodies to CsHSP17.5. The single band detected in all samples had the same apparent size as purified CsHSP17.5 (see Fig. 1). Approximately 25 μ g of total protein were loaded in each lane. Samples are numbered from 1 (January) to 12 (December). B, RNA gel-blot analysis of equivalent samples using the complete cDNA for CsHSP17.5 as probe. Approximately 10 μ g of total RNA were loaded per lane (besides ethidium bromide [*EtBr*] staining, even loading was routinely tested by hybridizing membranes with an 18S *rRNA* probe). Lane numbers are as in A. C, Monthly averaged maximal and minimal temperatures at the sample collection site during 1999 (data source: National Institute of Meteorology, Madrid).

during spring and fall, transcription was found to be maximal in late summer (July and August) and winter. The highest and lowest yearly temperatures at the sample collection site were recorded during these two periods, respectively (Fig. 4C; data for 1999). The presence of mRNA for CsHSP17.5 in the above extracts was verified by RT-PCR (see "Materials and Methods"). Even at low annealing temperatures a single 383-bp band was amplified in all preparations analyzed, the sequence of which was identical to the *Cshsp17.5* cDNA (nucleotides 130–512). Taken together, our results are indicative that CsHSP17.5 is constitutively expressed in chestnut stems. Furthermore, protein abundance is subjected to periodic (seasonal) variations, with the highest rates of accumulation being associated with fall and early summer.

Cold Exposure Activates *shsp* Gene Expression

Many genes induced during cold acclimation are responsive to low temperatures (Thomashow, 1999). This appears to be the case for at least some chestnut *shsp* genes, as judged by RNA gel-blot hybridization of samples from cold-exposed seedlings. As shown in Figure 5, the abundance of transcripts hybridizing with the *Cshsp17.5* cDNA increased drastically in stem and root tissues upon temperature shift. Moreover, comparison with the same organs of untreated plants (time 0) revealed that transcript accumulation was initiated within 1 d of cold treatment, increasing steadily thereafter. The induction rate in stems appeared to be higher than in roots, and the same is true for the levels of mRNA accumulation. Figure 5 also shows that untreated seedlings accumulate in their stems hybridizing transcripts identical in size to the *Cshsp17.5* mRNA, in agreement with former results suggesting constitutive expression in this organ (see above and Fig. 1). As before, the presence of transcripts for CsHSP17.5 was verified by RT-PCR (data not shown). For comparison purposes we also included in the blots equivalent RNA samples from heat-stressed vegetative organs, harvested from normally grown seedlings after 8 h exposure to 40°C. Taken together, these results confirm the cold-responsiveness of at least some chestnut *shsp* genes and suggest a possible relationship with protective mechanisms against the effects of winter temperatures.

Cryoprotective Activity and Cryostability of CsHSP17.5

Molecular chaperone activity has been previously reported for purified CsHSP17.5 (Collada et al., 1997).

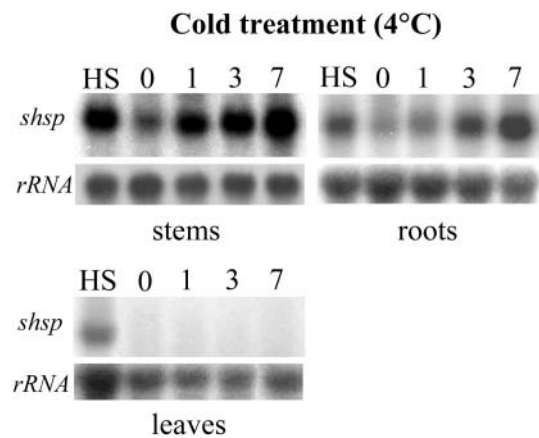
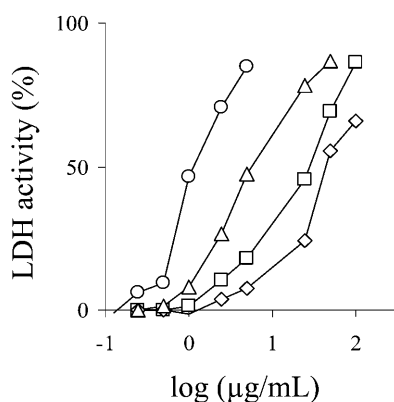


Figure 5. Influence of cold stress on the accumulation of *shSP* transcripts in vegetative organs. For these experiments, 30-week-old chestnut seedlings were shifted from normal temperature (22°C/18°C, day/night) to 4°C. Total RNA, extracted from roots, leaves, and stems at the times indicated (in days), was analyzed by northern hybridization using as a probe the *Cshsp17.5* cDNA. Equivalent RNA samples from heat-stressed (HS) roots, leaves, and stems were included in the gels for comparison. All hybridizing bands had approximately 750 nucleotides. As a control, the same filters were hybridized with a barley (*Hordeum vulgare*) 18S ribosomal probe.

Such ability to bind nonnative polypeptide chains, along with the expression patterns reported above, led us to investigate whether CsHSP17.5 could also prevent freeze-induced protein inactivation *in vitro*. We used as a model substrate for these experiments L-lactate dehydrogenase (LDH), a freeze-labile enzyme that loses most of its catalytic activity by a single freeze/thaw cycle (Lin and Thomashow, 1992). Figure 6 presents a comparison of the cryoprotective effects of CsHSP17.5 (purified from seeds) and those of several other proteins, chosen either for their similarity in size and pI to the sHSP (hen egg-white lysozyme) or randomly (ConA lectin, soybean trypsin inhibitor). In these assays CsHSP17.5 exhibited the highest efficiency in protecting LDH against freeze-inactivation, with a monomeric CP_{50} value (in $\mu\text{g mL}^{-1}$) between 5 and 37 times lower than those of the other proteins tested (Fig. 6, bottom). On a molar concentration basis, the sHSP complex is about 400 times more effective as cryoprotectant toward LDH than hen egg-white lysozyme. In these experiments 50% cryoprotection was obtained at a sHSP to LDH molar ratio (monomers) of approximately 1 (0.5 in protein weight). PsHSP18.1, a pea (*Pisum sativum*) sHSP highly similar to CsHSP17.5, can protect significantly LDH from

thermal aggregation at similar chaperone to substrate ratios (Lee et al., 1995).

We also tested the effects of repeated freezing/thawing on both the chaperone activity of purified CsHSP17.5 (prepared at 5 g L^{-1} in distilled water) and its ability to assemble into homododecameric complexes under nondissociating conditions. For the molecular chaperone activity assays, CsCh1, the major chestnut seed endochitinase (Collada et al., 1992), was chemically denatured in 6 M guanidine hydrochloride and then diluted 100-fold into a refolding buffer that was supplemented with or contained no CsHSP17.5 (frozen/thawed up to 10 times before addition to the mixture). CsCh1 was chosen because it is a putative *in vivo* substrate for CsHSP17.5, since both proteins accumulate abundantly and concomitantly in cotyledonary cells during seed development. As shown in Figure 7A, the presence of CsHSP17.5 resulted in refolding yields of CsCh1 about 2-fold higher than in control reactions after 120 min of incubation, with no appreciable effect of repeated freezing on chaperone activity. Analogous results were obtained when chemically denatured citrate synthase was used as substrate (data not shown). Figure 7B presents data indicating that the fraction of sHSP molecules able to form dodecameric complexes is not appreciably influenced by repeated freezing/thawing (up to 10 cycles, separated by 1-week storage at -20°C). No variations were found either in the total amount of soluble protein, thus confirming the cryostability of CsHSP17.5 in aqueous solution. In summary, CsHSP17.5 does not appear to be significantly influenced in its conformational stability, aggregation properties, and chaperone activity by alternated exposure to subzero and above-zero temperatures, a condition that plants often endure during winter in temperate climates.



	CP_{50} ($\mu\text{g/mL}$)	CP_{50} (nM)
sHSP	1.2	68
STI	6.3	196
HEWL	39.7	2268
ConA	44.6	405

Figure 6. Cryoprotection of LDH. This enzyme was subjected to one freeze/thaw cycle in the presence of varying amounts of purified CsHSP17.5 (sHSP), soybean trypsin inhibitor (STI), ConA lectin (ConA), hen egg-white lysozyme (HEWL), or buffer alone. In the latter case, the residual LDH activity was less than 10% relative to the activity before freezing (not shown). The graph shows the dependence of residual LDH activity (i.e. after freezing/thawing) on the amount of added protein. \circ , sHSP; Δ , STI; \square , HEWL; \diamond , ConA. The CP_{50} values were determined as described in "Materials and Methods". The results shown are the average of four independent experiments. SE of the CP_{50} values were $\pm 10.4\%$ for sHSP, $\pm 9.7\%$ for STI, $\pm 11.8\%$ for HEWL, and $\pm 14.5\%$ for ConA.

DISCUSSION

Like other long-lived woody species native to temperate regions, *C. sativa* exhibits a remarkable freezing tolerance. Here we show that a member of the sHSP family is significantly up-regulated in stem tissues of this species as the winter approaches, reaching the highest abundance during the coldest period of the year (November-March). This protein has been identified by MALDI-TOF mass spectrometry and internal peptide sequencing as CsHSP17.5, a cytosolic class I sHSP previously isolated from mature chestnut cotyledons (Collada et al., 1997). Expression of the corresponding gene in stem tissues was confirmed through cDNA cloning and RT-PCR. The accumulation of identical sHSPs in seeds and vegetative organs had been formerly postulated in sunflower (*Helianthus annuus*) and pea on the basis of electrophoretic mobility (Almoguera et al., 1993; Wehmeyer et al., 1996). Protein and RNA analysis of samples harvested under field conditions (adult trees)

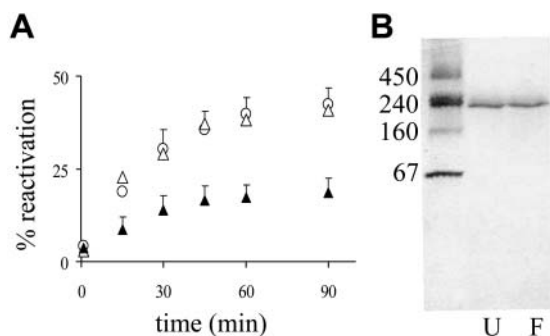


Figure 7. Cryostability of purified CsHSP17.5. For each freeze/thaw cycle, protein solutions (at 5 g L^{-1} in distilled water) were kept 1 week at -20°C before thawing at 25°C . For controls, lyophilized CsHSP17.5 was directly suspended in the appropriate buffer. A, Effects of CsHSP17.5 on the refolding of chemically denatured endochitinase CsCh1. The purified enzyme ($2.5 \mu\text{g}$) was denatured in 6 M guanidine hydrochloride and then placed under refolding conditions in the presence of equimolar amounts of CsHSP17.5 (dodecamer), either unfrozen (white circles) or after 10 freezing/thawing cycles (white triangles). As a negative control (black triangles), CsHSP17.5 was replaced by the same amount of BSA (in μg). At the times indicated, aliquots were assayed for chitinolytic activity. Enzyme reactivation is expressed as percentage relative to the activity of an equivalent amount of nondenatured CsCh1. Average values and SE of three independent replicate trials are shown. B, After freezing/thawing, the ability of soluble CsHSP17.5 to assemble into oligomeric complexes was analyzed by native PAGE (lane F corresponds to a sample frozen/thawed 10 times). Comparison with unfrozen CsHSP17.5 (lane U) showed that the amount of soluble protein or its ability to form complexes was essentially unaffected by freezing. Identical results were obtained for samples frozen two, four, six, and eight times. Before loading in native gels (Ready Gel 7.5%, Bio-Rad), each sample was suspended in 60 mM Tris-HCl, pH 6.8, 10% Suc, and centrifuged 10 min at $15,000\text{g}$ to eliminate insoluble protein.

suggest that CsHSP17.5 is expressed constitutively in stems (Fig. 4). In agreement with these findings, a single protein band with identical mobility to CsHSP17.5 was consistently immunodetected in stem tissues of nonstressed chestnut seedlings by antibodies raised against the seed sHSP (Fig. 1). The only evidence so far of plant sHSPs constitutively expressed in vegetative organs was obtained in the resurrection plant *Craterostigma plantagineum*, a model system for molecular studies of desiccation tolerance (Alamillo et al., 1995). Although proteins immunologically related to CsHSP17.5 were not observed in roots or leaves of nonstressed chestnut seedlings, they were induced in both organs after a short exposure to 38°C (Fig. 1). This treatment also stimulated sHSP expression in stems.

Cold acclimation is characterized in trees by increased cold tolerance in fall, reaching a maximum in winter and then decreasing rapidly in spring (Weiser, 1970; Sakai and Larcher, 1987). During the same time as hardiness develops, deciduous species also enter into endodormancy. Differential expression studies in a number of long-lived woody plants have identified proteins related temporally with these processes,

especially dehydrins and vegetative storage proteins (Howe et al., 1999). The association of HSPs with cold acclimation and/or endodormancy, however, has scarcely been studied in trees. In the first of such studies, Wisniewski et al. (1996) analyzed the seasonal patterns of HSP70 homologs in four species, finding winter-specific induction only in *Prunus persica* (peach). More recently, expression of the endoplasmic reticulum-localized sHSP WAP20 was also associated with cold acclimation in mulberry trees (Ukaji et al., 1999), and the same was true for stem sHSPs of *A. platanoides*, *S. nigra*, and the liana *A. macrophylla* (Lubaretz and zur Nieden, 2002). Except for the May to August period (see below), the seasonal expression patterns of CsHSP17.5 do not differ substantially from those found for other proteins induced during cold acclimation in trees (Wetzel et al., 1989; Wisniewski et al., 1996; Artlip et al., 1997; Ukaji et al., 1999). Since low temperature is a major factor triggering this process, we analyzed the effects of cold stress on *shsp* gene activity. After temperature downshift rapid induction of transcripts hybridizing with the *Cshsp17.5* cDNA was observed in stems and roots, but not in leaves (Fig. 5). It is noteworthy that after only 2 to 3 d of cold stress, the levels of hybridizing transcripts in stems were comparable to those produced by a severe heat shock on control plants (8 h at 40°C). Besides confirming the cold responsiveness of CsHSP17.5, these results may help explain the previous finding that keeping chestnut seedlings at 4°C for several weeks resulted in enhanced levels of *shsp*-like transcripts (Soto et al., 1999). It is tempting to speculate that the lack of cold- but not heat-responsiveness observed in chestnut leaves may be related to the deciduous habit of this species. By contrast to the fall-winter period, the expression patterns of CsHSP17.5 in spring and summer differ substantially from those reported for most cold acclimation-associated proteins. Thus, CsHSP17.5 is strongly up-regulated during this period, reaching another maximum at the warmest time of year (July-August). It seems likely that the thermal stress characteristic of this period plays a key role in such induction, a hypothesis supported by protein and RNA analyses of heat-stressed chestnut seedlings (Figs. 1 and 6). High-level accumulation of sHSPs has been reported in a wide variety of plant species as a consequence of heat treatment (Vierling, 1991).

The precise reasons why CsHSP17.5 is constitutively expressed in chestnut stems are not yet known, and the same is true for its periodic changes of abundance. It is now well established that sHSPs have a high capacity to bind nonnative polypeptides, and current models propose that they cooperate with other cell chaperones to promote proper protein folding and assembly (van Montfort et al., 2001). In addition, recent data suggest that at least some sHSPs can stabilize cell membranes against the effects of thermal fluctuations (Török et al., 2001; Tsvetkova et al., 2002). Different lines of evidence support a role for plant

sHSPs in the acquisition of thermotolerance (see van Montfort et al., 2001). Such a role might contribute to explain the increasing abundance of CsHSP17.5 as summer progresses. In fact, the ability of CsHSP17.5 to enhance cell viability at elevated temperatures has been substantiated through bacterial expression (Soto et al., 1999), and analogous results have been reported for other sHSPs (e.g. Yeh et al., 1997). In addition, evidence bringing together chaperone activity and cell thermotolerance has recently been obtained for a cyanobacterial sHSP (Giese and Vierling, 2002).

Some stresses characteristic of winter can also augment the cellular requirements for chaperones. Low temperature directly affects the stability and solubility properties of many globular proteins, making thermodynamically favorable the exposure of nonpolar side chains to the aqueous solvent (Pace, 1990; Privalov, 1990). The occurrence of cold labile proteins in planta has been confirmed by analyzing extracts of [³⁵S]Met pulse-labeled spinach leaves (Guy et al., 1998). These authors also showed that low nonfreezing temperatures caused increased association of some cell proteins with two chaperones of the HSP70 family. Although sHSPs were not analyzed, there are no reasons a priori to rule out their participation in protective chaperone networks acting at low temperatures. On the contrary, the finding that CsHSP17.5 enhances cell survivability at 4°C when expressed in *Escherichia coli* (Soto et al., 1999) lends support to this hypothesis. Other authors have observed a correlation between the accumulation of sHSPs and the acquisition of chilling tolerance in heat-stressed tomato fruits (Sabehat et al., 1998). But the protective function of sHSPs may not be restricted to chilling temperatures. Analysis of stem samples from adult trees clearly indicates that both gene activity and protein abundance are maximal during the coldest time of year, when subzero temperatures are frequently recorded (Fig. 4). These results led us to test whether purified CsHSP17.5 might act as a protein cryoprotectant in vivo, by using a standard in vitro cryoprotection assay. Our data indicate that CsHSP17.5 is rather effective in preventing freeze-induced inactivation of the cold-labile enzyme LDH (Fig. 6). While a general nonspecific stabilizing effect can be expected from increased protein concentration, the relevant finding is that the CsHSP17.5 complex is considerably more effective than all the other proteins tested (about 400-fold on a molar basis compared to hen egg-white lysozyme [HEWL], which is very similar in size and pI). In addition, the in vitro chaperone activity of CsHSP17.5 toward two different protein substrates was not significantly affected after exposure to repeated freezing and thawing (Fig. 7). We also verified that repeated freezing did not affect the ability of CsHSP17.5 to assemble into homo-oligomeric complexes under nondissociating conditions. sHSP oligomeric stability is thought to be a requisite for cellular function (van Montfort et al., 2001; Giese and Vierling, 2002).

In summary, the biochemical data and the expression patterns reported here for CsHSP17.5 are strongly supportive of a protective role in vivo at periods when thermal extremes often occur. On the other hand, the constitutive expression observed in stems also suggests a role in normal cell maintenance. Virtually identical sHSP expression patterns have been observed in a preliminary study of phylogenetically distant woody species, and the characterization of a *Cedrus atlantica* polypeptide is now under way (R. Casado, I. Allona, C. Collada, C. Aragoncillo, and L. Gomez, unpublished data). The ability of purified CsHSP17.5 to act as cryoprotectant in vitro has not been reported before for any member of this protein family. Such activity may help explain its strong up-regulation in association with the acquisition of cold hardiness in adult trees, and also its cold-responsiveness in seedlings. Nonetheless, the protection of cold-labile proteins is only a working model within the chaperone frame. Cell membranes are thought to be the primary site of freezing injury, and some sHSPs have been shown to stabilize the liquid-crystalline state of model bilayers (Török et al., 2001; Tsvetkova et al., 2002). Further studies will obviously be needed to determine the precise functions played by these proteins at low temperature, the cellular components with which they interact, and also their intriguing absence in vegetative tissues of most cold-acclimated herbaceous plants (Waters et al., 1996).

MATERIALS AND METHODS

Plant Material and Stress Conditions

Stem material (2-year-old branch internodes) and mature seeds of *Castanea sativa* Mill. were harvested from adult trees growing in Zarzalejo, Madrid (4°11' W, 40°35' N). Chestnut seedlings were kept in growth chambers as previously described (Soto et al., 1999). For cold treatment, temperature was downshifted to 4°C.

Protein Purification

Stem material was ground in liquid nitrogen to the consistency of flour. This powder was extracted (10:1, v/w) with 62.5 mM Tris-HCl, pH 6.8, 5% mercaptoethanol, 0.5% SDS, 1 mM phenylmethylsulfonyl fluoride for 60 min at 22°C. After centrifugation at 30,000g for 30 min, proteins in the supernatants were precipitated with 12% (w/v) TCA, washed three times with 80% acetone in water, and then analyzed by SDS-PAGE. For subsequent protein fractionation and purification, the extracted proteins (dried acetone pellets) were first suspended in 0.1 M Tris-HCl, pH 8.6, 2 mM EDTA, 2 mM DTT, 6 M guanidine hydrochloride and then carboxymethylated with 0.2 M iodoacetic acid for 30 min at 37°C. This step reduced significantly protein aggregation caused by phenolic compounds. The carboxymethylated proteins were dialyzed against water, lyophilized, and fractionated by size-exclusion HPLC on a Spherogel TSK-G column (3 × 215 mm) eluted with 50 mM ammonium acetate, pH 5.5, 0.1% SDS. The appropriate HPLC fractions were further fractionated by two-dimensional isoelectrofocusing (IEF) × SDS-PAGE on a Bio-Rad MiniProtein II system (Bio-Rad Laboratories, Hercules, CA). Seed CsHSP17.5 was purified to homogeneity as previously described (Collada et al., 1997).

Peptide Sequencing and MALDI-TOF Mass Spectrometry

Following IEF × SDS-PAGE, proteins were electrotransferred onto PVDF membranes (Immobilon-P, Millipore, Bedford, MA) on a Bio-Rad Mini

Trans-Blot cell. Selected protein spots were subjected to in situ digestion for 5 h at 37°C with endoproteinase Asp-N (Roche Diagnostics, Indianapolis) in 50 mM sodium phosphate, pH 8.0. The resulting peptidic fragments were eluted with 80% (v/v) formic acid in water and separated by reverse-phase HPLC on a Nucleosil C-4 column (4.6 × 250 mm) using a two-step gradient of 0.1% trifluoroacetic acid in acetonitrile (0%–70% acetonitrile in 70 min; 70%–100% acetonitrile in 10 min; flow rate 1 mL/min). The purified peptides were sequenced by standard methods using an Applied Biosystems (Foster City, CA) gas phase 470A sequenator. For MALDI-TOF analyses, peptide-mass fingerprints were obtained as in Pineiro et al. (2001).

cDNA Cloning and RT-PCR

A cDNA library was constructed in Lamda Uni-ZAP XR using chestnut stem poly(A⁺) RNA and the ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene, La Jolla, CA). The library was enriched in 2,000- to 400-bp fragments by gel filtration on Sepharose CL-2B (Amersham Biosciences, Piscataway, NJ). Approximately 10⁵ clones were screened using as probe the full-length cDNA for seed CsHSP17.5 (Soto et al., 1999). After in vivo excision of selected clones, the resulting pBluescript SK(-) phagemids were purified with the UltraClean mini plasmid prep kit (Mo Bio Laboratories, Carlsbad, CA). For RT-PCR analysis, cDNA was synthesized from 5 µg of stem poly(A⁺) RNA using Moloney murine leukemia virus reverse transcriptase (Stratagene) at 75 units per reaction and 2.8 µg of the 50-bp synthetic poly(dT)-containing primer of the Gigapack III Gold Cloning kit (Stratagene). PCR amplifications were then carried out for 25 cycles (94°C, 1 min; 48°C, 90 s; 72°C, 1 min) using two primers positioned in the coding region of the *Cshsp17.5* cDNA: 5'-GAGGGCTTCAGTGCTGTAGC-3' (forward primer, nucleotides 130–149), and 5'-GAGATCTCGATGGACTTGAC-3' (reverse primer, nucleotides 512–493).

Northern Hybridization

Nylon membranes (Magna, MSI, Minnetonka, MN) were prehybridized for 2 h at 42°C in 50% formamide, 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, pH 7, 1 mM EDTA), 5× Denhardt's solution, 0.2% (w/v) SDS, 10% dextran sulfate with denatured salmon sperm DNA at 0.1 g L⁻¹, and then hybridized for an additional 16 h in the same solution with ³²P-labeled probe (see below). After hybridization, membranes were washed at room temperature twice in 2× SSC (1× is 0.15 M NaCl, 15 mM sodium citrate, pH 7), 0.1% SDS for 15 min, twice in 1× SSC, 0.1% SDS for 15 min, and twice in 0.1× SSC, 0.1% SDS for 15 min (moderate stringency). The 660-bp *EcoRI-SalI* fragment from the *Cshsp17.5* cDNA was gel-purified and labeled with [α -³²P]dATP using the Boehringer Mannheim's random-primed DNA labeling kit.

Western Blotting

PVDF-adsorbed proteins were probed with purified polyclonal antibodies to CsHSP17.5 prepared as in Collada et al. (1997). Immunoreactive proteins were detected with goat anti-rabbit IgG coupled to alkaline phosphatase (Bio-Rad).

Cryoprotection Assays

The cryoprotective activity of CsHSP17.5 (purified as in Collada et al., 1997), hen egg-white lysozyme (Roche Diagnostics), soybean trypsin inhibitor (Sigma, St. Louis), and the ConA lectin from *Canavalia ensiformis* (Sigma) was assayed essentially as in Lin and Thomashow (1992). The freeze-labile enzyme LDH (EC 1.1.1.27; L-LDH from hog muscle, Roche Applied Science) diluted at 2.5 µg mL⁻¹ in 10 mM K-phosphate, pH 7.5, was used as substrate. The reaction mixtures were frozen at -20°C for 18 h, thawed at 25°C for 15 min, and then assayed for LDH activity. The CP₅₀ (50% cryoprotection) value of a given protein is the concentration necessary to obtain 50% residual LDH activity after a freeze/thaw cycle (the average residual activity in the absence of added protein was less than 10%).

Chaperone Activity Assays

Purified CsCh1, the major chestnut seed endochitinase (Collada et al., 1992) was used as substrate for these experiments. Chitinolytic activity was measured by a colorimetric method that uses CM-chitin-RBV (Loewe

Biochemica GmbH, Sauerlach, Germany) as substrate (see Garcia-Casado et al., 1998). Briefly, the CsCh1-containing mixtures (see below) were combined with one-half volume of aqueous CM-chitin-RBV. The resulting mixtures (400 µL) were incubated at 37°C for 10 min, and the reactions were stopped by adding 100 µL of 2N HCl. Nondegraded substrate fibers were precipitated 20 min on ice and then removed by centrifugation at 12,000g for 30 min. The amount of soluble dye released by the chitinolytic activity was estimated spectrophotometrically. At least three independent measurements of A₅₅₀ were taken for each sample. For the chaperone activity assays, CsCh1 was denatured in 6 M guanidine hydrochloride, 200 mM Na-acetate, pH 5.5, for 2 h at 25°C, and then diluted 100-fold into Na-acetate buffer supplemented with equimolar amounts of CsHSP17.5 (dodecamer). At the times indicated in Figure 7, 200-µL aliquots (containing 2.5 µg of CsCh1) were assayed for chitinolytic activity.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AJ582679.

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