Pergamon

PII: S0031-9422(98)00372-0

Phytochemistry Vol. 49, No. 8, pp. 2207–2213, 1998

Printed in Great Britain
0031-9422/98/$ - see front matter

A LOW TEMPERATURE INDUCED APOPLASTIC PROTEIN ISOLATED FROM *ARACHIS HYPOGAEA*

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(*Received in revised form* 27 *April* 1998)

Key Word Index—*Arachis hypogaea*; Fabaceae; groundnut; thaumatin-like proteins; apoplastic protein; cryoprotective protein; cold induced protein.

Abstract—We describe the isolation, characterization and identification of an *Arachis hypogaea* cold shock protein (AHCSP33). AHCSP33 is secreted into the leaf apoplast during low temperature exposure. N-terminal sequence of AHCSP33 shows homology to Thaumatin-Like (TL) protein family (also called Group5 Pathogenesis-related (PR) proteins). AHCSP33 shows strongest homology (55%) at the N-terminus with the Rye TL protein (M_r 25 k) which is an apoplastic protein and has antifreeze activity. Like several TL proteins, AHCSP33 is also targeted to the apoplast and persists for several days after low temperature treatment, although at reduced levels. AHCSP33 possesses intrachain disulfide bonds which is a well conserved feature of TL proteins. AHCSP33 might be involved in cryoprotecting proteins as it was shown to prevent freeze-induced denaturation of L-lactate dehydrogenase (LDH). Several features of AHCSP33 are consistent with its role in cryoprotection. It is a hydrophilic protein and is boiling stable. Hydrophilic amino acids constitute 80.4 mol%. Asp/Asn and Glu/Gln together constitute 20.8 mol%. AHCSP33 is glycosylated and exists as an oligomer in its native state.

INTRODUCTION

Secretion of stress-induced proteins into the leaf apoplast is a widespread phenomenon. Antifreeze proteins in winter and spring rye, winter and spring wheat, winter barley and spring oats are known to be secreted into the leaf apoplast of cold acclimated (CA) plants [1, 2]. Similarly, plants exhibiting hypersensitivity during an incompatible host–pathogen interaction secrete PR proteins into the leaf apoplast. For example, acidic PR proteins are secreted into the leaf apoplast of several plant species during viral, bacterial and fungal infections [3–5]. Stress induced apoplastic proteins play a crucial role in protecting the plants against stress-induced damage. For example, antifreeze proteins prevent physical damage to the tissues by modifying ice crystal growth and inhibiting the recrystallization of ice [1]. Similarly, the enhanced resistance (against further pathogenic attack) of uninfected parts of the plant following a hypersensitive reaction has been attributed to PR proteins [5].

Groundnut is grown in tropical and warmer temperate regions throughout the world. Groundnut plants show maximum growth at 28°, but experiences severe metabolic perturbations when exposed to temperatures below 12° [6]. However, the groundnut cultivar, TAG-24 (bunchtype developed at this Research Centre) is able to survive at 12° for several days without any apparent injuries. TAG-24 recovers rapidly from the stress and resumes normal growth at its optimal growth temperature (28°). In order to understand the basis for this tolerance we have studied low temperature induced secretion of proteins into the leaf apoplast. In this paper we report the isolation, characterization and identification of AHCSP33. N-terminal sequence of AHCSP33 shows homology to TL proteins. AHCSP33 possesses several of the well conserved features of TL proteins. We also present evidence that suggests a possible cryoprotective role for AHCSP33.

RESULTS AND DISCUSSION

AHCSP33, a boiling-stable protein was secreted into the leaf apoplast of CA plants. AHCSP33 was

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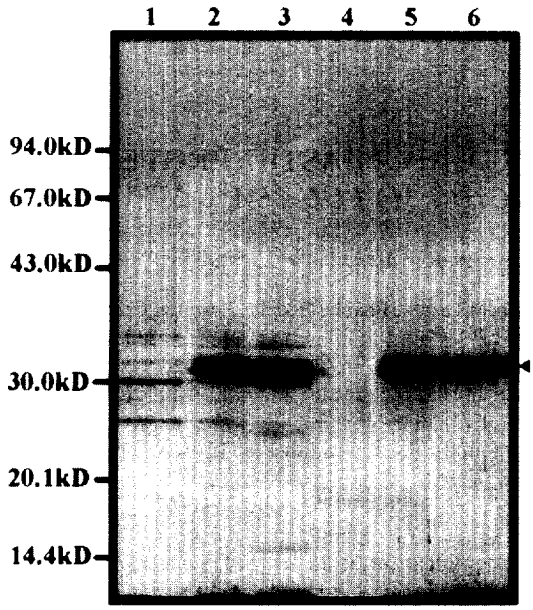

Fig. 1. Secretion of apoplastic proteins during low temperature stress. Apoplastic proteins from leaves of NA, CA and DA plants were resolved by SDS-PAGE. Lane 1–3 contain apoplastic proteins from NA, CA and DA plants while Lane 4–6 contain boiling stable apoplastic proteins from NA, CA and DA plants (5 μg/lane). Gel was silver stained. Arrowhead on the right indicates position of AHCSP33.

the most abundant apoplastic protein and only trace amounts of low M_r polypeptides (< 20 k) were similarly secreted. The secretion of AHCSP33 occurred to a lesser extent into the leaf apoplast of deacclimated (DA) plants. AHCSP33 was not detected in the leaf apoplast of nonacclimated (NA) plants (Fig. 1). AHCSP33 could be detected from 48 h after cold acclimation and up to at least day 7 of deacclimation. Boiling stable apoplastic proteins

Table 1. Accumulation of boiling stable apoplastic protein

Sample	Boiling stable apoplastic protein (μg/g leaf)	Fold increase over control
Control (NA)	0.95	–
CA 6 h	3.00	3.2
CA 2 days	6.80	7.2
CA 3 days	12.00	12.7
CA 6 days	19.52	20.7
DA 2 days	4.08	4.3
DA 4 days	9.50	10.1
DA 7 days	9.92	10.5

continued to accumulate steadily in the leaf apoplast during cold acclimation and by day 6 their concentration was 20.7 fold higher than control levels. During deacclimation (up to day 7) the amount of these proteins was reduced to almost half the level observed at the end of cold acclimation (Table 1). Since AHCSP33 was the most abundant apoplastic protein, therefore the accumulation of the boiling stable apoplastic proteins to a very large extent reflects the accumulation of AHCSP33. The prolonged persistence of AHCSP33 in the leaf apoplast during deacclimation could be the result of its resistance to degradation by proteolytic enzymes. PR proteins have a high resistance against degradation by proteolytic enzymes [3, 4, 7].

AHCSP33 was purified by ion-exchange chromatography. AHCSP33 eluted from the DEAE-Sephacel column at 0.1 M NaCl concentration. 2.7 mg of AHCSP33 was purified from 7.6 mg of boiling stable apoplastic proteins. The yield of this purification protocol was 35.8%. The protein was purified to near homogeneity.

N-terminal sequence of AHCSP33 shows homology to TL proteins [8], with the strongest homology to N-terminal sequence of Rye TL protein (M_r 25 k) [9] (Fig. 2). The acidic-neutral members

Apoplastic protein from *A. hypogaea*

AHCSP33	**A** Q **I** **T** L T **N** **K** A **S** **Y** **T** **V** T **P** P **A** Q A N A A D A	
Rye-TL (25 k)	**A** T **I** **T** V V **N** **K** F **S** **Y** **T** **V** X **P** G **A** L P G	55%
Barley-TL (S17573)	**A** T **I** **T** V V **N** R C **S** **Y** **T** **V** W **P** G **A** L P G	50%
Thaumatin Thm-1	**A** T F E I V **N** R C **S** **Y** **T** **V** W A A **A** S K G	35%
Osmotin	**A** T **I** E V R **N** N C P **Y** **T** **V** W A A S T P I	30%
PR-5	**A** T F D I V **N** Q C T **Y** **T** **V** W A A **A** S P -	30%

Fig. 2. Amino acid sequence alignment of AHCSP33. The N-terminus sequence (up to 20 residues) of AHCSP33 was compared with the N-terminal sequences of Rye TL-protein [9], Barley TL-protein [10], *Thaumatococcus daniellii* Thaumatin-1 protein [8], Tobacco Osmotin [15], Tobacco PR-5 protein [14]. Figures on the right indicate % homology.

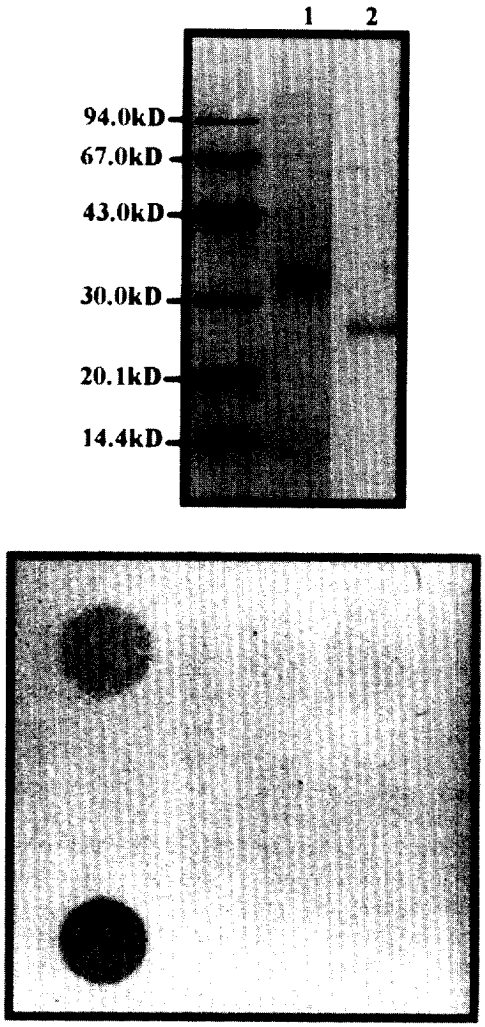

Fig. 3. (a) Detection of intrachain disulfide bonds. Purified AHCSP33 (50 μg/lane) was complexed with SDS in presence (Lane 1) or absence (Lane 2) of 2-mercaptoethanol and then resolved by SDS-PAGE. Gel was stained with Coomassie brilliant blue. (b) PAS-staining for detecting AHCSP33 glycosylation. Proteins were dot blotted onto nitrocellulose membrane and stained with PAS reagent. From top left (clockwise) AHCSP33 (50 μg), β-Lactoglobulin (+ve control), BSA (−ve control) and ovalbumin (+ve control) 100 μg each.

of TL protein family are known to be targeted to the apoplast [3,4]. Several TL proteins e.g., Rye TL [9], Barley TL [10] and Osmotin in *Solanum commersonii* [11] are expressed during cold acclimation.

AHCSP33 has intrachain disulfide bonds. AHCSP33 complexed with SDS in presence of 2-mercaptoethanol migrated slowly in comparison with AHCSP33 complexed with SDS in absence of 2-mercaptoethanol [Fig. 3(a)]. This anomalous migration results because complete unfolding of the polypeptide does not occur when the intrachain disulfide bonds are not reduced. Intrachain disulfide bonds is a recurring feature of the TL protein family members. For example, Thaumatin [8], Rye TL [12], PR-5 from tobacco [13], PR-R from tobacco [14] and Osmotin [15] have been reported to possess intrachain disulfide bonds.

AHCSP33 was relatively more effective (on a concentration basis) than BSA, ovalbumin and sucrose in stabilizing LDH during a freeze thaw cycle (Table 2). The 50% cryoprotection value (CP_{50}) for AHCSP33 was 2.5 times less than that of

Table 2. A comparison of cryoprotective effect of AHCSP33 and other cryoprotectants

Sample	M_r	CP_{50} (μg/ml)*	Protective effect
AHCSP33	32 974	20	1.0
BSA	66 000	50	2.5
Ovalbumin	45 000	110	5.5
Sucrose	342	7000	350.0

*CP_{50} values are average of two replicates.

Table 3. Amino acid composition analysis of AHCSP33

Amino acid	mol%
Asp/Asn	10.2
Glu/Gln	10.6
Ser	23.1
His	1.0
Gly	20.5
Thr	7.0
Arg	2.8
Cys	1.5
Ala	1.1
Tyr	0.2
Met	2.3
Val	5.9
Phe	5.4
Ile	2.7
Leu	2.4
Lys	3.5

BSA (Fig. 4). BSA is recognized as being an effective cryoprotectant and protein stabilizer [16, 17]. COR85, which is closely associated with the development of freezing tolerance during cold acclimation [18] was reported to have a CP_{50} value of 15 μg/ml [19]. CP_{50} value of COR85 is slightly less than the value estimated for AHCSP33. The unprocessed COR15a polypeptide was reported to have a very low CP_{50} value [20], but subsequent studies showed that COR15am (the processed form of COR15a) and BSA have similar cryoprotective effects [21]. CP_{50} values for several proteins have been found to range between 38 and 1820 μg/ml [19]. Thus it seems likely that the low CP_{50} value of AHCSP33 is an indication of its ability to stabilize and cryoprotect freeze labile proteins like LDH.

AHCSP33 is a hydrophilic protein, hydrophilic amino acids constitute 80.4 mol% (Table 3). The amino acid composition analysis of AHCSP33 revealed a compositional bias for Ser (23.1 mol%) and Gly (20.5 mol%). Asp/Asn and Glu/Gln constitute 20.8 mol%. It has been suggested that the relative level of Asp and Glu is a critical factor in the cryoprotective activity of a protein [19]. Na-glutamate was shown to be a very effective agent in cryoprotection of freezing sensitive enzymes. The ionized glutamate with a negative charge seems to play a crucial role in cryoprotection [16].

AHCSP33 possesses several biochemical properties of cryoprotective proteins. The activity of cryoprotective proteins remains unaffected by boiling [22–24]. The cryoprotective activity of AHCSP33 was also present after boiling (the assay was done on purified AHCSP33, and purification protocol involves heat treatment of the apoplastic protein extract). AHCSP33 exists as an oligomer in its native conformation. The M_r of the native protein was 159 k, although its subunit M_r is only 33 k (Fig. 5). A protein band that co-migrated with AHCSP33 was present in apoplastic protein extract which had not been subjected to boiling treatment. This indicates that the oligomerization is not the result of boiling treatment. COR85, COR15am and Cryoprotectin also exist as oligomers [19, 25, 26]. Maize G50 dehydrin which possesses cryoprotective activity is believed to exist as a dimer [27]. AHCSP33 is a glycoprotein. AHCSP33 and ovalbumin were stained with Periodic Acid Schiff's (PAS) reagent. Staining of β-lactoglobulin was very faint. The negative control did not stain at all

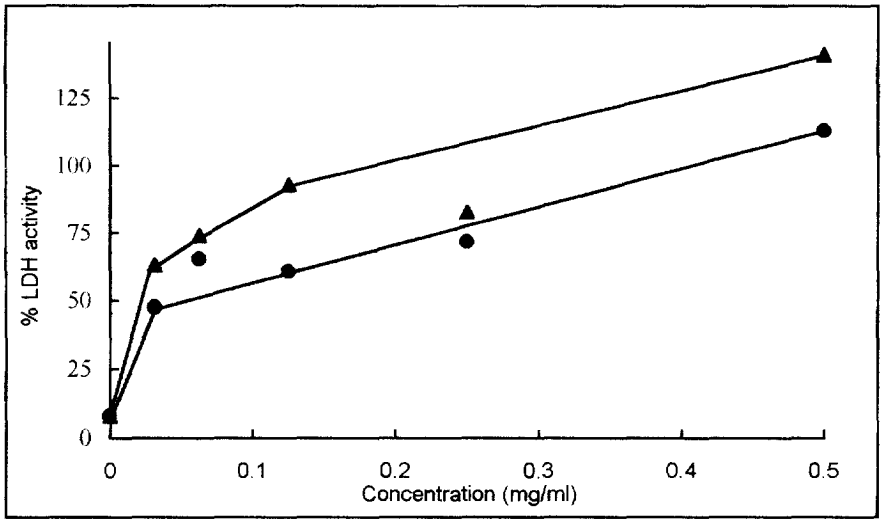

Fig. 4. A comparison of the cryoprotective effect of AHCSP33 and BSA. LDH solution (50 μg/ml) was frozen (−20° for 24 h) with different concentrations of AHCSP33 and BSA. Residual LDH activity after a freeze thaw cycle was plotted against concentration of AHCSP33 (▲) and BSA (●). CP_{50} was calculated by determining the concentration of the additive required to give 50% residual LDH activity. Each data point is the average of two replication.

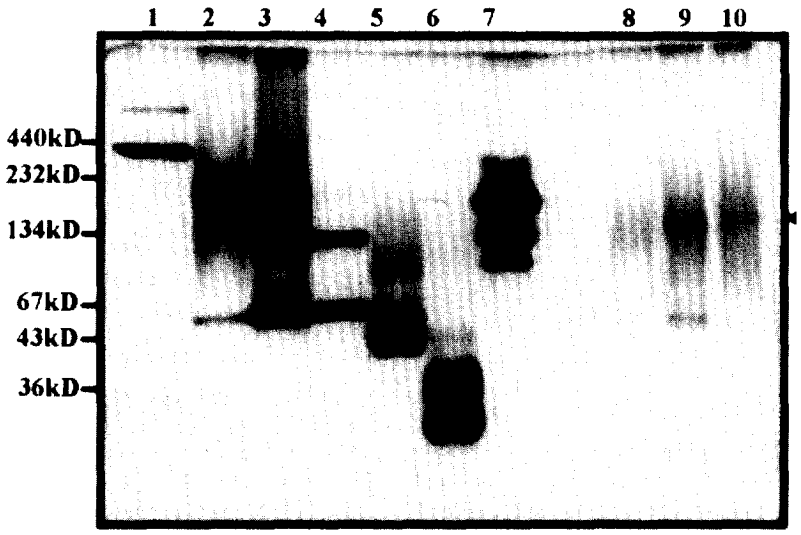

Fig. 5. M_r of native AHCSP33. Purified AHCSP33 was electrophoresed through a 5–20% polyacrylamide gel along with known protein M_r standards. Lane 1–7 contain (4 μg/lane) ferritin (440 k), catalase (232 k), albumin dimer (134 k), albumin monomer (67 k), ovalbumin (43 k), β-lactoglobulin (36 k) and carbonic anhydrase (30 k). Lane 8 contains 15 μg of purified AHCSP33. Lane 9 and 10 contain (5 μg/lane) apoplastic proteins and boiling stable apoplastic proteins, respectively. Arrowhead on the right indicates the position of AHCSP33.

[Fig. 3(b)]. AHCSP33 and ovalbumin had a carbohydrate content of 8.07% and 3.08%, respectively. The reported value for ovalbumin is 3.2% [28]. Like AHCSP33, Cryoprotectin has also been reported to be glycosylated [26].

Low temperature induces changes in protein folding that may result in aggregation [29]. Physiological changes at low temperature can also favor protein denaturation [30, 31]. Cryoprotective proteins are believed to provide stabilizing influences to proteins and cellular structures. These proteins might assist in either driving a partially unfolded protein back into a folded state or at least inhibit further denaturation (like chaperones). In *Saccharomyces cerevisiae* a direct correlation between the degree of heat shock protein (M_r 97, 85 and 70 k) induction and protection against freezing and thawing injury was observed. These proteins cryoprotected the cell by decreasing the denaturation (Delta-H) of total cellular proteins [32].

The mode of action of cryoprotective proteins is only now beginning to be understood. Cryoprotective proteins might function like chaperones [27, 32]. They might also interact with cellular membranes by reducing membrane permeability during freezing and increasing membrane expandability during thawing [22, 23, 33]. Groundnut being a tropical crop is unlikely to adapt to extremely low temperatures. We believe that dehydration is associated with a low temperature stress and many physiological features of dehydration are also associated with low temperature stress.

TL proteins possess several common features, yet diverse functions have been attributed to them e.g., antifungal, antifreeze, etc. AHCSP33 shares these conserved features and hence we propose that it is a member of the TL protein family. AHCSP33 is likely to have a role in cryoprotection and protein stabilization. Many of its properties are consistent with this finding. The inclusion of AHCSP33 into the TL protein family adds one more function to the already diverse functions attributed to its members. The diversity of functions and at the same time possession of several well conserved features seems to indicate that TL proteins have had a common evolutionary origin and are now diverging to generate new functions.

EXPERIMENTAL

Plant material and growth conditions

Groundnut cultivar, TAG-24 was grown at 28° for 20 days (12 h L/12 h D) and then CA at 12° for 10 days (12 h L/12 h D). CA plants were DA at 28° for 2 days (12 h L/12 h D). NA plants were grown at 28° (12 h L/12 h D) for the same duration as the CA and DA plants.

Apoplastic protein extraction

Apoplastic proteins were extracted from leaves of NA, CA and DA plants by vacuum infiltration with a buffer (20 mM Tris–HCl, pH 8.0 containing 2 mM EDTA, 1 mM 2-mercaptoethanol and 1 mM PMSF). Infiltrated leaves were gently packed in the barrel of a 50 ml syringe and placed in a centrifuge tube. The buffer which had infiltrated the leaf apoplast was recovered by centrifuging at low speed

(1000*g*; 4°; 15 min). Apoplastic fluid thus obtained was heated at 100° for 15 min and then centrifuged (12 000*g*; 15 min; 4°) to separate the denatured proteins. Boiling stable apoplastic proteins were precipitated from the supernatant with $(NH_4)_2SO_4$ at 85% saturation and dialysed against 20 mM Tris–HCl, pH 8.0.

Protein estimation

Protein concentration was estimated by the Bicinchoninic acid assay [34] using BSA as a standard.

Polyacrylamide gel electrophoresis

Proteins were electrophoresed in polyacrylamide gels containing SDS [35]. Proteins were denatured by incubating at 100° in the sample buffer (0.1 M Tris–HCl, pH 7.6 containing 1% SDS, 5% 2-mercaptoethanol and 20% glycerol) for 2–5 min. Proteins were electrophoresed along with protein M_r standards at constant voltage (150 V) for about 675 V h. Gel was stained with either Coomassie brilliant blue R-250 or $AgNO_3$ [36].

M_r estimation of the native protein

AHCSP33 was electrophoresed through a gel containing a gradient of polyacrylamide (5–20%) along with known protein M_r standards. A calibration curve was plotted to estimate the M_r of the native protein.

AHCSP33 purification by ion-exchange chromatography

Boiling stable apoplastic protein extract was first subjected to cation exchange chromatography (CM-Sepharose CL-6B). Unbound proteins (including AHCSP33) were then subjected to anion exchange chromatography (DEAE-Sephacel). Regenerated resin was packed into a column and then equilibrated with 20 mM Tris–HCl, pH 8.0. The protein sample was loaded onto the column in the equilibration buffer and eluted from the column using a step gradient (0.1–1 M NaCl) prepared in the equilibration buffer. $A_{280\ nm}$ was used to monitor protein elution. Individual fractions were analyzed by SDS-PAGE.

PAS-staining for detecting AHCSP33 glycosylation

Protein samples (50–100 μg) in 500 μL Tris buffered Saline (50 mM Tris–HCl, pH 7.5 containing 200 mM NaCl; TBS) were dot blotted onto 0.22 μm nitrocellulose membranes. The protein dot blot was placed in an oxidizing solution (0.8% periodic acid in 0.3% aq. NaOAc) for 5 min and then washed twice in distilled H_2O. The blot was stained with PAS reagent (in dark and at room temp) for *ca.* 10 min. After staining the blot was rinsed with 3% HNO_3 thrice (5 min/wash) and then with 0.01 M HCl for less than 1 min.

Estimation of carbohydrate content of AHCSP33

Phenol-H_2SO_4 assay was used to estimate the carbohydrate content of AHCSP33 and ovalbumin. To 1 ml of AHCSP33 (67 μg) and ovalbumin (200 μg) solution 1 ml 5% (w/v) phenol and 5 ml conc. H_2SO_4 was added. $A_{490\ nm}$ was determined against blank. A standard curve was plotted using glucose as a standard.

Amino acid composition of AHCSP33

AHCSP33 (400 μg) was hydrolysed in 1 ml of 6 M HCl containing 1% phenol at 110° for 24 h in an evacuated thick-walled glass hydrolysis tube. OPA derivatives of amino acids in the hydrolysate (except Pro) were generated immediately before injection. The amino acid derivatives were separated on a precalibrated reverse-phase C18 (octadecyl silyl) column in the Pharmacia FPLC system. Amino acid elution from the column was monitored by UV $A_{340\ nm}$ [37].

N-terminal sequencing of AHCSP33

AHCSP33 was electroblotted onto PVDF membrane [38]. Electroblotting was done in a semi-dry electroblotter at 12 V (a constant voltage power supply was used), 0.2 A for 24 h at 4°. The protein band on the membrane was visualized by staining with PonceauS. Edman degradation reaction [39] was performed on purified AHCSP33 electroblotted onto PVDF membrane in a Shimadzu PPSQ gas phase protein sequencer.

Cryoprotection assay

Cryoprotection assay [20] was used to compare the cryoprotective effect of AHCSP33 with BSA, ovalbumin and sucrose. LDH solution (400 units/mg; 50 μg/ml) was frozen (−20°; 24 h) with different concentrations of AHCSP33, BSA, ovalbumin and sucrose. LDH (50 μg/ml) stored at 4° for 24 h was used as a positive control and LDH (50 μg/ml) solution subjected to the freeze thaw cycle (−20°; 24 h) was used as a negative control. Residual LDH activity was estimated immediately after thawing [16]. The decrease in $A_{340\ nm}$ was measured between 30 and 90 s from the start of the reaction. The assay was done in duplicate.

Acknowledgements—R.S.D. was supported on a CSIR Fellowship. We thank D. M. Kale for providing TAG-24 seeds, Dr A. S. Bhagwat and Dr K. N. Susheelan for help in amino acid composition analysis and Dr M. Mathur for help in sequence analysis. N-terminal sequencing was done at IIT, Mumbai. N-terminal sequence reported appears in the Swiss-Prot and PIR protein databanks under the accession number P80926.

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