Phytochemistry, Vol. 25, No. 11, pp. 2497–2500, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

A CRYOPROTECTIVE POLYPEPTIDE ISOLATED FROM *NOTHOFAGUS DOMBEYI* SEEDLINGS

ANNELORI ROSAS, MIREN ALBERDI*, MICHEL DELSENY† and LUIS MEZA-BASSO‡

Instituto de Bioquímica, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile; *Instituto de Botánica, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile; †Laboratoire de Physiologie Végétale UA 565 du CNRS, Université de Perpignan, Perpignan, France

(*Received* 6 *January* 1986)

Key Word Index—*Nothofagus dombeyi*; Fagaceae; cold resistance; cryoprotective polypeptide.

Abstract—A comparative study of soluble proteins of *N. dombeyi* seedlings showed significant differences between electrophoretic patterns of cold-conditioned samples (48 hr at 0°) and control seedlings kept at 18°. A cold-induced polypeptide with apparent M_r of 35 k was isolated. The polypeptide showed an ability to protect isolated thylakoid membranes against freezing in comparison to low M_r cryoprotectants. The isolated protein may play a role in the development of freezing tolerance.

INTRODUCTION

Non-acclimated potentially hardy plants sustain injury when they are frozen below a certain temperature. It is generally accepted that the primary sites of freezing injury are at the membrane level [1, 2], resulting in, among other effects, an irreversible uncoupling of photophosphorylation from electron transport in chloroplasts [3, 4].

Plants which survive temporary freezing must be able to protect the frost-sensitive membranes during the freezing process. Based on existing data, it seems that proteins are involved in several mechanisms for increasing cold tolerance in higher plants. A general increase of soluble proteins has been observed as the temperature drops [5] and depending on the species, changes in non enzymic proteins, isoenzyme composition of a number of enzymes or enzyme conformation have been found as important modifications during cold acclimation [6].

In this work, we report the isolation of a polypeptide from cold-conditioned *Nothofagus dombeyi* (MIRB). Oerst seedlings, a woody evergreen species from the Chilean rain forest, showing an effective capacity *in vitro* in protecting thylakoid membranes against freezing damage.

RESULTS AND DISCUSSION

During protein extraction, cell compartments are broken, proteinases mixed with the cell content and degradation products are released in the extract. Thus, additional bands or spots appear on electrophoresis gels, that have no direct physiological or genetic significance [7]. In order to minimize the hydrolytic effect of proteinases on crude extracts, the rate of hydrolysis was determined by using different extracting buffer systems. Experiments were performed with proteins obtained from seedlings grown either at 18° or 0°.

Table 1 identifies buffers used, their pH range and data obtained from triplicate determinations. Two major peaks of proteolysis were located at pH 4.0 and at pH 7.0. A smaller extent of proteolysis of ^{3}H-proteins is obtained by using Tris–HCl (pH 8.0), indicating that unwanted proteolysis can be limited by working at a pH where proteolytic activity is minimal. No detectable difference was found between the behaviour of proteolysis in plants grown at 18° or 0°. In this way more reliable information can be obtained about changes in the electrophoretic patterns of the soluble proteins when seedlings are shifted from 18° to 0°.

A comparative study on the electrophoretic pattern of soluble proteins of *N. dombeyi* seedlings is shown in Fig. 1.

Table 1. Hydrolysis of ^{3}H-protein from *N. dombeyi* seedlings* as a function of pH

pH	Buffer	% Hydrolysis/hr†
2	Phosphate–glycine	8.9 ± 0.9
3	Phosphate–glycine	8.7 ± 2.0
4	Citrate–phosphate	10.2 ± 1.8
4	Acetate	11.3 ± 1.8
5	Acetate	9.1 ± 2.4
6	Phosphate	8.1 ± 1.7
7	Phosphate	10.9 ± 1.3
7	Tris–HCl	11.2 ± 1.9
8	Tris–HCl	4.3 ± 1.1
9	Glycine–NaOH	6.9 ± 1.4

*Total soluble proteins were extracted by using 50 mM buffer solutions individually. The rate of hydrolysis was determined at 37°.

† % hydrolysis is expressed as the arithmetic mean ± standard deviation of the mean.

‡To whom correspondence should be addressed.

Abbreviations: SDS sodium dodecyl sulphate; PMSF, phenylmethylsulphonyl fluoride; Hepes, *N*-2 hydroethylpiperazine-*N*-2-ethane-sulphonic acid; BSA, bovine serum albumin.

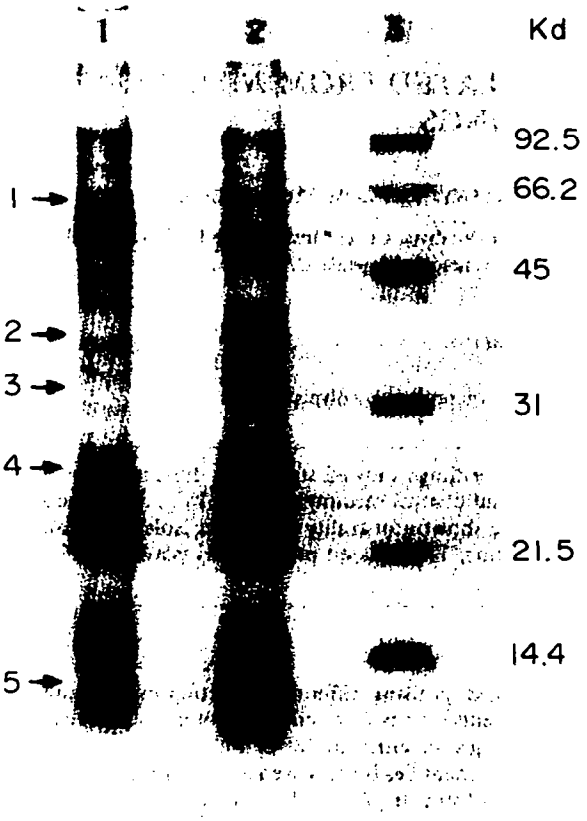

Fig. 1. SDS-polyacrylamide gel electrophoresis of total soluble proteins extracted from cotyledons of *N. dombeyi* seedlings. Electrophoresis was performed as described in the Experimental. Lane 1, soluble proteins of seedlings kept at 18°. Lane 2, soluble proteins of cold-conditioned seedlings (48 hr at 0°). Lane 3. M_r standards: phosphorylase B 92 500, BSA 66 200, ovalbumin 45 000, carbonic anhydrase 31 000, soybean trypsin inhibitor 21 500 and lysozyme 14 400. Arrows indicate the increase in intensity of some bands or the appearance of new polypeptides upon cold treatment.

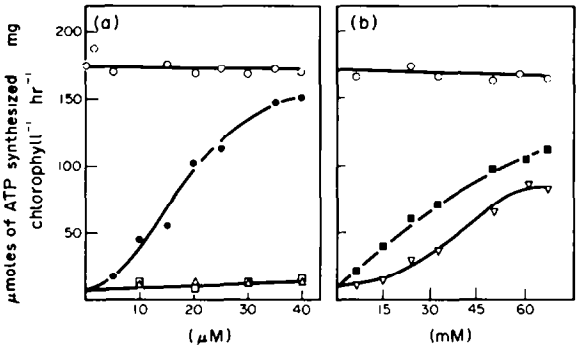

Fig. 2. Protection of *N. dombeyi* thylakoid membranes during freezing for 4 hr at −20° in the presence of increasing concentration of cryoprotectants. After thawing, the functional integrity of the membranes was measured as described in the Experimental. A, Protective effect of isolated polypeptides: M_r 41 k (Δ), 35 k (●) and 26 k (□). B, Protective effect of sucrose (■) and valine (▽). Unfrozen thylakoids kept at 0° (○) served as references.

Significant differences in the relative abundances of polypeptides can be detected when protein extracted from cold-conditioned seedlings and controls kept at 18° are compared. The appearance of new polypeptides or the increase in intensity of some bands upon cold treatment (bands 1–5) is evident. Polypeptides, whose accumulation appears to be strongly stimulated were isolated by preparative gel electrophoresis (bands 2–4). After isolation, SDS was removed from the polypeptides which were renaturated (see Experimental). The apparent M_rs of these polypeptides were 41, 35 and 26 k.

Figure 2A shows that freezing of thylakoids for 4 hr at −20° results in an almost complete inactivation of cyclic photophosphorylation in comparison to membranes kept at 0°. The addition of increasing amounts of one of the cold-induced polypeptide (M_r 35 k) protects the membrane against freezing inactivation. The effect seems to be specific, because enriched-fractions containing the polypeptide of M_r 41 k or the polypeptide of M_r 26 k obtained also from cold-treated seedlings, had little cryoprotective activity.

Volger and Heber [8] isolated two highly protective polypeptides from frost-resistant spinach leaves. They were found at very low levels in non-hardy tissue. These results along with ours suggest that the specific synthesis of new polypeptides has a role in the acquisition of frost tolerance in plants.

It should be emphasized that the polypeptide found in *N. dombeyi* failed to show any cryoprotective capacity when it was tested in its denatured form. The biological activity was achieved only after liberation of SDS and recovery of a structure closer to native configuration. The low protective level obtained by polypeptides of M_r 41 k and 26 k could be due to an incomplete or inappropriate renaturation process.

Figure 2B shows another aspect of membrane response towards freezing in the presence of naturally-occurring low M_r metabolites. Preliminary experiments have indicated that leaves of *N. dombeyi* at every stage of development, exhibit a specific increase of sucrose and valine during the acquisition of frost resistance. The addition of increasing amounts of sucrose or valine partially prevents the inactivation of photophosphorylation by freezing. On a molar basis, sucrose was a more efficient cryoprotector agent than valine.

Results in Table 2 attempt to demonstrate the specificity of the cryoprotective effect caused by different metabolites and macromolecules. It is apparent that sucrose is a better protector than any other sugar tested. Similarly, Lineberger and Steponkus [9] showed that glucose, sucrose and raffinose possess different effectiveness in protecting spinach thylakoid membranes against freezing. These results appear to be mainly due to non-ideal activity–concentration profiles by sugars during freezing.

Besides sugars, other compounds, e.g. amino acids, proved to be effective in the protection of thylakoid membranes during freezing. Valine and proline can act as cryoprotectants, being on a molar basis more efficient than glycine and alanine.

The effect of individual amino acids in protecting spinach thylakoids has been investigated [10]. These results are in part in agreement, under comparable

Table 2. Extent and specificity of the cryoprotector effect on isolated thylakoids during freezing

Treatment	% Cyclic photophosphorylation
Control at 0°	100
Frozen, 4 hr at −20°	4
Frozen in the presence of:	
40 μM polypeptide M_r 35 k (*N. dombeyi*)	83
60 mM valine	48
60 mM proline	45
60 mM glycine	17
60 mM alanine	21
60 mM sucrose	65
60 mM raffinose	28
60 mM glucose	14
40 μM polypeptide M_r 35 k + 60 mM sucrose + 60 mM valine	92
20 μM ribulose carboxylase (*Z. mays*)	6
50 μM ribosomal protein L10 (*E. coli*)	6
80 μM polypeptide M_r 41 k (*N. dombeyi*)	8
80 μM polypeptide M_r 26 k (*N. dombeyi*)	10

The data in the table are taken from a representative experiment. Experimental variation was within 7% of the data reported.

conditions, with results obtained during the freezing of *N. dombeyi* thylakoid membranes in the presence of either proline, alanine or glycine. The discrepancy is supported on the protecting ability of valine. Our data indicate that valine is a more efficient protector than any other amino acid tested; however this amino acid has been classified as a toxic solute on spinach thylakoid membranes [10]. This paradox could be due to the fact that even potentially toxic solutes, such as valine, can contribute to colligative protection, during freezing, below a damaging level [11]. At low concentration, valine increases protection although at a concentration higher than 100 mM it causes membrane inactivation (data not shown).

Table 2 also demonstrates that the protective ability of the polypeptide of M_r 35 k is specific. Ribulose carboxylase from *Z. mays*, ribosomal protein L10 of *E. coli* as well as polypeptides of M_r 41 or 26 k, isolated from cold-treated seedlings, fail to prevent thylakoid membrane inactivation. On the other hand, the active polypeptide produces its effect at very low concentration, being on molar basis 2500–3500 times more efficient than sucrose and valine. Combinations of polypeptide (M_r 35 k), sucrose and valine were more effective in preventing inactivation of photophosphorylation by freezing than either cryoprotectant alone. Furthermore the biological activity of this polypeptide can be achieved only after the polypeptide recovers a certain structural organization, closer to its native configuration. These observations would suggest that protection occurs through a mechanism different from those which can be attributed to colligative means.

The evidence obtained in this study and from earlier investigation [8] strongly suggest that the deleterious effects of freezing can be minimized if cryoprotectants such as specific polypeptides, some carbohydrates, certain amino acids, and other membrane-compatible solutes are simultaneously present in sufficient amounts during the freezing process. Among different physiological cryoprotectants, the high effectiveness of the polypeptide, indicates that proteins seem to play a role in the acquisition of cold tolerance.

EXPERIMENTAL

Plant material. N. dombeyi seeds and juvenile plants were obtained from Botanical Garden of Universidad Austral de Chile in Valdivia. Seeds were stratified in wet sand at 4° for 3 months and germinated at 18°.

Cold treatment. After 7 days of growth, half of the seedlings were transferred to a cold room in the dark at 0° for 48 hr. Control seedlings were kept in the dark at 18°. At the end of the treatment, total soluble proteins were extracted from cotyledons.

^{3}H-Protein hydrolysis as a function of pH. Batches of five seedlings were incubated in Petri dishes containing 100 μCi of L-[2,3-^{3}H]-proline (30.4 Ci/mmol) in 10 mM NaPi buffer (pH 6.5) either at 0° or 18° during the last 6 hr of the 48 hr treatment period. Cotyledons were removed and individual samples ground in a mortar in the presence of liquid N_2. Powders were suspended individually in 1 ml of 50 mM buffers at pH ranging between 2 and 9. Mixtures were shaken vigorously for 1 min and centrifuged at 27000 *g* at 2–4° for 15 min. Supernatants containing labeled proteins were saved and stored at −20°.

^{3}H-Proteins were incubated at 37° in the presence of each individual buffer (see Results). The kinetics of hydrolysis was followed for 180 min, withdrawing 10 μl every 30 min containing 20000–30000 cpm. The radioactivity incorporated into protein was determined according to ref. [12]. Decrease in TCA-insoluble radioactivity was a measure of hydrolysis rate.

Protein isolation. Around 100 cold-conditioned seedlings (48 hr at 0°) and controls kept at 18° were processed essentially as above, except that after grinding in liquid N_2, powders were resuspended in 10 vols of extraction buffer containing: 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 10% sucrose, 1% 2-mercaptoethanol and 50 μg/ml of PMSF. Mixtures were shaken vigorously for 1 min, centrifuged as above and super-

natant precipitated in the presence of 8 vols of cold Me_2CO. After centrifugation, pellets were dried down under vacuum and dissolved in Laemmli buffer [13]. The protein concn was determined by the method of ref. [14].

Gel electrophoresis. Samples for analysis containing 150 μg of protein were applied to one dimensional 12.5% polyacrylamide gels containing 0.4% SDS and subjected to electrophoresis with a constant current of 10 mA. The gel and buffer solns were prepared as described by ref. [13].

Preparative gel electrophoresis was performed essentially as above, except that 1 mg of protein was loaded and samples were not heat-treated. After separation, two thin slices were cut from the block and rapidly stained and destained to locate the position of individual proteins bands. Some of them were electrophoretically eluted according to the method of ref. [15]. The eluted proteins were liberated from SDS according to the procedure of ref. [16]. Polypeptides were then dissolved in a buffer containing 20 mM Tris–HCl (pH 7.8), 10 mM NaCl, 1 mM 2-mercaptoethanol and 8 M urea. The resulting solns were dialysed against 10 vols of the same buffer as above, except urea, with constant stirring for 36 hr and four changes of dialysing buffer. After dialysis, proteins were stored at −20°.

Test for cryoprotective activity. Thylakoid membranes were isolated from fresh *N. dombeyi* leaves from juvenile trees as described by ref. [17]. Thylakoids were then stored at 0° or frozen at −20° for 4 hr in the absence or presence of different amount of substances, whose cryoprotective activities were to be measured. After a rapid thawing in a water bath, the functional integrity of the membranes was checked by measuring the remaining activity of cyclic photophosphorylation according to the procedure of ref. [3]. Unfrozen controls served as references.

The inorganic phosphate uptake was determined as described in ref. [18]. Chlorophyll contents were measured as previously described [19].

Acknowledgements—In Chile this work as supported by grants from Dirección de Investigación y Desarrollo, UACH (S-84-29, RS-83-19) and FONDECYT 1212-84. In France by grants from CNRS (U.A. 565 and A.I.P. 95 31 67). The authors wish to thank Dr. Claudio Donoso, Universidad Austral de Chile for the generous gift of seeds. Thanks are also due Mrs. M. Angélica Espinoza and Mr. Risnel Bustos for secretarial assistance.

REFERENCES

1. Garber, M. P. and Steponkus, P. L. (1976) *Plant Physiol.* **57**, 673.
2. Schmitt, J. M., Schramm, M. J., Pfanz, H., Coughlam, S. and Heber, U. (1985) *Cryobiology* **22**, 93.
3. Santarius, K. A. (1971) *Plant Physiol.* **48**, 156.
4. Thebud, R. and Santarius, K. A. (1981) *Plant Physiol.* **68**, 1156.
5. Brown, G. N. (1978) in *Plant Cold Hardiness and Freezing Stress. Mechanisms and Crop Implications* (Li, P. H. and Sakar, A., eds) pp. 153–164. Academic Press, New York.
6. Graham, D. and Patterson, B. D. (1982) *Ann. Rev. Plant Physiol.* **33**, 347.
7. Zivy, M., Thiellement, H., De Vienne, D. and Hoffmann, J. P. (1983) *Theor. Appl. Genet.* **66**, 1.
8. Volger, H. and Heber, U. (1975) *Biochim. Biophys. Acta* **412**, 335.
9. Lineberger, R. D. and Steponkus, P. L. (1980) *Plant Physiol.* **65**, 298.
10. Heber, U., Tyankova, L. and Santarius, K. A. (1971) *Biochim. Biophys. Acta* **241**, 578.
11. Heber, U. and Santarius, K. A. (1976) in *Ecological Studies. Analysis and Synthesis* (Lange, O. L., Kappen, L. and Schulze, E. D., eds) Vol. 19, pp. 253–267. Springer, Berlin.
12. Mans, R. J. and Novelli, G. D. (1961) *Arch. Biochem. Biophys.* **94**, 48.
13. Laemmli, U. K. (1970) *Nature* **227**, 680.
14. Luck, J. M. (1958) *J. Biol. Chem.* **233**, 1407.
15. Vera, J. C., Concha, I. I. and Burzio, L. O. (1982) *IRCS Med. Sci.* **10**, 34.
16. Konigsberg, W. H. and Henderson, L. (1983) in *Methods in Enzymology* (Hirs, C. H. W. and Timasheff, S. N., eds) Vol. 91, pp. 254–259. Academic Press, New York.
17. Santarius, K. A. (1982) *Cryobiology* **19**, 200.
18. Fiske, C. H. and SubbaRow, Y. (1929) *J. Biol. Chem.* **81**, 629.
19. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1.