Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 794 (2003) 109–114
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Purification and characterization of a small (7.3 kDa) putative lipid transfer protein from maize seeds

Mariana S. Castro[a,*], Isabel R. Gerhardt[c], Stefania Orrù[d], Piero Pucci[d], Carlos Bloch Jr.[b]

[a]*Laboratório deToxinologia, Departamento de Ciências Fisiológicas/IB, Universidade de Brasília, Brasília/DF, 70.910-900, Brazil*
[b]*Laboratório de Espectrometria de Massa, Embrapa/Cenargen, Brasília/DF, 70770-900, Brazil,*
[c]*Centro de Biologia Molecular e Engenharia Genética, Unicamp, Campinas/SP, Brazil*
[d]*Ceinge Biotecnologie Avanzate, University of Napoli Federico II, Naples, Italy*

Received 16 January 2003; received in revised form 6 May 2003; accepted 19 May 2003

Abstract

The present study reports, for the first time in literature, the purification and biochemical characterization of a small basic protein from maize seeds similar to plant lipid transfer proteins-2, named mLTP2. The mLTP2 consists of 70 amino acid residues and has an M_r of 7303.83, determined by electrospray ionization mass spectrometry. The primary structure of mLTP2 was determined by automated Edman degradation of the intact protein and peptides obtained from digestions with trypsin and by C-terminal sequencing using carboxypeptidase Y. The mLTP2 exhibits high sequence similarity (51–44% identical positions) with other plant LTP2s previously described.

Keywords: Purification; Characterization; Lipid transfer proteins

1. Introduction

Plant lipid transfer proteins have been isolated from several members of mono- and dicotyledonous species. Plant LTPs have some general characteristics: basic, high contents of proline, glycine, alanine and serine, low number of aromatic residues and four disulfide bridges. The LTPs are divided into two subfamilies with relative molecular masses (M_r) of 9 kDa (LTP1s) and 7 kDa (LTP2s) [1,2].

The three-dimensional structures of plant LTP1s have been determined by X-ray crystallography and NMR techniques [3–6]. All LTP1s are compact, single domain proteins with four helices and a long C-terminal tail, stabilized by four disulfide bridges. In addition, there is an internal hydrophobic cavity running through the whole molecule which is probably the binding site for phospholipids and fatty acid chains [7]. On the basis of their in vitro properties of shuttling lipids and binding acyl chains, it was suggested that plant LTP1s could be involved in membrane biogenesis and regulation of the intracellular fatty acid pools [1]. However, the fact that LTP1s are located extracellularly [8,9] made this suggested role inconsistent. Thus, different roles have been proposed for plant LTP1s considering their extracellular location and novel properties discovered in recent years: participation in the cutin

*Corresponding author. Tel.: +55-61-307-2160; fax: +55-61-274-1251.
E-mail address: mscastro@unb.br (M.S. Castro).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00423-9

layer synthesis and wax assembly by carrying acyl monomers [10,11]; defensive role against bacterial and fungal pathogens [12–14]; participation in *Rhizobium* symbiosis [15]; and adaptation to environmental conditions, such as cold [16], salt [17] and drought [18] stresses.

Few LTP2s have been isolated and characterized from different plant sources [19–22] and only the three-dimensional structure for rice LTP2 has been solved. The rice LTP2 is an α-helical protein formed by three prominent helices and two single-turn helices. Four disulfide bridges also stabilize the three-dimensional structure, but the disulfide bond organization in LTP2 differs from LTP1 at the C×C motif [23].

The LTP2s have had their lipid transfer activity tested and it was observed that LTP2s efficiently transferred lipid molecules [20,23]. For wheat LTP2, for example, the level of transfer activity is at least five times higher than that for wheat LTP1 [20]. This increase in the lipid transfer efficiency of LTP2s is probably the result of the presence of a more flexible cavity in LTP2s in contrast to LTP1s, which can facilitate lipid molecule binding and release [23].

The present study reports, for the first time in literature, the purification and biochemical characterization of a small (M_r 7300) basic protein from maize seeds similar to plant lipid transfer proteins-2, named mLTP2.

2. Materials and methods

2.1. Maize seeds

Maize (*Zea mays* L. cv. BR-451) seeds were obtained from Setor de Difusão e Tecnologia, Centro Nacional de Pesquisas de Milho e Sorgo, Embrapa (Sete Lagoas, MG, Brazil) and stored at 4 °C until utilization.

2.2. Reagents and solvents

Reagents and solvents for protein sequencing were purchased from Applied Biosystems (USA). Reagents, solvents and enzymes for proteolytic digests and chromatographic procedures were of sequanal grade from several sources. Peptide Marker Kit was purchased from Amersham Pharmacia Biotech (Sweden). Only analytical grade reagents from commercial suppliers were used and all solutions were prepared with Milli-Q water (Millipore Reagent Water System, USA).

2.3. Purification procedures

Small maize LTP (mLTP2) was isolated and purified according to a slightly modified previous procedure [24], involving homogenization, saline extraction, ammonium sulfate precipitation, pseudo-affinity and RP-HPLC chromatographies. RP-HPLC chromatographies were performed using a Series 200 LC pump/235C diode array detection system (Perkin-Elmer, USA) and reversed-phase C_{18} columns (Vydac 218TP510 and 218TP54, The Separations Group, USA).

2.4. Polyacrilamide gel electrophoresis

Tris–Tricine–SDS–PAGE [25], a suitable method for resolution of small peptides, was used to monitor the homogeneity of the fractions. Samples were dissolved in buffer containing 4% SDS and 2% β-mercaptoethanol and heated for 30 min at 40 °C. The electrophoresis run was performed in 1.0-mm slab gels at 100 V. Gels were fixed in methanol 50%, acetic acid 10% and proteins were visualized by Coomassie Brilliant Blue G250 staining.

2.5. Reduction and alkylation

Purified mLTP2 aliquots (500 μg) were reduced and S-pyridylethylated or carbamidomethylated [26] prior to mass spectrometric analysis, enzymatic digests and sequencing. Desalting was performed by RP-HPLC.

2.6. Mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) analyses of the native and carbamidomethylated mLTP2 were performed on a VG Bio-Q triple quadrupole mass spectrometer (Fisons, USA). Mass calibration was performed by means of multiple-charged ions from a separate injection of myoglobin

(average molecular mass, 16 950.5). All masses reported are average values.

2.7. Enzymatic digest

Reduced and alkylated mLTP2 was digested using trypsin (TPCK treated) from Sigma according to [27]. The resulting peptide mixture was fractionated using a HPLC system fitted to an analytical C_{18} reversed-phase column (Vydac 218TP54). A linear gradient of acetonitrile (containing 0.1% TFA) was applied and the effluent was monitored at 215 nm. Several peptides obtained by these procedures were sequenced in order to obtain the primary structure of the mLTP2.

2.8. Protein sequencing

Native protein and reduced and alkylated peptides were submitted to automatic Edman degradation using an Applied Biosystems 477A pulsed-liquid protein sequencer on line with an Applied Biosystems 120A HPLC apparatus, modified for better performance according to [28]. The chromatography system was calibrated with phenylthiohydantoin (PTH) amino acid standards prior to each analysis.

2.9. C-Terminal sequencing

The C-terminal sequencing was performed on the reduced protein using the Sequazyme™ C-Peptide Sequencing Kit (PerSeptive Biosystems, USA) and posterior analysis by matrix-assisted laser desorption-time-of-flight ionization mass spectrometry (MALDI-TOF MS) on a Voyager-DE™ STR BioSpectrometry Workstation (PerSeptive Biosystems).

2.10. Sequence analysis

The amino acid sequence similarity search was done using NCBI nonredundant database through the BLASTP 2.2.3 program [29] (http://www.ncbi.nlm.nih.gov/BLAST). Clustal W (1.82) [30] was used for multiple sequence alignment (http://www.ebi.ac.uk/clustalw). Theoretical p*I* and molecular mass were calculated from mLTP2 sequence using Compute p*I*/Mw Tool (http://www.expasy.ch/tools/pi_tool.html). Hydrophobicity and flexibility profiles were calculated using PROTSCALE (http://ca.expasy.org/cgi-bin/protscale.pl). Secondary structure prediction was performed by the GOR1 method [31] (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor.html).

3. Results and discussion

From molecular biology approaches, the existence of a class of small (7 kDa) lipid transfer proteins in plants was determined [32], but only few LTP2s have been isolated until now [19–22]. This article describes the purification and biochemical characterization of a small (7.3 kDa) putative lipid transfer protein from maize seeds, co-purified as a homogeneous fraction during the procedures related to the purification of γ-zeathionins.

The mLTP2 was obtained according to a previous procedure [24], with a minor modification. Saline extraction, precipitation with 5.0 *M* NaOH and ammonium sulfate, and pseudo-affinity chromatography using a Red-Sepharose CL-6B column (Amersham Pharmacia Biotech) were performed as described in [24]. The eluate from Red-Sepharose column was dialyzed against distilled water and lyophilized. Next, these samples were submitted to RP-HPLC. Each sample (5.0 mg) was dissolved in 200 μl of 0.1% aqueous TFA and applied on a semipreparative reversed-phase C_{18} column (Fig. 1A). Individual peak fractions, eluted at approximately 35% of the acetonitrile gradient, were manually collected, vacuum-dried in a Speed-Vac concentrator (Savant Instruments, USA), redissolved in 200 μl of 0.1% aqueous TFA and rechromatographed on an analytical reversed-phase C_{18} column (Fig. 1B). The homogeneity of the purified protein was analyzed by Tricine–SDS–PAGE (Fig. 1C).

The observed copurification of γ-thionins and lipid transfer proteins was probably due to similar biochemical properties of these two groups of plant proteins, such as low molecular mass and highly basic profile.

The primary structure of mLTP2 shown in Fig. 2 was determined by Edman degradation and confirmed by mass spectrometric analysis. The elucidation of the amino acid sequence was based on automated sequence analysis of the intact protein and

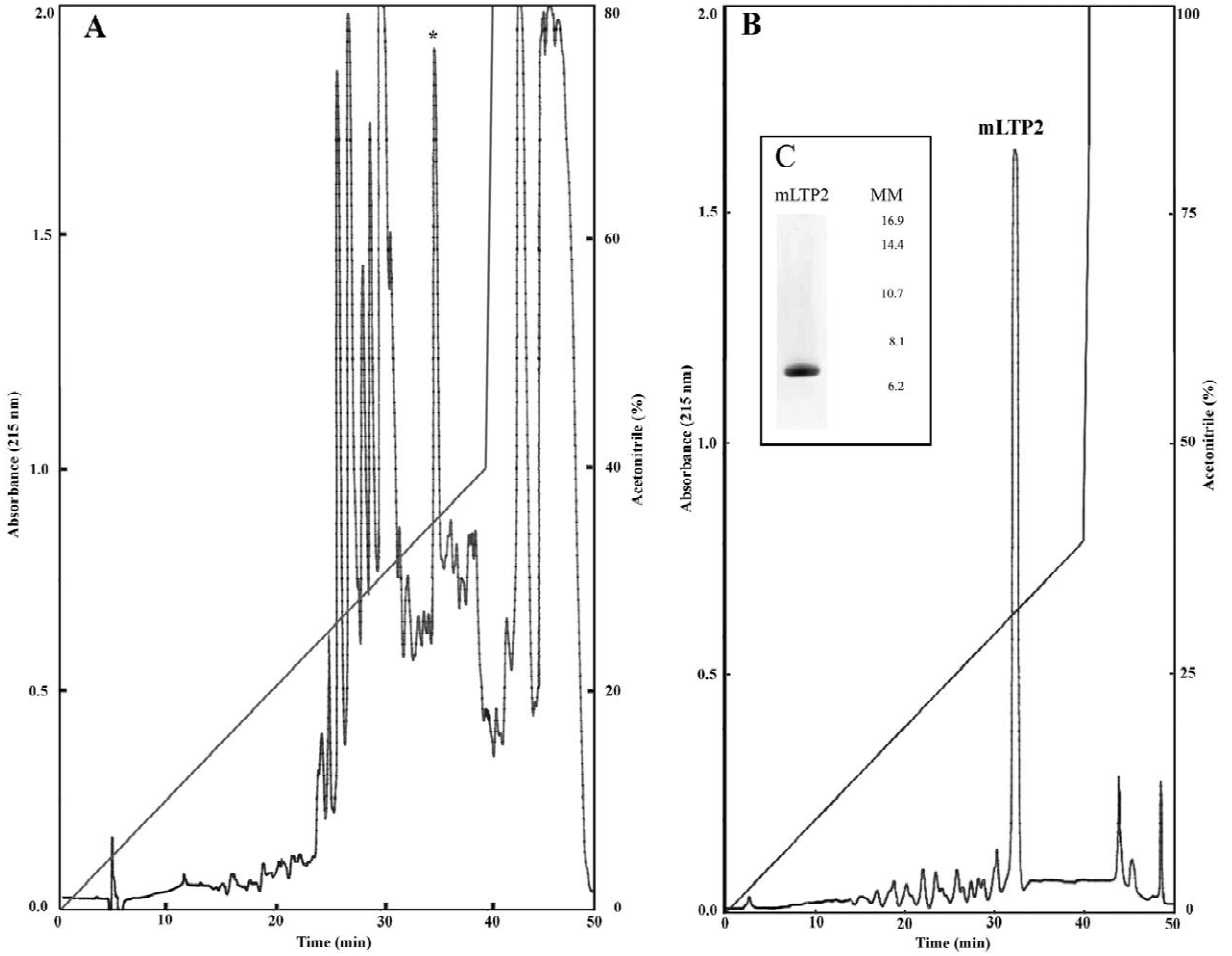

Fig. 1. (A) Preparative RP-HPLC. Aliquots of 5.0 mg of the fraction obtained from Red-Sepharose CL-6B chromatography were dissolved in 200 μl 0.1% TFA (v/v) and injected onto a C_{18} column (Vydac 218TP510, 250×10 mm, 5 μm) in equilibrium with 0.1% TFA (v/v). The column was eluted with a linear gradient of acetonitrile containing 0.1% TFA (0–40% in 40 min; 40–100% in 1 min and 10 min of wash with 100% acetonitrile) at a flow-rate of 2.5 ml/min and the effluent absorbance was monitored at 215 nm. The fractions were manually collected and vacuum-dried. (B) Marked peak was rechromatographed using an analytical C_{18} column (Vydac 218TP54, 250×4.6 mm, 5 μm) eluted with the same acetonitrile gradient at a flow-rate of 1 ml/min and monitoring absorbance at 215 nm. (C) Tricine–SDS–PAGE profile of mLTP2. MM, molecular markers (molecular masses in kDa of myoglobin fragments).

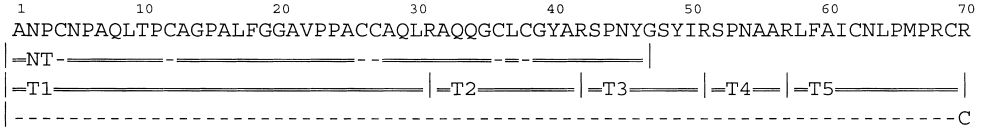

Fig. 2. Complete amino acid sequence of mLTP2. The double lines indicate the sequenced parts, whereas broken lines represent the parts of the protein that were not identified. The peptides are numbered according to their position in the sequence of the protein and letters indicate the type of cleavage reaction used for fragmentation: T, trypsin and C, carboxypeptidase Y. NT denotes sequence determined by Edman degradation of the intact protein.

peptides (after reduction and alkylation) obtained by enzymatic cleavage with trypsin-TPCK. C-Terminal sequencing with carboxypeptidase Y and posterior analysis by MALDI-TOF confirmed the C-terminal arginine residue.

The N-terminal sequence up to the 47th residue was determined by analysis of the intact protein, and the peptides from enzymatic digests were useful to provide an unambiguous alignment. The orientation of tryptic peptides T4 and T5 was determined by sequence homology.

A single sequence of 70 amino acid residues was obtained for mLTP2, with eight cysteines and a high content of alanine (17.1%), proline (14.3%) and glycine (8.6%). No tryptophan was detected in the mLTP2 sequence (Fig. 2). All these structural features are typical of proteins included in the plant LTP family, as described in Ref. [1].

ESI-MS analysis of the reduced and carbamidomethylated protein revealed a measured mass of 7769.26, i.e. 8×58 mass increment per each half-cystine residue, thus further confirming the sequence assignments. The mLTP2 mass measured by ESI-MS perfectly agreed with that predicted from the sequence data (mLTP2 predicted mass: 7311.54; observed: 7303.83), considering four disulfide bridges.

The sequence information obtained was used for a database search for identities or similarities through the BLASTP program. The mLTP2 exhibits high levels of sequence identity (51–44%) with other plant LTP2s (Fig. 3). It is interesting that, despite the lower level of sequence identity between mLTP2 and apricot LTP2 (aLTP2), these two proteins present a very similar distribution of proline residues along their sequences, as well as the presence of a leucine at the C×C motif instead of a phenylalanine. These structural features might be responsible for some modifications in the overall folding of these two LTP2s.

One important feature revealed by sequence analysis is that the eight cysteine residues are located according to the position pattern: **3**-C-**7**-C-**13**-CC-8-C-**1**-C-**23**-C-6-C-1, quite similar to those presented by 9000 LTPs **2**/**3**-C-**8**-C-**12**/**15**-CC-19-C-**1**-C-**21**/**23**-C-13-C-4/8 [1], as can be confirmed by looking at the bold numbers in those patterns.

The theoretical p*I* of 9.14 is in good agreement with other plant LTPs that present p*I* values in the range of 8.8–10 [1], evidencing the highly basic profile of this protein family. This characteristic is commonly observed among proteins that appear to be capable of destabilizing membrane permeability, resulting in leakage of electrolytes, such as thionins and cysteine-rich antimicrobial peptides [33,34]. A synergistic interaction between thionins and LTPs against bacterial and fungal pathogens has also been reported [35].

From GOR analysis, a percentage of 11.4% α-helix, 48.6% extended strand, 30.0% turns and 10.0% random coil was detected in mLTP2. These results are in concordance with secondary structure predictions for LTP1s, which commonly suggest the presence of α-helix and β-strands [36]. All LTPs structures determined by NMR or crystallography approaches reveal that they constitute an all-α class of proteins [3–6,23], not confirming the predicted structure. It is desirable to study the conformation presented by mLTP2 using spectroscopic techniques to verify the predicted results. The hydrophobicity and flexibility profiles revealed some highly hydrophobic regions similar to those obtained for other

```
LTP2        MULTIPLE SEQUENCE ALIGNMENT                                          %I
            1         10        20        30        40        50        60        70
mLTP2       ANPCNPAQLTPCAGPALFGGAVPP-ACCAQLRAQQGCLCGYARSPNYGSYIRSPNAARLFAICNLPMPRCR   100
rLTP2       -AGCNAGQLTVCTG-AIAGGARPTAACCSSLRAQQGCFCQFAKDPRYGRYVNSPNARKAVSSCGIALPTCH    51
wLTP2(G)    --ACQASQLAVCAS-AILSGAKPSGECCGNLRAQQGCFCQYAKDPTYGQYIRSPHARDTLTSCGLAVPHC-    49
wLTP2(P)    --ACQASQLAVCAS-AILSGAKPSGECCGNLRAQQPCFCQYAKDPTYGQYIRSPHARDTLQSCGLAVPHC-    47
aLTP2       -VTCSPVQLSPCLG-PINSGAPSPTTCCQKLREQRPCLCGYLKNPSLRQYVNSPNARKLASNCGVPVPQC-    44
               *   **: *    :  **     **  ** *: *:* : : *    *: **:*      * : :* *
```

Fig. 3. Comparison of the amino acid sequences between plant LTP2s from different sources and mLTP2. The letter w indicates wheat; r, rice and a, apricot. Gaps are included to achieve maximal sequence similarity. The symbol '*' denotes identical residues and conservative substitutions are indicated with ':'; %I is the percentage identity of mLTP2 with other plant LTP2s.

plant LTPs [36] and the presence of a rigid polypeptide chain, in accordance with the presence of four disulfide bonds connecting the whole molecule.

In the present study we purified and characterized a novel structurally-related plant LTP2 from maize seeds, named mLTP2. Further studies must be conducted in order to analyze its ability in carrying lipids as well as to evaluate its antimicrobial activity.

Acknowledgements

The authors thank Dr. Patrícia G. B de Carvalho for critical reading of the manuscript. This investigation was supported by FAPDF (Fundação de Amparo à Pesquisa do Distrito Federal) and FUB/UnB.

References

[1] J.C. Kader, Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 627.
[2] J.P. Douliez, T. Michon, K. Elmorjani, D. Marion, J. Cereal Sci. 32 (2000) 1.
[3] J.P. Simorre, A. Caille, D. Marion, D. Marion, M. Ptak, Biochemistry 30 (1991) 11600.
[4] D.H. Shin, J.Y. Lee, K.Y. Hwang, K.K. Kim, S.W. Suh, Structure 3 (1995) 189.
[5] J. Gomar, M.C. Petit, P. Sodano, D. Sy, D. Marion, J.C. Kader, F. Vovelle, M. Ptak, Protein Sci. 5 (1996) 565.
[6] J.Y. Lee, K. Min, H. Cha, D.H. Shin, K.Y. Hwang, S.W. Suh, J. Mol. Biol. 276 (1998) 437.
[7] J.C. Kader, Trends Plant Sci. 2 (1997) 66.
[8] S. Thoma, Y. Kaneko, C. Somerville, Plant J. 3 (1993) 427.
[9] J. Pyee, H. Yu, P.E. Kolattukudy, Arch. Biochem. Biophys. 311 (1994) 460.
[10] P. Sterk, H. Booij, G.A. Schellekens, A. Van Kammen, S.C. De Vries, Plant Cell 3 (1991) 907.
[11] B. Hollenbach, L. Schreiber, W. Hartung, K.J. Dietz, Planta 203 (1997) 9.
[12] A. Molina, A. Segura, F. García-Olmedo, FEBS Lett. 316 (1993) 119.
[13] F. García-Olmedo, A. Molina, A. Segura, M. Moreno, Trends Microbiol. 3 (1995) 72.
[14] K.K. Nielsen, J.E. Nielsen, S.M. Madrid, J.D. Mikkelsen, Plant Mol. Biol. 31 (1996) 539.
[15] A. Krause, C.J.A. Sigrist, I. Dehning, H. Sommer, W.J. Broughton, Mol. Plant–Microbe Interact. 7 (1994) 411.
[16] R.S. Pearce, C.E. Houlston, K.M. Atherton, J.E. Rixon, P. Harrison, M.A. Hughes, A. Dunn, Plant Physiol. 117 (1998) 787.
[17] S. Torres-Schumann, J.A. Godoy, J.A. Pintor-Toro, Plant Mol. Biol. 18 (1992) 749.
[18] M.B. Trevino, M.A. O'Connell, Plant Physiol. 116 (1998) 1461.
[19] B.L. Jones, L.A. Marinac, J. Agric. Food Chem. 48 (2000) 257.
[20] J.P. Douliez, C. Pato, H. Rabesona, D. Molle, D. Marion, Eur. J. Biochem. 268 (2001) 1400.
[21] A. Conti, D. Fortunato, C. Ortolani, M.G. Giuffrida, V. Pravettoni, L. Napolitano, L. Farioli, L. Perono Garoffo, C. Trambaioli, E.A. Pastorello, J. Chromatogr. B 756 (2001) 123.
[22] Y.J. Liu, D. Samuel, C.H. Lin, P.C. Lyu, Biochem. Biophys. Res. Commun. 294 (2002) 535.
[23] D. Samuel, Y.J. Liu, C.S. Cheng, P.C. Lyu, J. Biol. Chem. 277 (2002) 35267.
[24] M.S. Castro, W. Fontes, L. Morhy, C. Bloch Jr., Protein Pept. Lett. 3 (1996) 267.
[25] H. Schägger, G. von Jagow, Anal. Biochem. 166 (1987) 368.
[26] K.J. Wilson, P.M. Yuan, in: J.B.C. Findlay, M.J. Geisow (Eds.), Protein Sequencing—a Practical Approach, IRL Press, Oxford, 1989, p. 1.
[27] A. Aitken, M.J. Geisow, J.B.C. Findlay, C. Holmes, A. Yarwood, in: J.B.C. Findlay, M.J. Geisow (Eds.), Protein Sequencing—a Practical Approach, IRL Press, Oxford, 1989, p. 43.
[28] W. Fontes, R.B. Cunha, M.V. Sousa, L. Morhy, Anal. Biochem. 258 (1998) 259.
[29] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, J. Mol. Biol. 215 (1990) 403.
[30] J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic Acids Res. 22 (1994) 4673.
[31] J. Garnier, D.J. Osguthorpe, B. Robson, J. Mol. Biol. 120 (1978) 97.
[32] R. Kalla, K. Shimamoto, R. Potter, P.S. Nielsen, C. Linnestad, O.A. Olsen, Plant J. 6 (1994) 849.
[33] J.M. Caaveiro, A. Molina, J.M. Gonzalez-Manas, P. Rodriguez-Palenzuela, F. García-Olmedo, F.M. Goni, FEBS Lett. 410 (1997) 338.
[34] P.R. Shewry, J.A. Lucas, Adv. Bot. Res. 26 (1997) 135.
[35] A. Molina, A. Segura, F. García-Olmedo, FEBS Lett. 316 (1993) 119.
[36] S.M. Madrid, D. von Wettstein, Plant Physiol. Biochem. 29 (1990) 705.