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Primary structure of the N-linked carbohydrate chains of Calreticulin from spinach leaves

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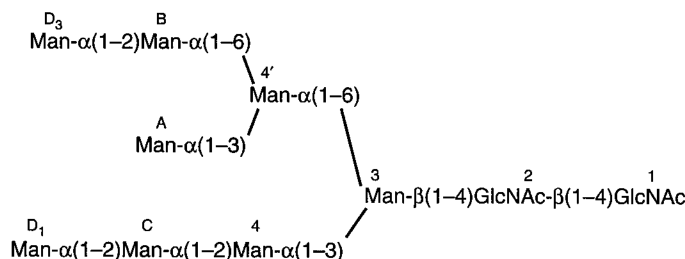
Calreticulin is a multifunctional Ca^{2+}-binding protein of the endoplasmic reticulum of most eukaryotic cells. The 56 kDa Calreticulin glycoprotein isolated from spinach (*Spinacia oleracea* L.) leaves was N-deglycosylated by PNGase-F digestion. The carbohydrate moiety was isolated by gel permeation chromatography and purified by high-pH anion-exchange chromatography. The fractions were investigated by 500 MHz ^{1}H-NMR spectroscopy, in combination with monosaccharide analysis and fast-atom bombardment-mass spectrometry. The following carbohydrate structure could be established as the major component ($Man_8GlcNAc_2$):



Heterogeneity was demonstrated by the presence of two minor components being $Man_7GlcNAc_2$ lacking a terminal residue (D_1 or D_3), compared to the major component. A cross-reactivity with an antibody against the endoplasmic reticulum retention signal HDEL was also found.

Keywords: Calreticulin, plant glycoproteins, N-linked carbohydrate chains, oligomannose-type, ^{1}H-NMR spectroscopy

Introduction

Calreticulin (CR) is a major Ca^{2+}-binding/storage protein mainly localized in the lumen of the endoplasmic reticulum (ER) of most eukaryotic organisms. In addition to Ca^{2+} buffering within the ER, recent findings suggest for CR other fundamental functions such as modulation of gene expression, interaction with integrins, as well as a molecular chaperone role [1, 2]. Despite the overwhelming data concerning CR reported in the past few years, the glycosylation patterns of CR have not been firmly established yet. Although most of the available amino acid sequences of animal CRs have a potential glycosylation site in the C-domain, the sites seem to be rarely occupied. To date, only CR from rat liver [3, 4], bovine liver [5] and bovine brain [6] have been shown to contain carbohydrate. In plant cells the characterization of CR proteins is only at the beginning. Nevertheless, there is growing evidence for the presence of carbohydrate moieties in plant CRs. Calreticulin from spinach leaves [7] and pea seeds [8] were shown to be stained by periodic acid Schiff. It has also been reported that tobacco CR [9]

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as well as maize CR [10] bind to Concanavalin A (Con A). Moreover, a glycosylated isoform of CR has recently been purified from spinach leaves by using Con A-Sepharose affinity chromatography [11]. In this paper, we report the monosaccharide composition and glycan structure of a plant CR.

Materials and methods

Isolation and purification of CR from spinach leaves

CR was isolated from spinach (*Spinacia oleracea* L.) leaves as described previously [11]. By repeatedly using this procedure 7.4 mg CR was isolated from 10 kg spinach leaves.

Concentration of CR sample and removal of methyl α-D-mannopyranoside

A sample of 7.4 mg CR, dissolved in 33 ml 10 mM sodium phosphate buffer, pH 6.8, containing 0.5 M NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, and 0.2 M methyl α-D-mannopyranoside, was concentrated using a Centriprep-3 device (15 ml, Amicon). A stepwise centrifugation (60 min, 3500 × **g**) with intermediate removal of the filtrate and replenishment of the retentate resulted in a final volume of 6 ml. A decrease in the methyl α-D-mannopyranoside concentration from 0.2 M to about 0.02 M was achieved by additional centrifugations and replenishment of the retentate with water. Methyl α-D-mannopyranoside was completely removed by gel permeation chromatography on a Sephadex G-25 HiTrap^{tm} desalting column (4 × 5 ml columns, Pharmacia) using 5 mM ammonium bicarbonate as elution solvent with a flow rate of 2 ml min^{-1}. The eluate was monitored at 206 nm. The void volume peak, containing CR, was collected. The complete removal of methyl α-D-mannopyranoside was checked by GLC. To this end, an aliquot of the CR-containing fraction was lyophilized and then trimethylsilylated. GLC analysis (monosaccharide GLC conditions) demonstrated the absence of the trimethylsilyl derivative of methyl α-D-mannopyranoside.

Protein determination

Protein concentration was determined according to [12], using Coomassie Protein Assay Reagent (Pierce). Bovine serum albumin (Pierce) and Ribonuclease B (Sigma) were used as standard proteins.

Carbohydrate determination

Carbohydrate content determinations were performed with the phenol-sulfuric acid assay [13].

Monosaccharide analysis

Monosaccharide composition and carbohydrate content determinations were carried out by gas-liquid chromatography on a capillary DB-1 column (30 m × 0.32 mm, J. & W. Scientific), using a Perkin Elmer 8010 gas chromatograph (temperature programme 130–240 °C at 4° min^{-1}) and flame-ionization detection. Trimethylsilylated methyl glycosides were prepared by methanolysis (1.0 M methanolic HCl, 24 h, 85 °C), re-*N*-acetylation and per-*O*-trimethylsilylation. Identification of the monosaccharide derivatives was confirmed by gas-liquid chromatography-mass spectrometry (GLC-MS) on a Fisons Instruments GC 8060/MD 800 System (Interscience) [14].

Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to [15] on 10% polyacrylamide gels. Gels were stained for proteins with Coomassie Blue (Pierce) or Stains All (Bio-Rad). Protein transfer to nitrocellulose membranes and immunostaining of blots were carried out as described [11]. The anti-HDEL monoclonal antibody 2E7 [16] was used.

Liberation and isolation of the carbohydrate chains

The N-linked carbohydrate chains were enzymatically released from the glycoprotein according to a modified version of a previously described protocol [17]. Calreticulin (5.6 mg) was dissolved in 1.5 ml 50 mM Tris HCl, pH 7.2, containing 50 mM EDTA, and 1% (v/v) β-mercaptoethanol. Denaturation of the sample was carried out by heating for 4 min at 100 °C in the presence of 0.5% (w/v) SDS. After cooling to room temperature the solution was incubated, in the presence of 1% (w/v) decanoyl-*N*-methylglucamide (MEGA 10), with 5 U PNGase-F (recombinant peptide-N (*N*-acetyl-β-glucosaminyl) asparagine amidase F from *Flavobacterium meningosepticum* expressed in *E. coli* (EC 3.5.1.52), Boehringer Mannheim) for 16 h at room temperature in an end-over-end mixer. After heating for 2 min at 100 °C, a fresh aliquot of 5 U PNGase-F was added and the incubation was continued for another 16 h. The digestion was monitored with SDS-PAGE. The ultimate mixture was diluted with 1.5 ml 150 mM ammonium bicarbonate/HCl, pH 7.1, and fractionated on a Superdex 75 column (60 × 2.6 cm, Pharmacia), using 150 mM ammonium bicarbonate/HCl, pH 7.1, at a flow rate of 3 ml min^{-1} (Pharmacia FPLC system). The elution was monitored at 206 nm. Definite fractions were collected and aliquots stained for carbohydrate with orcinol/H_2SO_4. The carbohydrate-positive fractions were lyophilized several times with intermediate dissolving in fresh water and then purified on a Bio-Gel P-2 column (47 × 1.6 cm, Bio-Rad) eluted with 5 mM ammonium bicarbonate at a flow rate of 12 ml h^{-1}. A further purification of the oligosaccharide fraction SII was obtained by gel filtration on a Bio-Gel P-4 column (46 × 1 cm, Bio-Rad) at a flow rate of 5 ml h^{-1}.

Fast protein liquid chromatography (FPLC)

The purified carbohydrate-containing Superdex 75 fraction was analysed on a Resource Q anion-exchange column (30 × 6.4 mm, Pharmacia). The column was first eluted with 2 ml H_2O, followed by linear concentration gradients of 0–50 mM NaCl in 8 ml H_2O, 50–250 mM NaCl in 8 ml H_2O, and 250–500 mM NaCl in 4 ml H_2O, at a flow rate of 4 ml min^{-1} (Pharmacia FPLC system). The elution was monitored at 214 nm.

High-pH anion-exchange chromatography (HPAEC)

The carbohydrate-containing fraction obtained after Bio-Gel P-4 was subfractionated by HPAEC on a CarboPac PA-100 column (4 × 250 mm, Dionex), using a Dionex LC system. Detection was performed with a gold electrode and triple-pulse amperometry. The column was eluted with a 25–75 mM NaOAc linear gradient in 0.1 M NaOH in 20 min, at a flow rate of 1.0 ml min^{-1}.

Fast-atom bombardment-mass spectrometry (FAB-MS)

Positive-ion mass spectra were obtained with a Jeol JMS/SX102/102A four-sector instrument using MS1. Xenon was used as the FAB gas; the gun was operated at 6 kV and a 5 mA discharge current. Thioglycerol was used as liquid matrix. The spectra were recorded over a mass range of *m/z* 10–1800.

500 MHz ^{1}H-NMR spectroscopy

The oligosaccharide samples were repeatedly treated with ^2H_2O (99.96 atom% ^{2}H, Isotec Inc.) with intermediate lyophilization. High resolution ^{1}H-NMR spectra were recorded on a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy) at a probe temperature of 300 K. The spectral width was 5 kHz. Suppression of the ^{2}HOH signal was achieved by applying an advanced WEFT pulse sequence [18]. Resolution enhancement of the spectra was achieved by a Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a fourth-order polynomial fit. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225) [19].

Results

Protein determinations before and after the Centriprep-3 and Sephadex G-25 HiTrap treatment revealed a recovery of 6.3 mg CR (yield 85%). Monosaccharide analysis of CR (Table 1) revealed a carbohydrate content of 4.7% (w/w), consisting of mannose and *N*-acetylglucosamine in a molar ratio of 8:2, suggesting the presence of oligomannose-type carbohydrate chains only, which are thus N-linked. A carbohydrate content of 4.2% (w/w) was determined by the phenol/H_2SO_4 assay.

For a more detailed knowledge of the structure of the carbohydrate chains, CR was N-deglycosylated by PNGase-F. Coomassie Blue staining of SDS-PAGE gels (Fig. 1) showed that the material originally present as a band with an apparent molecular mass of 56 kDa

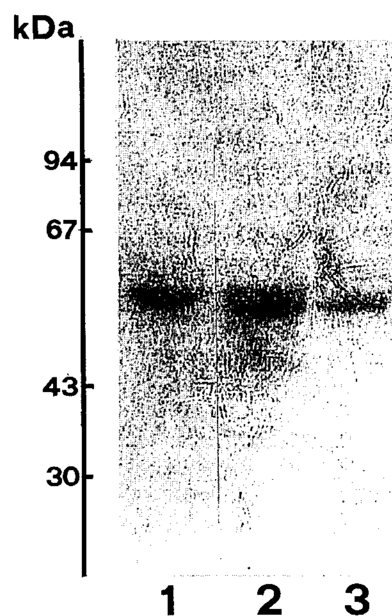

Figure 1. Digestion of spinach CR with PNGase-F. SDS-PAGE (10%) gel was stained with Coomassie Blue. Key to lanes: (1) CR before digestion (2 μg); (2) CR digested with 5 U PNGase-F for 16 h (2 μg); (3) CR digested with 10 U PNGase-F for 32 h (2 μg).

Table 1. Monosaccharide analysis of spinach CR and derived fractions

	Molar ratio[a]						
	CR	*Superdex 75*		*Bio-Gel P-4*	*CarboPac PA-100*		
Monosaccharide[b]		*SI*	*SII*	*BIII*	*CI*	*CII*	*CIII*
Man	7.6	7.5	7.8	7.9	6.7	6.8	7.5
GlcNAc	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Carbohydrate content (%, w/w)	4.7	<0.5	n.d.[c]	n.d.	n.d.	n.d.	n.d.

[a]GlcNAc taken as 2.0; [b]In all samples, Xyl and Glc are present as contaminants but they are not constituents of the carbohydrate chains of CR as will be demonstrated in this paper; [c]n.d. means not determined.

appeared as one band with reduced molecular mass of about 54 kDa after treatment with 10 U PNGase-F for 32 h. The released oligosaccharides were separated from the protein by gel permeation chromatography on Superdex 75 (Fig. 2). Two carbohydrate-positive fractions (orcinol/H_2SO_4 test), denoted SI and SII were collected. Monosaccharide analysis of the Superdex 75 void-volume peak (SI) showed the presence of <0.5% (w/w) carbohydrate on the remaining protein, indicating that >90% (w/w) of the carbohydrate had been cleaved by PNGase-F. The monosaccharide composition (Table 1) of the carbohydrate chains not released by PNGase-F was identical to that of native CR.

The carbohydrate-containing fraction SII (after desalting on Bio-Gel P-2) was further purified by gel filtration on a Bio-Gel P-4 column (Fig. 3). The void-volume fraction BI contained peptide material. The carbohydrate-positive fraction BIII revealed the monosaccharide composition Man:GlcNAc = 7.9:2.0 (Table 1). Fraction BIII was then subjected to anion-exchange chromatography on a Resource Q column (Fig. 4). The first peak, representing the neutral fraction, turned out to contain carbohydrate (orcinol/H_2SO_4 test). ^{1}H-NMR spectroscopy of this fraction showed the occurrence of a mixture of oligomannose-type of glycans, comprising of $Man_8GlcNAc_2$ as the major component, together with a small amount of $Man_7GlcNAc_2$. This carbohydrate-containing fraction was separated by HPAEC on a CarboPac PA-100 column, yielding three subfractions denoted CI, CII and CIII, and these were investigated by monosaccharide analysis (Table 1). The subfractions exhibited different retention times compared to the references $Man_6GlcNAc_2$ and $Man_9GlcNAc_2$, respectively (Fig. 5). ^{1}H-NMR spectroscopic analysis of the subfractions CI-CIII revealed the oligomannose-type glycan structures as depicted in Fig. 5A. The ^{1}H-NMR spectrum of fraction CIII is shown in Fig. 6. The presence of $Man_7GlcNAc_2$ in fractions CI-CII was confirmed by FAB-MS analysis. The mass spectrum (not depicted) showed pseudo-molecular ions $(M + H)^+$ and $(M + Na)^+$ at *m/z* 1559 and 1581, respectively, indicating a molecular mass of 1558 kDa. The relative amounts of the compounds

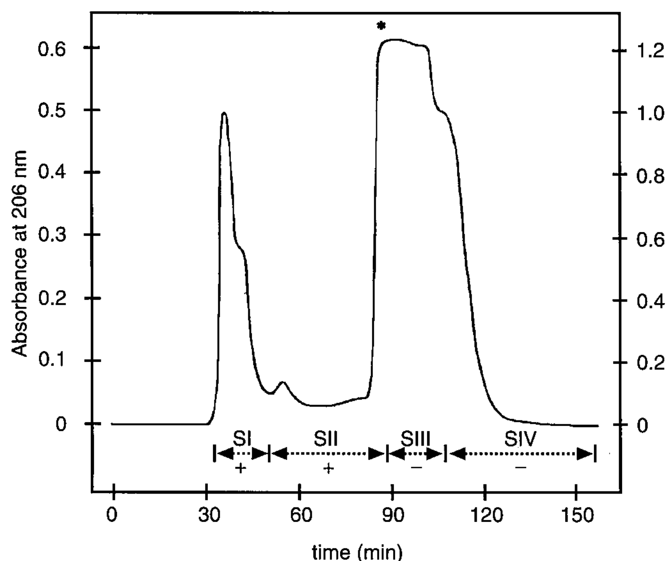

Figure 2. Elution profile of PNGase-F digest on Superdex 75. The column (60 × 2.6 cm) was eluted with 150 mM ammonium bicarbonate/HCl, pH 7.1, at a flow rate of 3 ml min^{-1}. Fractions were collected as indicated. + means carbohydrate positive and – means carbohydrate negative, according to orcinol/H_2SO_4 test. Asterisk (*) means attenuation change.

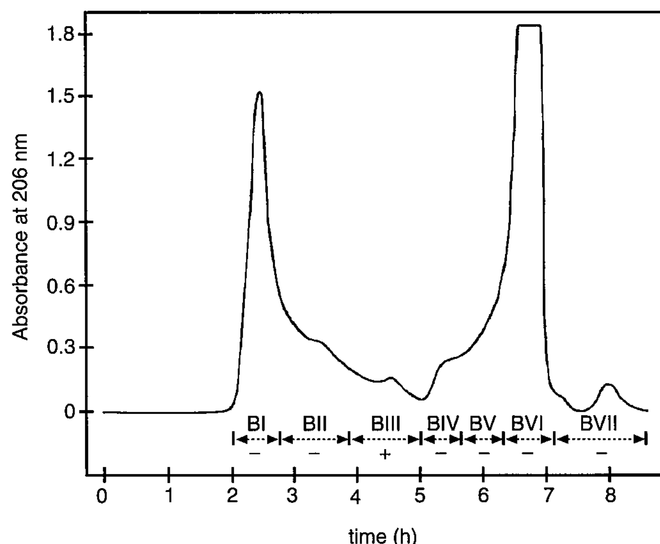

Figure 3. Elution profile of carbohydrate-containing Superdex 75 fraction SII on Bio-Gel P-4. The column (46 × 1 cm) was eluted with 5 mM ammonium bicarbonate at a flow rate of 5 ml h^{-1}. Fractions were collected as indicated. + means carbohydrate positive and – means carbohydrate negative, according to orcinol/H_2SO_4 test.

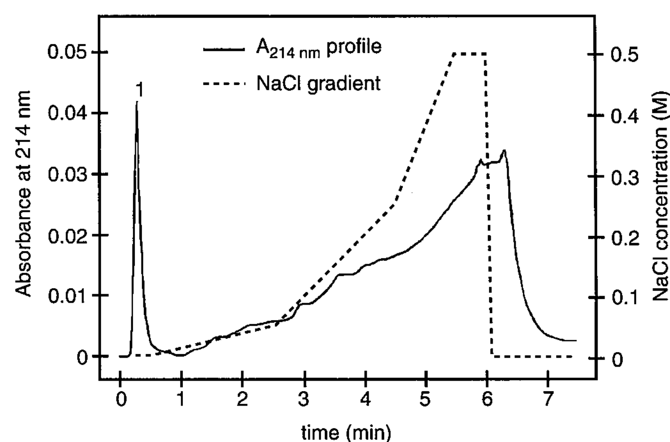

Figure 4. Elution profile of the oligosaccharide fraction derived from CR on Resource Q. The column (30 × 6.4 mm) was eluted with a NaCl gradient as indicated, at a flow rate of 4 ml/min. Peak 1 indicates the presence of only neutral material.

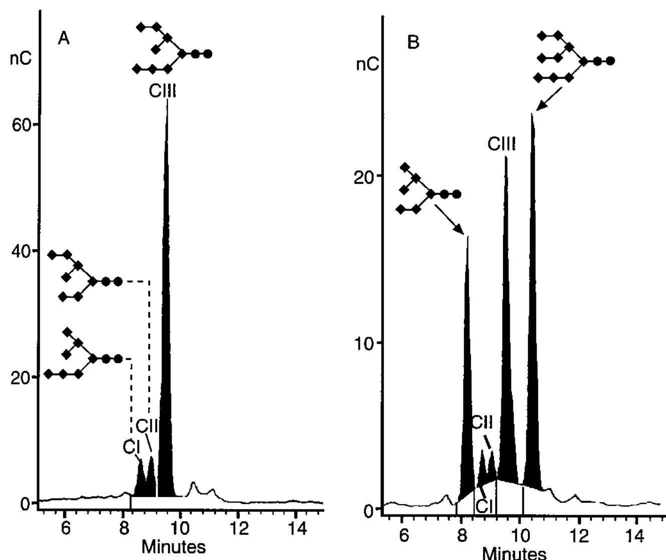

Figure 5. (A) Elution profile of the carbohydrates derived from CR on CarboPac PA-100. The column (4 × 250 mm) was eluted with 0.1 M NaOH using a NaOAc gradient as indicated, at a flow rate of 1 ml min^{-1}. Fraction CI, CII and CIII were collected; (B) Elution profile of the total carbohydrate moiety on the same column, co-injected with two reference compounds ($Man_6GlcNAc_2$ and $Man_9GlcNAc_2$). Compounds are represented by the following short-hand symbolic notation: ◆, D-Man; ●, D-GlcNAc.

(CI:CII:CIII = 10:10:80) were calculated by integration of the HPAEC peaks and from the relative intensities of the anomeric signals in the ^{1}H-NMR spectrum of the oligosaccharide mixture. For the identification of the different oligosaccharide structures use was made of literature data [19–21] and a ^{1}H-NMR database computer program [22]. The most relevant structural reporter-group signals of CIII ($Man_8GlcNAc_2$) are well defined, namely ManA H-1, ManB H-1, ManC H-1, $ManD_1$ H-1 and $ManD_3$ H-1 at δ 5.088, δ 5.145, δ 5.304, δ 5.042 and δ 5.042, respectively (Fig. 6). For CI, ManB H-1 has shifted to δ 4.908, indicating the lack of $ManD_3$, and for CII, ManC H-1 has shifted to δ 5.055, indicating the lack of $ManD_1$.

It is clear that the most abundant carbohydrate chain present in spinach CR showed a $Man_8GlcNAc_2$ structure, lacking the terminal α(1–2)-linked Man residue (D_2), when compared with the $Man_9GlcNAc_2$ structure.

In order to check if spinach CR, as all plant CRs so far investigated, contains the C-terminal ER retention sequence HDEL, we used the anti-HDEL monoclonal antibody 2E7 [16]. Figure 7 shows that the 56 kDa spinach CR is recognized by this specific antibody.

Discussion

The presence of two CR isoforms of 56 kDa and 54 kDa in spinach leaves has been previously reported [11]. The larger protein was shown to be a glycoprotein containing N-linked carbohydrate chain(s), on account of ligand blot techniques with Con A and affinity chromatography on Con A-Sepharose. After deglycosylation of the 56 kDa CR by PNGase-F, the remaining protein had an apparent molecular mass of about 54 kDa. A reduction of 2 kDa indicated a carbohydrate content of about 3%, which is in reasonable agreement with the carbohydrate content of 4.2–4.7% found by the phenol/H_2SO_4 assay and by monosaccharide analysis, respectively. The carbohydrate moiety of CR consists predominantly of carbohydrate chain(s) having a $Man_8GlcNAc_2$ structure. The minor $Man_7GlcNAc_2$ components may be stemming from biosynthetic intermediates.

The comparison of the available amino acid sequences of plant CRs (barley [23]; tobacco [9]; maize [10]) shows the presence of a potential glycosylation site at the same position (Asn32) of the N-domain. This site is different from that (Asn327) detected in most of the animal CRs sequenced so far [1, 2]. However, this site is only rarely glycosylated, with the rat liver CR [3, 4] as the only example up to now. More interestingly, it was demonstrated that the oligosaccharide moiety of bovine brain CR [6] is attached to Asn162, which is located in the middle of the peptide chain. The bovine brain protein has been shown to contain an oligomannose structure ($Man_5GlcNAc_2$), whereas the carbohydrate moiety of rat liver CR turned out to be of a hybrid type with terminal galactoses, a type of glycosylation unusual for ER resident proteins.

The oligomannose type of oligosaccharide found in spinach CR, together with the observed cross-reactivity with the anti-HDEL monoclonal antibody, are typical features of ER resident proteins. Calreticulin from barley [23], tobacco [9] and maize [10] have been shown to contain the ER retention signal motif HDEL, suggesting that they are indeed ER lumenal proteins. Moreover, immunofluorescence staining on maize root cells and tobacco cells has confirmed that CR is localized in the ER, in addition to the nuclear envelope. In pea seeds, CR has been found in microsomes enriched for ER [8].

The structure elucidation of the carbohydrate chains of CR can provide relevant information about a possible post-ER transit and retrieval to the ER. Our results do not indicate, however, the occurrence of any sugar residue typically added at the Golgi level [24]. Taken together, these data support the possibility that the glycosylated isoform of spinach CR is indeed a reticuloplasmin [25], i.e. a soluble ER resident protein. However, recent evidence indicates that CR can also be found outside of the ER compartment in some animal cell types [26–29].

The possibility that most plant CRs may be glycosylated is supported by our recent findings that CRs from other plant sources (e.g. pollen and ovary from

Figure 6. 500 MHz ^{1}H-NMR spectrum of fraction CIII. The denotation of the protons refer to the corresponding residues in the structure. The ^{2}HOH signal (4.7–4.8 ppm) has been omitted. '3' indicates the position of Man3 H-1 under the omitted ^{2}HOH signal. GN1 and GN2 refer to GlcNAc1 and GlcNAc2, respectively. The relative intensity scale of the *N*-acetyl region differs from that of the other part of the spectrum, as indicated.

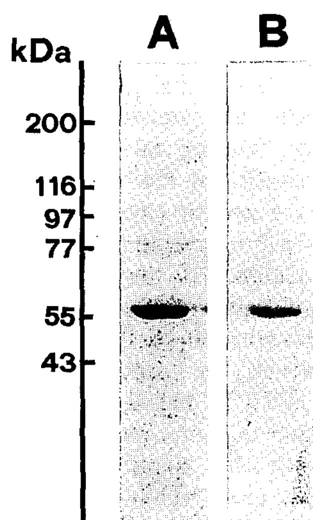

Figure 7. Immunoblot of spinach CR with the anti-HDEL monoclonal antibody 2E7. Samples of spinach CR (4 μg) were electrophoresed. Half of the gel was stained with Stains All (A) and the other half (B) was transferred onto nitrocellulose and then incubated with 2E7 (diluted 1:200).

Liriodendron tulipifera L.) interact with Con A [Navazio et al. unpublished observations]. This marks an important difference from animal CRs and can represent a distinctive feature of these plant Ca^{2+}-binding proteins.

Acknowledgements

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