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Evidence that Spinach Leaves Express Calreticulin but Not Calsequestrin[1]

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The presence of either calreticulin (CR) or calsequestrin (CS)-like proteins in spinach (*Spinacia oleracea* L.) leaves has been previously described. Here we report the purification from spinach leaves of two highly acidic (isoelectric point 5.2) Ca^{2+}-binding proteins of 56 and 54 kD by means of DEAE-cellulose chromatography followed by phenyl-Sepharose chromatography in the presence of Zn^{2+} (i.e. under experimental conditions that allowed the purification of CR from human liver). On the other hand, we failed to identify any protein sharing with animal CS the ability to bind to phenyl-Sepharose in the absence of Ca^{2+}. Based on the N-terminal amino acid sequence, the 56- and 54-kD spinach Ca^{2+}-binding proteins were identified as two distinct isoforms of CR. Therefore, we conclude that CR, and not CS, is expressed in spinach leaves. The 56-kD spinach CR isoform was found to be glycosylated, as judged by ligand blot techniques with concanavalin A and affinity chromatography with concanavalin A-Sepharose. Furthermore, the 56-kD CR was found to differ from rabbit liver CR in amino acid sequence, peptide mapping after partial digestion with *Staphylococcus aureus* V8 protease, pH-dependent shift of electrophoretic mobility, and immunological cross-reactivity with an antiserum raised to spinach CR, indicating a low degree of structural homology with animal CRs.

There is growing evidence that Ca^{2+} homeostasis in plant cells (Bush, 1993; Gilroy et al., 1993) shares mechanisms, membrane systems, and specific classes of Ca^{2+}-binding proteins with animal cells. In these latter cells, the intracellular distribution of Ca^{2+} is controlled primarily by the ER. This is best exemplified in the skeletal muscle cells, in which a specialized form of ER, the SR, exerts a dual control on the intraluminal accumulation of Ca^{2+} and on Ca^{2+} release into the myoplasm, thus regulating Ca^{2+} transients in relation to the contraction-relaxation cycle. In animal cells, the ER/SR intralumenal Ca^{2+}-binding proteins involved in Ca^{2+} storage belong to the CS and CR families (Pozzan et al., 1994). Two isoforms of CS, which are the products of two distinct genes, are expressed at different levels in muscle tissues, i.e. in cardiac and smooth muscle, in addition to skeletal muscle. The only nonmuscle cells in which an authentic CS was shown to be expressed are chicken cerebellum Purkjnie neurons. CR, although ubiquitous in eukaryotic cells, varies considerably in the level of expression, being highest in hepatocytes, neurons, and fibroblasts. It was also reported to be expressed in multiple isoforms, depending on the existence of either more than one gene or alternatively spliced products of the same gene.

In the last few years there have been several attempts to identify and characterize Ca^{2+}-binding proteins in plant tissues that are homologous proteins to animal CS and CR. Krause et al. (1989) first reported in *Streptanthus tortuosus* cells and spinach (*Spinacia oleracea* L.) leaves the presence of a Ca^{2+}-binding protein sharing electrophoretic and immunologic properties with animal CS. "CS-like proteins" were also described in *Pistia stratiotes* (Franceschi et al., 1993) and in red beet and cucumber cells (Xing et al., 1994). In contrast, Menegazzi et al. (1993), Denecke et al. (1993, 1995), and Hassan et al. (1995), investigating the identity of Ca^{2+}-binding proteins in spinach leaves, tobacco seedlings, and pea seeds, respectively, found sequence homologies with animal CR. Chen et al. (1994) reported the identification of cDNA clones encoding CR in barley. Although it seems to be uncontroversial that CR is expressed in plant cells, it is still an open question whether previous identifications of CS in the same cells might depend on some wrong assumptions. The main reason for the controversy could lie in the fact that CS and CR, although structurally different (Michalak et al., 1992), are similar in apparent molecular mass on SDS-PAGE, Stains-All staining, and Ca^{2+}-binding properties. Such shared features were the source of much confusion in the identification of these proteins in early studies on their distribution in animal tissues. Therefore, it is mandatory that additional, more rigorous criteria must be used to distinguish between these two main classes of Ca^{2+}-binding proteins, such as, for instance, the ability of CS to bind to phenyl-Sepharose in

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Abbreviations: CR, calreticulin; CS, calsequestrin; HRP, horseradish peroxidase; SR, sarcoplasmic reticulum.

the absence of Ca^{2+} (Cala and Jones, 1983), as opposed to the ability of CR, which lacks a Ca^{2+}-regulated hydrophobic site (Damiani et al., 1989), to bind to phenyl-Sepharose in the presence of Zn^{2+} (Heilmann et al., 1993).

Along this line, we have undertaken a deeper biochemical characterization of the Ca^{2+}-binding proteins previously identified by some of us (Menegazzi et al., 1993) in spinach leaves. Here we provide conclusive evidence that CR, and not CS, is expressed in spinach leaves. Moreover, we report the purification and partial characterization of two CR isoforms.

MATERIALS AND METHODS

Preparative Procedures

Spinach (*Spinacia oleracea* L.) plants obtained from a local market and the livers of adult male New Zealand albino rabbits were used in this study.

Homogenates of spinach leaves and of rabbit liver were obtained and fractionated by an ammonium sulfate precipitation procedure (Slupsky et al., 1987) under experimental conditions identical to those described by Damiani et al. (1989).

In some experiments, we used a fraction of SR terminal cisternae, obtained from rabbit soleus (a slow-twitch muscle) by isopicnic Suc-density centrifugation, according to Saito et al. (1984), with slight modifications (Damiani and Margreth, 1994). Terminal cisternae are enriched in the content of both skeletal and cardiac isoforms of CS (Damiani et al., 1990) and were used as positive controls in immunoblot experiments with polyclonal antibodies to animal CS.

Protein concentration was determined using the Protein Assay Reagent (Bio-Rad), which is based on the method of Bradford (1976), using BSA as standard.

DEAE-Cellulose Chromatography

DEAE-cellulose (Whatman) column chromatography was carried out under experimental conditions identical to those reported by Damiani et al. (1989).

Phenyl-Sepharose Chromatography

Phenyl-Sepharose (Pharmacia) column chromatography was carried out using either the procedure described by Heilmann et al. (1993) for purification of CR from human liver (procedure 1) or that described by Damiani et al. (1990) for purification of CS from rabbit skeletal muscle (procedure 2).

Procedure 1

DEAE-cellulose fractions collected between 0.2 and 0.4 M NaCl were pooled and dialyzed overnight against 10 mM Mops, pH 6.9, 0.5 M NaCl, 0.5 mM $ZnCl_2$ (buffer A). The protein sample was applied to a phenyl-Sepharose CL-4B (5 mL) column equilibrated with buffer A. After extensive washing (10 column volumes) to remove unbound proteins, the column was eluted by 10 mM Mops, pH 6.9, 0.5 M NaCl, 3 mM EDTA (buffer B).

Procedure 2

DEAE-cellulose fractions collected between 0.2 and 0.4 M NaCl were pooled and dialyzed overnight against 50 mM Mops, pH 7.1, 0.5 M NaCl, 1 mM EGTA (buffer A'). Equilibration and washing of the column was performed with buffer A', and elution was with 50 mM Mops, pH 7.1, 0.5 M NaCl, 10 mM $CaCl_2$ (buffer B').

Con A-Sepharose Chromatography

DEAE-cellulose fractions collected between 0.2 and 0.4 M NaCl were applied to a Con A-Sepharose (Pharmacia) column (2 mL) previously equilibrated with 10 mM sodium phosphate buffer, 0.5 M NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, pH 6.8. The column was washed with several volumes of equilibration buffer and then Con A-binding glycoproteins were eluted upon addition of 0.2 M methyl-α-D-mannopyranoside in the same buffer.

Preparation of Antiserum to Spinach CR

Antiserum against spinach CR was raised in adult rabbits by four weekly intramuscular injections of 0.2 mg of Con A-Sepharose-purified 56-kD CR, emulsified with an equal volume of Freund's complete adjuvant in the case of the first two injections and of Freund's incomplete adjuvant in the case of the latter two. After 35 d from the beginning of immunization, a booster injection was given, and the animal was bled after 1 week.

Gel Electrophoresis and Western Blot Techniques

One-dimensional SDS-PAGE was carried out according to Laemmli (1970), using 7.5 to 10% polyacrylamide linear gradient gels.

Two-dimensional gel electrophoresis was carried out (a) according to Michalak et al. (1980), by running the first dimension at neutral pH (7.0) in 10% polyacrylamide-SDS gels, as described by Weber and Osborn (1969); or (b) according to O'Farrell (1975), with IEF in the first dimension in the presence of 2% Ampholines (Pharmacia). The pH range was usually from 3.5 to 10. For both types of electrophoresis, the second dimension was a standard 7.5 to 10% polyacrylamide-SDS gel. Slab gels were stained with Coomassie blue (Bio-Rad), partially destained, and then restained with Stains-All (Bio-Rad), according to Damiani and Margreth (1994), or were stained with silver nitrate by the method of Morrissey (1981), as indicated in the legends to the figures

Electrophoretic transfer of proteins onto nitrocellulose was carried out as described by Towbin et al. (1979). Blots were used for three different types of ligand overlay.

^{45}Ca Overlay

^{45}Ca overlay of blots was carried out under experimental conditions identical to those described by Zorzato and

Volpe (1988). ^{45}Ca-labeled proteins were visualized by autoradiography (Amersham).

Immunoblot

Blots were incubated as described by Damiani et al. (1990) with (a) polyclonal antibodies to rabbit fast-twitch skeletal and dog cardiac CS (Damiani et al., 1990) or (b) the antiserum raised against spinach CR. In preliminary experiments, it was verified that the preimmune serum did not react with any protein of the several fractions tested. Immunoenzymic staining of blots was carried out using an HRP/diamino benzidine system.

Overlay with HRP-Con A

Blots were incubated with 0.9% NaCl, 3% BSA (Sigma) for 1 h and then with 1 μg/mL HRP-Con A (Sigma) for 1 h at room temperature in the presence of 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. After washing four times per 5 min with 0.9% NaCl, 0.1% Tween, Con A-binding proteins were detected using a HRP/diamino benzidine system.

One-Dimensional Peptide Mapping

One-dimensional peptide mapping was carried out as described by Cleveland et al. (1977) on CR protein bands excised from preparative SDS gels and digested with 5 μg of *Staphylococcus aureus* V8 protease (Sigma). Proteolytic fragments were resolved using 12.5 to 19% polyacrylamide linear gradient gels.

Sequence Analysis

For N-terminal sequence analysis, proteins were electroblotted onto a polyvinylidene difluoride membrane under conditions identical to those described by Matsudaira (1987). Proteins were sequenced on an Applied Biosystems 476A sequencer with on-line phenylthiohydantoin detection.

For internal amino acid sequencing, the 56-kD protein was electroblotted onto a polyvinylidene difluoride membrane as described above. After Coomassie blue staining, the protein band was cut in 1- to 2-mm squares and the Coomassie blue was eliminated by a 2-min wash in 70% acetonitrile. The in situ digestion was performed by covering the pieces of membrane with 10 μL of 25 mM ammonium bicarbonate, 1% D-octyl-glucopyranoside, 10% methanol, and 0.1 μg/μL trypsin. After 18 h of incubation, formic acid was added to a final concentration of 50% to allow release of peptides bound to the membrane. The extraction with formic acid was repeated a second time. The digestion mixture was resolved by reversed-phase HPLC using an Aquapore RP300 (Brownlee Laboratories, Santa Clara, CA) column (100 × 1 mm, 7 μm). The buffers used were: A, 0.1% TFA; and B, 0.08% TFA, 70% acetonitrile.

RESULTS

Purification and Biochemical Characterization of Spinach Ca^{2+}-Binding Proteins

Homogenates of spinach leaves were fractionated on DEAE-cellulose using a linear gradient of 0.05 to 1 M NaCl. Figure 1A shows the electrophoretic pattern of a DEAE-cellulose fraction eluted between 0.2 and 0.4 M NaCl after staining with Stains-All. In agreement with our previous results (Menegazzi et al., 1993), two major blue-staining proteins of 56 and 54 kD were identified, together with two additional blue-staining protein components of about 25 kD. Figure 1B shows that the blue-staining 56- and 54-kD proteins were both identifiable as Ca^{2+}-binding proteins, as judged by the $^{45}Ca^{2+}$-ligand overlay technique.

DEAE-cellulose fractions enriched in 56- and 54-kD Ca^{2+}-binding proteins were pooled and applied to phenyl-Sepharose in the presence of $ZnCl_2$, as described for purification of CR from human liver (Heilmann et al., 1993). Upon addition of 3 mM EDTA, a protein peak was eluted (Fig. 2A) that specifically contained the 56- and 54-kD Ca^{2+}-binding proteins (Fig. 2B). In contrast, when an identical sample was applied to phenyl-Sepharose in the presence of 1 mM EGTA and subsequently eluted with 10 mM $CaCl_2$, i.e. under experimental conditions identical to those employed for purification of CS from animal tissues (Damiani et al., 1990), neither the 56- nor the 54-kD spinach Ca^{2+}-binding protein could be purified (Fig. 3).

Figure 4 shows that phenyl-Sepharose-purified 56- and 54-kD spinach Ca^{2+}-binding proteins failed to react with polyclonal antibodies specific for the skeletal (Fig. 4B) and the cardiac (Fig. 4C) isoforms of mammalian CS as demonstrated by western blot techniques.

The 56- and 54-kD Ca^{2+}-binding proteins exhibited an acidic pI of 5.2 when analyzed in two-dimensional electrophoresis after IEF in the first dimension (O'Farrell, 1975) (Fig. 5).

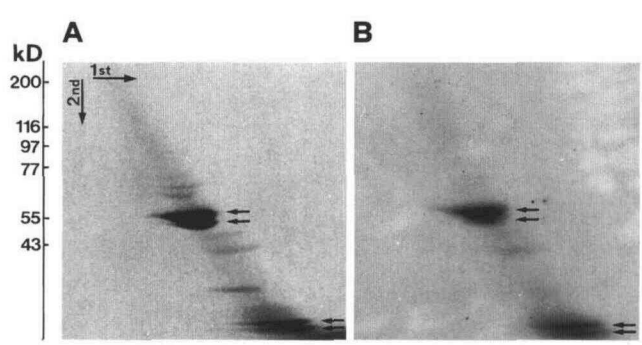

Figure 1. Identification of spinach Ca^{2+}-binding proteins by Stains-All staining and $^{45}Ca^{2+}$-ligand overlay. Protein fractions, eluted between 0.2 and 0.4 M NaCl from a DEAE-cellulose column, were analyzed by two-dimensional SDS-PAGE, according to Michalak et al. (1980), by running the electrophoresis in a disc gel at pH 7.0 in the first dimension and in a Laemmli slab gel at pH 8.8 in the second dimension. Slab gels were stained with Stains-All (A) or transferred onto nitrocellulose and then incubated with $^{45}CaCl_2$ (B). ^{45}Ca-labeled proteins were visualized by autoradiography (5-d exposure). About 50 μg of protein were loaded per gel. Arrows indicate blue staining, Ca^{2+}-binding proteins.

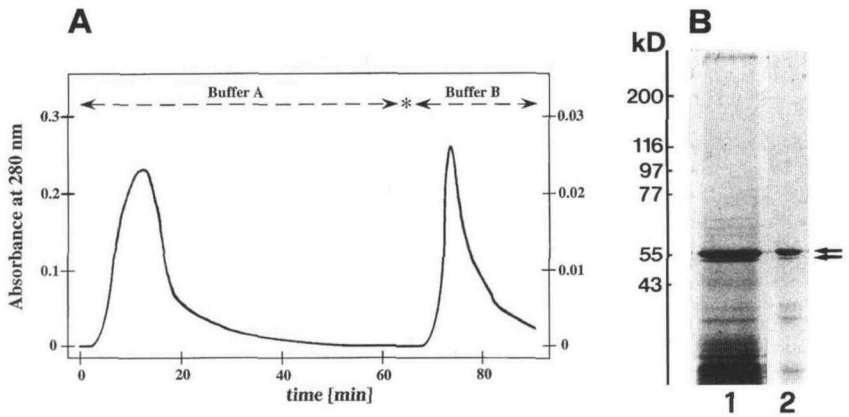

Figure 2. Purification of 56- and 54-kD spinach Ca^{2+}-binding proteins by phenyl-Sepharose chromatography in the presence of Zn^{2+}. A, A_{280} profile. An asterisk (*) indicates a change in the A_{280} scale to that listed on the right. B, Stains-All-stained SDS-PAGE gel of spinach protein fractions obtained from phenyl-Sepharose chromatography. Lane 1, Protein sample applied to phenyl-Sepharose (40 μg); lane 2, eluate with EDTA (4 μg). Arrows indicate 56- and 54-kD blue-staining, Ca^{2+}-binding proteins.

In a previous study (Menegazzi et al., 1993), by using the periodic acid Schiff staining, we suggested that the 56-kD CR was glycosylated, whereas the 54-kD isoform was not. We have reinvestigated this problem by using a more sensitive ligand blot technique, employing peroxidase-labeled Con A. The 56-kD spinach protein was found to be heavily labeled with Con A, whereas the 54-kD protein did not bind Con A at all (Fig. 6A).

Furthermore, we found that, when DEAE-cellulose fractions from spinach leaves were applied to a Con A-Sepharose column, the only protein that was retained and subsequently eluted by methyl-α-D-mannopyranoside was the 56-kD protein. (Fig. 6B).

Amino Acid Sequence Analysis of Spinach Ca^{2+}-Binding Proteins

Table I shows the N-terminal sequences of spinach 56- and 54-kD Ca^{2+}-binding proteins. The N-terminal sequence obtained for the 56-kD Ca^{2+}-binding protein was found to correspond to that previously reported by Menegazzi et al. (1993) for CR-like protein from spinach leaves. The comparison between 56- and 54-kD protein N-terminal sequences showed that they were not identical, i.e. in the 54-kD protein a Phe was replaced by a Glu in position 4, and a Glu was replaced by an Ala in position 6. However, it is noteworthy that the net charge in this first region was not changed in the two proteins. Table I also shows that the 56- and 54-kD spinach proteins share a considerable degree of homology with the N terminus of both plant and animal CRs, and that they can be deemed authentic CR proteins.

In addition, we were successful in sequencing three internal fragments obtained by tryptic digestion of the 56-kD spinach CR. Fragment 1 could be aligned with the region between amino acids 57 and 69 of different CRs, and it was found to be 92% identical with both barley CR (Chen et al., 1994) and tobacco CR (Denecke et al., 1995). The degree of identity with the published sequence for rabbit CR (Fliegel et al., 1989) was lower (67%). Fragment 2 also displayed a high degree of identity with barley CR (78%) and tobacco CR (89%), as well as with rabbit CR (67%). Fragment 3 (XIVFQPN) could not be aligned with any sequence of CR, indicating that it might be located in a less-conserved re-

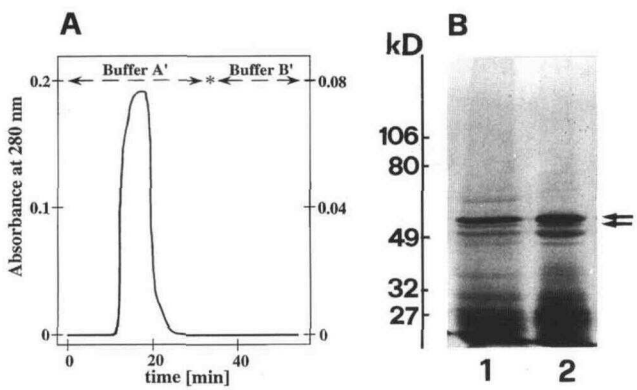

Figure 3. Phenyl-Sepharose column chromatography, in the presence of EGTA, of DEAE-cellulose fractions from spinach leaves. A, A_{280} profile. An asterisk (*) indicates a change in the A_{280} scale to that listed on the right. B, Stains-All-stained SDS-PAGE gel of the protein sample applied to the column (lane 1, 50 μg) and of void volume (lane 2, 55 μg). Arrows indicate the 56- and 54-kD Ca^{2+}-binding proteins.

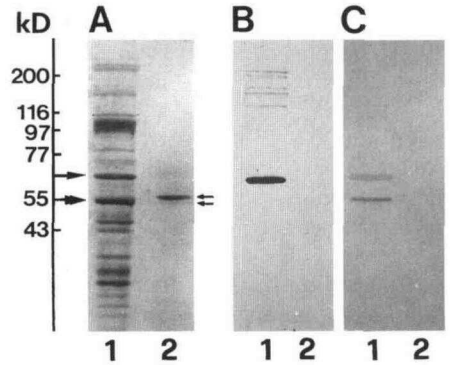

Figure 4. Immunostaining of purified 56- and 54-kD spinach Ca^{2+}-binding proteins with antibodies to skeletal and cardiac muscle CS. Blots were stained with Ponceau red (A) and then incubated with polyclonal antibodies to rabbit skeletal CS (B) or to dog cardiac CS (C). Lanes 1, SR terminal cisternae from rabbit slow-twitch muscle (30 μg), used here as a control; lanes 2, spinach proteins purified by phenyl-Sepharose chromatography (4 μg). Arrowhead, 64-kD rabbit skeletal CS. Double arrowhead, 55-kD rabbit cardiac CS. Arrows, 56- and 54-kD spinach Ca^{2+}-binding proteins.

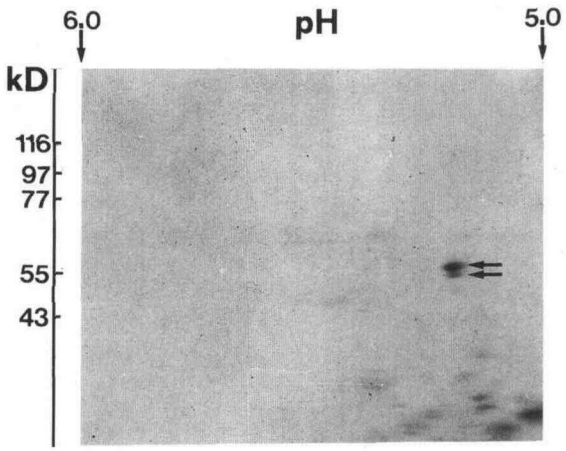

Figure 5. IEF of the DEAE-cellulose fraction from spinach leaves. The slab gel was stained with Stains-All. About 50 μg of protein was loaded. Only the region between pH 5.0 and 6.0 is shown. Arrows indicate 56- and 54-kD blue-staining, Ca^{2+}-binding proteins.

gion of the protein. The lower molecular mass band has not yet been microsequenced due to the limited amount of protein available.

As further evidence of the close structural interrelation, but not complete identity, of the 56- and 54-kD spinach CRs, we found that Cleveland peptide maps of both proteins, obtained by partial digestion with *S. aureus* V8 protease, were very similar but not identical (Fig. 7).

Comparison of CR Proteins Purified from Spinach Leaves and Rabbit Liver

CR was obtained from rabbit liver by phenyl-Sepharose chromatography in the presence of Zn^{2+}, as described above. Table II summarizes the general properties of CR from spinach leaves and rabbit liver.

Figure 8 shows the co-electrophoresis, using the two-dimensional gel system of Michalak et al. (1980), of rabbit liver CR purified by phenyl-Sepharose chromatography and of spinach CR purified by Con-A-Sepharose chromatography. Rabbit liver CR had an apparent molecular mass (60 kD) higher than that of spinach CR (56 kD). It also exhibited a significant pH-dependent shift in the electrophoretic migration, as already shown for human liver CR (Damiani et al., 1989) and rat liver CR (Treves et al., 1990), whereas the 56-kD spinach CR was found to stay on the diagonal line.

Rabbit liver 60-kD CR and spinach 56-kD CR were digested with *S. aureus* V8 protease as described by Cleveland et al. (1977). The peptide composition patterns of CR from rabbit liver and spinach leaves were found to be different, even though some shared peptides could be observed (data not shown).

Figure 9 shows that an antiserum raised against the 56-kD spinach CR strongly reacted with the spinach 56- and 54-kD CR isoforms (Fig. 9B, lane 2). On the other hand, there was no detectable cross-reaction of our antiserum to the 60-kD CR from rabbit liver (Fig. 9B, lane 1).

Furthermore, we found that rabbit CR failed to bind Con A, as judged by the Con A-overlay technique, and it could not be purified by Con A-Sepharose column chromatography (data not shown), confirming previous data of Khanna et al. (1987).

DISCUSSION

In this paper we report the isolation from spinach leaves, purification, and biochemical characterization of two main Ca^{2+}-binding proteins that are structurally unrelated to the CS family and that can be grouped together with animal CRs because of their inability to interact with phenyl-Sepharose in the absence of Ca^{2+} coupled with their ability to interact with phenyl-Sepharose in the presence of Zn^{2+}. This behavior, first described for human liver CR by Heilmann et al. (1993), is also shared by rabbit liver CR (this paper).

The two Ca^{2+}-binding proteins of spinach leaves described here have apparent molecular masses on SDS-PAGE of 56 and 54 kD. Based on amino acid sequence analysis at the N terminus, the 56-kD protein appears to be identical to the protein of the same size described by Menegazzi et al. (1993). We also find extensive identities in amino acid sequences between tryptic fragments of the 56-kD spinach protein and the corresponding sequences obtained from barley (Chen et al., 1994) and tobacco (Denecke et al., 1995) CRs, as well as from rabbit CR (Fliegel et al., 1989). On the other hand, we failed to obtain evidence that a protein sharing biochemical properties with animal CS exists in spinach leaves. Therefore, we conclude that CR, but not CS, is expressed in spinach cells. Although CR and CS, given their high Ca^{2+}-binding capacity coupled with low affinity for Ca^{2+} (Michalak et al., 1992), seem to be particularly good candidates for functioning as Ca^{2+}-storage proteins in the lumen of intracellular

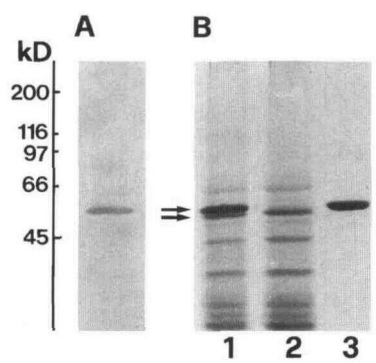

Figure 6. Identification of Con A-binding proteins by Con A-overlay (A) and affinity chromatography on Con A-Sepharose (B). A, Samples of DEAE-cellulose fractions (30 μg) from spinach were electrophoresed and transferred onto nitrocellulose. Blots were probed with Con A conjugated with peroxidase. B, DEAE-cellulose fractions were loaded onto a Con A-Sepharose column. Bound proteins were eluted with 0.2 M methyl-α-D-mannopyranoside. Samples were resolved by SDS-PAGE, and the gel was stained with Stains-All. Lane 1, DEAE-cellulose fraction applied to Con A-Sepharose (40 μg); lane 2, void volume (40 μg); lane 3, eluate (4 μg). Arrows indicate 56- and 54-kD Ca^{2+}-binding proteins.

Table I. *Amino acid sequence analysis of the N termini and tryptic fragments of spinach 56- and 54-kD Ca^{2+}-binding proteins*

Comparison with the amino acid sequence of different CRs. X, Undetermined amino acid residue.

Sample	Sequence	Reference
N-terminal sequences		
Spinach 56 kD	K V F F E E R F E D	This work
Spinach 54 kD	K V F E E A R F E D	This work
Spinach	K V F F E E R F E D	Menegazzi et al. (1993)
Pea	K V F F E E R F E D	Hassan et al. (1995)
Barley	D V F F Q E K F E D	Chen et al. (1994)
Tobacco	E V F F E E S F N D	Denecke et al. (1995)
Rabbit	E P V V Y F K E Q F L D	Khanna et al. (1987)
Human	E P A V Y F K E Q F L D	McCauliffe et al. (1990)
Tryptic fragments		
	57 69	
Spinach 56 kD (fragment 1)	X Y A I S A E F P E F S N	This work
Barley	F Y A I S A E Y P E F S N	Chen et al. (1994)
Tobacco	F F A I S A E F P E F S N	Denecke et al. (1995)
Rabbit	F Y A L S A R F E P F S N	Fliegel et al. (1989)
	73 81	
Spinach 56 kD (fragment 2)	T L V F Q F S V K	This work
Barley	T L V L Q F T V K	Chen et al. (1994)
Tobacco	N L V F Q F S V K	Denecke et al. (1995)
Rabbit	P L V V Q F T V K	Fliegel et al. (1989)

organelles regulating Ca^{2+} homeostasis, our study does not exclude the possibility that other Ca^{2+}-binding proteins might exist in spinach cells.

The spinach 54-kD protein, although indistinguishable from the major 56-kD protein by pI value as well as antigenically, does not represent a proteolytic product, because of the presence of two amino acid substitutions in the N-terminal sequence (see Table I) and the differences in Cleveland peptide maps after partial digestion with *S. aureus* V8 protease (Fig. 7). For the same reason, the previous hypothesis (Menegazzi et al., 1993), that the band of 54 kD might be simply an unglycosylated form of the 56-kD protein, can now be discarded. The presence of more than one CR isoform in spinach leaves is not surprising, since two cDNAs of CR were also sequenced in barley (Chen et al., 1994), and in this case they were identical in the pre-

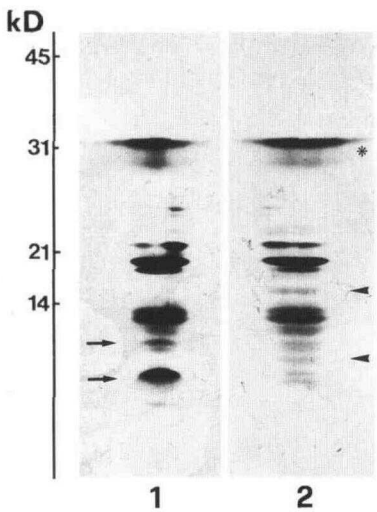

Figure 7. Peptide mapping of spinach 56- and 54-kD CR proteins. One-dimensional peptide mapping of CR was carried out according to Cleveland et al. (1977) using CR protein bands excised from preparative slab gels. Proteins were digested with *S. aureus* V8 protease, and fragments were resolved using 12.5 to 19% SDS-PAGE. The slab was stained with silver nitrate. About 2 μg of protein were loaded per lane. Lane 1, 56-kD CR; lane 2, 54-kD CR. Arrows and arrowheads indicate peptides specific to 56- and 54-kD Ca^{2+}-binding proteins, respectively. The asterisk (*) indicates the protein doublet corresponding to the protease.

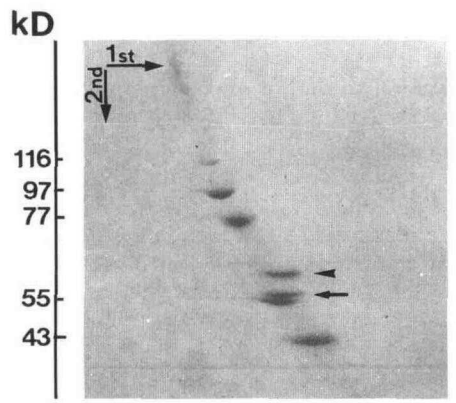

Figure 8. Co-electrophoresis of CR proteins from spinach leaves and rabbit liver. Two-dimensional SDS-PAGE of 56-kD CR purified from spinach leaves by Con A-Sepharose chromatography and of CR purified from rabbit liver by phenyl-Sepharose chromatography was carried out according to Michalak et al. (1980). Gels were stained with Stains-All. The diagonal line of molecular mass standards includes β-galactosidase (116 kD), phosphorylase *b* (97 kD), ovotransferrin (77 kD), glutamate dehydrogenase (55 kD), and ovoalbumin (43 kD). Arrowhead, 60-kD rabbit CR (3 μg); arrow, 56-kD spinach CR (3 μg).

dicted mature protein but differed in the N-terminal signal sequence.

Our present evidence that only the 56-kD isoform of spinach CR is glycosylated means that there is an important difference not only from the 54-kD isoform but also from rabbit liver 60-kD CR. The evidence is 2-fold: the differential ability of the 56-kD protein to bind Con A on ligand blots and to be purified using Con A-Sepharose affinity chromatography. This procedure appears to be an ideal method for obtaining bulk quantities of the spinach 56-kD CR, especially given the low recovery of the same protein following binding to phenyl-Sepharose in the presence of Zn^{2+}. It is a point of interest that among animal CRs so far investigated, very few are glycosylated (bovine liver, Khanna et al., 1987; rat liver, Van et al., 1989; Peter et al., 1992; bovine brain, Matsuoka et al., 1994), and these have different oligosaccharides.

Despite the existence of differences in molecular size, pH-dependent electrophoretic behavior, peptide map composition, and glycosylation, a property by which animal CRs and at least those plant CRs investigated so far (Chen et al., 1994; Denecke et al., 1995) could be linked together in a single family is the presence of a KDEL or HDEL sequence at the C terminus, which is believed to be responsible for the retention of proteins in the ER (Nash et al., 1994). However, at the present stage any conclusion about the subcellular localization of CR proteins in spinach leaves would be premature. Although in plant cells the vacuole, together with the cell wall (Gilroy et al., 1993; Seals et al., 1994), is generally regarded to be the main site of accumulation of Ca^{2+}, there is equal evidence that the ER may play an important role in Ca^{2+} homeostasis (Bush et al., 1989, 1993). ER-enriched microsomal fractions from celery were shown to contain otherwise uncharacterized Ca^{2+}-binding proteins with apparent molecular masses of 55 and 58 kD (Randall, 1992). Further immunocytochemical and biochemical work, carried out on microsomal fractions isolated by differential centrifugation, is required to firmly establish the subcellular localization of CR proteins in spinach as well as in other plant cells. On the basis of recent findings in animal cells (Burns et al., 1994; Dedhar, 1994), one cannot exclude the additional presence of CR in the nucleus and/or in the cytosol of plant cells.

Table II. *Comparison of biochemical properties of CR proteins from spinach leaves and rabbit liver*

+, Present. –, Absent.

Property	Spinach CR		Rabbit CR
Molecular mass[a] (kD)	56	54	60
Stains-All staining	Blue	Blue	Blue
pH-dependent shift of electrophoretic mobility	–	–	+
pI	5.2	5.2	4.7[b]
Zn^{2+}-regulated hydrophobic site	+	+	+
Ca^{2+}-regulated hydrophobic site	–	–	–[c]
Con A binding	+	–	–

[a] Estimated by SDS-PAGE, according to the method of Laemmli (1970). [b] Data from Pozzan et al. (1994). [c] Data from Damiani et al. (1989).

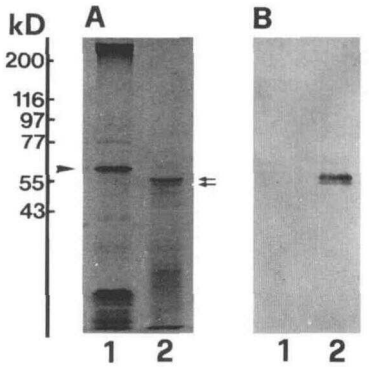

Figure 9. Immunological cross-reactivity of CR proteins with antiserum to the spinach 56-kD CR. Samples of DEAE-cellulose fractions from rabbit liver (lane 1, 5 μg) and from spinach leaves (lane 2, 8 μg) were electrophoresed. Half of the gel was stained with Stains-All (A) and the other half of the gel was transferred onto nitrocellulose. Blots were incubated with anti-spinach 56-kD CR antiserum (diluted 1 to 2000) (B). Arrowhead, 60-kD rabbit CR; arrows, 56- and 54-kD spinach CRs.

This study provides useful information for the evaluation of the degree of structural interrelation between animal and plant CRs and suggests that CR has been conserved during evolution even in organisms that are phylogenetically very distant.

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