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CALRETICULIN IS THE MAJOR Ca^{2+} STORAGE PROTEIN IN THE ENDOPLASMIC RETICULUM OF THE PEA PLANT (*PISUM SATIVUM*)

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A 56kDa protein with high similarity in its N-terminal amino acid sequence to animal calreticulin and 100% homology with the N-terminal amino acids of spinach calreticulin has been identified in seeds of the pea plant (*Pisum sativum*). A new purification procedure is described by which the calreticulin-like protein was selectively solubilized by incubation with deoxycholate and $HgCl_2$ from microsomes enriched for endoplasmic reticulum. Following Mono Q ion exchange chromatography of the deoxycholate extract by fast protein liquid chromatography, the calreticulin-like protein was obtained in nearly pure form. This purified protein is similar to animal calreticulin in apparent mass, characteristic blue staining with Stains-all dye and calcium-binding ability. In addition, this protein is recognized only by affinity purified antibodies against rabbit calreticulin and is not recognized by anti-calsequestrin antibodies. Our data suggested that calreticulin rather than calsequestrin functions as the Ca^{2+}-storage protein in the endoplasmic reticulum of pea plants.

In animal and plant cells, calcium is now generally recognized as an intracellular second messenger (1). To serve this role, intracellular calcium levels should be maintained within strict limits. Consequently, there is a requirement for an effective intracellular calcium buffering mechanism. In the sarcoplasmic reticulum (SR) of cardiac and skeletal muscle, calsequestrin (CSQ) is the major Ca^{2+}-binding protein; its primary physiological function is to sequester large amounts of Ca^{2+} in the lumen of the SR, contributing to the regulation of the lumenal concentration of free Ca^{2+} (2). Ca^{2+} storage, release and uptake in nonmuscle cells are largely controlled by proteins of the endoplasmic reticulum (ER) (1) in a manner similar to muscle SR membranes. However, in nonmuscle ER membranes, calreticulin (CRT), not calsequestrin, is the major Ca^{2+}-binding (storage) protein (3).

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The abbreviations used are: sarcoplasmic reticulum, SR; calsequestrin, CSQ; calreticulin, CRT; endoplasmic reticulum, ER; sodium dodecyl sulfate, SDS; 5-bromo-4-chloro-3-indolyl phosphate, BCIP; nitro blue tetrazolium, NBT; deoxycholate, DOC; polyacrylamide gel electrophoresis, PAGE; periodic acid/Shiff, PA/S.

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CRT and CSQ share several biochemical properties such as staining blue with the carbocyanine dye Stains-all and having clusters of acidic amino acid residues at their carboxyl terminus (2, 4, 5). Both CSQ and CRT have been reported to have a high-capacity, low affinity Ca^{2+}-binding domain (2,4). In addition, CRT also has a high-affinity, low capacity Ca^{2+}-binding domain (4). High-affinity, low capacity Ca^{2+}-binding sites may be consistant with inducing a conformational change in CSQ by the mechanism proposed by He *et al.* (6), but the role of these sites in CRT is unknown. It is, however, the low-affinity, high capacity Ca^{2+}-binding sites (40-50 Ca^{2+} per molecule of CSQ (2) and 30-40 Ca^{2+} per molecule of CRT (4)) that allow these proteins to function in Ca^{2+} storage and regulation.

Recently, increasing attention has been directed towards identifying the Ca^{2+} storage protein in plants. Some controversy exists whether CSQ or CRT serves as the major Ca^{2+} storage protein in plant cells, particularly within the plant ER. Calsequestrin-like proteins have been reported in extracts of *Streptanthus tortuosus* cell culture and in spinach leaves (7) and in the crystal idioblast cells of *Pistia stratiotes* (8). However, a protein with an N-terminus similar to animal CRT was recently purified from spinach leaves (9) and two cDNA clones from barley encoding a calreticulin-like protein have been reported (10). In the present study we show that CRT is the major Ca^{2+} binding protein in the ER of peas. This data supports the suggestion that CRT, not CSQ, is the major Ca^{2+}-storage protein in the plant ER.

MATERIALS AND METHODS

Affinity purified goat anti-rabbit CRT antibodies were provided by Dr. M. Michalak (University of Alberta, Edmonton, Canada). Sheep anti-rabbit skeletal muscle CSQ antibodies were prepared by our laboratory. Rabbit anti-canine cardiac CSQ antibodies were provided by Dr. L. Jones (Indiana School of Medicine, Indianapolis, IN). Alkaline phosphatase-conjugated secondary antibodies utilized were either swine anti-goat (Tago, Burlingame, CA), donkey anti-sheep (Sigma, St. Louis, MO) or goat anti-rabbit (Tago, Burlingame, CA). Sodium dodecyl sulfate (SDS), acrylamide, N,N-methylenebisacrylamide, and 2-mercaptoethanol were purchased from Bio-Rad (Hercules, CA). The cationic carbocyanine dye Stains-all, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), Nitro Blue Tetrazolium (NBT), deoxycholate (DOC) and all other reagent grade chemicals were purchased from Sigma (St. Louis, MO).

Plant material: Seeds of *Pisum sativum*, cv. *Columbia*, were obtained from Dr. David Oliver (University of Idaho). The dry seeds were sterilized with 10% bleach for 15 min, rinsed with water and allowed to soak overnight in running tap water.

Protein isolation: Isolation of a calreticulin-like protein from pea seeds was carried out using a modification of the procedure developed by White *et al.* (11). Pea seeds (50g) were homogenized with 4 volumes of extraction buffer (0.25 M sucrose/10 mM Tris, pH 7.4) in a Waring blender using three 30-sec bursts, each separated by 30 sec. The homogenate was passed through four layers of cheesecloth and centrifuged at 3000 x g for 5 min to remove debris and larger organelles. The supernatant was centrifuged at 10,000 x g for 20 min and the pellet discarded. This supernatant was then centrifuged at 110,000 x g for 30 min. The resulting microsomal pellet obtained was resupended in 15 ml of 0.6 M KCl/10 mM Tris, pH 7.4, using a glass/teflon Dounce homogenizer and the suspension was centrifuged at 110,000 x g for 30 min. The ER enriched pellet was suspended in 0.1 M KCl/5 mM $MgCl_2$/10 mM Tris, pH 7.4 to a concentration of 5-10 mg protein/ml and incubated for 10 min at 0°C with 0.1 mg DOC/ mg protein. $HgCl_2$ was added to a concentration of 5 mM and the preparation incubated for an additional 10 min at 37°C. After centrifugation at 110,000 x g for 30 min, the clear supernatant was dialyzed overnight against 0.1 M KCl/5 mM $MgCl_2$/10 mM Tris, pH 7.4 and applied to a Mono Q HR5/5 column (Pharmacia, Piscataway, NJ) equilibrated at 4°C with 0.1 M KCl/5 mM $MgCl_2$/10 mM Tris, pH 7.4. Proteins were eluted with a linear gradient from 0.1 to 0.8 M KCl in 5 mM $MgCl_2$/10 mM Tris, pH 7.4. This procedure provides a new, relatively rapid and efficient method for the purification of CRT from plant tissue.

Biochemical analysis: Polyacrylamide gel electrophoresis (PAGE) of the proteins was carried out as described by Laemmli (12). Gels were stained with Coomassie Blue or with Stains-all (13).

$^{45}Ca^{2+}$ ligand overlay was carried out on purified protein (4μg) or the deoxycholate/$HgCl_2$ extract of microsomal membranes electroblotted to nitrocellulose membranes and incubated with 100 μCi of ^{45}Ca as described by Maruyama *et al.* (14).

NH_2-terminal sequence analysis of CRT was carried out on protein purified from a Mono Q HR5/5 column. Purified protein was electroblotted onto PVDF membrane according to the procedure of Matsudaira (15). Automated sequence analyses were performed on an Applied Biosystems Model 475A gas-liquid phase protein sequencer.

For immunoblot analysis, proteins resolved by SDS-PAGE were transferred to nitrocellulose, blocked with 3% BSA in PBS and probed separately with 1) anti-rabbit CRT (1: 2000 dilution), 2) anti-rabbit skeletal muscle CSQ (1:8000 dilution) and 3) anti-canine cardiac CSQ (1:2000 dilution). After washing, the membrane was incubated with the appropriate alkaline phosphatase conjugated secondary antibody. Positive reactions were visualized by color development using NBT and BCIP as described by Blake *et al.* (16).

Glycoprotein detection was carried out according to the procedure of Stromqvist and Gruffman (17) except that nitrocellulose membrane was used instead of PVDF membrane. Protein concentration was determined according to the method of Bradford (18), using bovine serum albumin as a standard.

RESULTS

A new procedure was developed to purify CRT from pea microsomes. Total microsomal protein (Fig. 1A, Lane 2) was extracted with DOC/$HgCl_2$ as described in Methods. This extraction solubilized CRT protein (Fig. 1A, Lane 4) but resulted in a vast majority of microsomal proteins remaining in the insoluble pelleted fraction (Fig. 1A, Lane 3).

Many calcium binding proteins can be detected after SDS-PAGE gel resolution by staining the gel with the cationic carbocyanine dye Stains-all. Acidic proteins which bind Ca^{2+}, such as CSQ and CRT, will stain blue with Stains-all, whereas other proteins stain pink (13). We have employed this technique to identify a Ca^{2+}-binding protein in the deoxycholate/$HgCl_2$ extract of ER

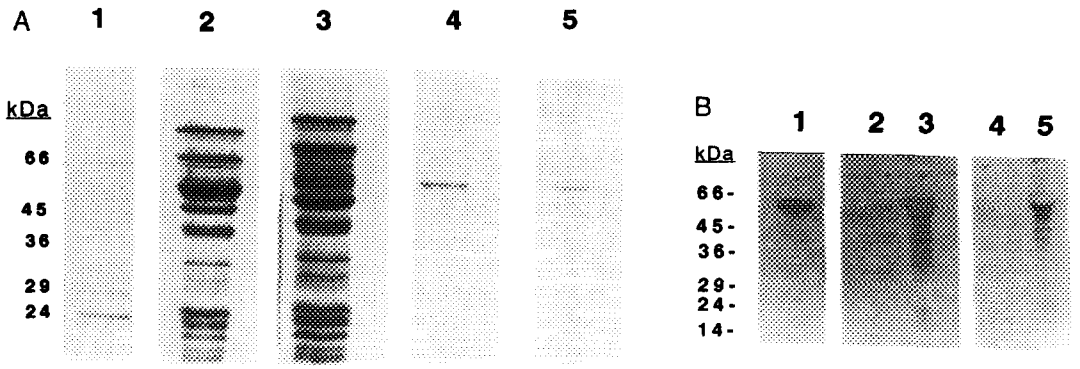

Figure 1. Panel A. Coomassie blue and Stains-all staining of the DOC/$HgCl_2$ extract of pea microsomes. Lane 1: molecular weight standards; Lane 2: total protein profile of pea microsomes; Lane 3: insoluble pellet after DOC/$HgCl_2$ extraction; Lane 4: soluble extract after DOC/$HgCl_2$ treatment; Lane 5: Stains-all staining of soluble DOC/$HgCl_2$ extract. Panel B. Western blot immunodetection with anti-CRT and anti-CSQ antibodies. Lane 1: pea microsome DOC/$HgCl_2$ extract proteins detected with anti-rabbit CRT antibody; Lanes 2 and 4: pea microsome DOC/$HgCl_2$ extract proteins detected with anti-skeletal muscle CSQ and anti-cardiac CSQ antibodies, respectively; Lanes 3 and 5: skeletal muscle CSQ detected with anti-skeletal muscle CSQ and anti-cardiac CSQ antibodies, respectively.

enriched microsomes from pea seeds. Stains-all staining revealed two polypeptides of 56kDa and 30kDa that stained blue (Fig. 1A, Lane 5).

More direct evidence that the 56kD protein was indeed a Ca^{2+}-binding protein was obtained from a $^{45}Ca^{2+}$-overlay experiment (cf. below, Fig 2). Proteins from a deoxycholate/$HgCl_2$ extract were resolved by SDS-PAGE, electroblotted onto nitrocellulose and probed with $^{45}Ca^{2+}$ as described by Maruyama *et al.* (14). $^{45}Ca^{2+}$ bound strongly to the 56kD protein.

Because the 56kDa Ca^{2+}-binding protein has a molecular mass close to that of CSQ and CRT, it was of interest to determine whether this protein was antigenically related to CSQ or CRT. To test this, the soluble DOC/$HgCl^2$ extract of pea microsomal membranes was probed with antibody to CRT, skeletal muscle CSQ and canine cardiac CSQ . As shown in Fig. 1B , the 56kDa plant protein was recognized by anti-CRT antibody (Fig. 1B, Lane 1), but was not recognized with anti-CSQ antibody raised against either skeletal or cardiac forms of the protein (Fig 1B, Lane 2 and 4, respectively). Control reactions, (Fig. 1B, Lanes 3 and 5) demonstrated that the two anti-CSQ antibodies appropriately recognized skeletal muscle CSQ as well as some CSQ degradation products.

To further characterize the microsomal Ca^{2+}-binding protein from pea seeds and its possible relation to CRT, the 56kDa protein was further purified using Mono Q FPLC ion exchange chromatography. A major elution peak appearing at about 0.46 M KCl appeared to consist only of the purified 56kDa protein. Fig 2, Lane 1, shows Stains-all staining of the fraction eluting at 0.46 M KCl; only one blue band was observed. An autoradiograph of a $^{45}Ca^{2+}$ overlay (Fig. 2, Lane 2), using the same Mono Q elution fractions, confirmed that the 56kDa blue staining protein from the ER-enriched microsomes of pea seeds was indeed a Ca^{2+} binding protein. Skeletal muscle CSQ, used as a control, was also observed to bind $^{45}Ca^{2+}$ (Fig. 2, Lane 3) Some CSQ degradation products, which also bind $^{45}Ca^{2+}$, were observed as a ladder below the main CSQ band.

To test whether the protein purified from pea was glycosylated, the purified protein was blotted onto nitrocellulose and subjected to periodic acid /Schiff (PA/S) staining (17). Fig. 2, Lane 4, shows that the 56kDa protein is PA/S positive, indicating that the purified protein is a glycoprotein.

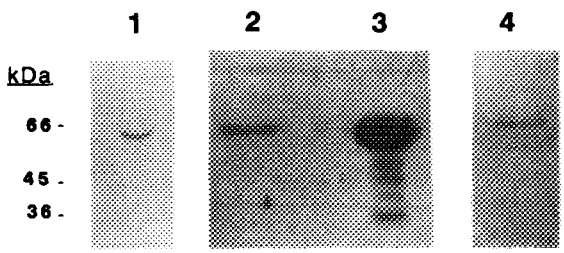

Figure 2. Analysis of the purified CRT isolated from pea seed microsomes. 2.4 µg/lane of purified CRT or 12.5 µg/lane of CSQ (as a control) were resolved on a 10% SDS-polyacrylamide gel and analyzed for blue-staining protein bands by Stains-all staining (Lane 1), for $^{45}Ca^{2+}$ binding by CRT (Lane 2) and CSQ (Lane 3) using Ca^{2+}-overlay techniques and by PA/S staining for glycoproteins (Lane 4).

To confirm that the protein identified in plant is calreticulin, an NH_2-terminal amino acid sequence analysis of the purified protein was performed. Table 1 shows the NH_2-terminal amino acid sequence obtained for the 56kDa protein and for calreticulin from different sources. The NH_2- terminal sequence (11 amino acids) of the 56kDa pea plant protein exactly matched (100% homology) that of spinach CRT and corresponded closely to that of rabbit and human calreticulin.

DISCUSSION

In the present study we have shown that CRT is present in ER-enriched microsomes of pea seeds. CRT was identified in these membranes by Western blot analysis, $^{45}Ca^{2+}$-overlay, Stains-all staining, and NH_2-teminus amino acid sequencing.

The localization of the calreticulin-like protein in the ER of pea seeds is not surprising in the light of a recent finding that two cDNA clones (CRH1 and CHR2), identified in barley and shown to be similar to the animal CRT, contain an ER retention signal motif, HDEL (10). The presence of ER retention signal suggests that the gene products of these two clones are likely to be ER lumenal proteins. Similarly, CRT in animals is found mainly in the ER, although CRT has also been detected in the nucleus and nuclear envelope (19). The purified calreticulin-like protein from pea and that of spinach were found to be similar by several criteria. First, both proteins migrated on SDS-PAGE with an apparent molecular mass of 56kDa. Second, the NH_2-terminal amino acid sequence of purified spinach and pea proteins were found to be 100% identical for the first 11 amino acids residues. Finally, the two proteins were shown to be glycoproteins. Although the available sequence of CRT from different sources contains one potential site for glycosylation, the purified protein from mouse or rabbit muscle and chicken liver contains no carbohydrate (20). However, bovine liver calreticulin also appears to be glycosylated (20).

These results suggested that the calreticulin-like proteins from spinach and pea plants are highly similar and may be identical. The striking similarity between the NH_2-terminal amino acid sequence of the pea CRT and the available sequences of spinach (9), barley (10), rabbit (21) and human CRT (22) indicated that CRT is conserved in plant and animal species and support the suggestion that this protein might have a fundamental physiological function(s) in a variety of cells.

Earlier reports have suggested that a calsequestrin-like protein is present in plant cells of spinach (7), *Streptanthus tortuosus* (Brassicaceae) (7) and the water lettuce, *Pistia stratiotes* (8). Uncertainty as to the identity of the major plant Ca^{2+} binding appears to have be centered around two points. First, Menegazzi *et al.* (9) reported, using spinach plants and techniques similar to

Table 1. NH_2-terminal amino acid sequence of pea calreticulin aligned with the published NH_2-terminal sequences for calreticulin and calsequestrin

Ca^{2+} Binding Protein	NH_2 Terminus	Reference
Calreticulin (pea)	KVFFEERFEDG	(this work)
Calreticulin (spinach)	KVFFEERFEDG	(9)
Calreticulin (rabbit liver)	EPVVYFKEQFLDG	(21)
Calreticulin (human liver)	EPAV-FKEVFLDG	(22)
Calsequestrin (rabbit skeletal)	EEGLDFEYDGVDR	(5)
Calsequestrin (canine cardiac)	GGGLNFPTYDGKD	(24)

those used by Krause *et al.* (7), that the major Ca^{2+} binding protein was CRT. Second, Parys *et al.* (23) reported that the antibody used by Krause *et al.*. (7) cross-reacted with both CSQ and CRT. It appears that these previous papers describing a CSQ-like protein from plants did not consider CRT as the possible identity of the ER Ca^{2+}-binding protein and no amino acid sequence data was presented. However, in the cases where the protein was purified and subjected to N-terminal amino acid sequence analysis (9, and this study), similarity was seen with CRT but not with CSQ. This information, combined with our results which show immuno-recognition of the pea plant ER Ca^{2+}-binding protein by anti-CRT antibodies, but not by anti-CSQ antibodies, suggests that CRT rather than CSQ functions as the major ER Ca^{2+} strorage portein at least in the pea plant and possibly in plants in general.

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REFERENCES

1. Walz, B. and Baumann, O. (1989) Topics Histochem. Cytochem. 20, 1-20.
2. MacLennan, D.H., Campbell, K.P., and Reithmeier, R.A.F. (1983) in *Calcium and cell Function*, vol. IV, Cheung, W.Y., ed (New York, Academic Press), pp. 151-173.
3. Milner, R.E., Bahsh, S., Shemanko, C., Carpenter, M.R., Smillie, L., Vance, J.G., Opas, M., and Michalak, M. (1990) J. Biol. Chem. 266, 7155 - 7165.
4. Michalak, M., Milner, R.E., Burns, K., and Opas, M. (1992) Biochem. J. 285, 681-692.
5. Fliegel, L., Ohnishi, M., Carpenter, M.R., Khanna, V.K., Reithmeier, R.A.F., and MacLennan, D.H. (1987) Proc. Natl. Acad. Sci. 84, 1167-1171.
6. He, Z., Dunker, A.K., Wesson, C.R. and Trumble, W.R. (1993) J. Biol. Chem. 30, 24635-24641.
7. Krause, K.-H., Chou, M., Thomas, M. A., Sjolund, R.D., and Campbell, K.P. (1989) J. Biol. Chem. 264, 4269-4272.
8. Franceschi, V.R., Li, X., Znang, D., and Okita, T. W. (1993) Proc. Natl. Acad. Sci. USA. 90, 6986-6990.
9. Menegazzi, P., Guzzo, F., Baldan, B., Matiani, P., and Treves, S. (1993) Biochem. Biophys. Res. Commun. 190, 1130-1135.
10. Chen, F., Hayes, P. M., Mulrooney, D. M., and Pan, A. (1994) The Plant Cell 6, 835-843.
11. White, D. M., Colwyn, R.T., and Denborough, M. A. (1983) Biochem. Biophys. Acta., 744, 1-6.
12. Laemmli, U.K. (1970) Nature 227, 680-685.
13. Campbell, K. P., MacLennan, D. H., and Jorgensen, A. O. (1983) J. Biol. Chem. 258, 11267-11273.
14. Maruyama, K., Mikawa, T., and Ebashi, S. (1984) J. Biochem. 95, 511-519.
15. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035-10038.
16. Blake, M .S., Johnston, K. H., Russel-Jones, G. J., and Gotschich, E. C. (1983) Anal. Biochem. 136, 175-179.
17. Stromqvist, M., and Gruffman, H. (1992) Biotechniques 13, 744-749.
18. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.
19. Opas, M., Dziak, E., Fliegel, L., and Michalak, M. (1991) J. Cell Physiol. 149, 160-171.
20. Khanna, N.C., Tokuda, M., Waisman, D.M. (1987) Biochem. J. 242, 245-251.
21. Treves, S., DeMattei, M., Lanfredi, M., Villa, A., Green, N.M., MacLennan, D.H., Meldolesi, J., and Pozzan, T. (1990) Biochem. J. 271, 473-480.
22. Heilmann, C., Spamer, C., Leberer, E., Gerok, W., and Michalak, M. (1993) Biochem. Biophys. Res. Commun. 193, 611-616.
23. Parys, J. B., McPherson, M., Mathews, L., Campbell, K. P., and Longo, F. J. (1994) Develop. Biol. 161, 466-476.
24. Scott, B.T., Simmerman, H.K.B., Collins, J.H., Nadal-Ginard, B., and Jones, L.R. (1989) J. Biol. Chem. 263, 8958-8964.