Plant Physiol. (1991) 95, 1077–1083
0032-0889/91/95/1077/07/$01.00/0

Received for publication July 13, 1990
Accepted December 7, 1990

Purification and Immunohistochemical Detection of an Embryogenic Cell Protein in Carrot[1]

Tomohiro Kiyosue*, Shinobu Satoh, Hiroshi Kamada, and Hiroshi Harada

Institute of Biological Sciences, University of Tsukuba, Tsukuba-shi, Ibaraki, 305, Japan

ABSTRACT

An embryogenic cell protein from carrot (*Daucus carota* L.), designated ECP31 for embryogenic cell protein and with a relative mass of 31,000, was purified by sequential column chromatographies. Its apparent relative mass was estimated to be 120,000 by gel filtration. Immunoblotting and immunohistochemical studies showed that ECP31 was preferentially localized in the peripheral cells of clusters of embryogenic cells in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) and disappeared during the course of somatic embryogenesis in the absence of 2,4-D. ECP31 began to accumulate on the 33rd day after initiation of cultures of hypocotyl segments on Murashige-Skoog medium with 2,4-D, when callus began to appear on the segments. In dry seeds, lower amounts of ECP31 were located throughout the entire zygotic embryos but not in endosperm. ECP31 was also detected in provascular tissue of malformed somatic embryos.

Somatic embryogenesis in carrot, which can readily be induced by the transfer of embryogenic cells from auxin-containing medium to auxin-free medium (14), is known as a useful model system for investigation of the morphological, biochemical, and molecular events that take place during the early develpment of plants (11, 16, 18). However, little information has been obtained about the events that occur when somatic cells become competent to produce somatic embryos. One of the reasons for this lack of information is the paucity of data on the isolation and characterization of markers for embryogenic cells.

Recently, we isolated a monoclonal antibody (1D11) that recognizes an antigen with a M_r of 31,000 in carrot embryogenic cells but not in nonembryogenic cells and developed somatic embryos (7), though the function of the antigen in somatic embryogenesis is not known. To study the function of a protein, it is a very important step to know its localization. However, the monoclonal antibody (1D11) was not suitable for immunohistochemical analysis (7). We, therefore, need to prepare polyclonal antibodies against ECP31. In this report, we describe the purification of the antigen (ECP31) and its immunohistochemical localization in carrot cells with antiserum raised in rabbit against ECP31.

[1] This research was supported in part by Grants-in-Aid for Special Research on Priority Areas (Project No. 01660002, Cellular and Molecular Basis for Reproductive Processes in Plants) from the Ministry of Education, Science and Culture, Japan.

MATERIALS AND METHODS

Plant Materials and Cell Cultures

Embryogenic cell clusters of carrot, *Daucus carota* L. cv US-Harumakigosun, were obtained from 1-month-old nodular calluses which had been formed by culturing segments of 1-week-old hypocotyls on MS[2] agar medium (10) that contained 4.5×10^{-6} M 2,4-D. Nonembryogenic cells were obtained from the same plant material as described in Satoh *et al.* (12). Cultures of both embryogenic and nonembryogenic cells were maintained by transfer at intervals of 2 weeks to fresh MS liquid medium supplemented with 4.5×10^{-6} M 2,4-D. Somatic embryos were produced by the transfer of embryogenic cells to auxin-free MS medium, as described in Satoh *et al.* (12).

Carrot plants were grown from seeds in pots in a greenhouse.

Carrot crown galls and hairy roots were induced by the inoculation of axenic carrot seedlings with *Agrobacterium tumefacience* C58C1 that harbored pTiB6S3 (wild-type octopine Ti plasmid) (6) and with *Agrobacterium rhizogenes* strain A4 that harbored the Ri plasmid (pRiA4) (19), respectively, grown on YEB agar medium.

Purification of ECP31

During the entire procedure for purification of ECP31, the detection of ECP31 was performed by immunoblotting with the monoclonal antibody (1D11), as described before (7), and all steps in the purification procedure were carried out at 4°C. Embryogenic cells (200 g fresh weight) were harvested, 14 d after inoculation, by filtration on two sheets of Miracloth (Calbiochem, La Jolla, CA) and homogenized with a glass-Teflon homogenizer in 5 volumes of extraction buffer (200 mM Tris-HCl [pH 8.0], 1 mM diisopropyl fluorophosphate, and 1 mM EDTA). The homogenate was filtered through two sheets of Miracloth and cellular debris was removed by centrifugation at 8900*g* for 30 min.

The supernatant was mixed with sufficient $(NH_4)_2SO_4$ to give 40% saturation, and the resulting precipitate was collected by centrifugation at 8900*g* for 30 min. Protein pellets were dissolved in 50 mM Tris-HCl (pH 8.0) that contained 16% saturated $(NH_4)_2SO_4$ and the solution was centrifuged at 12,000*g* for 30 min.

[2] Abbreviations: MS, Murashige-Skoog; FPLC, fast-phase liquid chromatography; TFA, trifluoroacetic acid; TBS, Tris-buffered saline; PEMs, proembryogenic masses; LEA, late embryogenesis abundant.

The supernatant was loaded on a column (4.5 cm i.d. × 28 cm) of butyl-Toyopearl 650M (Tosoh, Tokyo, Japan) which had been equilibrated with 50 mM Tris-HCl (pH 8.0) that was 18% saturated with $(NH_4)_2SO_4$. The column was eluted with a linear gradient of $(NH_4)_2SO_4$, from 18 to 0% saturation, in 50 mM Tris-HCl (pH 8.0), at a flow rate of 3 mL/min. The proteins eluted by the 12 to 4% saturated solution of $(NH_4)_2SO_4$ were precipitated with a 40% saturated solution of $(NH_4)_2SO_4$ and collected by centrifugation at 12,000*g* for 30 min.

The pellet was dissolved in 50 mM Tris-HCl (pH 8.0) that contained $(NH_4)_2SO_4$ to 20% saturation and centrifuged at 12,000*g* for 30 min. The supernatant was loaded on a column (3.2 cm i.d. × 5 cm) of phenyl-Toyopearl 650M (Tosoh) equilibrated with 50 mM Tris-HCl (pH 8.0) that contained $(NH_4)_2SO_4$ to 20% saturation. The proteins were eluted with 50 mM Tris-HCl (pH 8.0) at a flow rate of 2 mL/min and precipitated in a 60% saturated solution of $(NH_4)_2SO_4$.

Protein pellets were suspended in 4 mL of 50 mM Tris-HCl (pH 8.0) and applied to a column (1.7 cm i.d. × 71.5 cm) of Sephacryl S-300 (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl (pH 8.0) that contained 50 mM NaCl and proteins were eluted with the same buffer at a flow rate of 2.5 mL/h. The fractions (15 mL) corresponding to a mol wt of approximately 120,000 were collected.

The collected fractions were applied to a column (2.5 cm i.d. × 10 cm) of DEAE-Toyopearl 650M (Tosoh) equilibrated with 50 mM Tris-HCl (pH 8.0) and eluted with 500 mM NaCl at a flow rate of 1 mL/min. The eluate was brought to 20% saturation with $(NH_4)_2SO_4$ and centrifuged at 12,000*g* for 30 min.

The supernatant was applied to a column of phenyl-Superose HR5/5 (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 8.0) that contained $(NH_4)_2SO_4$ to 20% solution using a FPLC system (Pharmacia) and the protein was eluted with a linear gradient of a 20 to 0% saturated solution of $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 8.0) at a flow rate of 0.5 mL/min. The fractions containing ECP31 were pooled and then desalted on PD-10 columns (Pharmacia).

The desalted fraction was loaded on a Mono Q HR5/5 column (Pharmacia), which had been equilibrated with 50 mM Tris-HCl (pH 8.0), and eluted at a flow rate of 1 mL/min with a linear gradient of 0 to 800 mM NaCl in 50 mM Tris-HCl (pH 8.0).

The eluate from Mono Q was applied to a column of Superose-6 (Pharmacia), which had been equilibrated with 50 mM Tris-HCl (pH 8.0) that contained 100 mM NaCl, and protein was eluted with the same buffer at a flow rate of 0.5 mL/min. ECP31-rich fractions were pooled and stored at −20°C.

Gel Electrophoresis

SDS-PAGE (12% acrylamide) was performed according to the method of Laemmli (8), and 40% urea (w/v) was added in separation gel in the case of urea/SDS-PAGE.

The plant material was homogenized with an equal volume of 60 mM Tris-HCl buffer (pH 6.8) that contained 2% SDS, 0.02% bromphenol blue, 10% glycerol, and 5% 2-mercaptoethanol, boiled for 5 min in a water bath, and centrifuged at 10,000*g* for 5 min. The resulting supernatant was applied to the gels.

Purity Check of ECP31 and Production of Antiserum

An ECP31-rich fraction (200 μg protein) was subjected to SDS-PAGE and the proteins on the gel were stained with Coomassie brilliant blue. The gel band corresponding to ECP31 was cut out, crushed, dialyzed against PBS, and subcutaneously injected with Freund's complete adjuvant into female New Zealand rabbits. Injections were repeated at 14 d; after 50 d the serum was collected and used for further experiments.

For purity check of the protein, the ECP31 gel band was cut out, the protein was eluted etectophoretically, dialyzed against 20 mM Tris-HCl (pH 8.0) with 0.1% SDS, lyophilized, dissolved in 0.1% TFA in water/acetonitrile, 95:5(v/v), and was applied to a C8 column (COSMOSIL packed column 5C8-300, 4.6 mm i.d. × 150 mm, nacalai tesque, Tokyo, Japan) equilibrated with 0.1% TFA in water and proteins were eluted at a flow rate of 1 mL/min with a linear gradient of 0 to 100% acetonitrile with 0.1% TFA.

Immunoblotting

Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters in a solution of 25 mM Tris, 192 mM glycine, and 20% methanol at 30 V for 1 h at 4°C. This procedure was followed by staining with 0.1% amido black 10B dissolved in a solution of 45% methanol and 20% acetic acid in water. For immunodetection, the nitrocellulose filter was agitated in 10% newborn calf serum in PBS (NPBS) for 1 h at room temperature, and then in 0.2% (v/v) ECP31-specific antiserum in NPBS for 1 h. The blot was washed with 0.2% Tween 20 in PBS, and then agitated in a 0.02% solution (w/v) of horseradish peroxidase-conjugated antibodies against rabbit IgG raised in goat (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in NPBS for 1 h at room temperature. The blot was visualized by placing it in PBS that contained 0.03% 3,3-diaminobenzidine and 0.003% H_2O_2.

Tissue Fixation and Cryomicrotomy

The embryogenic cell clusters, somatic embryos, and dry seeds were immersed in periodate-lysine-paraformaldehyde solution (9), aspirated for 5 min and then allowed to stand at 4°C for 12 h. After standing in PBS that contained 0.1 M sucrose and 4% paraformaldehyde at 4°C for 6 h, they were immersed in PBS that contained 1.0 M sucrose and 4% paraformaldehyde at 4°C for 24 h, embedded with O.C.T. compound (Miles Scientific, Naperville, IL) and frozen at −25°C. Sections (15–20 μm) were made at −25°C on a Coldtome CM-41 (Sakura Seiki Co., Tokyo), mounted on cover glasses, which had been coated with a solution of 1 mg/mL poly-L-lysine, and then immersed immediately in methanol.

Immunohistochemistry

The sections were immersed in methanol that contained 0.3% H_2O_2 for 30 min to inactivate endogenous peroxidases

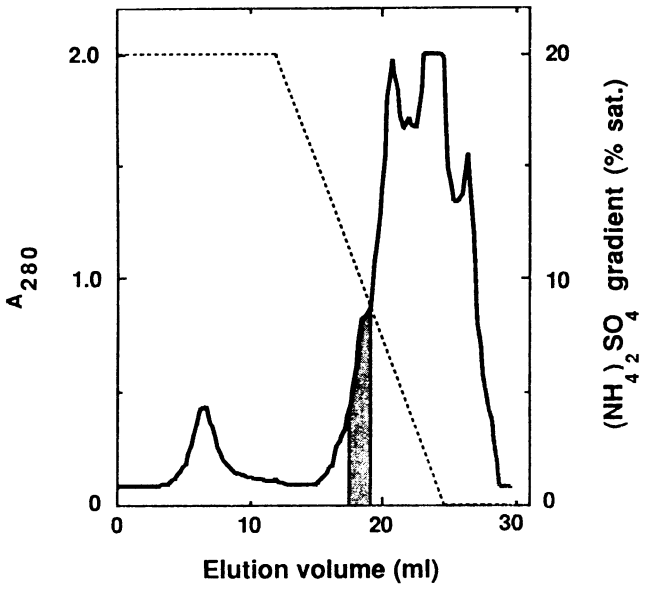

Figure 1. Hydrophobic chromatography on phenyl-Superose of ECP31 extracted from carrot embryogenic cell clusters. The eluate from DEAE-Toyopearl (see "Materials and Methods") was applied to the column and eluted with a gradient of a solution that was 20 to 0% saturated with $(NH_4)_2SO_4$. The shaded area represents the ECP31-containing fractions used for further purification.

(15), and then in PBS that contained 4% paraformaldehyde for 10 min. They were then rinsed sequentially for 10 min with PBS, with two changes of TBS for 20 min, and then with 10% newborn calf serum in TBS (NTBS) for 30 min. The ECP31-specific antiserum (0.2% [v/v]) in NTBS was overlaid on the sections and they were then incubated for 3 h in a humid chamber. The preimmune serum was used as a negative control. After rinsing for 30 min with three changes of TBS that contained 0.1% Tween 20 (TTBS) and then with TBS alone, sections were overlaid with 0.02% peroxidase-conjugated antiserum against rabbit IgG raised in goat (Jackson) in NTBS and incubated for 1 h in a humid chamber. The sections were sequentially rinsed for 40 min with four changes each of TTBS and TBS, and then they were immersed in PBS that contained 0.03% 3,3-diaminobenzidine and 0.003% H_2O_2 for 5 to 10 min. After the color had developed, the sections were rinsed for 30 min with two changes of distilled water and mounted with 50% glycerol that contained 7% gelatin. Photographs were taken by using a light microscopy, Vanox model AHBT (Olympus, Tokyo, Japan). Image scanning was performed with ARGUS system (Hamamatu-photonics, Hamamatu, Japan) that connected to Nikon OPTIphoto (Nikon, Tokyo, Japan) equipped with 490 nm band pass filter (Nihon-shinkuu-kougaku., Tokyo, Japan).

RESULTS

Purification of ECP31

As described in "Materials and Methods," the precipitate obtained at 40% saturation with $(NH_4)_2SO_4$ from an extract of carrot embryogenic cells was sequentially applied to columns of butyl-Toyopearl, phenyl-Toyopearl, Sephacryl S-300, and DEAE-Toyopearl. The fraction containing ECP31 was then sequentially applied to a column of phenyl-Superose (Fig. 1) and Mono Q (Fig. 2). ECP31 was eluted at the shoulder of the peak of absorbance at 280 nm from both columns. Then the ECP31-containing fraction was finally applied to a colunn of Superose 6 and the M_r of ECP31 was estimated to be about 120,000 (Fig. 3). When the final eluate from Superose 6 was subjected to SDS-PAGE, electrophoretically transferred to a nitrocellulose filter, and stained with amido black, a band at 31 kD corresponding to ECP31 and other contaminating bands were observed (Fig. 4). The 31-kD gel band was composed of one peptide demonstrated by the profile of a reverse phase column (Fig. 5), and was used for production of antiserum.

Detection of ECP31 by Immunoblotting

The distribution of ECP31 among various cultured cells was examined by immunoblotting. As shown in Figure 6, a large amount of ECP31 was detected in embryogenic cell clusters and much less was found in somatic embryos. None was detected in nonembryogenic cells, crown galls, and hairy roots. An additional band corresponding to a protein with an apparent M_r of 60,000 was also detected with ECP31-specific antiserum, though it was not observed with using urea/SDS-PAGE in place of SDS-PAGE (data not shown).

Figure 7 shows the time course of the appearance of ECP31 after initiation of cultures of hypocotyl segments on MS agar medium supplemented with 2,4-D. ECP31 began to accumulate on d 33 after the initiation of the culture, when the formation of callus was just visible on the segments.

The distribution of ECP31 in the entire carrot plant was

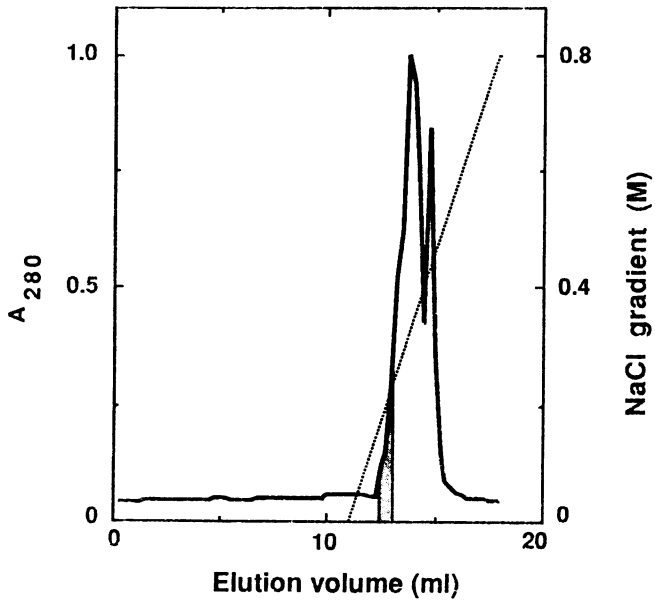

Figure 2. Ion-exchange chromatography on Mono Q of ECP31. The eluate from phenyl-Superose (Fig. 1) was applied to the column and eluted with a gradient of 0 to 0.8 M NaCl. The shaded area represents the ECP31-containing fractions used for further purification.

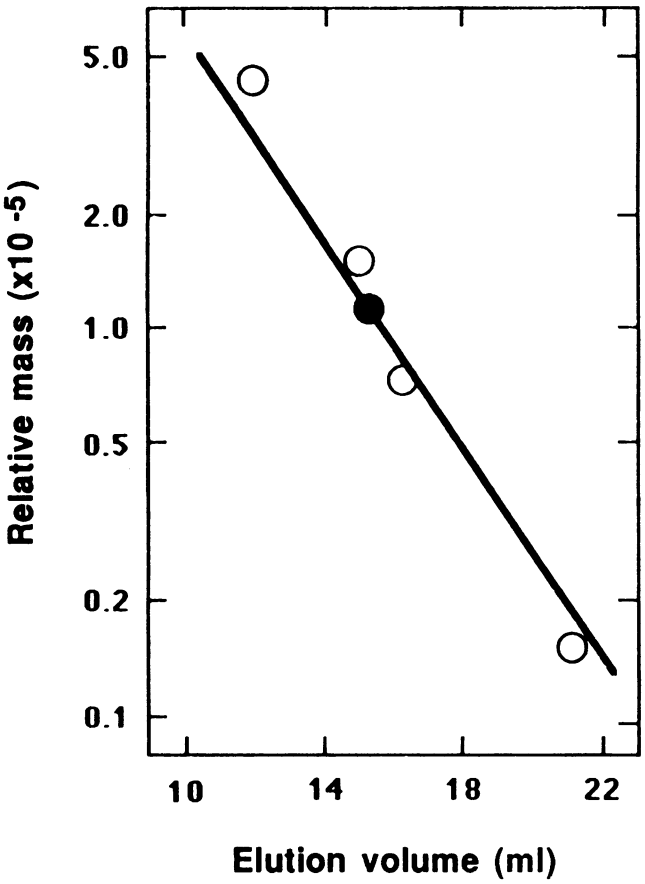

Figure 3. Gel filtration of ECP31 on a column of Superose 6. The eluate from the Mono Q column (Fig. 2) was applied to the column. The closed circle represents the elution of ECP31 and the open circles represent the elution of the following marker proteins: ferritin (M_r, 450,000), aldolase (M_r, 158,000), BSA (M_r, 68,000), and ribonuclease A (M_r, 13,700).

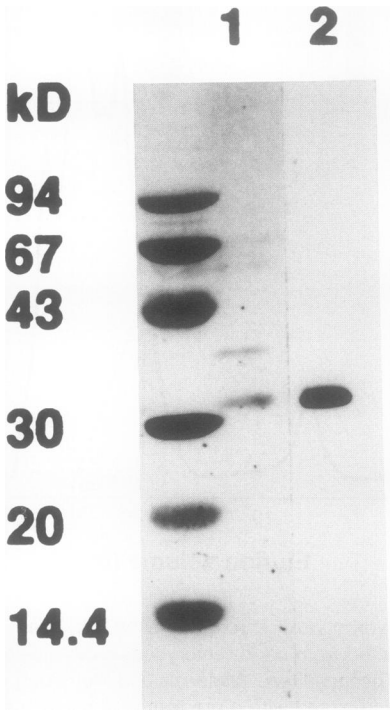

Figure 4. SDS-PAGE and immunoblotting of purified ECP31. The eluate from the column of Superose 6 (Fig. 3) was subjected to SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane, and stained either with amido black (lane 1) or immunologically with a monoclonal antibody (1D11); (lane 2).

then examined. ECP31 was not found in any parts of the carrot plant, such as flowers, leaves, petioles, cotyledons, shoot meristems, and tap roots (data not shown), and was only found in dry fruits (seeds) (Fig. 6, lane 6). ECP31 began to appear in fruits on d 28 after flowering and disappeared on the fourth day after sowing when equal numbers of fruits or seedlings were used in Western blot analysis (data not shown). These results were the same as those obtained with monoclonal antibody 1D11 (7), supporting the idea that ECP31 is identical to the antigen recognized by 1D11.

Immunohistochemical Localization of ECP31

Because immunoblot analysis indicated that ECP31 was present in embryogenic cell clusters, somatic embryos, and fruits, we examined its localization by indirect immunohistochemistry. Red-brownish deposits were abundant in the peripheral cells of embryogenic cell clusters cultured in the presence of 2,4-D (Fig. 8B), the site at which the PEMs are known to originate (3, 4). Using an image-scanning technique, we estimated the signal for ECP31 in peripheral cells to be about seven- to eightfold stronger than that in the central cells

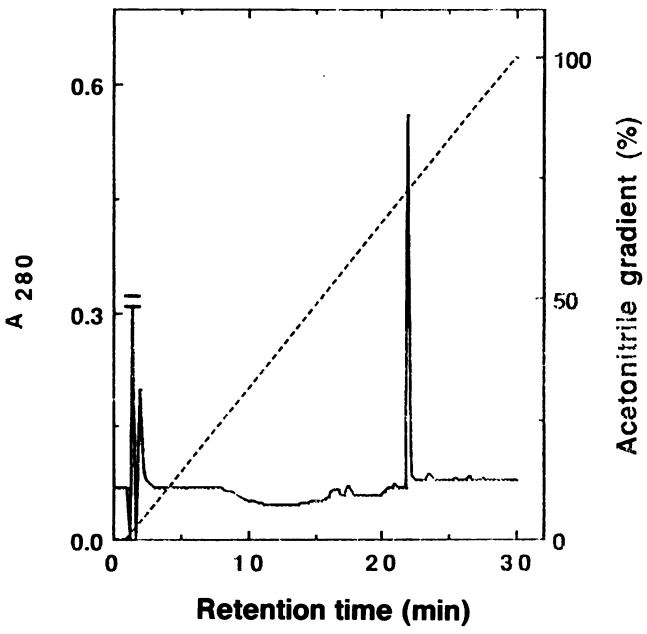

Figure 5. Purity check of ECP31 with reverse phase chromatography. The eluate of ECP31 gel band was applied to the C8 column and eluted with a gradient of 0 to 100% acetonitrile. The major peak corresponded to ECP31 and several minor peaks corresponded to ghost peaks which were seen in the case without sample.

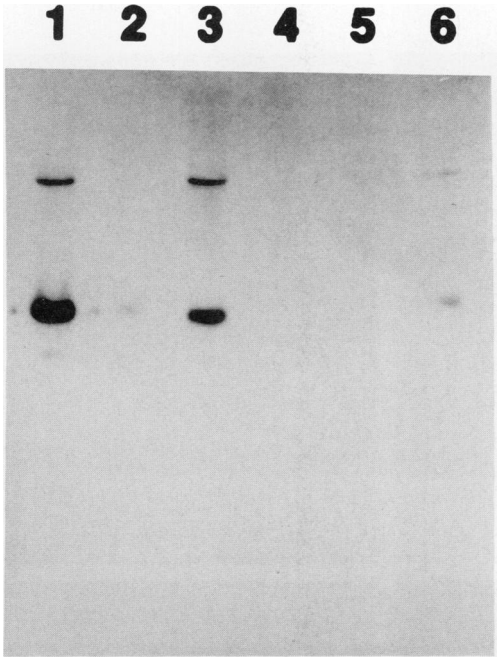

Figure 6. Detection of ECP31 in carrot cell cultures. Extracts of embryogenic cell clusters (lane 1), nonembryogenic cells (lane 2), a mixture of heart- and torpedo-shaped somatic embryos (lane 3), crown galls (lane 4), hairy roots (lane 5), and dry seeds (lane 6) were subjected to SDS-PAGE and immunoblotting using rabbit antiserum against ECP31. Protein equivalent to 4 mg of cells was loaded in each well.

of the embryogenic cell clusters (Fig. 8C). In the course of somatic embryogenesis in the absence of 2,4-D, ECP31 was found in the middle region of globular embryos (Fig. 8D) at much lower levels than in embryogenic cells, but it was not found in embryos at the torpedo stage (Fig. 8E). By contrast, in malformed embryos which originated from older embryogenic cell clusters after repeated subculture, ECP31 was detected in what was possibly provascular tissue (Fig. 8F). In dry carrot fruits, ECP31 was detected only in entire zygotic embryos and was not found in endosperm (Fig. 8, H and I).

DISCUSSION

ECP31, a protein with M_r of 31,000 from carrot embryogenic cells, was purified by sequential steps of column chromatography and following SDS-PAGE. The purified ECP31 in the gel was used for immunization after the evaluation of the purity by a reverse phase chromatography (Fig. 5).

The antiserum sometimes showed a 60 kD band in addition to a 31 kD band (Fig. 6). This additional band probably corresponded to a dimer, because it disappeared in the presence of urea in separation gel (data not shown). The relative mass of the native protein was estimated to be about 120,000 by gel filtration (Fig. 3). These results suggest that ECP31 is present as oligomers in the cell.

The amount of ECP31 accumulated in various types of cultured cell (Fig. 6) was as follows: embryogenic cells $\gg$ somatic embryos $\gg$ nonembryogenic cells $>$ crown gall and hairy root, and the amount seems to be correlated with the competence of the cells to form somatic embryos. This view is also supported by the coincidence in timing of the accumulation of ECP31 and of the appearance of embryogenic callus on hypocotyl segments cultured on MS agar medium with 2,4-D (Fig. 7), as well as by immunohistochemical data (Fig. 8) demonstrating that ECP31 is preferentially localized in peripheral cells of embryogenic cell clusters, where PEMs were originated.

ECP31 was found in both zygotic and some types of somatic embryos (Figs. 6, 7). The reasons for the differences in the distribution of ECP31 among various types of embryo, its absence in torpedo-shaped somatic embryos, its presence in the provascular tissues of malformed embryos, and its presence in entire zygotic embryos, are not known yet. Provascular tissue is known to be stained by active staining for esterases, demonstrating the presence of an early histochemical marker of commitment to stele differentiation (1). However, no esterase activity was detected in the ECP31-rich fraction (data not shown). One may explain the accumulation of the protein in zygotic and malformed somatic embryo as follows: both embryos would have higher embryogenic tendency; for instance, both embryos can produce embryogenic callus readily or even produce somatic embryos directly.

Recently, Thomas and Wilde, as well as de Vries *et al.*, demonstrated that mRNA that corresponded to a cDNA clone, Dc3, expressed at high levels in somatic embryos and PEMs but not in nonembryogenic cells, that this expression was not related to the presence of 2,4-D in the medium (2, 17, 18, 20). This situation was similar to that of ECP31, as described above, although the accumulation of ECP31 in embryogenic cell clusters was much higher than that in so-

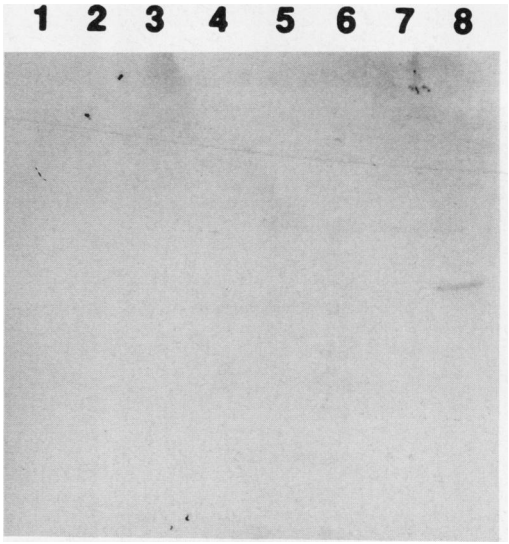

Figure 7. Time course of accumulation of ECP31 in cultured segments of hypocotyl. Hypocotyl segments were axenically cultured on MS medium with 4.5×10^{-6} M 2,4-D for 0 d (initial) (lane 1), 1 d (lane 2), 4 d (lane 3), 8 d (lane 4), 11 d (lane 5), 17 d (lane 6), 23 d (lane 7), and 33 d (lane 8). Protein equivalent to 4 mg of cells was loaded in each well and subjected to SDS-PAGE and immunoblotting with ECP31-specific antiserum.

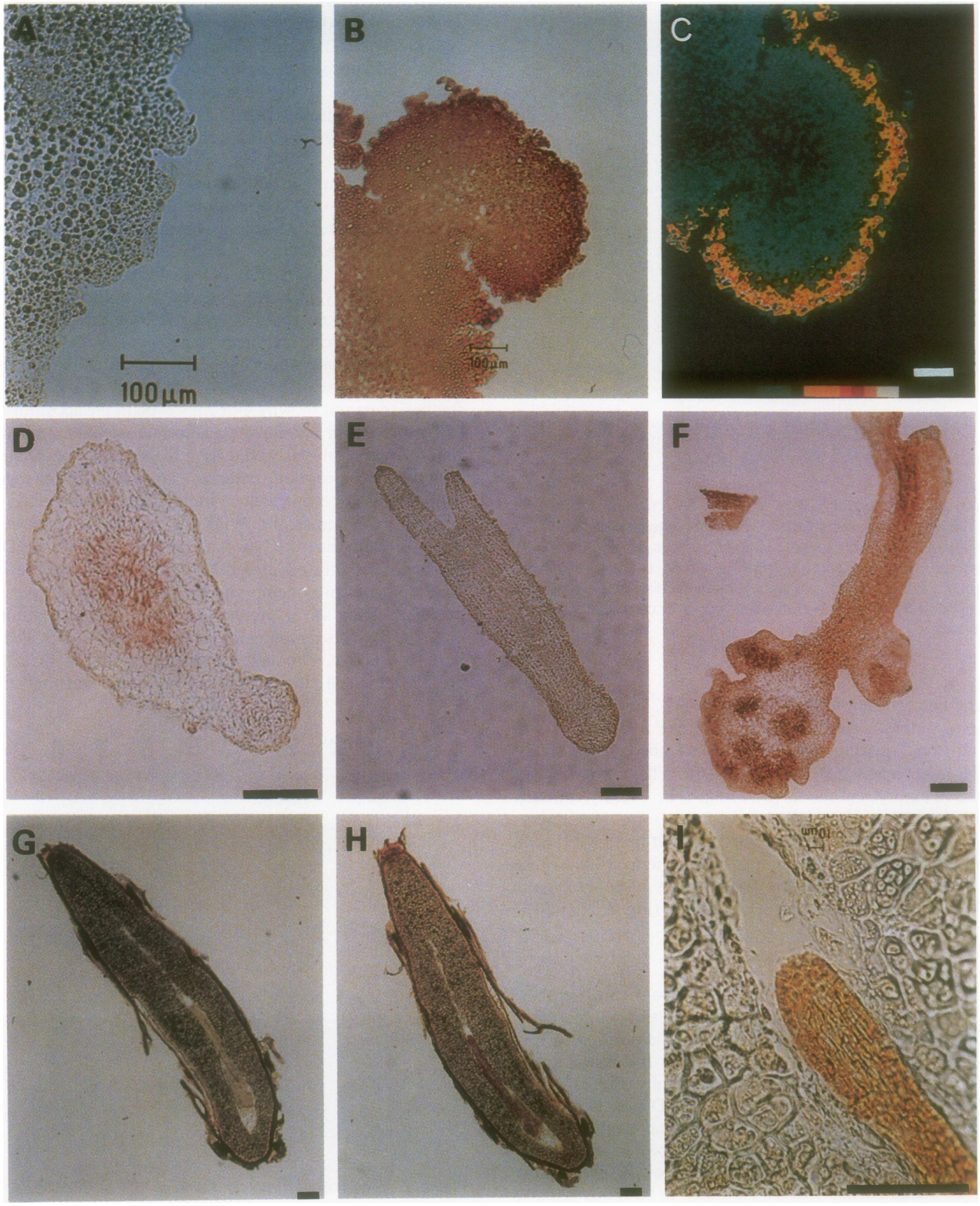

Figure 8. Immunohistochemical detection of ECP31 in cultured cells and dry carrot fruits. Sections from an embryogenic cell cluster (A, B, C), a globular somatic embryo (D), a torpedo-shaped somatic embryo (E), a malformed somatic embryo (F), and a dry fruit (G, H, I) were treated with ECP31-specific antiserum (B, C, D, E, F, H, I) or preimmune serum (A, G). Bar = 100 μm.

matic embryos. Indeed, there are no amino acid sequence homologies between ECP31 and the product of Dc3 (preliminary results). Dc3 was reported to encode a protein that was homological to a member of LEA proteins (3). LEA proteins were found to accumulate the late embryogenesis stages in many higher plants and may be universal in occurrence in plant seeds (3). The main function of the proteins were proposed to protect plant cells to desiccation or water stress (3, 13). Judging from the accumulation pattern of ECP31 in fruits (7), we may classify this protein as a LEA protein. However, our results obtained in culture system imply the possibility that ECP31 performs some role in the induction and maintenance of embryogenic competence. Further characterization of ECP31 is required to define its importance in somatic embryogenesis.

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