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Immunocytochemical Localization of Vicilin in Endosperm Cells of *Panax ginseng* C.A. Meyer

Lee, Chang-Seop, Seong Cheol Yu and Woo Kap Kim
(Department of Biology, Korea University, Seoul)

인삼 (*Panax ginseng* C.A. Meyer) 배유세포내 Vicilin의 면역세포화학적 분포

李 昌 燮·劉 成 哲·金 宇 甲
(高麗大學校 生物學科)

ABSTRACT

The endosperm protein, vicilin, of ginseng (*Panax ginseng* C.A. Meyer) was purified by ammonium sulfate precipitaion, gel permeation and ion exchange column chromatography. Vicilin is a glycoprotein composed of 2 subunits with molecular masses of 55,000 (large subunit) and 44,000 (small subunit).

The anti-vicilin antibody was raised in rabbit, and purified by DEAE Affi-Gel Blue affinity chromatography. The endosperm cells of the seed were reacted with this anti-vicilin antibody and colloidal gold conjugated secondary antibody. Gold particles were labelled on the elaborating granules of Golgi complex, electron-dense granules and protein bodies in the endosperm cells.

These results indicated that the vicilin, which was synthesized in rough endoplasmic reticulum and transported to Golgi, was elaborated in saccules of the Golgi and then transported into protein bodies by electron-dense granules.

INTRODUCTION

The storage proteins in mature seeds are found mainly in specialized organelles, the protein bodies. The economic importance of these storage proteins and the physiological role of these organelles in endosperm cells of seeds lead to publication of numerous papers concerned with these proteins in the seeds of cereals and legumes (Krishnan *et al.*, 1986; Bollini and Chrispeels, 1978, 1979; Baumgartner *et al.*, 1980; Craig and Miller, 1984; Craig and Goodchild, 1984a, 1984b).

On the basis of their solubility, the various proteins of seeds are classified as four categories of globulins, albumins, prolamines, and glutelins. Vicilin, 6.9 S protein is one of the globulin category. This protein consists of 3 or 4 non-identical polypeptides which are glycosylated (Bollini and Chrispeels, 1979). The vicilin is biosynthesized in endosperm cells during seed development and maturation. Although the physiochemical nature of vicilin of legumes and their patterns of synthesis during seed development have been studies, there is very little information relating to that of ginseng.

Therefore, using immunocytochemistry, we carried out this study with *Panax ginseng* to elucidate the biosynthesis, the accumulation pathway, and the storage of vicilin.

MATERIALS AND METHODS

Plants. Seeds of 4 year old ginseng (*Panax ginseng* C.A. Meyer) were obtained from Kang Wha cultivate.

Protein extraction. Proteins were extracted from dry seeds by the method of Bollini and Chrispeels (1978). The seeds were homogenized in cold 20 mM sodium boratc, pH 9.0, in a prechilled motar using 8 ml of medium

per gram of tissue. The homogenate was centrifuged at 10,000×g for 10 min and the residue was reextracted with 4 ml of medium per gram of tissue. The combined extracts were applied to ammonium sulfate precipitation.

Ammonium sulfate precipitation. Ammonium sulfate was added to the extract with constant stirring to give 40% saturation. After 30 min the precipitate was removed by centrifugation at 5,000×g for 10 min and discarded. The supernatant was adjusted to 70% saturation and after 30 min stirring the precipitate was collected by centrifugation. It was redisolved in minimum volume of 25 mM Tris-HCl, pH 7.5.

Further purification was performed by use of Sephadex G-50 gel permeation column chromatography.

Sephadex G-50 gel permeation column chromatography. The protein extract was loaded on a Sephadex G-50 column (2 cm I.D.×50 cm length) equilibrated with 25 mM Tris-HCl, pH 7.5 (buffer A). The column was eluted with buffer A and the cloudy fractions containing the protein in the excluded volume were combined and constitute the protein extract.

DEAE cellulose ion exchange column chromatography. The protein extract was loaded on a DEAE cellulose (DEAE-DE 52) column equilibrated with buffer A, and the column was eluted with 200 ml of a 0 to 400 mM NaCl linear gradient in buffer A. The fractions were analyzed by absorbance at 280 nm. After the fractions in each peak were pooled and dialyzed against buffer A, each fraction was loaded again on a DEAE cellulose column and eluted with 0, 150, and 300 mM NaCl step gradient in buffer A. The fractions in each peak were pooled, dialyzed again and concentrated with centriprep-30 concentrator (Amicon).

Polyacrylamide gel electrophoresis (PAGE). The proteins of each fraction were analyzed by native-PAGE (7% gels) and SDS-PAGE (10% gels) according to Laemmli (1970).

Antibodies and Western blotting. Antibodies to the vicilin were raised in rabbits according to Bollini and Chrispeels (1979). The immunoglobulin G (IgG) of the sera was purified by DEAE Affi-Gel Blue affinity chromatography (Bio-Rad).

Proteins were transferred from SDS gels onto nitrocellulose filters with the glycine electrode buffer of Towbin *et al.* (1979) in a TE22 mini apparatus (Hoefer Scientific Instruments).

Western blotting of electrophoresed vicilins was carried out using anti-vicilin antibodies (Krishnan *et al.*, 1986). Immune complexes were detected using peroxidase conjugated goat anti-rabbit IgG and diaminobenzidine as a substrate.

Preparation for electron microscopy. Endosperm cells of the seed were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.8, for 2 h at 4℃, washed in buffer, and then dehydrated through a graded ethanol series and embedded in either Epon-Araldite or Spurr's resin (Spurr, 1969).

Sections were collected on collodion-coated copper grids, counterstained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and viewed in a JOEL JEM 100 CX-II electron microscope at 80 kV.

Immunocytochmical labelling. Sections of glutaraldehyde-fixed tissue were collected on collodion-coated nickel grids, and floated for 20 min on 20 μl of PBST-ween (15 mM, pH 7.2, 0.2% Tween-20) containing 1% bovine serum albumin. The grids were incubated for 30 min on 20 μl of anti-vicilin antibodies diluted 1 : 100 with PBST-BSA. Non-specifically binding antibodies were removed by washing the sections 6 times on a drop of PBST. The sections were then incubated for 20 min in goat anti-rabbit IgG conjugated with 30 nm gold particles (GAR-G30; Janssen pharmaceuticals) diluted 1 : 20 in PBST-BSA. After washing in PBST and distilled water, sections were stained with 2% aqueous uranyl acetate. Some were also stained with lead citrate for 2 min.

For control treatment, the grids were incubated with diluted preimmune serum under the same conditions. Stained grids were viewed in a JOEL JEM 100 CX-II electron microscope at 80 kV.

RESULTS AND DISCUSSION

Vicilin purification and PAGE. The protein extract of ginseng endosperm cells purified through ammonium sulfate precipitation and sephadex gel permeation chromatography, was loaded on a DEAE-cellulose ion exchange chromatography (Fig. 1).

Peak B represented the salt soluble globulin fraction. The protein fractions were separated by native-PAGE and stained with Coomassie blue and periodic acid-Shiff's reagent (PAS) (Fig. 2).

The salt soluble globulin was viewed as a single band, and stained with PAS, which indicated that it was a glycoprotein. Analysis of the polypeptides by SDS-PAGE showed that the salt soluble globulin contained 2 kinds of polypeptides with molecular masses of 55,000 (lare subunit) and 44,000 (small subunit) (Fig. 3). But the amount of the large subunit was more than that of the

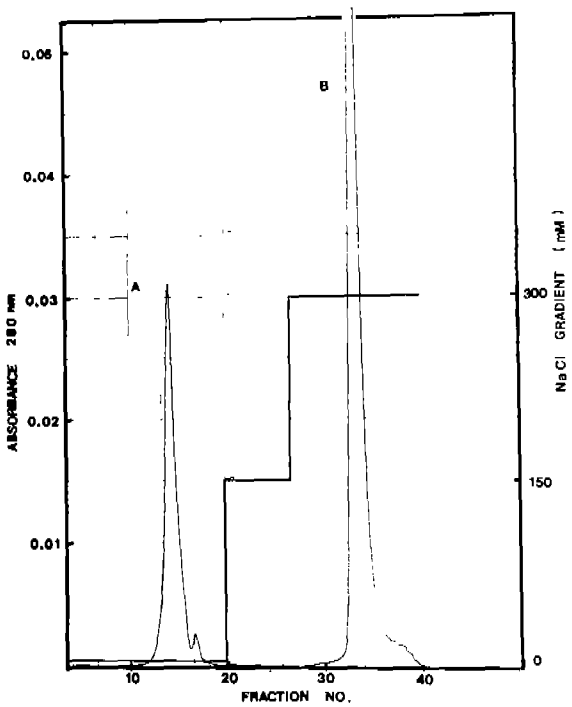


Fig. 1. Fractionation of ginseng endosperm proteins on DEAE cellulose. The column was eluted with a 0 to 0.3 M NaCl step gradient. Peak A represents the unabsorbed proteins, while peak B contains the salt-soluble globulin.

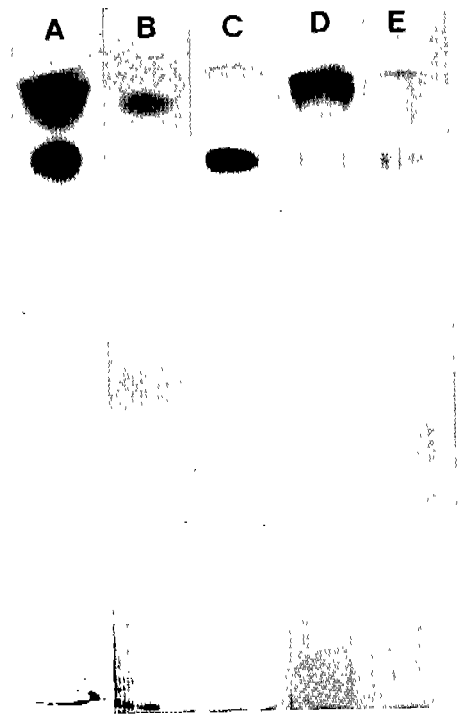


Fig. 2. Analysis of proteins by native-PAGE. A-C, CBB staining gel; D-E, PAS staining gel. A, D: Fraction of sephadex column. B: Peak A fraction of DEAE-cellulose column. C, E: Purified vicilin (Peak B fraction of DEAE-cellulose column).

small subunit. The salt soluble globulin was referred to as vicilin (Bollini and Chrispeels, 1978).

From these results, vicilin of ginseng endosperm proteins was a glycoprotein composed of 2 subunits with molecular masses of 55,000 and 44,000. In legumes, however, vicilin contained 3 polypeptides with molecular masses of 52,000, 49,000 and 46,000 (Bollini and Chrispeels, 1978; McLeester *et al.*, 1973).

Antibodies and Western blotting. The anti-vicilin antibody was raised in rabbit and the immunoglobulin G was separated from the antiserum (Fig. 5).

A Western blot of vicilin separated by SDS-PAGE resulted in cross-reactivity with anti-vicilin antibodies (Fig. 4).

Immunocytochemical localization of vicilin

Vicilin synthesis. The goat anti-rabbit conjugated gold particles were labelled on protein bodies in vacuoles and on saccule of Golgi complex (Figs. 6-8A). Protein accumulation in vacuoles occurred near rER and Golgi complex (Fig. 6). In developing seeds, reserve protein can be detected microscopically first as clumped deposits

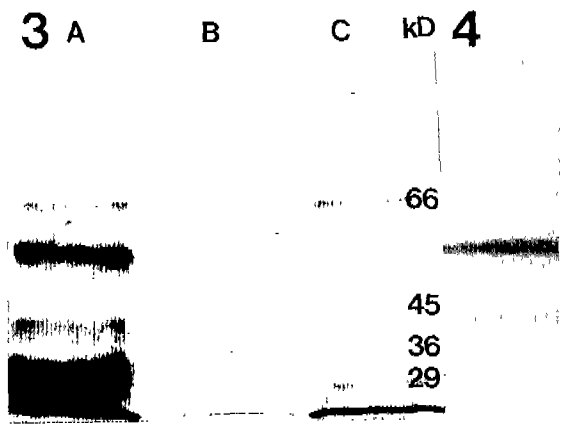


Fig. 3. Separation of polypeptides by SDS-PAGE. A: Fraction of sephadex column. B: Purified vicilin. C: Standard mixture.

Fig. 4. Western transblot probed with antibodies produced from vicilin.

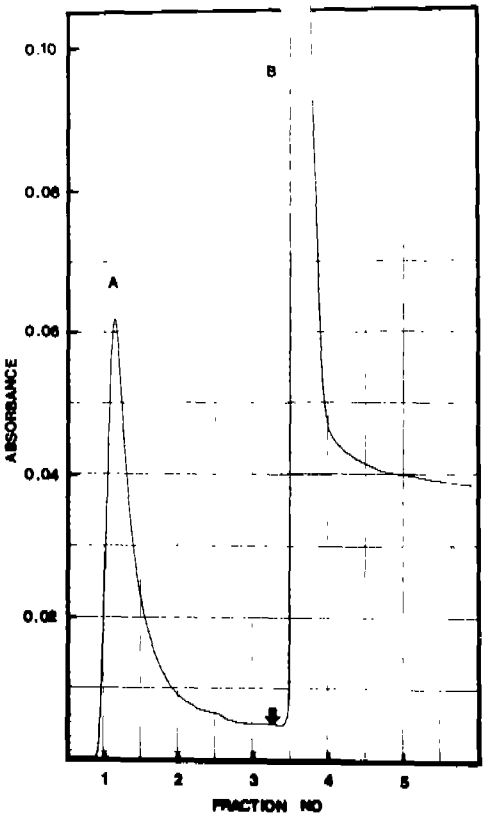


Fig. 5. Affinity chromatograph of antiserum on a DEAE Affi-Gel Blue gel. Peak A represents IgG. Peak B shows the remaining serum proteins. The arrow indicates the point of buffer exchange.

at the periphery of large vacuoles (Craig and Millerd, 1980) which fragment give rise to the protein bodies as development processes (Craig and Miller, 1980).

Small protein bodies were newly synthesized in rER, transported to large protein bodies, and then fused together (Fig. 7). Bollini and Chrispeels (1979) and Baumgartner *et al.* (1980) observed that rER was the site of vicilin synthesis in developing bean cotyledons. Vicilin was elaborated in the Golgi complex (Fig. 8A). According to Craig and Miller (1984), vicilin was detected within the Golgi cisternae and vicilin was shown within the Golgi vesicles (Craig and Goodchild, 1984a). Vicilin was reacted with PAS (Fig. 2) and elaborated vicilin in the Golgi complex was labelled with gold particles (Fig. 8A), which indicated that vicilin was glycosylated. Craig and Miller (1980) suggested that at least the glycosylated polypeptides of vicilin would pass through the Golgi complex. Therefore, from all these results, vicilin is synthesized in rER, glycosylated in the Golgi complex, and passes through the Golgi complex.

Vicilin transport. Vicilin was also associated with the electron dense granules ranging in size from 0.1 to 0.35 μm. The electron dense granules were distributed over the cytoplasm and also labelled with gold particles (Figs. 8 and 9), viewed in enlarged micrograph upper right-hand corner of Fig. 9. The electron dense granule containing elaborated vicilin was formed at the Golgi complex (Fig. 8A), and transported toward the protein bodies (Fig. 8B-D). The transported electron dense granules were finally incorporated into the protein body (Fig. 8D). In the legume seed, vicilin was associated with electron dense Golgi vesicles which were suggested to be a vehicle by which newly synthesized protein was relocated into the vacuoles (Craig and Goodchild, 1984a).

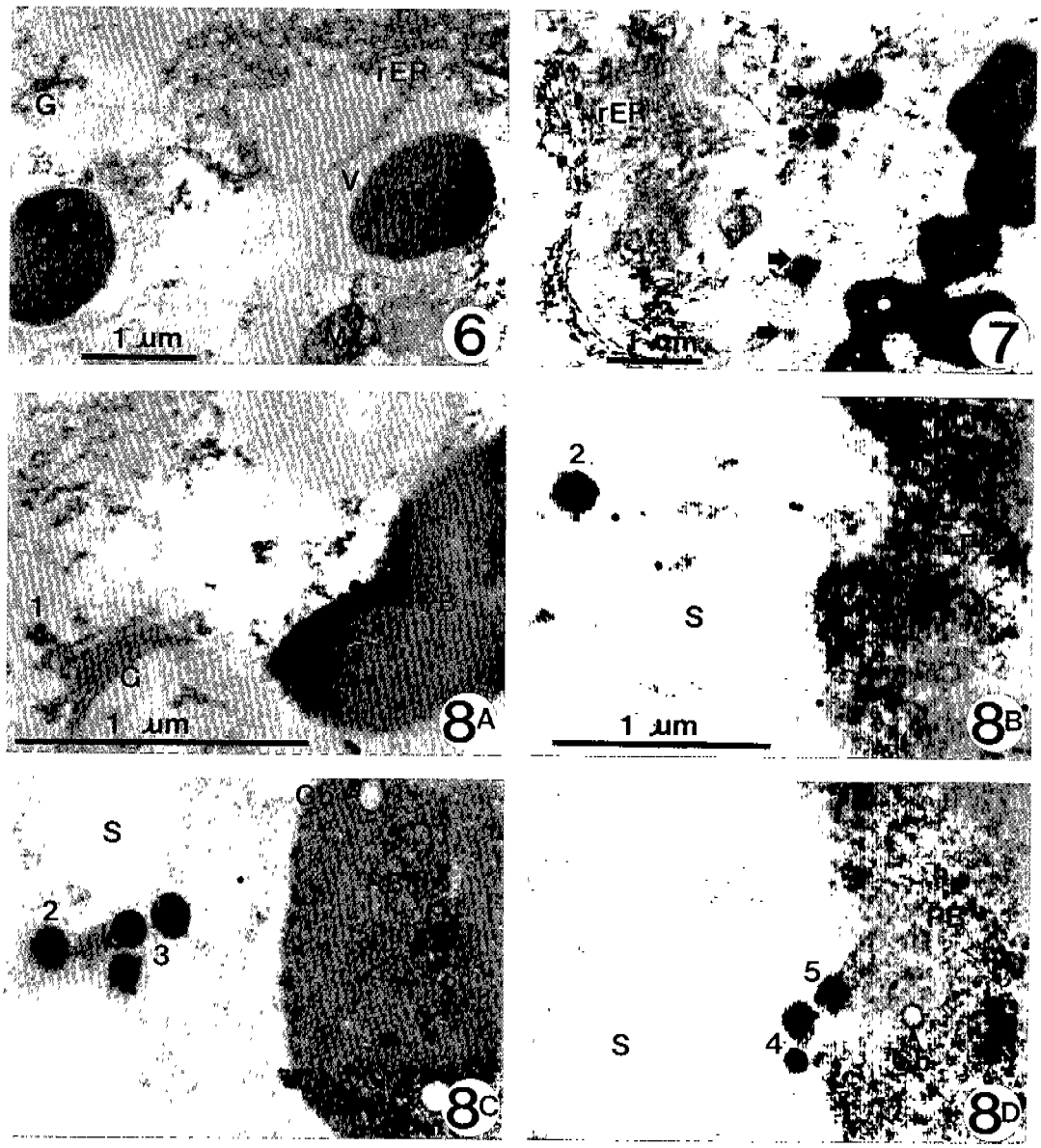
Vicilin preservation. The protein bodies distributed over the cytoplasm were labelled uniformly with gold particles (Figs. 9-11). Electron dense granules were observed and also labelled with gold particles (viewed in upper right-hand corner of Fig. 9). Very few gold particles were observed in the cytoplasm. Therefore, vicilin synthesized in rER was preserved in protein bodies. These results coincided with the works of Baumgartner *et al.* (1980) and Craig and Goodchild (1984a) who postulated that newly synthesized vicilin was initially sequestered within the ER and then transported to the protein bodies. Embedded with Epon-Araldite, fine structural preservation was improved (Fig. 9), whereas the amount of gold bound to the protein bodies was decreased. When the endosperm cell of Fig. 10 was embedded with Spurr's resin, the fine structural preservation was decreased, whereas the amount of gold labelling was increased.

Irregularly shaped protein bodies were also observed in Figs. 11 and 12. Gold particles were also labelled on these irregularly shaped protein bodies. As the endosperm cells were maturated, these irregularly shaped protein bodies were aggregated together to form the large spherical protein bodies.

Control experiments were carried out by treating the sections with preimmune serum followed by gold-conjugated goat anti-rabbit IgG. Very few gold particles were visible on the sections treated in this manner (data not shown), indicating that preimmune rabbit IgG did not bind nonspecifically to the sections.

적 요

인삼(*Panax ginseng* C.A. Meyer) 종자단백질인 vicilin을



Figs. 6-8. Immunoelectron micrographs of ginseng endosperm cells. Fig. 6. Vicilin synthesized in rER was deposited in vacuoles (V) to form protein bodies (PB). Protein accumulation into vacuoles occurred near rER and Golgi complex (G). M, mitochondria. Fig. 7. Small protein bodies (arrows) newly synthesized in rER were transported to large protein bodies (PB). Fig. 8. Transportation of electron dense granules from Golgi (G) to large protein bodies (PB). A, Elaboration of vicilin in Golgi complex and formation of electron dense granule containing elaborated vicilin. B, Transportation of electron dense granules toward the protein bodies. C, The granules were transported near protein bodies. D, Incorporation of transported granules into the protein bodies. All the granules were labelled by gold particles. The numbering indicates the transportation process of electron dense granules from Golgi to protein bodies. Gb, globoid; S, spherosome.

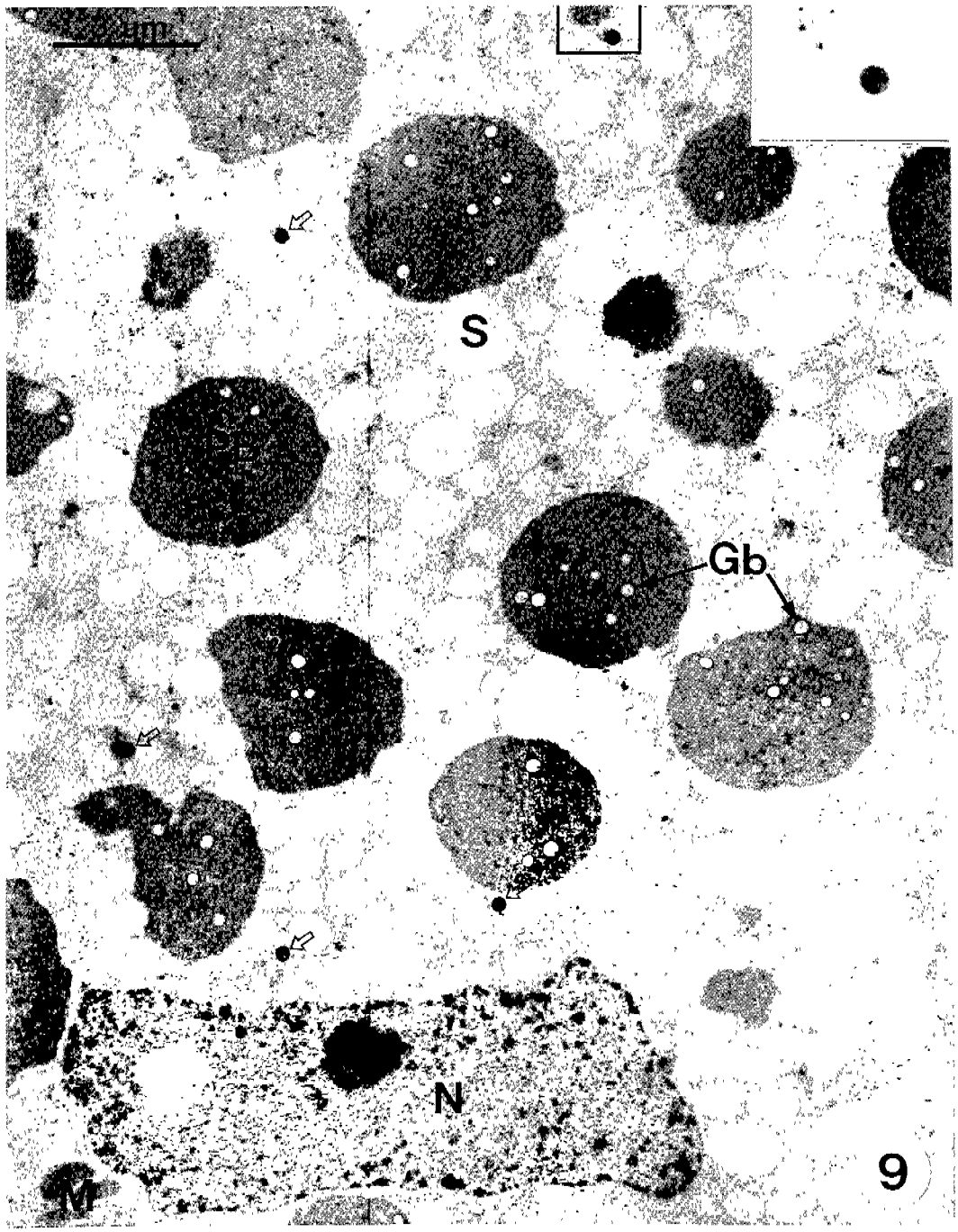
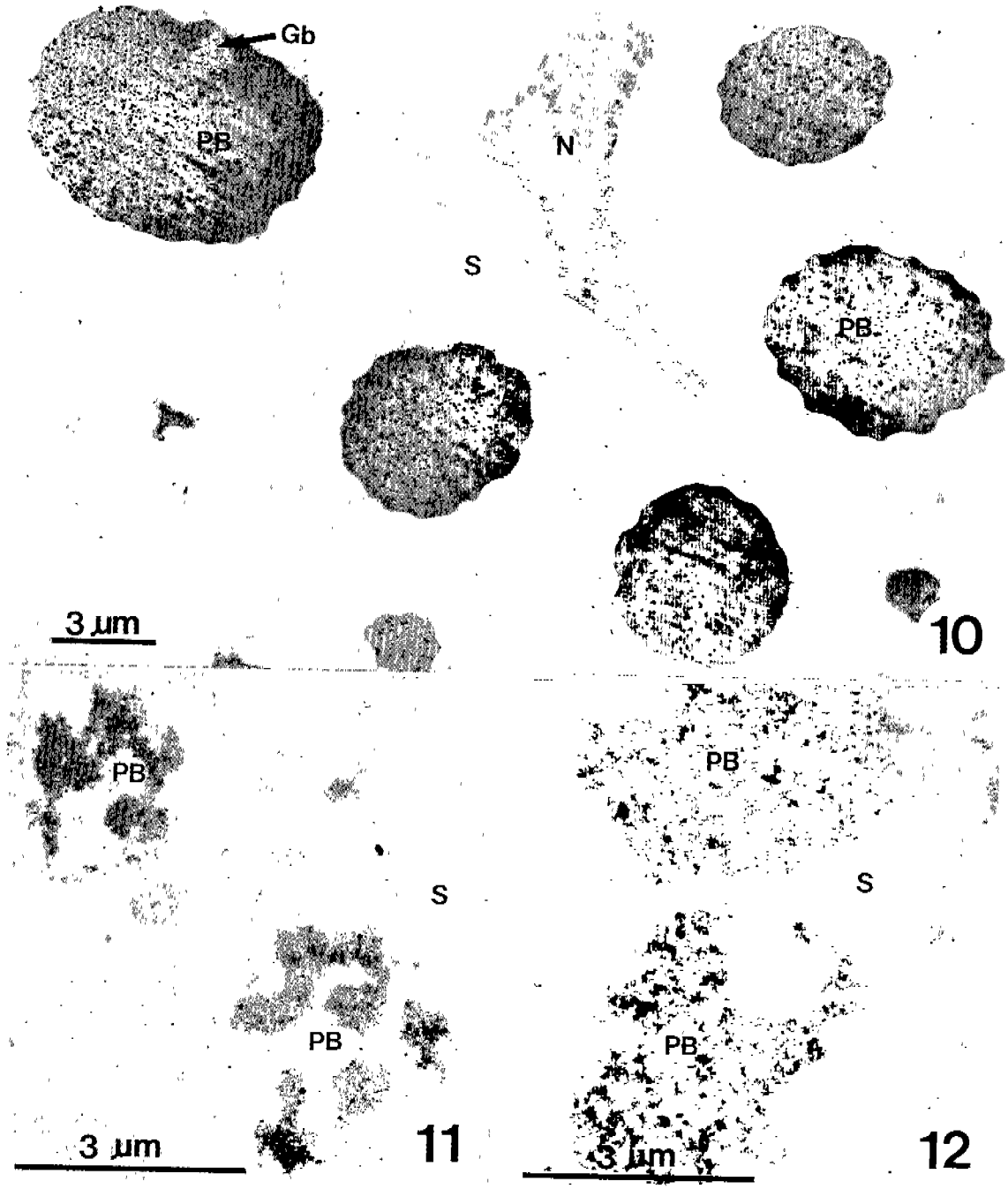


Fig. 9. Immunogold labelled electron micrograph of endosperm cell. Colloidal golds were labelled on the protein bodies (PB). Protein bodies were dispersed through the cytoplasm. Embedded with Epon-Araldite, fine structural preservation was improved, whereas the amount of gold bound to the protein bodies was decreased. Electron dense granules (arrows) distributed over the cytoplasm were also labelled with gold (viewed in upper right-hand corner). Gb, globoid; M, mitochondria; N, nucleus; S, spherosome.



Figs. 10-12. Immunoelectron micrographs of ginseng endosperm cells. Fig. 10. The endosperm cell embedded with Spurr's resin. The fine structural preservation was decreased, but the amount of gold labelling was increased. Gb, globoid; N, nucleus; PB, protein body; S, spherosome. Figs. 11 and 12. Irregularly shaped protein bodies (PB). S, spherosome.

ammonium sulfate 침전, gel permeation 및 이온 교환 크로마토그래피로 정제하였다. Vicilin은 분자량 55,000(큰 소단위) 및 44,000(작은 소단위)인 두 종의 소단위를 포함하는 당단백질이다.

Vicilin에 대한 항체를 토끼에서 형성시켜 DEAE Affi-Gel Blue affinity 크로마토그래피로 정제하였다. 이 항체와 금 입자가 결합된 2차 항체를 종자의 배유세포에 반응시켰다. 금 입자는 배유세포내의 단백질체, 전자밀도가 높은 과립 및 골지체의 elaborating 과립에 표지되었다.

이러한 결과는 조면소포체에서 합성되어 골지체로 수송된 vicilin이 골지의 소포내에서 공정과정을 거쳐 전자밀도가 높은 과립이 된 다음 단백질체로 수송됨을 나타낸다.

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