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Title: α' subunit of soybean β-conglycinin forms complex with rice glutelin via a disulfide bond in transgenic rice seeds

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Running title: Formation of complex between β-conglycinin and glutelin

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Abstract

We expressed the α' and β subunits of soybean β-conglycinin in rice seeds to improve the nutritional and physiological properties of rice as a food. The α' subunit accumulated in rice seeds at a higher level than the β subunit, but no detectable difference in mRNA transcription level between subunits was observed. Sequential extraction results indicate that the α' subunit formed one or more disulfide bonds with glutelin. Electron microscopic analysis showed that the α' subunit and the β subunit were transported to PB-II in analogy with glutelin. In mature transgenic seeds, the β subunit accumulated in low electron density regions in the periphery of PB-II, whereas the α' subunit accumulated together with glutelin in high-density regions of the periphery. The subcellular localization of mutated α' subunits lacking one cysteine residue in the N-terminal mature region (α'ΔCys1) or five cysteine residues in the pro and N-terminal mature regions (α'ΔCys5) were also examined. Low-density regions were formed in PB-II in mature seeds of transgenic rice expressing α'ΔCys5 and α'ΔCys1. α'ΔCys5 was localized only in the low-density regions, whereas α'ΔCys1 was found in both low- and high-density regions. These results suggest that the α' subunit could make complex via one or more disulfide bonds with glutelin and accumulated together in PB-II of transgenic rice seeds.

Introduction

Rice storage proteins are composed of glutelin (acid/alkaline-soluble), prolamin (alcohol-soluble), globulin (saline-soluble), and albumin (water-soluble). Glutelins, account for about 80 % of the total proteins in rice seeds (Ogawa et al., 1987; Li and Okita, 1993; Cagampang et al., 1966). They are synthesized as a precursor which is further processed proteolytically into acidic and basic chains connected with a disulfide bridge like 11S globulin. Rice proteins are rich in sulfur-containing amino acids, but are deficient in lysine. In contrast, β-conglycinin (7S globulin of soybean) and glycinin (11S globulin of soybean) are rich in lysine but are poor in sulfur-containing amino acids (Utsumi, 1992). Moreover, their physiological benefits to humans have been established. β-conglycinin lowers plasma cholesterol and triglyceride levels in human (Sirtori et al., 1995; Aoyama et al., 2001). The α' subunit of β-conglycinin has been reported to have LDL-cholesterol-lowering activity (Sirtori and Lovati, 2001) and phagocytosis-stimulating activity (Tsuruki et al., 2003). Therefore, an accumulation of β-conglycinin in rice seeds could lead to the development of a product with high nutritional value, several important physiological activities and useful physicochemical properties.

β-Conglycinin has a trimeric structure similar to other 7S globulins. The α and α' subunits contain an N-terminal extension in addition to a core region common to all the subunits. The β subunit consists of only the core domain (Maruyama et al., 1998). The extension regions of the α and α' subunits

probably protrude from the molecular surface of the core domains and have a minor role in proper folding and assembly (Maruyama et al., 2001). The α/α' subunits and the β subunit are synthesized on polysomes as prepro- and pre-forms, respectively. The signal peptides are cotranslationaly removed, the polypeptides are N-glycosylated with high-mannose glycans and assemble into trimers in the ER (Yamauchi and Yamagishi, 1979; Utsumi, 1992). The resultant α/α' subunits and the β subunit are in pro-form and mature form, respectively. Each trimer is transported from the ER to the protein storage vacuoles through the Golgi apparatus (Mori et al., 2004). The pro regions of the α and α' subunits are proteolytically processed to give their mature forms. Both the α and α' subunits contain four cysteine residues (Cys) in their pro regions and one Cys in the mature extension region (Cys13).

In rice seeds, there are two types of protein bodies, PB-I and PB-II. PB-I, derived directly from the ER, contains mainly prolamin (Tanaka et al., 1980; Yamagata et al., 1986). Binding protein (BiP) facilitates folding and assembly of prolamin polypeptides (Li et al., 1993). PB-II, derived from the vacuoles, have non-spherical structure and primarily contain glutelin and globulin (Tanaka et al., 1980). Both proteins are synthesized on the rER, assembled in the ER, transported to the Golgi apparatus, and sorted to the vacuole. Both glutelin and globulin are transported from the Golgi apparatus to storage vacuoles by the so-called dense vesicles (Krishnan et al., 1986, 1992). However, glutelin and globulin accumulate in different regions of PB-II (Krishinan and White, 1995). Globulin accumulates in the

periphery region of the PB-II (Krishnan et al., 1992; Krishinan and White, 1995).

To develop rice having a high nutritional value and physiological function, we introduced and expressed the α' and β subunits of soybean β-conglycinin in rice seeds. Further, we analyzed the traffic and accumulation behavior of the α' and β subunits in rice seeds. Our results indicate that the α' subunit accumulates at a higher level than the β subunit and that the α' subunit forms a complex via one or more disulfide bonds with glutelin in transgenic rice seeds.

Materials and methods

Construction of binary vectors and transformation

cDNAs encoding the α' and β subunits were modified to remove a *Sac* I cleavage site in the coding sequence while retaining the actual amino acid sequence, and to introduce the *Sac* I site at the downstream of the stop codon by PCR using the following pairs of oligonucleotide primers : 5'-gatatgaacgagggggctctttttctgcca -3' and 5'- cacaacactgaggaagacatccaagtcc -3' for α';

5'-ggatatcaacgaaggcgctcttcttctacc-3' and 5'-acagaactgaggaagatatccaagtcccg-3' for β;

5'-atgatgagagcgcggttcccattac-3' and 5'-catcatgcgagctctcagtaaaaagccctc-3' for α';

5'-atgatgagagtgcggtttcctttg-3' and 5'-catcatgcgagctctcagtagagagcacctaag-3' for β (underlines indicate *Sac* I cleavage site). The resulting modified α' and β cDNAs were digested by *Sac* I. A *Sal* I cleavage site was

introduced at 5' ends of glutelin promoters *GluB-1* and *GluB-2*, which direct the expression at the periphery of the endosperm (Takaiwa et al., 1996, Wu et al., 1998), by PCR using the following primers: 5'-agctatttgtacttgcttatggaagc-3' and 5'-acttcacaaagtagtagtcaacc-3' for *GluB-1* promoter; 5'-agctattagcagttgctaatggaaac-3' and 5'-gaggaatagagataaggttgaggag-3' for *GluB-2* promoter (underlines indicate *Sal* I cleavage site). The modified promoter regions were digested by *Sal I*. The 2.3 kb *GluB-1* and 2.4kb *GluB-2* promoter regions were ligated with the α' and β cDNAs between *Sal* I and *Sac* I cleavage sites of pBluescript SK (Stragegene, La Lolla, CA, USA), respectively. The DNA sequences of promoter and cDNA regions inserted into pBluescript SK were confirmed by DNA sequencing using the following primers: 5'-ccaaggaaaagctcgtattagtgag-3', 5'-gacgcgggagccgtcctaggtgcaccgg-3', 5'-tggaaaaattacatacaccaatatg-3' for *GluB-1*; 5'-atgatgagagcgcggttccc-3', 5'-cgaagacataagaataagaaccc-3', 5'-gtttcttcctatctagcac-3', 5'-aaacctttcaacttgagaagcc-3' for α'; 5'-attaccatccccataccagaaactc-3', 5'-aagttgtgagtgtgacttca-3', 5'-acacaacaaatttgaatgtttccag-3', 5'-ggcaagacacatactaaaagtatgg-3' for *GluB-2*; 5'-atgatgagagtgcggtttcc-3', 5'-gccatacccgtcaacaaacctggca-3', 5'-ggatatcaacgaaggagctcttcttctacc-3', 5'-tgggtctgcacaagatgttgagagg-3' for β. For α'Δcys1, the codon of Cys13 was replaced with that of Ser in the pBluescript SK using the following primers: 5'-gtggaggaagaagaagaaagcgaagaagg-3' and 5'-cttaaggaggttgcaacgagcgtgg-3'. For α'Δcys5, the pro-region of α' was deleted and the codon of Cys13 was replaced with that of Ser in the pBluescript SK using the following primers:

5’-gtggaggaagaagaagaaagcgaagaagg-3’ and 5’-aatgccaaatgagacagaaactgatgc-3’. The DNA region coding *GluB-1*-α’, -α’ ΔCys1 and -α’ΔCys5 in pBluescript SK was cleaved out by *Sac* I and *Sal* I, and these DNA fragments were inserted into a binary vector pGTV-HPT (Becker et al., 1992), where *Sac* I linker was introduced, to construct pGTV-HPT/α’, pGTV-HPT/α’ΔCys1 and pGTV-HPT/α’ΔCys5, respectively. *GluB-2* promoter and β cDNA were digested by *Sac* I, because there is a *Sac* I cleavage (-1759) site at upstream of *GluB-2* promoter, and the resulting DNA fragment was inserted into pGTV-HPT cleaved by *Sac* I to give pGTV-HPT/β. The direction of the insert was checked by DNA sequencing using the following primer: 5’-tgggtctgcacaagatgttga-3’.

pGTV-HPT/α’, pGTV-HPT/α’ΔCys1, pGTV-HPT/α’ΔCys5 and pGTV-HPT/β were introduced into *Agrobacterium tumefaciens* (EHA105) (Hood et al., 1993) by electroporation as described previously (Goto et al., 1999). Rice (cv. Kitaake) calli were infected with these *Agrobacterium*. Hygromycin-resistant calli were selected and plants were regenerated in the presence of 50 mg/l hygromycin.

Antibodies

Antisera against α’ subunit (Nishizawa et al., 2003) and β subunit (Maruyama et al., 1998) of β-conglycinin, rice proglutelin (Katsube et al., 1999), 10kDa and 16kDa prolamins (will be described elsewhere) and soybean BiP (Mori et al., 2004) raised in rabbit were used.

Quantification of expression levels of β-conglycinin in the rice seeds

Primary transgenic rice plant seeds (T_1) were dehulled, and ground separately with a mortar and pestle. Proteins were extracted with 45 mM Tris-HCl (pH 6.8) containing 2 % (w/v) SDS, 30 % glycerol, and 0.1 M ME (2-mercaptoethanol). Aliquots (1μg) of protein were then spotted on a nitrocellulose membrane and β-conglycinin was detected immunologically with either anti-α' or anti-β sera (Nishizawa et al., 2003; Maruyama et al., 1998). The accumulation levels of α' and β subunits were estimated by comparing the densitometric signals obtained from the extracts prepared from the transgenic plants with those obtained from the extract prepared from the non-transgenic plant containing a known amount of purified α' or β from soybean seeds. Six individual seeds were analyzed from individual transgenic plants and the maximum accumulation level within six seeds was used to compare expression levels between constructs.

SDS-PAGE and Western blotting

SDS-PAGE was done using 11% polyacrylamide gel. Proteins were stained with coomassie brilliant blue R-250. Western blotting was done after SDS-PAGE using 11% polyacrylamide gel. The separated proteins on gels were transferred electrophoretically to nitrocellulose membrane (0.45 μm; Schleicher and Schuell Inc., Dassel, Germany) and recombinant proteins were detected with anti-α', and/or anti-β sera followed by a goat anti-rabbit IgG-alkaline phosphatase conjugate (Promega, Madison, WI, USA).

2D SDS-PAGE

Two-dimensional (without/with ME) SDS-PAGE was done using 7.5 % gels. Sample lanes from the first dimension (without ME) were cut off from the gel and incubated with SDS-PAGE running buffer containing 0.1 M ME for 1 hr at room temperature, subjected to SDS-PAGE in the second dimension (with ME) and analyzed by Western blotting.

Comparison of transcription levels of α' and β mRNAs in rice seeds

Total RNA was extracted from immature homozygous T_2 seeds (about 15 days after pollination). Six seeds were ground with a mortar and pestle in phenol solution (phenol: chloroform: isoamyl alcohol = 25:24:1). After addition of TE buffer (0.1 M Tris-HCl (pH 9.0), 1 % (w/v) SDS, 0.1 M NaCl, 5 mM EDTA), total RNA was purified by lithium chloride precipitation and DNase digestion (Goto et al., 1999).

Transcription levels of α' and β mRNAs were measured by real-time PCR. This assay was carried out in the ABI-PRISM 7000 (Applied Biosystems) using the Taqman system (Applied Biosystems) in a final volume of 50 μl. The reaction mixture including 5.5 mM $MgCl_2$, 0.3 mM each of dATP, dCTP, and dGTP, 0.8 mM dUTP, 0.2 μM forward and reverse primers (5'-ttgtttgagattaccccagagaaaa-3'and 5'-cctcgttcatatccacaacactga-3' for α' subunit, 5'-gactaccggattgtccagtttca-3' and 5'-aatcggcgtcagcatggt-3' for β subunit, and 5'-cgaggcgcagtccaaga-3' and 5'-cccagttgctgacgatacca-3' for rice actin gene (RAc1) as an internal standard), 12.5 U reverse transcriptase, 20 U RNase inhibitor, 1.25 U DNA polymerase, and 0.1

μM Taqman probe. The probes for α' and β subunits were 5'-FAM-ccctcagcttcgggacttggatgtct-TAMRA-3' and 5'- FAM-tcaaaacccaacacaatccttctcccc-TAMRA-3', respectively. The probe for RAc1 was 5'-VIC-tatcttgaccctcaagtaccccatcgag-TAMRA-3'. These primers and probes were designed by Primer Express ver 2.0 (Applied Biosystems). Conditions for amplification were 30 min at 48 °C, 10 min 95 °C, and 40 cycles of 15 sec at 95 °C and 1min at 60 °C. Results were analyzed using a sequence detection system (ABI prism 7000 SDS system) provided by Applied Biosystems. The cycle number at which the ΔRn of a given reaction crossed the half height bar was denoted Ct. The Ct data from each sample were normalized to the internal standard (RAc1) Ct using the formula (ΔCt=target Ct – internal standard Ct). The ΔCt values of β were compared with the ΔCt value of α' using the formula (ΔΔCt=ΔCt (α') - ΔCt (β)). Relative quantification (RQ) and relative gene expression were measured according to the formula ($RQ=2^{\Delta\Delta Ct}$).

Gel-filtration chromatography

The molecular assembly of the α' and β subunits and modified α' subunit expressed in rice seeds was analyzed by gel-filtration using a Hi-Prep 26/60 Sephacryl S-300 HR column, equilibrated with buffer A (35 mM sodium phosphate (pH 7.6), 0.4 M NaCl, 1 mM EDTA, 0.02 % (w/v) NaN_3) containing 10 mM ME at a flow rate of 0.5 ml/min.

Endo H and PNGase F digestion

Five seeds from transgenic rice expressing α' or β subunit were ground with a mortar and pestle, and proteins were extracted with denaturing buffer (0.5 % SDS, 1 % ME, 100mM Tris-HCl, pH 8.0). Purified α' and β subunits from soybean were denatured with denaturing buffer. The final concentrations of extracted proteins and purified α' and β subunits were adjusted to 0.25 mg/ml with denaturing buffer. Samples were split into three tubes and incubated with 30 mU of endoglycosidase H (Endo H; Bio Labs) and storage buffer of Endo H (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) as a control, or 30 mU of peptide:N-glycanase F (PNGase F; Bio Labs) and storage buffer of PNGase F (20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, 50 % glycerol) as a control, at 37 °C for 3 hr. Total protein was then precipitated adding 1 volume of 30 % trichloroacetic acid, and the protein pellet was washed twice with ice-cold acetone and dissolved with 45 mM Tris-HCl (pH 6.8) containing 2 % (w/v) SDS, 30 % glycerol, and 0.1 M ME). Samples were analyzed by SDS-PAGE followed by Western blotting.

Sequential extraction

Five seeds from each of the transgenic T_1 plants were ground with a mortar and pestle and sequentially extracted with three types of solutions at seed/solvent ratio 1:10 (w/v), each extraction being followed by centrifugation at 30,000 x g for 15 min. The following solutions were used in two separate extraction series: buffer A without ME, eight extractions; 1% lactic acid, four extractions; SDS buffer (45 mM Tris-HCl, pH 6.8, containing 2 % SDS, 30 % glycerol and 0.1 M ME). The difference between the

first and the second extraction series is the presence or absence of 20 mM ME in buffer A.

Electron microscope immunocytochemical localization

Mature and developing rice seeds were cut into 1.5 to 2.0 mm sections and fixed for 5 h in 1.5 % (v/v) glutaraldehyde solution at 4 °C. Tissue sections were washed three times with buffer B (50 mM sodium phosphate, pH 7.2), dehydrated (the ethanol wash, starting with 50%, 60% to 98 %, 1 hr for each wash) followed by 100 % propylene oxide at –20 °C (three washings, 1 hr for each). The mature seed samples were placed in epoxy resin (Quetol-812)/propylene oxide 1:3 (v/v) for 2 days, resin/propylene oxide 3:1 (v/v) for 2 days, and finally 100 % resin for 2 days. Polymerization was done at 45 °C for 1 day, and at 60 °C for 2 days. The developing seed samples were placed into LR white overnight at 4 °C and transferred to beam capsules (NISSHIN EM, TOKYO) filled with freshly prepared resin. The resin was allowed to polymerize for 2 days under indirect UV light at 4 °C.

Ultrathin sections (60-80 nm) were obtained with a glass knife and placed onto formal/carbon-coated grids. The sections were blocked with 5 % (w/v) BSA-PBS and then incubated for 1 hr at room temperature on a drop of anti- α', anti-β, anti-globulin, anti-glutelin, anti-10kDa prolamin and 16kDa prolamin serum diluted 1/1000, 1/4000, 1/1000, 1/200, 1/200 1/10000 in 1 % (w/v) BSA-PBS, respectively. The sections were washed six times for 5 min each on a drop of 1 % (w/v) BSA-PBS and then incubated on a drop of goat anti-rabbit IgG conjugated to 15-nm or 5-nm gold particles (H+L, Auro Probe

EM, Amersham) diluted 1/25 in 1 % (w/v) BSA-PBS for 30min at room temperature. After washing with PBS, sections were washed twice with distilled water. The sections were stained for 25 min with 2 % (w/v) uranyl acetate followed by incubation with 80 mM lead nitrate for 25 min. The grids were examined and photographed using an electron microscope (model H-700H, Hitachi, Tokyo).

Seeds of three independent plants were observed for all constructs and the data was similar among three independent plants. Representative images were shown as a figure.

Statistical analysis

Student t-tests (two-tailed, unequal variance) were performed using MS Excel. The term statistical difference is used to indicate differences for which $P<0.05$.

Results

Transgenic rice seeds expressing α' and β subunits

β-Conglycinin α' and β cDNAs were driven by the rice glutelin *GluB-1* and *GluB-2* promoter regions in pGTV-HPT, respectively (Fig.1). The resulting pGTV-HPT/α' and pGTV-HPT/β were introduced into rice calli by *Agrobacterium tumefaciens*-mediated transformation. Eleven and 22 independent lines expressing α' and β, respectively, were regenerated. Total protein extracted from T_1 seeds of the transgenic plants was spotted onto nitrocellulose membranes and the accumulation levels of

the α' and β subunits were estimated immunologically (Fig. 2). The accumulation levels of the α' subunit was higher than those of the β subunit and the average levels of the α' and β subunits were 3.9% and 2.0%, respectively. The accumulation level between them was statistically difference. The lines with the highest accumulation levels of the α' and β subunits were subjected to subsequent analysis.

Comparison of transcription levels of α' and β subunits in rice

A difference in the transcription levels or translational efficiencies of α' and β mRNAs might be a reason for the difference in accumulation levels of the α' and β subunits (Fig. 2). To verify this possibility, we compared the quantities of both mRNAs by real-time PCR. The top lines having a single copy were self-pollinated to obtain homozygous lines. The accumulation levels of the α' and β subunits in homozygous lines were 7.9 ± 0.7 and 4.4 ± 0.8% in total proteins, respectively (data not shown). In the homozygous line seeds, the α' subunit accumulated about twice as much as the β subunit, similarly with the T1 seeds. The total RNAs from 15 days-after-flowering (15 daf) seeds of α'6-2 and β5-4 homozygous lines were subjected to real-time PCR. Ct values of α'6-2 and β5-4 collected from half height bar crossed each ΔRN were 18.9 and 18.5, respectively (Table 1). To correct the experimental error between the α' and β subunits, we utilized the rice actin gene RAc1 as the internal standard. The Ct values of RAc1 in transgenic rice seeds expressing the α' and β subunits were 28.5 and 28.1, respectively. To compare their transcription levels, the Ct values were normalized. The results indicate that the transcription level of

α'6-2 was close to that of β5-4, although the accumulation level of α'6-2 was about twice as much as that of β5-4. Thus, the transcription level was not reason for the difference in the accumulation levels of both proteins in rice seeds. Thus, transcription levels do not explain the difference in the accumulation of the two proteins in rice seeds. Translational efficiencies or protein stability may therefore account for the difference in the accumulation level between the α' and β subunit.

Post-translational modification of α' and β subunits in rice seeds

The bands for the α' and β subunits were clearly detectable when total seed proteins from transgenic rice were subjected to SDS-PAGE followed by western blotting (Fig. 3). No degradation products of both the α' and β subunits were found by western blotting. Thus, both the α' and β subunits stably accumulated in the rice seeds. The α' and β subunits extracted from transgenic rice seeds were eluted at the same positions (100 min and 124 min, respectively) as α' and β homo-trimers purified from soybean seeds on gel filtration chromatography (Fig. 4). This indicates that both the α' and β subunits from rice seeds form trimers.

Both the α' and β subunits are N-glycosylated with high-mannose type glycans in soybean (Yamauchi and Yamagishi, 1979). To examine whether the α' and β subunits synthesized in rice seeds were N-glycosylated, they were subjected to digestion either by PNGase F (hydrolyzes almost all N-glycans, excluding the core-fucosylated complex N-glycan) or by Endo H (primarily hydrolyzes high-mannose

glycan but not complex glycan). As a result of the digestion by PNGase F as well as by Endo H, both the α' and β subunits gave a single band of molecular mass lower than that of the intact subunit (Fig. 5). Thus, both the α' and β subunits were glycosylated with high-mannose N-glycan, and not with complex glycan, indicating that the N-glycan modification of the α' and β subunits in rice is similar to those in soybean seeds (Yamauchi and Yamagishi, 1979). Complex glycan modification of the α' and β subunits does not occur in soybean and rice seeds, although most of them traffic through the Golgi apparatus indicating limit access to modifying enzymes to these proteins in developing seeds.

Interaction of α' and β subunits with rice seed storage proteins

To examine whether the α' subunit containing Cys13 in the mature subunit interacts with glutelin in transgenic rice seeds via a disulfide bridge, we conducted sequential extractions of the seeds with buffer A without ME, lactic acid and SDS buffer. The same extractions were done with transgenic rice seeds expressing the β subunit. The extracts were subjected to SDS-PAGE in the absence of ME followed by western blotting (Fig. 6A-I, II, III). Most (84 %) of the β subunit detected as a single band was extracted in fractions 1 and 2 **(**Fig. 6A-III**)**. In contrast, a large amount of the α' subunit (39 %) was extracted with lactic acid in fraction 9 (Fig. 6A-I). When the extracts were subjected to SDS-PAGE in the presence of ME followed by western blotting, the α' subunit in fraction 9 as well as in fraction 1 showed a single band corresponding to the α' monomer (Fig. 6A-II). These results suggest that a part of mature α' subunit is

linked with rice acid-soluble proteins (mainly glutelin) via a disulfide bond.

To further characterize the interaction of rice proteins with the α' subunit, we subjected fraction 9 to two-dimensional (without/with ME) SDS-PAGE (Fig. 6B) followed by western blotting in the second dimension using anti-glutelin (Fig. 6B-III) and anti-α' (Fig. 6B-IV) sera. Most of the coomassie-stained proteins (first dimension) were identified in the second dimension as glutelin acidic chains (Fig. 6B-III**)**. One of the major bands of the α' subunit found in the second dimension migrated as α' monomer (Fig. 6B-IV; asterisk). Another major bands of the α' subunit might correspond to the complex of the α' subunit and rice acid-soluble proteins (Fig. 6B-IV; double asterisk). Other weak bands of the α' subunit were also detected in the high-molecular mass region (Fig. 6B-IV). Glutelins were detected in a region of molecular mass higher than the α' monomer, suggesting that a part of the α' subunit forms one or more disulfide bonds with glutelin.

In another experiment, we used a similar scheme of thirteen sequential extractions but added ME in buffer A for extractions from fractions 5 to 8 (Fig. 7). The extracts were subjected to SDS-PAGE in the presence of ME followed by western blotting. As expected, most of the β subunit was found in the first two fractions. However, only about half (51 %) of the α' subunit extracted by buffer A without ME corresponded to the α' trimers (Fig. 6 A-II). Most of the rest of the α' subunit (41 %) was extractable only by buffer A with ME. These results together with Fig. 6A-II indicate that the α' subunit is linked with

glutelin by one or more disulfide bonds.

Transgenic rice seeds expressing mutated α' subunit

To study the role of Cys residues of the α' subunit (four residues in the pro-region and Cys13 in the mature subunit) in the accumulation behavior of the α' subunit in rice seeds, cDNAs for the deletion mutants driven by the *GluB-1* promoter were introduced into rice calli (α'ΔCys1 and α'ΔCys5; see Fig. 1A). The pro region of α'ΔCys1 is supposed to be removed in the vacuoles. The average accumulation levels of α'ΔCys1 and α'ΔCys5 in total rice seed proteins were 3.2 and 2.5%, respectively (Fig. 2). A statistical difference of the accumulation level between α' and α'ΔCys5 could not be found. This indicates that the higher accumulation of the α' subunit with respect to the β subunit might not be due to interactions with glutelin by a disulfide bond. Both the substitution of Cys13 and the deletion of the pro region did not affect the self-assembly of α'ΔCys1 and α'ΔCys5 (Fig. 4B). Similar to the β subunit, more than 90 % of the total α'ΔCys1 and α'ΔCys5 from transgenic rice seeds were extractable with buffer A without ME (Fig. 7).

Subcellular localization of β-conglycinins in transgenic mature seeds

The subcellular localization of the α'and β subunits, α'ΔCys1 and α'ΔCys5 in transgenic rice seeds was studied by immunocytochemical analysis (Fig. 8). The electron density of PB-II was uniform in mature non-transgenic seeds (Fig. 8A). In contrast, low electron density regions at the periphery of PB-II

were detected in mature seeds of transgenic rice expressing the β subunit. The β subunit localized in low electron density regions (Fig. 8C). Remarkably, in the case of mature seeds of transgenic rice expressing the α' subunit, the electron density of the entire PB-II was high, although the α' subunit was detected only in the peripheral region (Fig. 8B). On the other hand, low-density regions were formed in PB-II in mature seeds of transgenic rice expressing α'ΔCys5 and α'ΔCys1 (Fig. 8D-F). α'ΔCys5 was localized only in the low-density regions (Fig. 8D), whereas α'ΔCys1 was found in both low- and high-density regions (Fig. 8E and F). These results indicate that the pro region of the α' subunit plays a fundamental role in the localization within PB-II.

Subcellular localization and trafficking of β-conglycinin in developing seeds

To investigate the trafficking of the α' subunit in rice seeds, developing seeds (10 days after flowering, 10 daf) of transgenic rice expressing the α' subunit were analyzed by electron microscopy (Fig. 9). In the early stage of PB-II formation, the α' subunit localized in the peripheral regions of PB-II (Fig. 9A). Low electron density regions of PB-II were not observed in immature seeds of transgenic rice expressing the α' subunit similar to mature seeds of transgenic rice expressing the α' subunit. Vesicles possibly budding from the Golgi apparatus were labelled by gold particles against the α' subunit antibody (Fig. 9B). Glutelin was also transported to PB-II by vesicles through the Golgi apparatus (Fig. 9C) as reported previously (Krishnan et al., 1986). Moreover, glutelin was observed in regions where the α'

subunit accumulated (Fig. 9D). This observation is consistent with the result that the α' subunit interacted with glutelin within rice seeds by a disulfide bond (Fig. 6).

In the developing seeds of transgenic rice expressing the β subunit, some PB-II had high and low electron density regions in analogy with mature seeds of transgenic rice expressing the β subunit. The gold particles against anti-β serum existed primarily in the low electron density regions of PB-II (Fig. 10A). On the other hand, gold particles against anti-glutelin serum were not observed in the low electron density regions of PB-II (Fig. 10B). Further, the β subunit was also observed in dense vesicles (Fig. 10C) and in morphologically different compartments (Fig. 10D). They were surrounded by ribosomes just like precursor accumulating (PAC) vesicles which were reported to carry storage proteins from the ER to the vacuoles bypassing the Golgi apparatus (Hara-Nishimura et al., 1998). Binding protein (BiP), a chaperon located in the ER, was observed in PAC-like vesicles (Fig. 10E). Further, electron-dense aggregate was also observed in the ER lumen, and prolamin could be barely observed in this aggregate (Fig. 10F). These results indicate that the PAC-like vesicle was different from PB-I, although it was derived from the ER in analogy with PB-I. This suggests that the PAC-like vesicle transported a part of the β subunit from the ER to the vacuole.

Developing seeds of transgenic rice expressing α'ΔCys5 had two electron density regions in PB-II in analogy with transgenic rice seeds expressing the β subunit. α'ΔCys5 existed mainly in the low

electron density region (Fig. 11A). On the other hand, glutelin accumulated in high electron density regions, but not in low electron density regions (Fig. 11B). Thus, α'ΔCys5 and glutelin also separately accumulated in PB-II. These phenomena are very similar to those of the β subunit in rice seeds of transgenic rice expressing the β subunit. Further, α'ΔCys5 was also observed in the Golgi apparatus and the dense vesicles (Fig. 11C). However, PAC-like vesicles were not observed in transgenic rice seeds expressing α'ΔCys5, although low electron density regions were observed in PB-II.

α'ΔCys1 accumulated in both high and low electron density regions in PB-II in developing seeds (Fig. 12A, B) and the PAC-like vesicles were not observed. In low electron density regions, glutelin was not observed in analogy with the case of β subunit and α'ΔCys5 (data not shown). Moreover, glutelin was also observed in high electron density regions where α'ΔCys1 was localized (Fig. 12C). These results with sequential extraction experiment suggest that the α' subunit interacts with glutelin via the pro region in rice seeds and that a disulfide bond plays an important role on the interaction.

Discussion

Complex formation of the α' subunit and glutelin in transgenic rice seeds

In this study, we introduced α' and β cDNAs of soybean β-conglycinin driven by glutelin promoters into the rice genome to investigate their accumulation behavior. Transcription levels of the α' and β

cDNAs in developing seeds of transgenic rice were found to be similar to each other (Table 1). Further, both α' and β subunits were shown to undergo posttranslational modification in rice seeds similar to those in soybean seeds (N-glycosylation by high-mannose glycans and detachment of N-terminal pro region from the α' precursor) (Fig. 5). The accumulation level of the α' subunit in mature rice seeds was about two-times higher than that of the β subunit (Fig. 2). The α' subunit and glutelin co-localized in high electron density regions in PB-II in developing seeds (Fig. 9), whereas the β subunit localized only in a low electron density region (Fig. 10). A sequential extraction experiment showed that the α' subunit could form a disulfide bond with glutelin (Fig. 6). Further, we examined the behavior of two kinds of modified α' subunit (α'ΔCys5 devoid of all Cys by means of removal of its pro region and substitution of Cys13 with serine, and α'ΔCys1 containing intact pro region and substituted Cys13) in transgenic rice seeds to elucidate the role of Cys residues in the accumulation of the α' subunit. Both α'ΔCys5 and α'ΔCys1 , similar to α' subunit, formed trimers (Fig. 4). The α'ΔCys5 localized in low-density regions in PB-II similarly to the β subunit, whereas α'ΔCys1 localized in low- and high-density regions (Fig. 8). These results suggest that the α' subunit makes a complex with glutelin via one or more disulfide bonds in rice seeds. Previous reports suggest that there could be a "dominant" effect of the storage proteins in directing foreign proteins to the vacuole in seed cells (Arcalis et al. 2004; Drakakaki et al. 2006). Stabilization by disulfide bonds might contribute a tendency for heterotypic interactions of storage proteins.

Trafficking of β-conglycinin in rice seed

Although PAC-like vesicles were observed in late developing stage (15 daf) of non-transgenic rice seeds (Takahashi et al., 2005), in analogy with pumpkin seeds (Hara-Nishimura et al., 1998), they were not observed in the nontransgenic rice of this study (10 daf). This suggests that the traffic ability of early developing rice seed is sufficient to transport storage proteins from the ER to the vacuoles through the Golgi apparatus. However, PAC-like vesicles were observed in the early developing stage (10 daf) of transgenic rice seeds expressing the β subunit, although the accumulation level of the β subunit was lower than that of the α'subunit. This suggests that introduction of the β gene in the rice induced the PAC-like vesicle formation in the early developing stage.

There have been reports on the direct pathway from the ER to the vacuole, introduced by transgenes. Introduction of a gene of sulfhydryl-endopeptidase (SH-EP) which has a KDEL-tail, papain-type vacuolar proteinase of germinated *Vigna mungo* seeds in *Arabidopsis* resulted in the formation of KDEL-vesicle, transported to the vacuoles by a Golgi-independent. Such vesicle was not observed in *Arabidopsis* seeds expressing SH-EP mutant lacking the KDEL-tail and nontransgenic *Arabidopsis* seeds (Okamoto et al., 2003). When a gene for KDEL-tagged β-phaseolin, 7S storage protein of French bean (*Phaseolus vulgaris*), was introduced into tobacco leaf protoplasts, KDEL-tagged β-phaseolin was

transported to the vacuole but the complex glycan was not formed, although normal β-phaseolin had the complex glycan. These suggest that the KDEL-tagged β-phaseolin was transported to the vacuoles directly from the ER (Frigerio et al., 2001). When human serum albumin containing N-terminal signal sequence and C-terminal KDEL tag was introduced into wheat and expressed in seeds, it was also transported to the vacuoles through the Golgi-independent route (Arcalis et al., 2004). It is likely that the overexpression of transgenes contained the KDEL-tail coding sequence in the ER caused ER stress, and that the ER stress induced the expression of some kind of molecular chaperons or transmembrane proteins resulting in a Golgi-independent pathway. The β subunit does not contain the KDEL sequence, but a direct transport pathway from the ER to the vacuoles was induced. In soybean seeds, ER-derived protein bodies (PBs) were observed at high frequency in the mutant line containing glycinin composed of only group I subunits the solubility of which was lower than that of the normal glycinin (Mori et al., 2004). Consequently, there is a possibility that the induction of PAC-like vesicle in rice seeds also depends on physicochemical properties, such as solubility and surface hydrophobicity, of the β subunit. The pH in the ER is estimated to be approximately 7.1 in resting HeLa cells (Kim et al., 1998). The α' subunit is soluble at μ= 0.08 and pH 7.1, whereas the β subunit is insoluble under these conditions (Maruyama et al., 1998, Maruyama et al., 2002). These findings suggest that the β subunit is insoluble in the ER lumen environment of rice seed and partly aggregates. In contrast, the α' subunit, α'ΔCys1 and α'ΔCys5, containing the hydrophilic domain

(extension domain), are soluble in the ER lumen environment, so all of them could be transported to the vacuoles via the Golgi apparatus. Recently, we showed that aggregated-type of red fluorescent protein forms ER-derived compartment in soybean and *Arabidopsis* seeds (Maruyama et al., 2008). Application of aggregated-type of red fluorescent protein could further elucidate a formation of ER-derived compartment in rice seeds.

Development of highly-physiologically functional rice to improve human health

β-Conglycinin has many physiological functions. It has been reported that the α' subunit decreases plasma cholesterol and triglyceride levels of rat when rats were fed 20 mg (kg body weight·day) of the α' subunit (Duranti et al., 2004). Thus, 1.2 g (60kg body weight·day) of α' subunit are needed for possible effective physiological functions in human. The maximum accumulation level of the α' subunit in total rice seed proteins was about 8%, and rice seed proteins account for 7% of total dry weight of rice seed. If one considers that average daily consumption of rice in Japan is 150g, which would contain about 0.84 g of α', increasing the accumulation levels of α' subunit by a factor of 1.5 is necessary to confer physiological functions to rice seeds.

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Figure Legends

Figure 1. Schematic presentation of the structure of wild-type and mutated β-conglycinin subunits (A) and fusion genes used for rice transformation (B). A; wild-type α' and β subunit and α' modified versions α'ΔCys1 and α'ΔCys5. Positions of Cys residues (SH) are shown. B, the α', β, α'ΔCys1 and α'ΔCys5 fusion genes. *GluB-1*, glutelin B-1 gene; g7, gene 7; HPT, hygromycin phosphotransferase.

Figure 2. Comparison of the accumulation levels of α' and β subunits, α'ΔCys1 and α'ΔCys5 in respective transgenic rice seed determined by dot immunoblotting. Total protein was extracted from each of the transgenic seeds with SDS buffer. Aliquots (1 μg/1 μl) were spotted on a nitrocellulose membrane and the recombinant proteins were detected immunologically with either anti-α' or anti-β sera. Accumulation levels of recombinant proteins were expressed as a percentage of respective total seed protein. Each mark represents the accumulation level in an independent transgenic plant.

Figure 3. Detection of α' and β subunits from transgenic rice seeds by SDS-PAGE and western blotting. Total seed protein extracted with SDS buffer was subjected to SDS-PAGE (lanes 1-3, CBB staining) and western blotting (lanes 4, 5). Lane M, molecular mass markers; lane 1, non-transgenic seeds; lanes 2 and 4, transgenic seeds expressing α' subunit; lane 3 and 5, transgenic seeds expressing β subunit. An arrow

indicates position of glutelin precursor.

Figure 4. Molecular assembly of recombinant proteins analyzed by gel filtration followed by western blotting. A. Purified α' and β homo-trimers from soybean seeds and bovine serum albumin (66 kDa) used as molecular mass markers were subjected to gel filtration on Sephacryl S-300 HR column and detected by A_{280}. B, α' and β subunits and α'ΔCys1 and α'ΔCys5 were extracted from transgenic rice seeds with buffer A containing ME and subjected to gel filtration using the same column as in A. Fractions collected every four minute were subjected to SDS-PAGE followed by Western blotting.

Figure 5. Analysis of N-glycosylation of α' and β subunits extracted from transgenic rice seeds. α' and β subunits extracted from rice seeds were incubated in the absence (-) or presence (+) of either Endo H or PNGase F at 37℃ for 3 h. Reaction mixtures were subjected to SDS-PAGE followed by western blotting.

Figure 6. Sequential extraction of α' and β subunits from transgenic rice seeds. A. Seeds were treated with buffer A containing no ME (eight extractions, lanes 1-8), then with 1 % lactic acid (four extractions, lanes 9-12), and finally with SDS buffer (lane 13). Each extract (fraction) was subjected to SDS-PAGE in the absence (I) or presence (II, III) of ME followed by western blotting with anti-α' (I, II) and anti-β (III).

Single and double asterisks indicate positions of α' monomer and dimer, respectively. B, Two-dimensional SDS-PAGE and western blot analysis of the fraction 9 of Fig. 6A-II. Arrow and arrow box indicate directions of electrophoresis in first (-ME) and second (+ME) dimensions, respectively. First dimension: CBB stained molecular mass markers (M), α' subunit purified from soybean seeds (I) and the fraction 9 (II). Second dimension, western blot analysis of fraction 9: immunoreactions with anti-glutelin (III) and anti-α' (IV) sera. Closed and open arrowheads indicate the position of glutelin precursor and glutelin acidic polypeptides, respectively.

Figure 7. Sequential extraction of recombinant α' and β subunit and α'ΔCys1 and α'ΔCys5 from respective transgenic rice seeds. Seeds were treated with buffer A containing no ME (four extractions, lanes 1-4), then with buffer A containing ME (four extractions, lanes 5-8), with 1 % lactic acid (four extractions, lanes 9-12), and finally with SDS buffer (lane 13). Each extract (fraction) was subjected to SDS-PAGE in presence of ME followed by Western blotting using with anti-α' (α', α'ΔCys1 and α'ΔCys5**)** and anti-β (β**)** sera**.**

Figure 8. Electron micrographs of mature seeds of non-transgenic and transgenic rice. A, non-transgenic seed non-treated with anti-serum. Immunoreactions were done with anti-β serum for transgenic seed

expressing the β subunit (C) and anti-α' serum for transgenic seeds expressing the α' subunit (B), α'ΔCys5 (D), and α'ΔCys1 (E, F). Gold particles, 15 nm; bars, 0.5 μm.

Figure 9. Electron micrographs of developing seeds (10 daf) of transgenic rice expressing the α' subunit. Immunoreactions were done with anti-α' (A, B) and anti-glutelin (C). Double immunoreactions were done with anti-α' (5nm) and anti-glutelin (15nm) (D) sera**.** GA indicates Golgi apparatus. Arrowheads indicates the position of α'. Bars = 0.5 μm.

Figure 10. Electron micrographs of developing seeds (10 daf) of transgenic rice expressing the β subunit. Immunoreactions were done with anti-β (A, C, D), with anti-glutelin (B), anti-BiP (E) and anti-prolamin (F) sera. Bars = 0.5 μm.

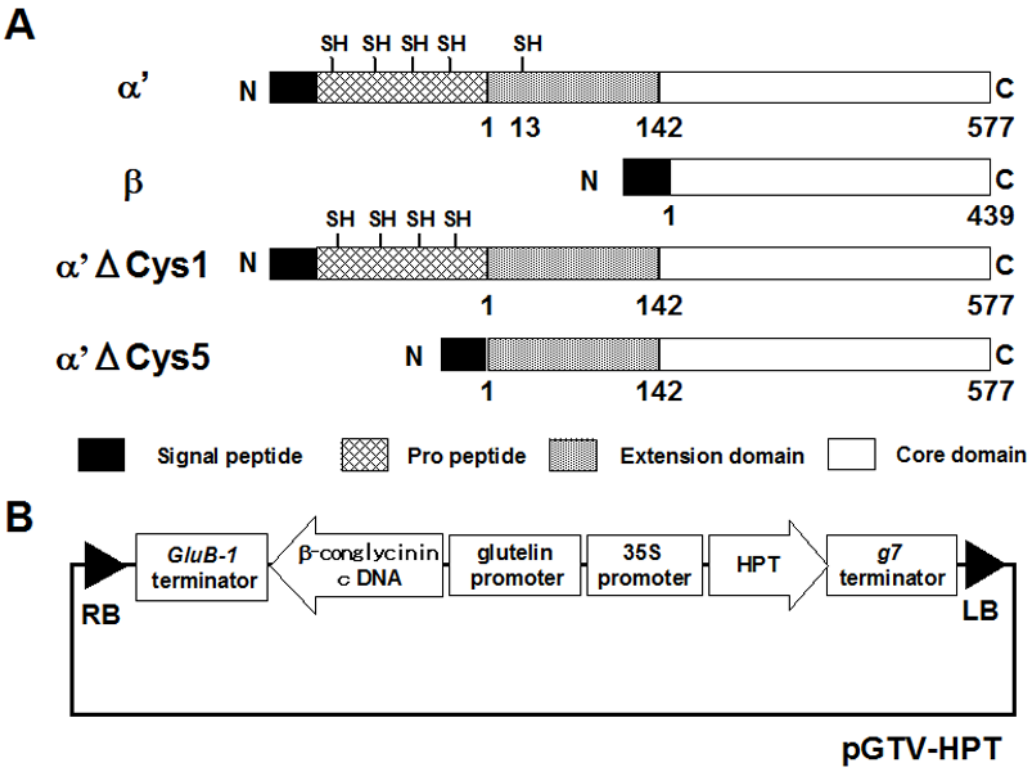
Figure 11. Electron micrographs of developing seeds (10 daf) of transgenic rice expressing α'ΔCys5. Immunoreactions were done with anti-α' (A and C) and anti-glutelin (B) sera. Bars = 0.5 μm.

Figure 12. Electron micrographs of developing seeds (10 daf) of transgenic rice expressing α'ΔCys1. Immunoreactions were done with anti-α' sera (A, B). Double immunoreaction was done with anti-α' (5nm) and anti-glutelin (15nm) (C) sera. Bars = 0.5 μm.

Table 1. Comparison of transcription levels of α' and β subunits in rice seeds.

	Target Ct	Internal standerd Ct (RAc1)	ΔCt	ΔΔCt	Relative quantification
α'6-2	18.9	28.5	9.60	0	1
β5-4	18.5	28.1	9.57	-0.03	0.98

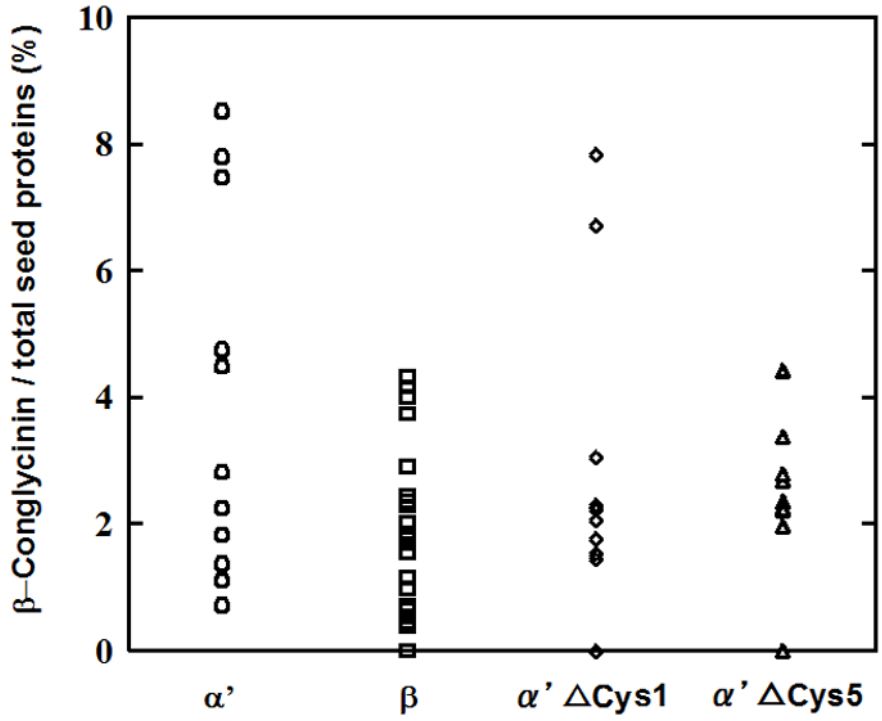
Fig. 1


A
α'
SH SH SH SH SH
N
C
1 13 142 577
β
N
C
1 439
α'ΔCys1
SH SH SH SH
N
C
1 142 577
α'ΔCys5
N
C
1 142 577
Signal peptide
Pro peptide
Extension domain
Core domain
B
GluB-1 terminator
β-conglycinin c DNA
glutelin promoter
35S promoter
HPT
g7 terminator
RB
LB
pGTV-HPT

1

2

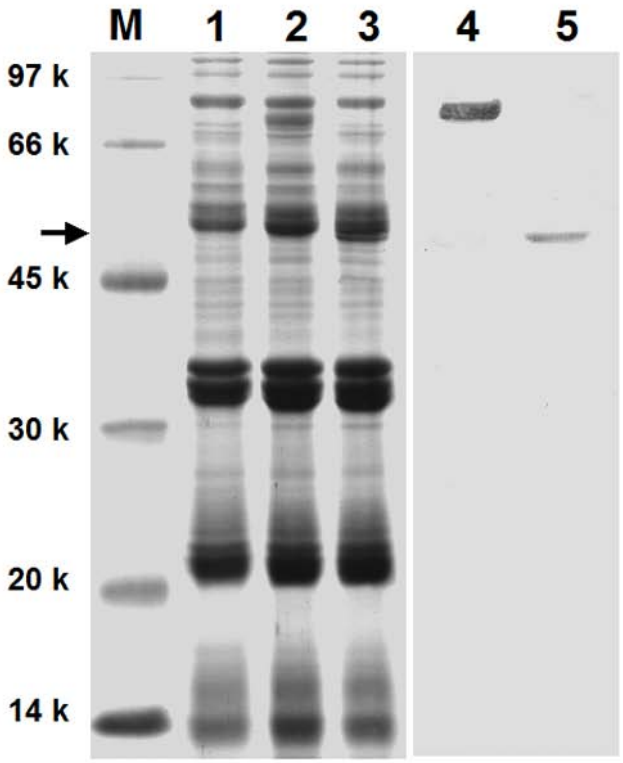
Fig. 2


β–Conglycinin / total seed proteins (%)
10
8
6
4
2
0
α'
β
α' ΔCys1
α' ΔCys5

1

2

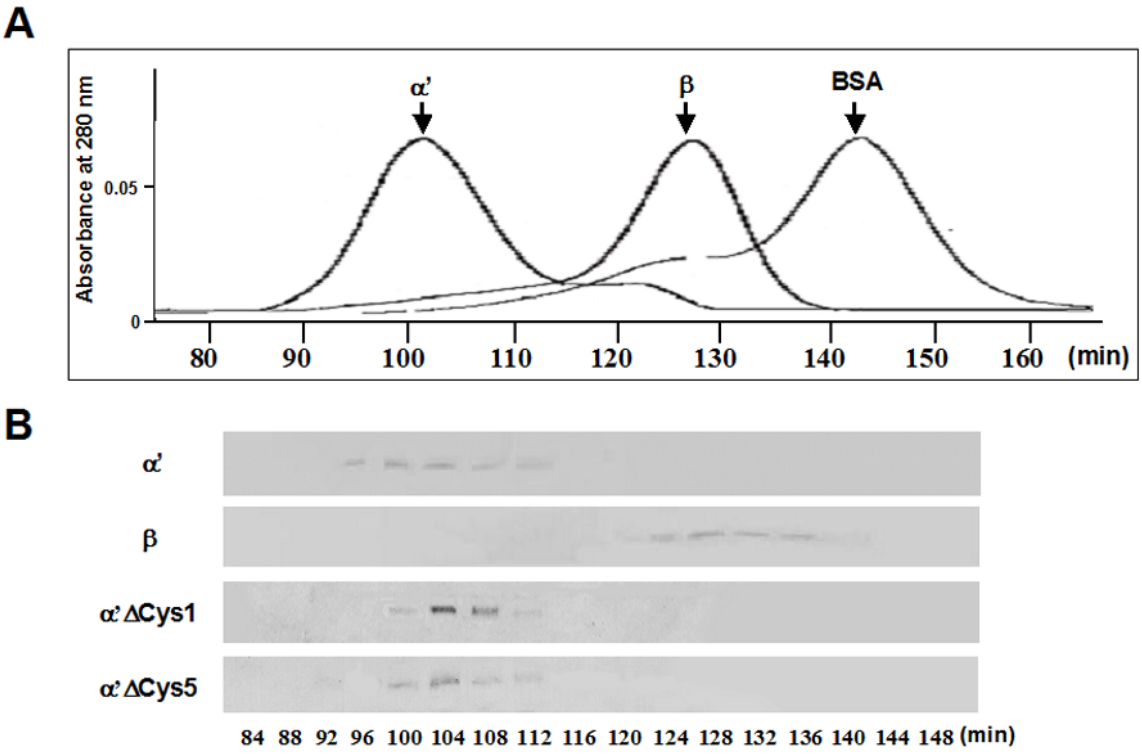
Fig. 3



1

2

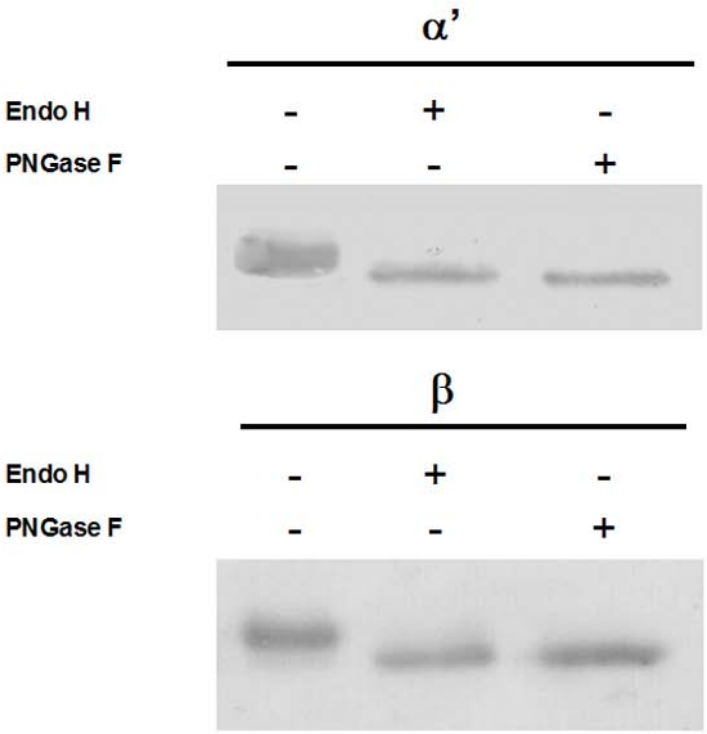
Fig. 4


A
Absorbance at 280 nm
0.05
0
α'
β
BSA
80
90
100
110
120
130
140
150
160
(min)
B
α'
β
α'ΔCys1
α'ΔCys5
84 88 92 96 100 104 108 112 116 120 124 128 132 136 140 144 148 (min)

1

2

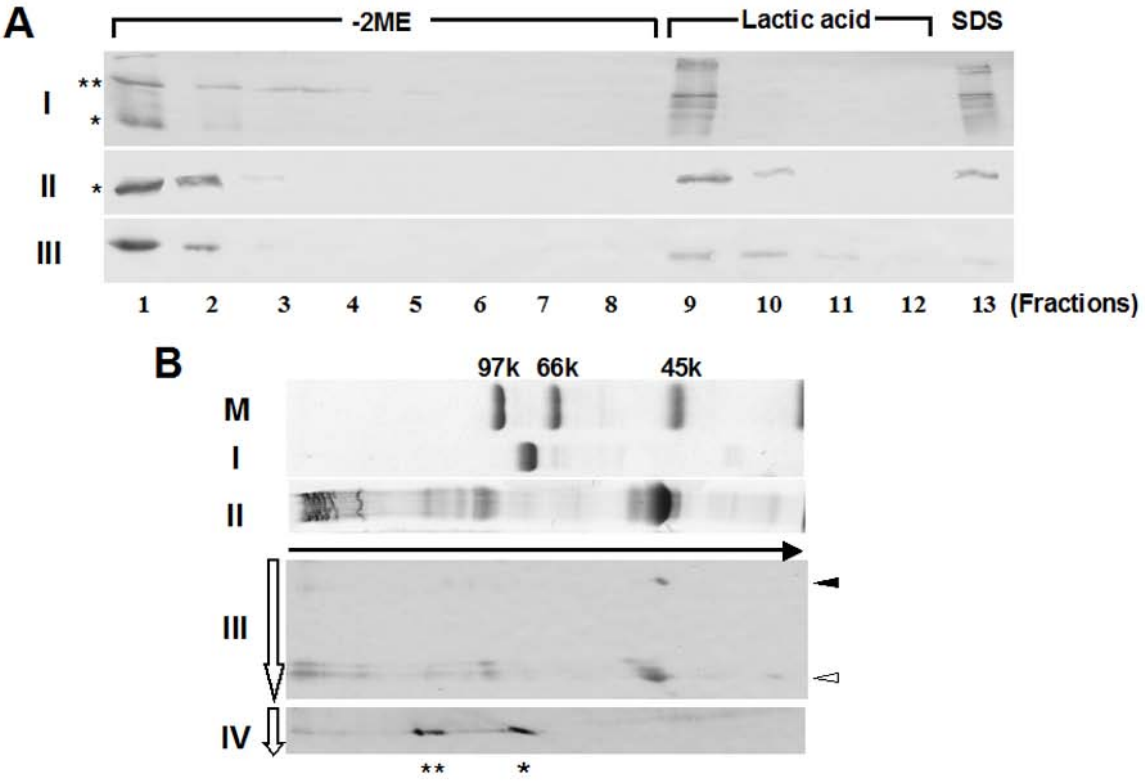
Fig. 5



1

2

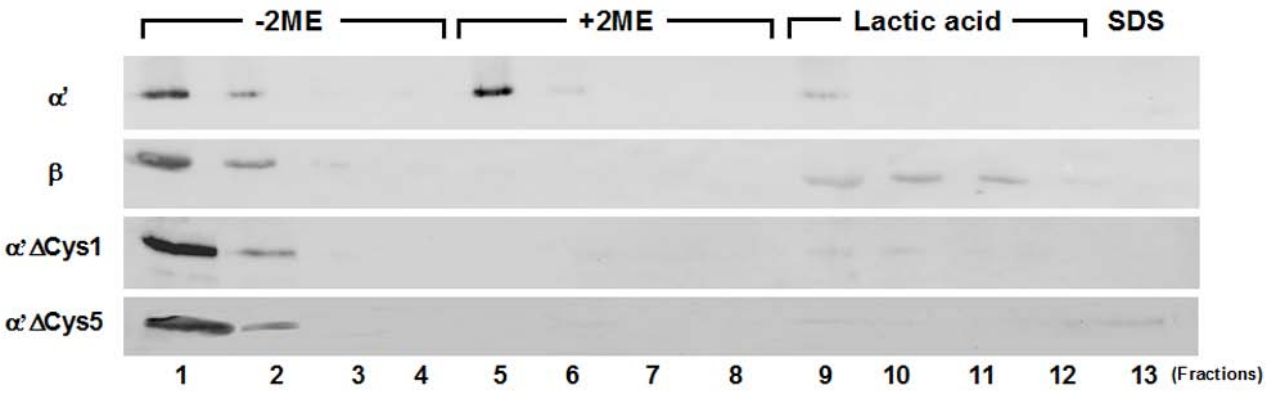
Fig. 6


A
-2ME
Lactic acid
SDS
I
II
III
**
*
*
1 2 3 4 5 6 7 8 9 10 11 12 13 (Fractions)
B
97k 66k 45k
M
I
II
III
IV
**
*

1

2

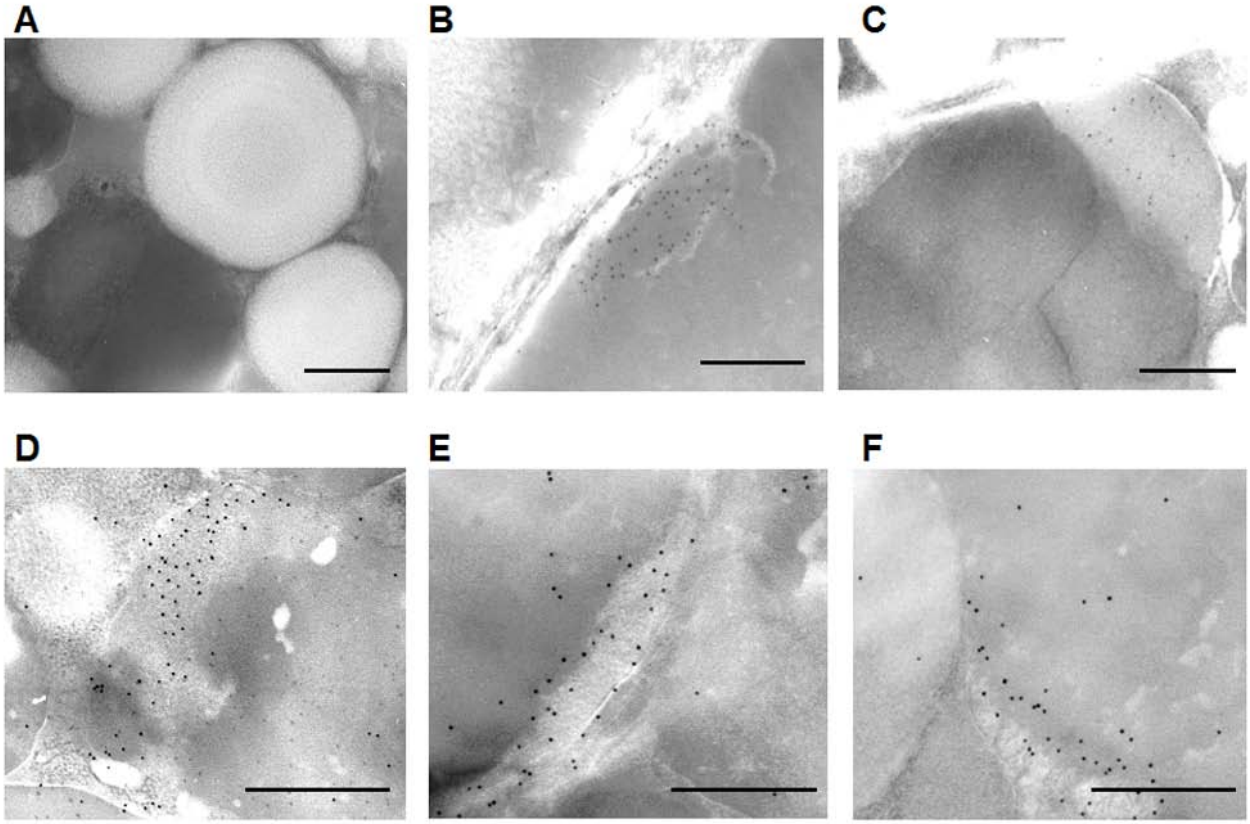
Fig. 7


-2ME
+2ME
Lactic acid
SDS
α'
β
α'ΔCys1
α'ΔCys5
1 2 3 4 5 6 7 8 9 10 11 12 13 (Fractions)

1

2

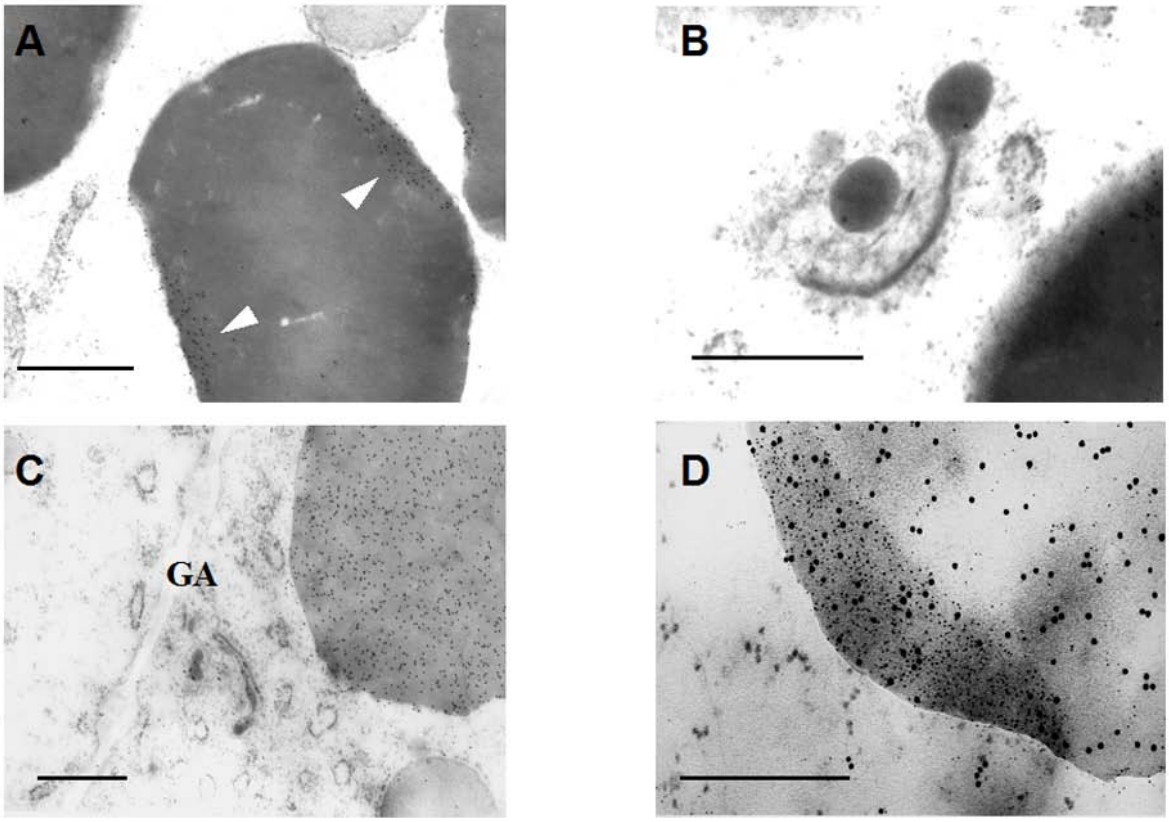
Fig. 8



1

2

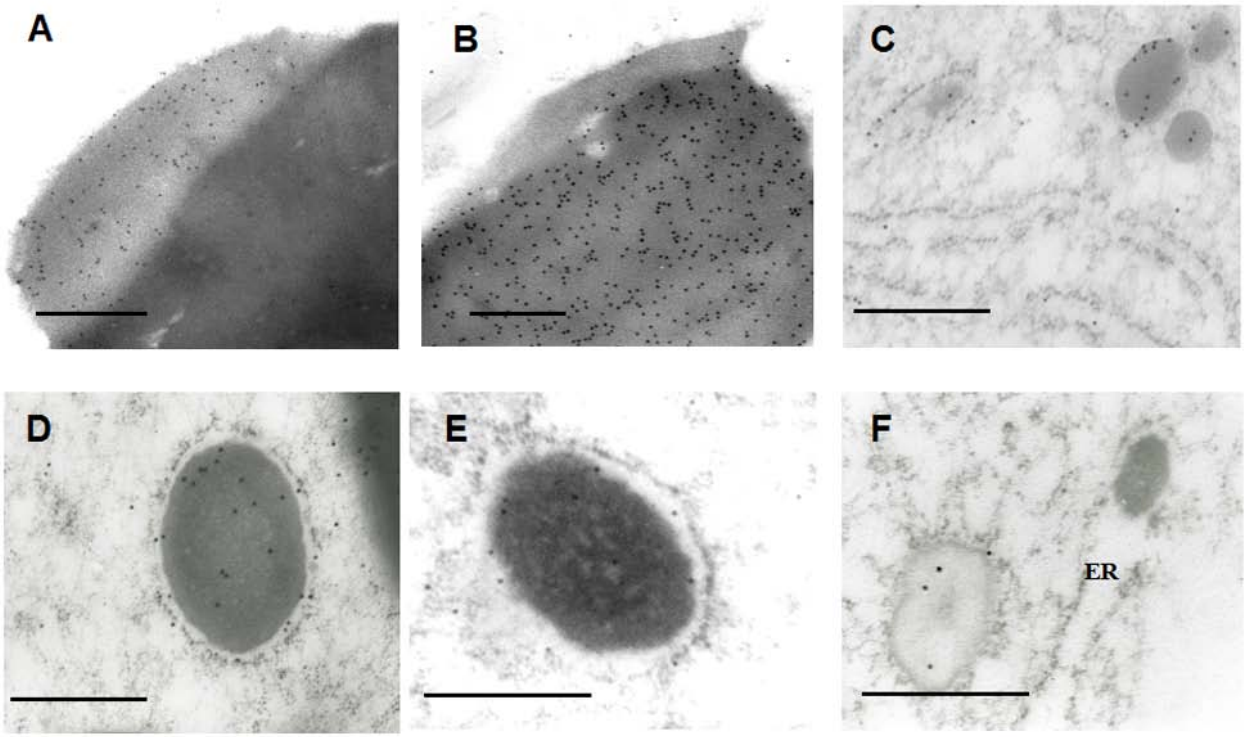
Fig. 9



1

2

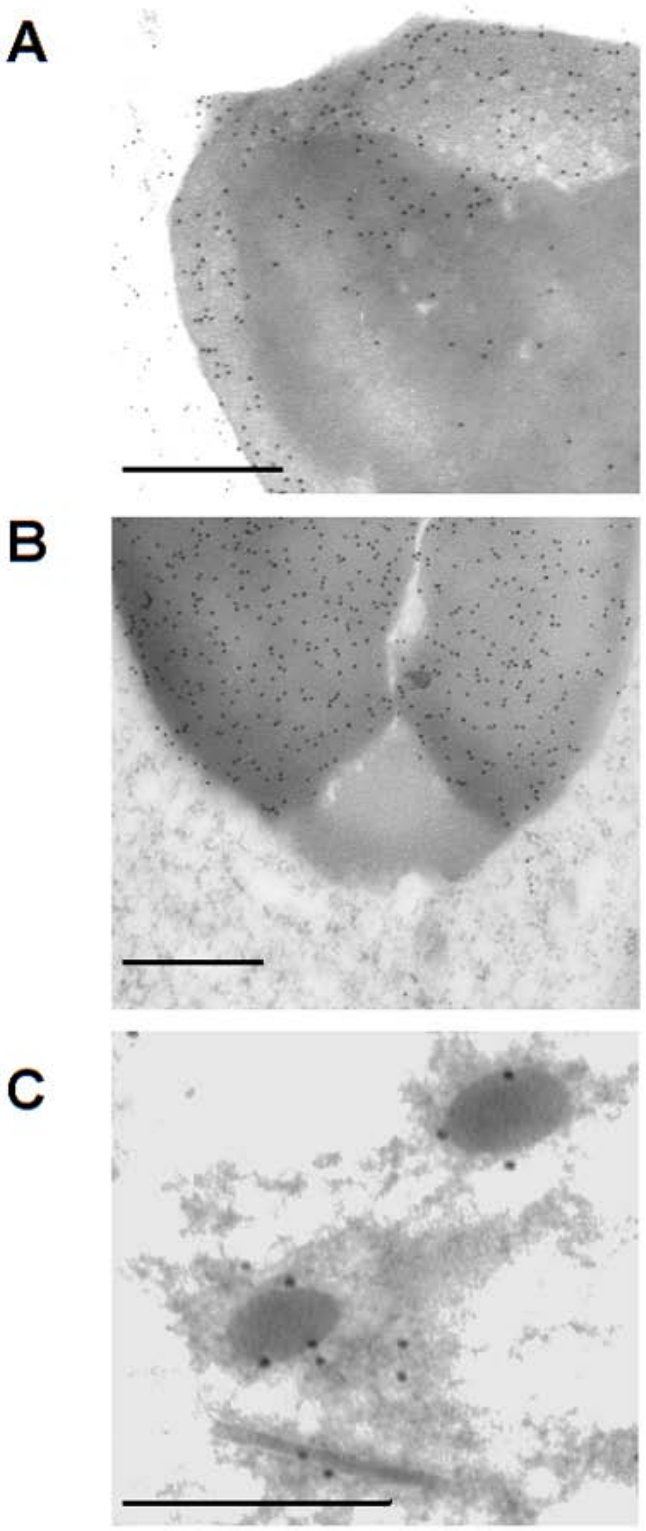
Fig. 10



1

2

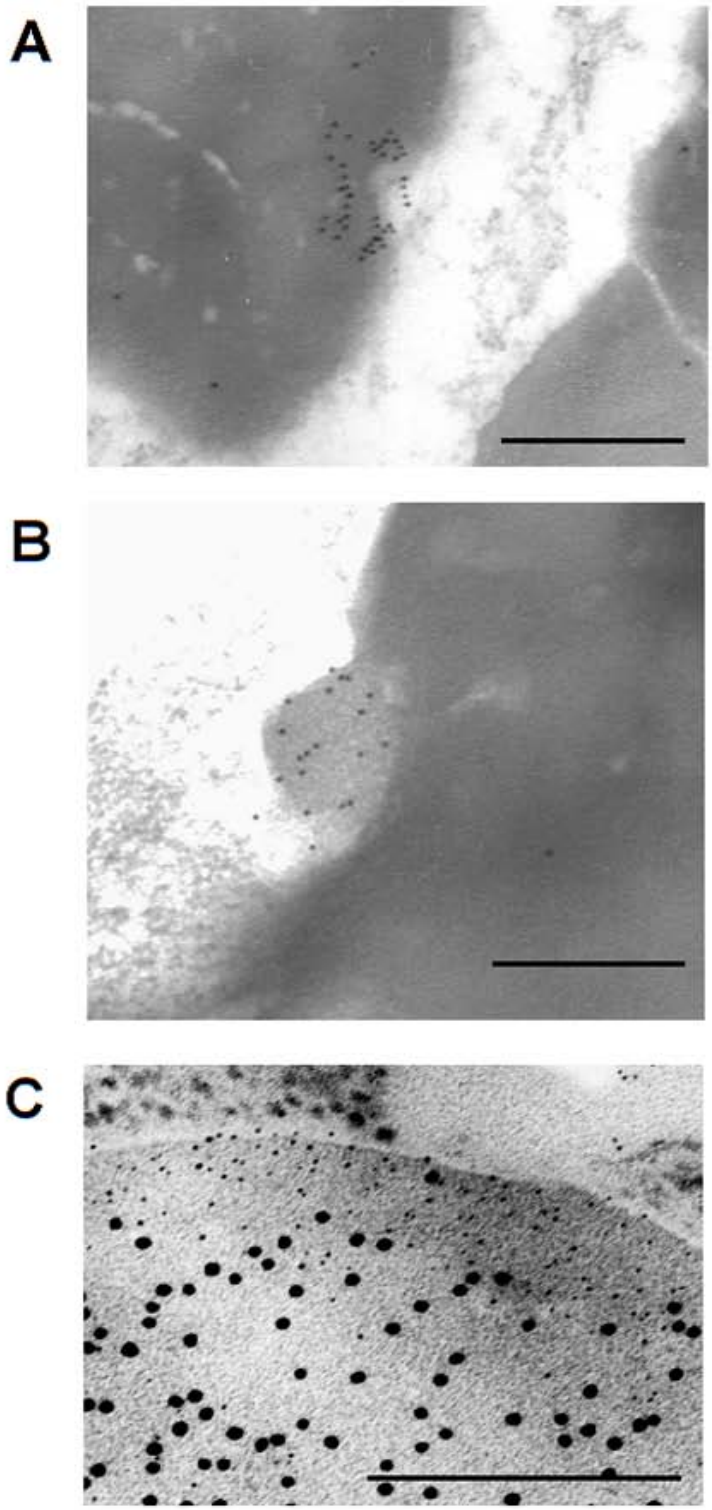
Fig. 11



1

2

Fig. 12



1