*Bot. Bull. Academia Sinica* 27: 147-162 (1986)

# CHARACTERIZATION OF STORAGE PROTEINS IN *INDICA* RICE[1,2]

SHU-CHEN GRACE CHEN and MING-CHIH CHENG

*Institute of Botany, Academia Sinica*
*Nankang, Taipei, Taiwan 11529, Republic of China*

(Received March 13, 1986; Accepted April 18, 1986)

**Abstract**

The storage protein in the endosperm of rice (***Oryza sativa*** L.) has been isolated by sequential extraction into albumin, membraneous protein, globulin, prolamin, glutelin and residual protein. The major storage protein glutelin constituted approximately 80% of total protein and was only solubilized under highly denaturing conditions. The polypeptide components in the solubility fractions of three ***indica*** cultivars, two ***japonica*** cultivars, and a Taiwan wild rice were analyzed by SDS-PAGE. Glutelin was heterogeneous and mainly composed of two polypeptide groups, 21-22 KD and 33-35 KD. The change of polypeptide patterns in endosperm of developing rice (***indica***) at 10, 17, 24, 31 days after flowering were also investigated by SDS-PAGE. The two major polypeptide groups of glutelin from cultivar Taichung Sen 3 (***indica***) were purified by Sephadex G-150 gel filtration and their antisera were raised in rabbits separately. High titers of 8,000 and 10,000 were obtained for anti 21-22 KD and anti 33-35 KD, respectively. Anti 21-22 KD crossreacted slightly with 33-35 KD, 55 KD and 14-17.5 KD polypeptides. Similarly anti 33-35 KD crossreacted slightly with 55 KD, 21-22 KD and 14-17.5 KD polypeptides. By immunoblotting assay, both antibodies were found to strongly crossreact with the proteins from cultivar Taichung Native 1 and Sen 10 (***indica***), cultivar Tainung 67 and Taichung 65 (***japonica***), as well as Taiwan wild rice. These observations suggest high homology in primary structure of glutelin among rice varieties.

**Key words:** Rice storage proteins; glutelin; antisera; immunochemical crossreactivity.

## Introduction

Rice, along with wheat and maize, is one of the three on which the human species largely subsists. Milled rice is the major source of protein in the diets of

[1] Paper No. 308 of the Scientific Journal Series, Institute of Botany, Academia Sinica, Taipei, Taiwan, Republic of China.

[2] This work was supported by research grants from National Science Council of Republic of China and Academia Sinica to Shu-chen Grace Chen.

tropical Asia. The protein content in milled rice varies from approximately 6% to 13% of dry weight among varieties (Juliano, 1972). Most of the storage proteins in the mature rice caryopsis is deposited in the form of protein bodies which begin to appear 7 days after flowering (DAF) in the developing grains (Mitsuda *et al.*, 1967; Del Rosario *et al.*, 1968). Three types of protein bodies (PB I, PB II, and PB III) were found both in normal-protein-content rice and high-protein-content rice endosperms (Harris and Juliano, 1977; Bechtel and Juliano, 1980). The rice protein can be extracted consecutively with water, saline, alcohol and alkali to solubilize albumin, globulin, prolamin and glutelin, respectively (Juliano, 1972; Padhye and Salunkhe, 1979). The major storage proteins of most cereal grains are prolamin and glutelin. However in rice grains, glutelin is the only major protein which constitutes approximately 80% of endoperm protein. Tanaka *et al.* (1980) reported that PB I contained prolamin and PB II which formed faster than PB I (Yamagata *et al.*, 1982) was rich in glutelin and globulin. The storage protein in rice endosperm begins to accumulate about 5 DAF and continued to increase up to 20 DAF (Cagampang *et al.*, 1976; Yamagata *et al.*, 1982). Amino acid analysis of total protein showed a progressive decrease in lysine and threonine among the essential amino acids during grain development.

Several research groups have characterized the polypeptide components of the four solubility fractions from developing and mature rice grains by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Discrepancy in electrophoretic composition of each fraction and estimated molecular size of individual polypeptide are observed (Sawai *et al.*, 1970; Cagampang *et al.*, 1976; Juliano and Boulter, 1976; Mandac and Juliano, 1978; Villareal and Juliano, 1978; Padhye and Salunkhe, 1979; Yamagata *et al.*, 1982; Damardjati *et al.*, 1985). Villareal and Juliano (1978) reported glutelin was majorly composed of three polypeptide subunits with estimated molecular weight of 38, 25 and 16 kilodaltons (KD). However, investigation by Yamagata *et al.* (1982) indicated two polypeptide groups 22 to 23 KD and 37 to 39 KD were the major components of glutelin. Padhye and Salunkhe (1979) reported 29.2, 15.3, 9.9, 9.2, and 7.7 KD as the molecular weights of five major polypeptides of glutelin. This inconsistency could be largely due to variations in the methods employed for protein extraction, fractionation and polyacrylamide gel electrophoresis. Disagreement regarding the molecular size and number of the constituents in the other three solubility fractions were also observed in these studies. Because of the insolubility except in dilute alkali or highly denaturing solvent and the heterogeneity, very little progress has been reported regarding characterization of rice glutelin in recent years.

By a modified extraction method, we fractionated rice endosperm proteins into six fractions in which nature of the constituents were clearly characterized. The varietal differences in electrophoretic composition of rice endosperm proteins were

investigated. We have further purified glutelin subunits from a *indica* rice (*Oryza sativa* L. cv. Taichung Sen 3) and prepared rabbit antibodies directed against glutelin subunits. Investigation on the immunochemical crossreactivity of glutelin among rice varieties was also carried out in this report.

## Materials

Various cultivars of rice (*Oryza sativa* L.) chosen in this study were as follows: *indica* (Sen) rice including cv. Taichung Native 1, Taichung Sen 3 and Taichung Sen 10; *japonica* rice including cv. Tainung 67 and Taichung 65. Taiwan wild rice (*Oryza perrennis* var. *formosana*) was also used. The following chemicals and reagents obtained from the commercial sources were listed below: Tris base, EDTA, Triton X-100, sodium dodecyl sulfate (SDS), acrylamide, N,N′-methylene-bis-acrylamide, ammonium persulfate, ammonium sulfate, bovine plasma albumin, Coomassie Brilliant Blue R-250, bromophenol blue, glycerol, glycine, molecular weight standard kit (SDS-7), sodium chloride (Sigma Chemical Co., USA.); Sephadex G-150 (Pharmacia Fine Chemicals, Sweden); methanol, acetic acid, *n*-propanol, hydrogen peroxide (E. Merck); Coomassie Brilliant Blue G-250 reagent for protein assay, nitrocellulose membrane (0.45 micron), gelatin, Tween-20, goat anti-rabbit IgG conjugated to horseradish peroxidase, and peroxidase color development reagent (Bio-Rad Laboratories, USA).

## Methods

*Extraction of Rice Proteins*

For extraction of total protein from rice seeds, the endosperms were separated from the testae and embryos, then ground to very fine powder. The rice flour was extracted with buffer R (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 2% SDS, 0.6% 2-mercaptoethanol and 4 M urea for two hours at room temperature by shaking vigorously. The protein extracts were collected by centrifugation at 15,000 xg for 20 min. The extraction was repeated three times.

For fractionation of storage proteins from rice seeds, the rice endosperms were also ground into very fine powder and extracted sequentially with five volumes of the following solvents for two hours at room temperature: Buffer R to extract albumin, 1% Triton X-100 (v/v) in buffer R for membraneous protein, 0.5 M NaCl in buffer R for globulin, 60% *n*-propanol (v/v) in buffer R for prolamin, 2% SDS, 0.6% 2-mercaptoethanol in buffer R for glutelin, and 0.1 N NaOH for residual protein.

The protein content in each fraction was estimated by Coomassie Brilliant Blue G-250 binding method developed by Bradford (1976). Bovine plasma albumin was used as standard. Since SDS concentration higher than 0.1% and Triton X-100

concentration higher than 0.1% in the assay mixture will interfere the color development, the protein samples of glutelin or membranous protein fraction containing these interfering substances were always diluted before measurement and the standard curve were made by using the reagent containing these substances.

*Analysis of Rice Protein by SDS-PAGE*

SDS-PAGE was performed according to the method of Laemmli (1970). Polyacrylamide gels (17-cm wide, 14.5-cm long and 0.8-mm thick) composed of 5% stacking and 14% running gels were prepared in a slab gel apparatus. The electrode buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS (pH 8.3). The protein samples were mixed with equal volume of sample buffer containing 125 mM Tris-HCl (pH 6.75), 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 0.01% bromophenol blue and then heated at 100°C for 3 to 5 min before loading. The sample size in each lane was 20 to 25 $\mu$l. The starting current was applied at 7.5 mA until the tracking dye reached the running gel and increased to 25–30 mA. The electrophoresis was completed when the dye reached the bottom of the gel. Bovine serum albumin (66 KD), egg albumin (45 KD), glyceraldehyde-3-phosphate dehydrogenase (36 KD), carbonic anhydrase (29 KD), trypsinogen (24 KD), soybean trypsin inhibitor (20.1 KD), $\alpha$-lactalbumin (14.2 KD) were used as the molecular weight markers. After electrophoresis, the gels were stained with 0.12% (w/v) Coomassie Brilliant Blue R-250, 50% methanol and 10% acetic acid for two hours, then destained in 30% methanol and 10% acetic acid overnight with several changes. The gels were then dried on the filter paper under vacuum with low heat.

*Purification of Glutelin from Cultivar Taichung Sen 3*

The crude glutelin fraction extracted from Taichung Sen 3 was concentrated to 15–20 mg/ml by ultrafiltration using YM-10 membrane with Amicon ultrafiltration cells. Sample of 3 ml (approximately 45 mg protein) was loaded onto a Sephadex G-150 column (26×100 cm) and eluted with 50 mM Tris-HCl, pH 7.5, containing 0.5% SDS and 0.6% 2-mercaptoethanol at the flow rate of 0.4 ml/min. Each fraction of 4 ml was collected and the protein content was followed by measuring optical density at 280 nm. The protein composition of each fraction was checked by SDS-PAGE. The central peak fractions rich in 21–22 KD and 33–35 KD polypeptides (Fig. 4A, 4B) were pooled and concentrated by ultrafiltration. The concentrate was rechromatographed on Sephadex G-150 column (2.6×100 cm) under the same elution conditions described above. A single broad peak was obtained, in which the protein composition of each fraction was analyzed by SDS-PAGE. The beginning and the last several fractions of the peak contained pure 33–35 KD and 21-22 KD respectively were concentrated by ultrafiltration and used for rabbit antibody induction.

*Preparation of 21–22 KD Group and 33–35 KD Group Antibodies*

Proteins of 21–22 KD and 33–35 KD group in 50 mM Tris-HCl, pH 7.5, containing 0.1% SDS and 0.6% 2-mercaptoethanol were emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into rabbits (2.5–3 kg body weight, two in each group) distributed over 5–6 sites on the back. Each rabbit received 0.5 mg protein in the primary injection. Booster injections of 0.5 mg protein without Freund's adjuvant were given one month after first injection and thereafter regularly once every four weeks. Rabbits were bled from the ear vein one to two weeks after each injection. Titers of antisera were determined by enzyme immunoassay on the surface of nitrocellulose membrane. All the procedures were performed at 25°C. The antigens (21–22 KD and 33–35 KD) were spotted on squares of nitrocellulose membrane pretreated with TBS buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl). Each square (0.7×0.7 cm) contained 1 $\mu$g of antigen. The squares were incubated in blocking solution containing 3% gelatin in TBS for one hour with gentle agitation. After washing with TTBS buffer (0.005% Tween-20 in TBS), the squares were incubated with rabbit antisera to be determined of various dilution (from 1:100 to 1:10000) using 1% gelatin-TTBS as diluent. After two hours of gentle agitation, the squares were washed with TTBS to remove unbound antisera and then transferred to solution containing goat anti-rabbit IgG conjugated to horseradish peroxidase (1:3000 dilution). After one hour incubation, nitrocellulose squares were washed with TTBS and TBS sequentially to remove unbound second antibody and Tween-20, then subjected to color development. The color reaction is based on oxidation of 4-chloro-1-naphthol by $H_2O_2$ catalyzed by bound peroxidase. The reaction was terminated after 30 min by washing with water. The antisera of highest titers were purified by two ammonium sulfate precipitations at 33% saturation. The antibodies were then dissolved in 50 mM sodium phosphate buffer (pH 7.0) and stored in aliquots at -70°C.

*Immunoblotting Assay of Rice Proteins*

The method developed by Towbin *et al.* (1979) was employed to transfer separated proteins from SDS-polyacrylamide gel onto nitrocellulose sheet. The slab gel after electrophoresis was soaked in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS, pH 8.3) and equilibrated for 30 min, then subjected to electrophoretic transfer in blotting buffer. The tranfer was completed in 5 h using 40 V and 200 mA maximum. The nitrocellulose sheet was then washed with TBS buffer and subjected to immunological detection using antibody directed against 21–22 KD or 33–35 KD as described above. Anti 21–22 KD and anti 33–35 KD were used at 1:8000 and 1:10,000 dilution, respectively. Goat antirabbit IgG-horseradish peroxidase conjugate was used at 1:3000 dilution. After color development the blots were dried between filter papers and stored protected from light.

## Results

*Fractionation and Electrophoretic Analysis of Rice Endosperm Protein*

The mature rice grains of different varieties including 5 cultivars of Taichung Native 1, Taichung Sen 3 and Taichung Sen 10 (*indica*), Tainung 67 and Taichung 65 (*japonica*) and Taiwan wild rice were chosen to investigate protein content and composition. Sequential extractions with different solvents were employed to fractionate endosperm protein into albumin, membraneous protein, globulin, prolamin, glutelin and residual protein. The bottom line of Table 1 lists the content of total extractable protein (%). Among six varieties examined, cultivar Tainung 67 had the highest content of total protein (9.29%). Taichung Sen 3, Sen 10, Taichung 65 and wild rice contained medium level of protein (8.93–8.98%). Taichung Native 1 had the lowest protein content. Comparing the protein content in each solubility fraction, glutelin constituted approximately 73–82% of total extractable protein and is thus the major storage protein in all the rice varieties in which cv. Taichung Sen 3 was the relatively richest in glutelin (81.31%). The

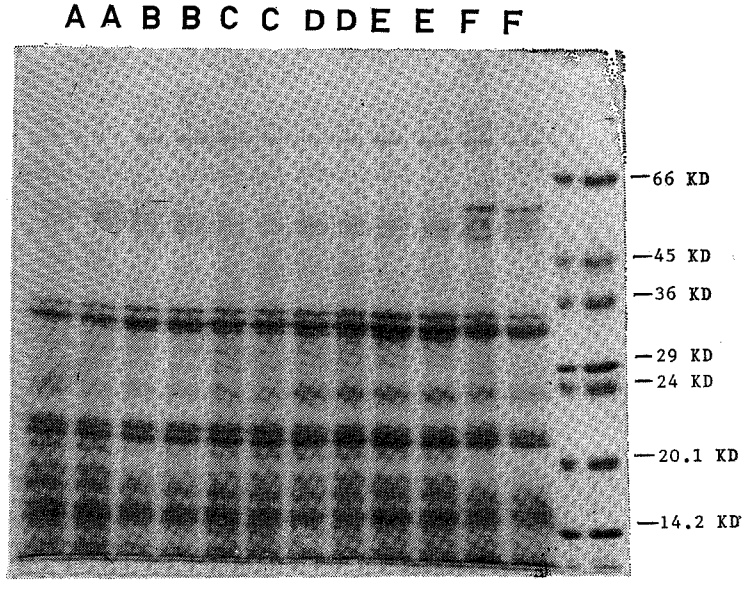


Fig. 1. Polypeptide patterns of total protein in endosperm of six rice varieties.

Endosperms of mature rice grains from six varieties were ground to fine powder and total proteins were extracted as described in Methods. Ten μl (25–30 μg) of extract was mixed with equal volume of sample buffer, heat treated, and separated by SDS-polyacrylamide gel (14%) electrophoresis. Each rice sample was run in duplicate lanes. The separated polypeptides were detected by Coomassie Brillant Blue R-250 staining. Lane A: Taichung Native 1, B: Taichung Sen 3, C: Taichung Sen 10, D: Taichung 65, E: Tainung 67, F: Taiwan wild rice.

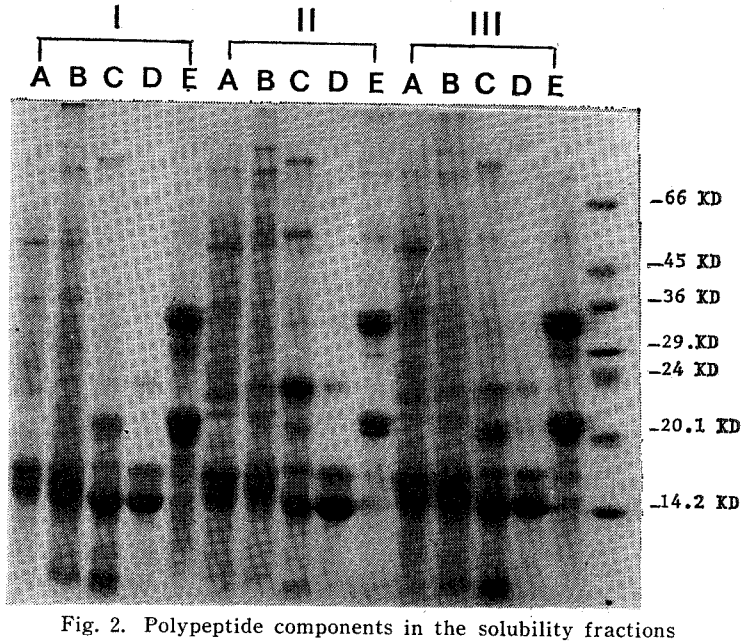


Fig. 2. Polypeptide components in the solubility fractions of three ***indica*** cultivars.

Endosperms of mature rice grains from three cultivars of ***indica*** were subjected to protein extraction into fractions. Approximately 20-30$\mu$g of each protein fraction was used for SDS-PAGE. I: Taichung Native 1, II: Taichung Sen 3, III: Taichung Sen 10. A: Albumin, B: Membraneous protein, C: Globulin, D: Prolamin, E: Glutelin.

**Table 1.** *Protein content in solubility fractions from six rice varieties*

Mature rice grains of six varieties were dehulled and the embryos were removed. After grinding into very fine powder, the rice flour was step-wise extracted into six solubility fractions as described in Methods. The protein content in each fraction was estimated by Coomassie Brilliant Blue G-250 binding Assay. The total extractable protein of each variety was the summation of the six solubility fractions.

| Fraction | Variety | | | | | |
|---|---|---|---|---|---|---|
| | Taichung Native 1 | Taichung Sen 3 | Taichung Sen 10 | Taichung 65 | Tainung 67 | Taiwan wild rice |
| | (% of total protein) | | | | | |
| Albumin | 3.58 | 3.62 | 3.84 | 4.08 | 4.22 | 4.58 |
| Membraneous protein | 2.18 | 2.01 | 2.54 | 3.13 | 3.68 | 3.12 |
| Globulin | 4.25 | 3.70 | 3.93 | 4.98 | 4.99 | 6.60 |
| Prolamin | 2.09 | 1.43 | 1.64 | 1.53 | 1.54 | 1.57 |
| Glutelin | 78.00 | 81.31 | 80.15 | 78.84 | 79.44 | 73.04 |
| Residual protein | 9.90 | 7.93 | 7.90 | 7.44 | 6.13 | 11.09 |
| | (g protein/100 g endosperms) | | | | | |
| Total protein | 7.52 | 8.93 | 8.98 | 8.94 | 9.29 | 8.97 |

protein content of albumin, membraneous protein, globulin and prolamin ranged from 1.5% to 6.6%.

To examine the polypeptide patterns of total protein from six rice varieties, SDS-PAGE was employed. The overall polypeptide compositions in six varieties were essentially similar, with major polypeptide bands at 33-35 KD, 21-22 KD, 14-16 KD, less intense bands at 81 KD, 50-55 KD, 23.7 KD, 17.5 KD, and some minor discrete bands as well as diffuse bands (Fig. 1). The relative intensity of the bands varied slightly among varieties. Some of the bands were unique in certain variety, such as 58 KD present only distinctly in wild rice, 18.8 KD present in Taichung Native 1, Sen 10, Taichung 65 and Tainung 67 but not in Taichung Sen 3 or wild rice. The 30 KD band was absent in wild rice.

Figure 2 shows the electrophoretic composition of five solubility fractions extracted from three cultivars of *indica* rice. Albumin comprised about 3.5 to 3.8% of total protein (Table 1), which revealed weak diffuse bands distributed from 81 KD to 14 KD. The major polypeptides of albumin moved electrophoretically in the region of 14.5-17 KD. Membraneous protein comprised 2-2.5% of total protein and electrophoretically distributed from larger than 100 KD to smaller than 10 KD,

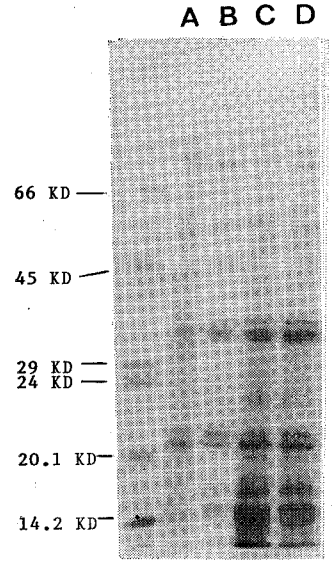


Fig. 3. Change of polypeptide components in rice endosperm during grain development.

The developing seeds of cv. Taichung Sen 3 were harvested at the desired times after flowering. The total protein in the immature starchy endosperm was extracted and analyzed by SDS-PAGE. The amount of sample loaded on each lane was equivalent to total protein extracted from 400 $\mu$g of endosperm at different developing stages. Lane A: 10 DAF, B: 17 DAF, C: 24 DAF, D: 31 DAF.

in which the major components also moved in 14.5–17 KD region. Globulin comprised 3.7–4.2% of total protein and was rich in polypeptides smaller than 24 KD, especially polypeptides of 14.5 KD and 21 KD. Prolamin is composed of 14.5 KD and 21 KD major polypeptides and 23.7 KD minor polypeptide, which constituted only 1.4–2.1% of total protein. Glutelin, the principal storage protein, consisted of two major polypeptide groups 21–22 KD and 33–35 KD as well as some minor bands.

The change of polypeptide components in rice endosperm during grain development of *indica* rice was studied. The total protein in the starchy endosperm at 10, 17, 24, and 31 days after flowering was extracted and analyzed by SDS-PAGE. Fig. 3 indicates that the amount of 81 KD polypeptide remained fairly constant at different stages of grain development. The amount of 50–55 KD polypeptides varied slightly. The accumulation of 33–35 KD and 21–22 KD which belong to glutelin increased rapidly from 10 DAF to 31 DAF. The accumulation of globulin and prolamin, i.e., polypeptides smaller than 21 KD was relatively low at 10 DAF but increased rapidly after 17 DAF.

*Purification of Glutelin and Antibody Preparation*

Because Taichung Sen 3 had the highest content of the major storage protein glutelin in the six varieties examined, this cultivar was adopted for further investigation.

Crude glutelin was prepared in large quantity from Taichung Sen 3 and purified by preparative Sephadex G-150 column. Fig. 4A shows the protein profile of effluent from the column. Three continuous protein peaks were obtained and each fraction was subjected to SDS-PAGE to examine the polypeptide composition. The two major polypeptide groups 21-22 KD and 33-35 KD were eluted in the central peak and thus combined for further purification. The third peak is majorly composed of 14.5 KD polypeptide which was originally contaminated from globulin or prolamin fraction (Fig. 4B). Although further purification on second Sephadex G-150 column did not widely separate 21–22 KD from 33–35 KD group but exhibited a single broad protein peak, the first and the last two fractions of the peak contained highly purified subgroups and therefore were collected for antibody preparation (Fig. 4C).

The antibodies against 21–22 KD group and 33–35 KD group were raised in two rabbits of each group, respectively. Since antigens 21–22 KD and 33–35 KD are only soluble in highly denaturing solvent such as buffer containing SDS or urea, standard immunodiffusion or immunotitration utilized for antiserum titration cannot be used. A very sensitive enzyme immunoassay after antigens were immobilized on nitrocellulose membrane was adopted for this purpose. The assay based on the following principle: Protein antigen is immobilized on nitrocellulose membrane and then reacts with rabbit antiserum in question of various dilution, followed by

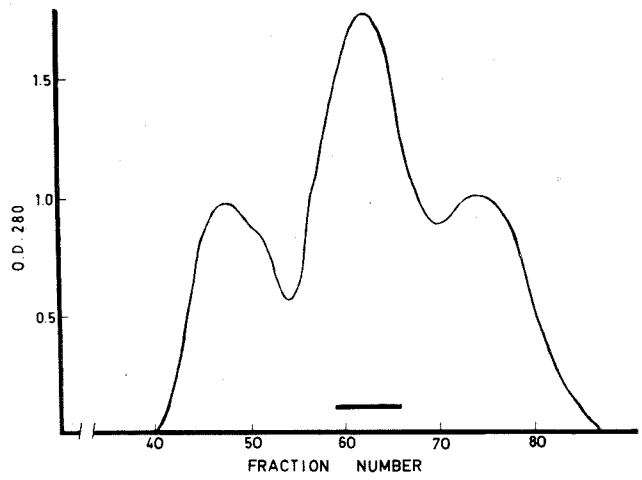


Fig. 4A. Preparative Sephadex G-150 column chromatography of rice glutelin.

Crude glutelin (45 mg in 3 ml) extracted from Taichung Sen 3 was loaded onto Sephadex G-150 (2.6 × 100 cm) column and eluted by 50 mM Tris-HCl (pH 7.5) containing 0.5% SDS and 0.6% 2-mercaptoethanol at the flow rate of 0.4 ml/min. Fractions of 4 ml each were collected. The fractions (No. 59-66) enriched in 21-22 KD and 33-35 KD polypeptides marked by horizontal black bar were combined and concentrated by ultrafiltration.

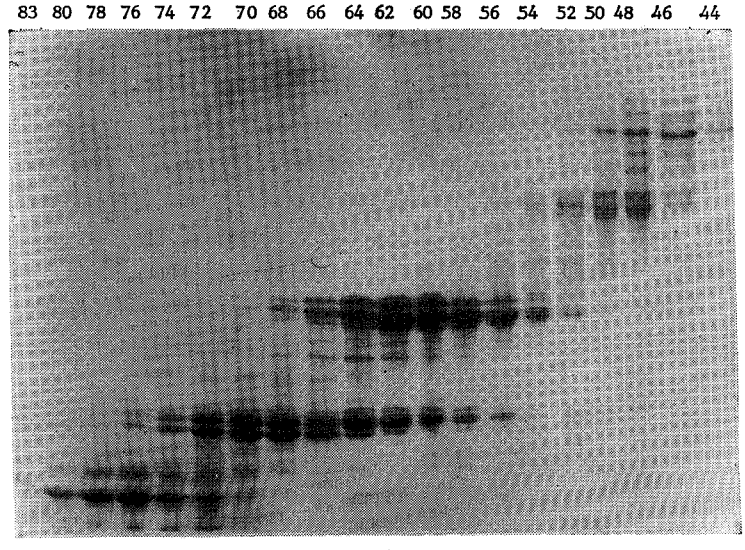


Fig. 4B. SDS-PAGE of effluent from first Sephadex G-150 column described in Fig. 4A.

Twenty μl of each fraction was used for gel electrophoresis and stained with Coomassie Brilliant Blue R-250. The number on top of each lane indicated the fraction number.

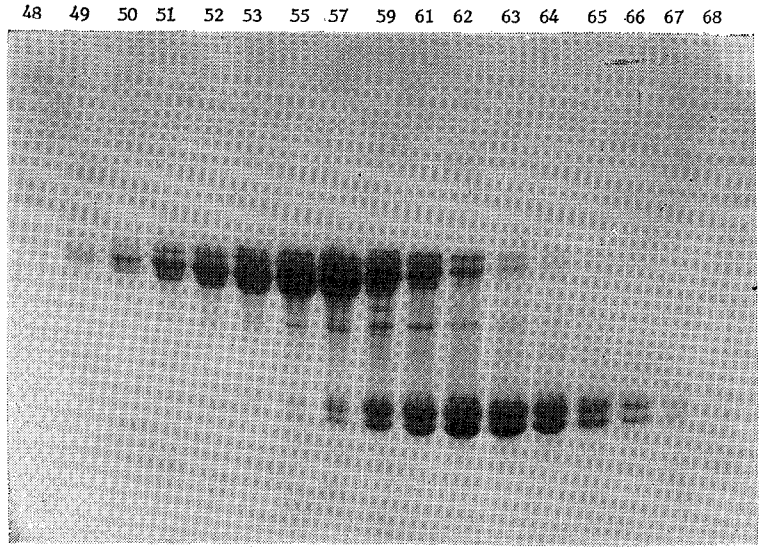


Fig. 4C. SDS-PAGE of effluent from second Sephadex G-150 column.

The concentrate of combined fractions 59-66 (3.5 ml) from first Sephadex G-150 column was rechromatographed on Sephadex G-150 column (2.6×100cm) under the same conditions as described in Fig. 4A. Each fraction was checked by SDS-PAGE as in Fig. 4B. Fractions 49-50 containing highly pure 33-35 KD polypeptides and fractions 66-68 containing highly pure 21-22 KD polypeptides were concentrated and utilized for antibody induction.

**Table 2.** *Titers of rabbit antisera directed against glutelin polypeptides 21-22 KD and 33-35 KD*

Purified 21-22 KD group and 33-35 KD group from cv. Taichung Sen 3 were emulsified with equal volume of Freund's adjuvant and injected subcutaneously into two rabbits in each group. Primary injections (0.5 mg) were followed by three booster injections (0.5 mg) as described in Methods. Titers of antisera were determined by enzyme immunoassay. The titers shown in the Table are defined as the highest reciprocals of antiserum dilution required to give positive color reaction. Rabbit No. 2 died during the experimental period.

| Group | Days after First Immunization | | | |
|---|---|---|---|---|
| | 45 | 72 | 92 | 102 |
| Anti 21-22 KD | | | | |
| Rabbit (1) | 1,000 | 4,000 | 8,000 | 8,000 |
| Rabbit (2) | 200 | 2,000 | — | — |
| Anti 33-35 KD | | | | |
| Rabbit (3) | 1,000 | 4,000 | 8,000 | 10,000 |
| Rabbit (4) | 400 | 3,000 | 8,000 | 8,000 |

reacting with second antibody conjugated to peroxidase. The antigen-antibody complex is then visualized by the color produced by the enzyme reaction. Table 2 shows three of the four rabbits developed very good antibodies except rabbit No. 2 of 21-22 KD group died sometimes after second injection. The antiserum against 21-22 KD of rabbit No. 1 reached a maximum titer of 8,000 after third booster injection. The antisera against 33-35 KD in rabbit No. 3 and No. 4 also attained maximum titer of 10,000 and 8,000 respectively 102 days after immunization. The antisera from the three rabbits were thus further purified by three ammonium sulfate precipitation.

*Immunochemical Reaction between Storage Proteins from Six Rice Varieties*

Total protein from mature endosperms of six rice varieties including Taichung Native 1, Taichung Sen 3, Taichung Sen 10, Taichung 65, Tainung 67 and Taiwan

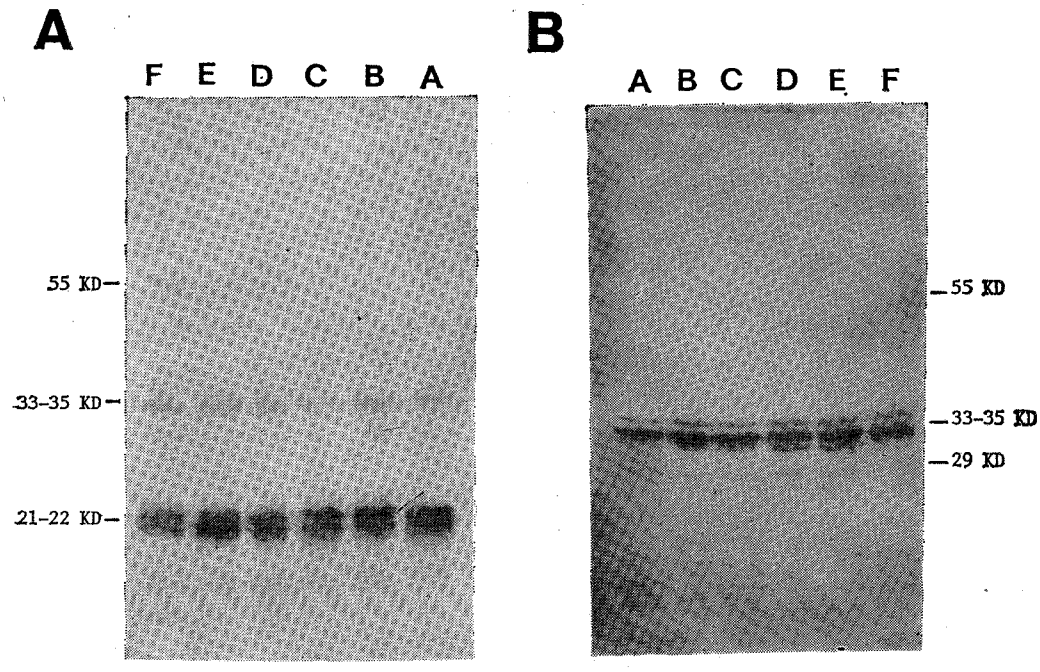


Fig. 5. Immunochemical reactions of antibodies directed against glutelin subfraction 21-22 KD and 33-35 KD with total proteins from six rice varieties.

Total storage proteins extracted from rice endosperms of six varieties were electrophoretically separated by SDS-PAGE in triplicate. Two of slab gels were blotted to nitrocellulose sheets. Immunochemical reactions were performed as in Methods. Fig. 5A was the blot incubated with antibody against 21-22 KD of Taichung Sen 3 (1:8000 dilution). Fig. 5B was the blot incubated with antibody against 33-35 KD of Taichung Sen 3 (1:10000 dilution). A: Taichung Native 1, B: Taichung Sen 3, C: Taichung Sen 10, D: Taichung 65, E: Tainung 67, F: Wild rice.

wild rice were extracted and separated on SDS-polyacrylamide gels in triplicate. The separated polypeptides on two of slab gels were electrophoretically transferred to nitrocellulose sheets and incubated with antibodies directed against 21–22 KD (1:8000 dilution) and 33–35 KD (1:10000 dilution) of Taichung Sen 3, respectively. The positive immunochemical reactions were visualized by the color produced by peroxidase reaction as described in Methods. The third duplicate gels was stained with Coomassie Brilliant Blue R-250. The polypeptide patterns of six varieties were the same as shown in Fig. 1. The results in track B of Fig. 5A and Fig. 5B indicate anti 21–22 KD at 1:8000 dilution crossreacted slightly with group 33–35 KD, 14–17.5 KD and 55 KD. Anti 33–35 KD at 1:10000 dilution crossreacted slightly with 29 KD and 14–17.5 KD. Both Fig. 5A and 5B show that antisera directed against glutelin subfraction 21–22 KD and 33–35 KD of Taichung Sen 3 strongly crossreacted with the corresponding proteins from wild rice and four other cultivars. These observations suggest the structural similarity of rice glutelin among varieties.

## Discussion

The rice proteins were ordinarily extracted on the basis of solubility into albumin, globulin, prolamin and glutelin (Juliano, 1972; Padhye and Salunkhe, 1979; Damardjati *et al.*, 1985). In this study, a modified sequential extraction method has been employed to extract protein from rice endosperm into six fractions. Buffer R containing 1% Triton X-100 was utilized to solubilize membraneous protein before saline extraction and Buffer R containg 2% SDS and 0.6% 2-mercaptoethanol was used to extract glutelin. The residual protein was finally extracted by 0.1 N NaOH. This method is more efficient in extracting rice protein and polypeptide composition in each fraction can be easily analyzed by SDS-PAGE. Table 1 lists the protein content in each fraction extracted from Taiwan wild rice, three *indica* cultivars and two *japonica* cultivars. The total extracted protein was the summation of the six fractions from each rice variety. The protein content in each fraction is basically consistent with the reports in literature with slight variation. Several extraction solvents have been tested to extract total protein from rice endosperm by one-step procedure. Tris buffer (pH 7.5) containing 2% SDS, 0.6% 2-mercaptoethanol and 4M urea turned out to be most adequate in term of extraction efficiency. However the yield of total protein by one-step procedure is still less than that by sequential extractions. The electrophoregram of total protein indicates the overall similarity of polypeptide patterns among rice varieties. Three typical polypeptide groups at 33–35 KD, 21–22 KD and 14–16 KD regions can be easily seen in the six varieties examined. Some unique protein bands were observed in certain variety, such as 58 KD polypeptide in wild rice (Fig. 1).

The electrophoregram of solubility fractions from three cultivars of *indica* rice demonstrated the detailed polypeptide composition in each fraction (Fig. 2). The major components of albumin appeard in 14.5-17 KD region, membraneous protein also in 14.5-17 KD region, globulin at 21 KD, 14.5 KD and 6 KD, prolamin at 14.5 KD, 17.5 KD and 23.7 KD, glutelin at 21-22 KD and 33-35 KD. The number and estimated molecular size of individual polypeptide of each fraction differ from the reports by Cagampang *et al.* (1976), Juliano and Boulter (1976), Mandac and Juliano (1978) and Yamagata *et al.* (1982). The inconsistency is apparently caused by different extraction and fractionation procedures employed by the investigators. For instance, one of the components 16 KD present in glutelin fraction reported by Juliano and Boulter (1976) is possibly 14.5 KD polypeptide of prolamin or globulin fraction (see lane C. D. E on Fig. 2) which has not been completely removed by saline or alcohol extraction and thus appeared in glutelin fraction.

The change of polypeptide components of *indica* endosperm during grain developmemt was examined from 10 DAF to 31 DAF. The continuous increases in accumulation of glutelin components 21-22 KD and 33-35 KD groups were parallel throughout the period and almost reached maximum at 31 DAF. The synthesis of globulin and prolamin was slower than that of glutelin, but increased rapidly during the period of 17 DAF to 24 DAF. The amounts of polypeptides 81 KD and 55 KD, components of globulin, remained fairly constant throughout the grain development. The polypeptide 55 KD is quite likely the 57 KD polypeptide designated by Yamagata *et al.* (1982), which is thought to be a precursor of glutelin subgroups 21-22 KD and 33-35 KD (Yamagata *et al.*, 1982). The biosynthesis and accumulation of rice glutelin seem similar to legumin synthesis in pea cotyledon where the precursor 60 KD is processed into 20 KD and 40 KD (Evans *et al.*, 1979; Croy *et al.*, 1980)

Crude glutelin from a cultivar of *indica* rice has been purified by two preparative Sephadex G-150 columns and pure 21-22 KD and 33-35 KD groups were obtained. The two polypeptide groups are heterogenous. More than four polypeptides and at least three polypeptides with very close electrophoretic mobilities are present in 33-35 KD and 21-22 KD group, respectively. Many efforts have been made to purify individual polypeptide from the two groups by high pressure liquid chromatography using gel filtration and reverse phase columns. However unsatisfactory results were obtained so far.

The immunochemical studies demonstrates that rabbit antibody raised against 21-22 KD crossreacts slightly with 33-35 KD, 14-17.5 KD, 55 KD polypeptides and anti 33-35 KD antibody crossreacts slightly with 29 KD and 14-17.5 KD polypeptides which are hardly seen on the photograph (Track B of Fig. 4A and 4B). At lower antibody dilution (1:8,000), anti 33-35 KD was clearly seen to crossreact with 21-22 KD and 55 KD (data not shown). These observations indicate partial homology among the primary structures of 55 KD, 33-35 KD, 21-22 KD and 14-17.5 KD

polypeptides. This observation is in agreement with the finding that 55 KD is a precursor of 21-22 KD and 33-35 KD.

The strong immunochemical crossreactions of antibodies against 21-22 KD and 33-35 KD of cultivar Sen 3 with the corresponding proteins isolated from other two cultivars of *indica*, two cultivars of *japonica*, and a Taiwan wild rice suggest high extent of structural similarity of glutelin among rice varieties. We have also investigated the immunochemical reaction of anti 21-22 KD and anti 33-35 KD with total protein extracted from four cereals maize, wheat, barley and millet by immunoblotting procedure. The primary results showed no crossreaction observed between rice glutelin and other cereal proteins. Prat *et al.* (1985) have reported several homologous sequence of maize glutelin, barley hordein, and wheat gliadin, suggesting these proteins belong to the same evolutionary family. Therefore, it is quite possible that rice glutelin has independently evolved and adopted the function of major storage protein in rice endosperm.

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# 籼稻種子貯藏性蛋白質的特性

陳 淑 真　鄭 明 琪

中央研究院植物研究所

利用連續性抽取法可把水稻種子胚乳貯藏性蛋白質依溶解度不同分離成六個部份，包括：albumin，膜蛋白質，globulin，prolamin，glutelin，以及殘留性蛋白質。Glutelin 爲主要的貯藏性蛋白質，約佔稻米胚乳總蛋白質的80%，必須用極强的變性溶劑來抽取。SDS 膠板電泳法可分析不同溶解度蛋白質抽出物內所含的多胜肽鏈組成。此類分析包括三種籼稻，二種粳稻及臺灣野生稻。Glutelin 爲非均質蛋白質，主要含有二個多胜肽羣 21～22 KD 以及 33～35 KD。本報告亦利用 SDS 膠板電泳分析籼稻開花後不同成熟時期胚乳蛋白質組成的變化。臺中籼三號 glutelin 之二個主要多胜肽羣已用重覆的 Sephadex G-150 柱體層析分離出來。它們的兎子抗體血清已成功的製備出來。抗 21～22 KD 之血清力價爲 8,000，抗 33～35 KD 之血清力價爲 10,000。抗 21～22 KD 之血清會低程度地與 33～35 KD，55 KD，14～17.5 KD 等多胜肽發生交叉免疫反應。相同的，抗 33～35 KD 血清會低程度地與 21～22 KD，55 KD 及 14～17.5 KD 等發生交叉反應。利用電泳轉移及酵素免疫測定，可測知臺中籼三號之抗 21～22 KD 血清以及抗 33～35 KD 血清會與臺中再來一號，臺中籼十號，臺農67號，臺中65號，以及臺灣野生稻之 glutelin 發生極强的交叉免疫反應。此結果表示不同品種的稻米，胚乳 glutelin 有極相似的蛋白質一級結構。