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Purification and some properties of storage proteins in *japonica* rice

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Abstract. Protein content of rice seeds from *Oryza sativa* L. *japonica* cv. Tainung 67 was 9.6% on a dry weight basis. Fractionation of rice proteins yielded albumin, globulin, prolamin and glutelin in the proportions of 1.6:9.6:5.5:83.3, respectively. The two major subunits of glutelin, acidic and basic polypeptides, were purified by Sephadex G-150 gel filtration and DEAE-fractogel chromatography under denaturing conditions. Antisera against the purified glutelin have been raised. The acidic polypeptides had molecular weight range of 28.5-35.0 kilodaltons and isoelectric point of 5.4-7.3. The basic polypeptides had molecular weight range of 17.8-19.2 kilodaltons and isoelectric point of 8.5-9.3. Both acidic and basic polypeptides appeared as early as 3 days after flowering, and the largest accumulation of these polypeptides occurred between 9 and 12 days after flowering.

Key words: Glutelin; *Oryza sativa*; Storage protein.

Introduction

Rice in contrast to other cereals exhibit a lower content of prolamins and a higher content of glutelins. Nearly 80% of rice storage proteins is glutelins, while prolamins only reach 8% of seed proteins (Pernollet and Mossé, 1983). Rice glutelins consist of a heterogeneous collection of polypeptides with the major components of 29-39 kDa (acidic subunit or α-subunit) and 20-23 kDa (basic subunit or β-subunit) (Yamagata *et al.*, 1982a; Luthe, 1983; Wen and Luthe, 1985; Zhao *et al.*, 1983). Yamagata *et al.* demonstrated that these two subunits were formed through post-translational cleavage of 57 kDa polypeptide (Yamata *et al.*, 1982a; Yamata *et al.*, 1982b). Zhao *et al.* (1983) showed that the amino acid sequence of a portion of the glutelin basic subunit is homologous to that of the β-subunit of pea legumin.

More recently, the complete amino acid sequence of rice glutelin precursor was deduced from the corresponding cDNA (Takaiwa *et al.*, 1986). The glutelin cDNA clones could be divided into two classes by restriction site polymorphism and sequence (Takaiwa *et al.*, 1987a). A gene encoding rice glutelin was isolated and its nucleotide sequence was determined. The cloned gene contains three short introns and codes for a prepro-glutelin protein of 499 amino acids identical with that deduced from the type II glutelin cDNA (Takaiwa *et al.*, 1987b).

Oryza sativa L. *japonica* cv. Tainung 67 is the most popularly cultivated subspecies of rice in Taiwan. Studies on the storage proteins of this subspecies at the molecular level would provide sound basis for further investigation on the

structure and function of the genes encoding these proteins. We report in this paper the purification and partial characterization of glutelins from this *japonica* cultivar and induction of antibodies against these glutelins.

Materials and Methods

Materials

Rice (*Oryza sativa* L. *japonica* cv. Tainung 67) was grown in a paddy at the Experimental Farm of the Institute of Botany, Academia Sinica. Developing seeds were harvested at the desired times after flowering and stored at −70°C before use. The following were purchased from commercial sources: dithiothreitol, sodium dodecyl sulfate, ammonium persulfate, phenylmethylsulfofluoride, N, N′-methylene-Bis-acrylamide, N, N, N′, N′-tetramethylene diamine (TEMED), Nonidet P-40 and 4-vinylpyridine (Sigma); urea, tris (hydroxymethyl) aminomethane, glycerol, β-mercaptoethanol, Fractogel TSK DEAE-650 (M), phenol reagent, sucrose (Merck); EDTA-Na_2, acrylamide, NaCl, acetone, methanol, sulfosalicylic acid, ethylacetate, acetic acid, formaldehyde (Wako); molecular weight calibration kits, pI marker kits, Sephadex G-150 and polybuffer exchanger (Pharmacia); nitrocellulose membrane, immunoblot assay kits and the dye-binding reagent for protein measurement (Bio-Rad); and Ampholine (LKB).

Extraction of Rice Storage Proteins

Rice seeds were dehulled and ground into 80 mesh flour with rice dehuller and blender. Total seed protein was extracted by stirring the flour (0.1 g flour/1 ml buffer) for 1 hr at room temperature in 4 M urea, 2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol and 5% (v/v) β-mercaptoethanol buffer. The protein extracts were collected by centrifugation with Sigma 2-MK centrifuge at 12,000 rpm for 20 min.

The rice flour was defatted by stirring in 2 volumes of ethylether for 2 h at room temperature, filtered through a Buchner funnel, and air-dried overnight. For fractionation of rice storage proteins, albumin, globulin, prolamin, and glutelin were sequentially extracted from defatted rice flour according to the method of Luthe (1983). Each fraction was dialyzed against distilled water, lyophilized, and stored at −20°C. The protein content of each fraction was determined by the Bradford method (1976). Nitrogen content was determined by the Kjeldahl method, converted to protein content using a conversion factor of 6.31 (Horwitz, 1970) and the protein content was expressed as percentage of the sample dry weight.

Glutelin Purification

The crude glutelin was purified by chromatography on a DEAE-Fractogel column (2.6×10 cm) equilibrated with a starting buffer containing 6 M urea, 2 mM carbonate buffer (pH 10.0). The crude glutelin (60 mg) was applied to the column. After washing with the start buffer, the column was eluted with a linear gradient (10×bed volume) of 0-1.0 M NaCl in the start buffer. The flow rate was 1 ml per min and fractions of 3.4 ml were collected. A_{280} of each fraction was measured. The polypeptide composition of the fractions was analyzed by SDS-PAGE. Fractions showing negligible contaminating proteins were pooled, dialyzed against sterilized water, lyophilized, and used for rabbit antibody induction.

Separation and Purification of Glutelin Acidic (α) and Basic (β) Subunits

Twenty mg of crude glutelin in 1 ml of the buffer containing 0.5% (w/v) SDS, 50 mM Tris-HCl (pH 8.5), and 50 mM β-mercaptoethanol was applied to a Sephadex G-150 column and eluted with the same buffer. The flow rate was 1.5 ml per h and the effluent was collected in 0.7 ml fractions. The polypeptide composition of the fractions was determined by SDS-PAGE. The fractions rich in acidic or basic subunits were pooled and concentrated. The concentrated acidic or basic subunits were

rechromatographed on Sephadex G-150 column under the same conditions. By rechromatography on the Sephadex G-150 column, electrophoretically homogeneous acidic and basic subunits were obtained.

Electrophoresis

Protein samples were analyzed by electrophoresis in SDS-polyacrylamide slab gel, consisting of a 5 to 20% (w/v) acrylamide gradient gel with the discontinuous buffer system as described by Laemmli (1970). High and low molecular weight standard protein kits were used as the markers. After electrophoresis, the gels were stained with 0.15% (w/v) Coomassie Brilliant Blue R-250, 12% (w/v) TCA, 3.6% (w/v) sulfosalicylic acid, and 30% (v/v) methanol for 2 h and destained with ethylacetate-acetic acid-ethanol-water (10:5:7:78, v/v) solution. Gels were dried by the method of Juang *et al.* (1984).

Isoelectric focusing was run using a horizontal LKB 2117 Multiphor System. Gels (26.5×12.5×0.2 cm) contained 4.9% acrylamide (29.1:0.9 weight ratio of acrylamide : bis), 6 M urea, 11.4% (v/v) glycerol, 5.2% (v/v) LKB Ampholines (0.3% pH 4-6.5, 0.3% pH 5-8, 0.6% pH 9-11, and 4% pH 3.5-10), 0.07% TEMED and 0.024% ammonium persulfate. Samples were dissolved in lysis buffer (O'Farrell *et al.*, 1977), absorbed into filter paper strips, and applied to the gel surface near the anode wick. The anode wick was soaked in 1 M H_3PO_4 and the cathode wick in 1 M NaOH. Electrophoresis was conducted at 30 W constant power at 5°C for 2 h. A pI marker kit (Pharmacia Fine Chemicals) was used as standard. After electrophoresis, the pH gradient of the gel was measured using a surface pH electrode (Broadley-James Co.) before the gel was fixed and stained.

Preparation of Antisera against Glutelins

Four New Zealand white rabbits designated as YT, YH, YHN and YN were used for raising antibodies against the purified total glutelins. YT and YH received a solubilized antigen without adjuvant in which glutelins were dissolved in 50 mM sodium phosphate buffer (pH 8.6) containing 6 M urea. 1.5 mg protein each was used for the first and second injections, and 1 mg each for the third and fourth injections. Intervals between injections were 3 weeks. YHN and YN were injected with a suspension antigen in which glutelins were suspended in 50 mM sodium phosphate buffer (pH 8.2). One mg of protein in 10% $KAl(SO_4)_2 \cdot 12H_2O$ was given in the initial injection. One mg protein with an equal volume of Freund's complete adjuvant and 0.6 mg protein without the adjuvant were given in the second and third booster injections respectively at one month interval.

Rabbits were bled from carotid artery one month after the final injection. The blood clot was centrifuged for 10 minutes at 3000 xg at 4°C, the clear serum was removed, lyophilized and stored at −70°C.

Immuno-blot Assay

The antiserum titers were determined by indirect method of enzyme immunoassay according to the method described in the Bio-Rad instruction manual with modifications. Two μg antigen was applied to the center of 1×1 cm square nitrocellulose membrane prewetted with TBS buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.5). After the squares were immersed in blocking solution (3% gelatin in TBS) for one hour, and washed with TTBS buffer (0.05% Tween-20 in TBS) twice for 5 min each, the squares were reacted with different concentrations of anti-glutelin sera (called first antibody) in antibody buffer (1% gelatin in TTBS buffer) for 2 hours. After washing with TTBS and TBS for 5 minutes each, the squares were incubated with the fresh HRP color development solution (0.05% 4-chloro-1-naphthol, 0.0015% H_2O_2, 0.17% methanol in 100 ml TBS) at 37°C for 2 hours. The reaction was terminated by washing with water for 10 minutes.

Western-blot analysis was performed as follows:

antigens were resolved with SDS-PAGE and the slab gel was equilibrated with trans-blotting buffer (0.1% SDS, 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 30 min. Trans-blot was performed as previously described (Burnette, 1981) and immuno-blot assay was carried out as described above.

Results and Discussion

Protein Content of Rice

Crude protein content of mature rice grains of different varieties including eight cultivars of *japonica* and six cultivars of *indica* were analyzed (Table 1). The protein content of rice was in the range of 7-16%. In general, *indica* rice has higher protein content than *japonica* rice. Since we are interested in the protein formation in the *japonica* rice cv. Tainung 67, the protein of this variety was further fractionated according to solubility. The protein content in each solubility fraction is: albumin, 1.61%; globulin, 9.55%; prolamin, 5.48%; and glutelin, 83.36%. These results were similar to those previously described (Larkins, 1981). The polypeptide patterns of the four solubility fractions are shown in Fig. 1. There are two major protein bands (around 18 kDa and 32 kDa) and two minor protein bands (around 14 kDa and 56 kDa) in the glutelin fraction. The two major proteins were identified as basic and acidic subunits in comparison with previous reports. The 56 kDa protein is presumably the precursor of acidic and basic subunits (Yamata *et al.*, 1982a; Yamata *et al.*, 1982b). The 14 kDa protein had mobility similar to those of the major proteins of prolamin fraction.

Table 1. *Crude protein content of rice**

Oryza sativa	Variety	Crude protein (%)
Japonica	Tainung 62	6.98
	Caloro	8.38
	Taichung 189	9.00
	Taichung 65	9.16
	Hsinchu 65	9.56
	Tainung 67	9.64
	Tainung 65	9.76
	Sha-tiao-tsao	10.35
Indica	Zenith	6.89
	NP-125	10.11
	Taichung Native 1	10.80
	Raminad Str. 3	15.11
	Dular	15.77
	Usen	15.79

* Determined by Kjeldahl method.
Crude protein (%) = %N × 6.31 (Horwitz, 1970)

Purification of Glutelin and its Subunits

The crude glutelin obtained from solubility fractionation was chromatographed on a DEAE-Fractogel column (2.6×10 cm). Three protein peaks designated I, II and III were eluted from the column (Fig. 2). Polypeptide components of these three peaks were analyzed by SDS-PAGE (Fig. 3). Peak II was shown to consist of almost only, if not exclusively, the acidic and basic subunits of glutelin. This fraction was thus used as antigen for induction of antibodies.

For separation of glutelin subunits, the crude glutelin of the solubility fraction was chromatographed on Sephadex G-150 column (1.0×93 cm).

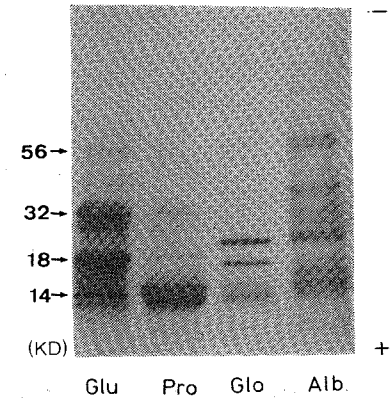

Fig. 1. SDS-polyacrylamide gel electrophoresis of albumin, globulin, prolamin and glutelin fractions of rice. Lanes are designated as follows: Alb, albumin; Glo, globulin; Pro, prolamin; Glu, glutelin.

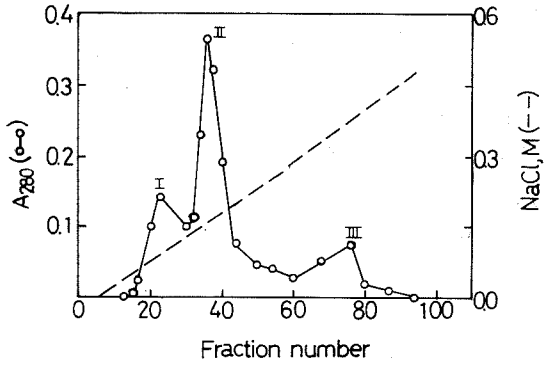

Fig. 2. Purification of rice glutelin by DEAE anion exchange chromatography. Glutelin (60 mg) was applied to DEAE-fractogel column (2.6 × 10 cm). After equilibrated with 6 M urea, 2 mM carbonate buffer (pH 10.0), the column was eluted with a linear gradient (10 × bed volumn) of 0-1.0 M NaCl in the equilibration buffer. Fractions of 3.4 ml were collected.

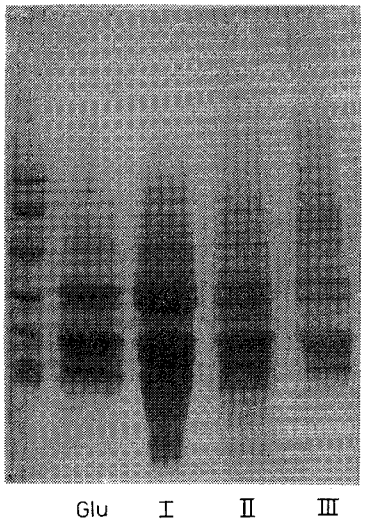

Fig. 3. SDS-polyacrylamide gel electrophoresis of peak I, II, and III fractions from the DEAE-fractogel column. (see Fig. 2).

Three protein peaks appeared in the elution profile (Fig. 4). Aliquots of some fractions were taken from these peaks and analyzed by SDS-PAGE (Fig. 5). It was found that the first peak contained the precursor of glutelin subunits and some other high molecular weight proteins. The second peak was predominatingly the acidic subunit. While fractions from the front of the third peak

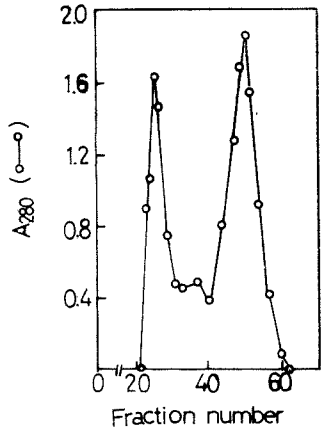

Fig. 4. Purification of rice glutelin by gel filtration. Glutelin (20 mg) was applied to a Sephadex G-150 column (1.0 × 93 cm). The column was eluted with 0.5% (w/v) SDS, 50 mM Tris-HCl (pH 8.5), and 50 mM β-mercaptoethanol buffer and fractions of 0.7 ml were collected.

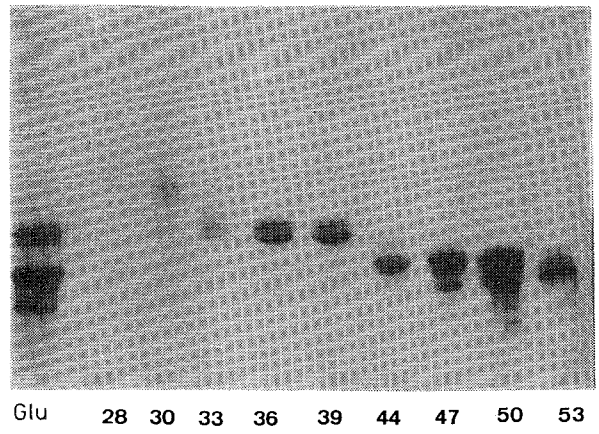

Fig. 5. SDS-polyacrylamide gel electrophoresis of glutelin and selected fractions from the Sephadex G-150 gel filtration described in Fig. 4. Glu: glutelin.

were mainly the basic subunit and fractions from the back of the peak contained both the basic subunit and prolamin. Fractions of the second and third peaks were pooled and rechromatographed individually on the same Sephadex G-150 column. The elution profile of acidic subunit is shown in Fig. 6 and protein components of some fractions from the peak were examined by SDS-PAGE (Fig. 7). It appeared that fractions 30 to 42 were

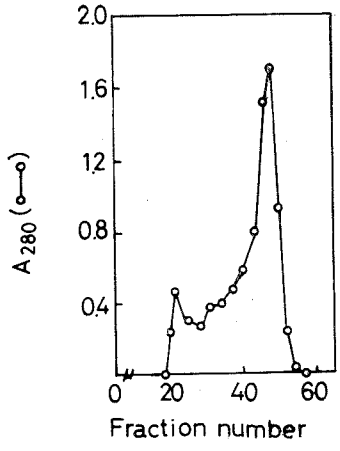

Fig. 6. The second separation of acidic subunit enriched fractions on Sephadex G-150 column. Experimental conditions were essentially the same as those described in Fig. 4. Fractions 33-40 from Fig. 4 was applied.

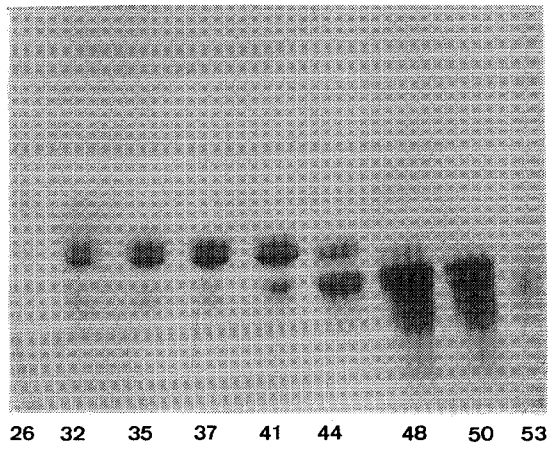

Fig. 7. SDS-polyacrylamide gel electrophoresis of selected fractions from the Sephadex G-150 gel filtration described in Fig. 6.

composed of mainly the acidic subunit and small amount of basic subunit. Rechromatography of these fractions on the same column yielded highly purified acidic subunit in the second peak and practically pure basic subunit from the front of the third peak (Fig. 8). Electrophoretically homogeneous acidic subunit was obtained by rechromatography of the second peak fractions of Fig. 8 on the same column (data not shown).

Fractions 43-50 obtained from the Sephadex G-150 column (Fig. 4) were rechromatographed on the same column (Fig. 9). Purities of the fractions were analyzed by SDS-PAGE. It appeared that fractions from the front of the major peak were mainly composed of the basic subunit and that from the back were predominatingly prolamin (data not

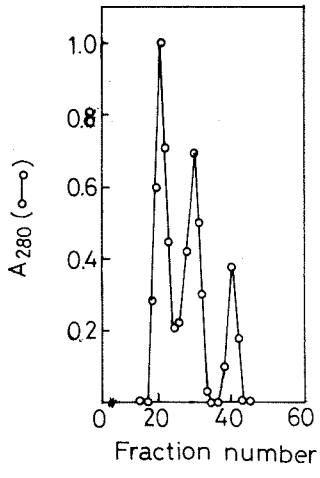

Fig. 8. The third separation of acidic subunit enriched fractions on Sephadex G-150 column. Experimental conditions were essentially the same as those described in Fig. 4. Fractions 30-42 from Fig. 6 was applied.

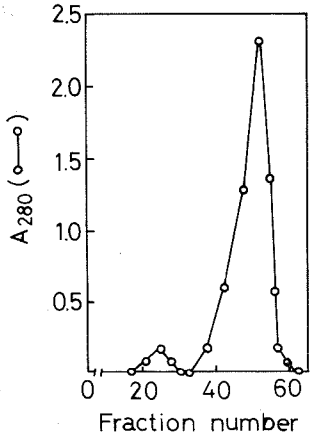

Fig. 9. The second separation of basic subunit enriched fractions on Sephadex G-150 column. Experimental conditions were essentially the same as those described in Fig. 4. Fractions 43-50 from Fig. 4 was applied.

shown). Electrophoretically homogeneous basic subunit was obtained by 70% ethanol extraction to remove prolamin from the major peak in Fig. 9 followed by rechromatography of the 70% ethanol insoluble proteins on the Sephadex G-150 column (data not shown).

Molecular Weight

The molecular weights of acidic and basic subunits were 28.5-35.0 and 17.8-19.2 kilodaltons, respectively, as determined by SDS-PAGE (5-20%) gradient gel electrophoresis (Figs. 10 and 11). These figures are similar to that deduced from glutelin cDNA sequence in which the acidic subunit has Mr 32,489 and basic subunit has Mr 19,587 (Takaiwa, 1986).

Isoelectric Points

Isoelectrofocusing analysis showed that the acidic subunits had pI in the range 5.4 to 7.3 and the basic subunits had pI 8.5-9.3 (Figs. 12 and 13). Our results are somewhat different from those

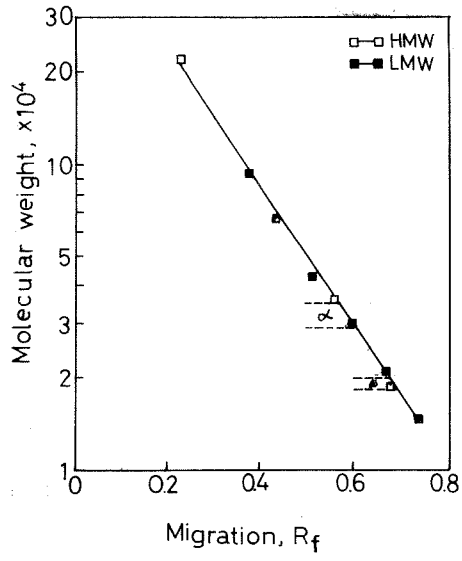

Fig. 11. Standard curve for molecular weight determination by SDS-PAGE on linear gradient (5-20%) polyacrylamide gels.
(□-□ High molecular weight markers)
(■-■ Low molecular weight markers)

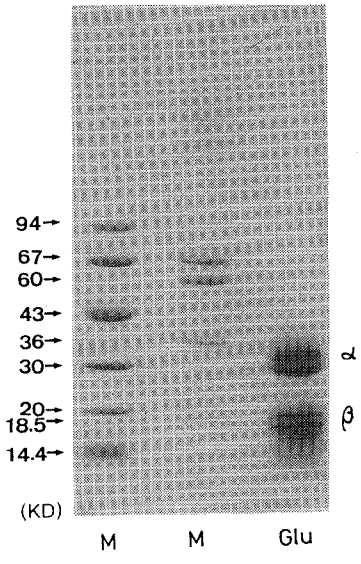

Fig. 10. SDS-polyacrylamide gel electrophoresis of purified rice glutelin and molecular weight markers. Numbers in the left-hand margin refer to the molecular weights in kilodaltons of the marker polypeptides. M: molecular weight marker, Glu: glutelin.

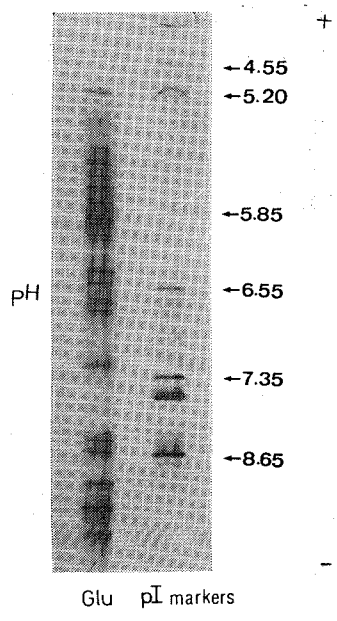

Fig. 12. Horizontal isoelectric focusing of purified glutelin and pI markers. Numbers in the right-hand margin refer to the pI of the marker polypeptides. Glu: glutelin.

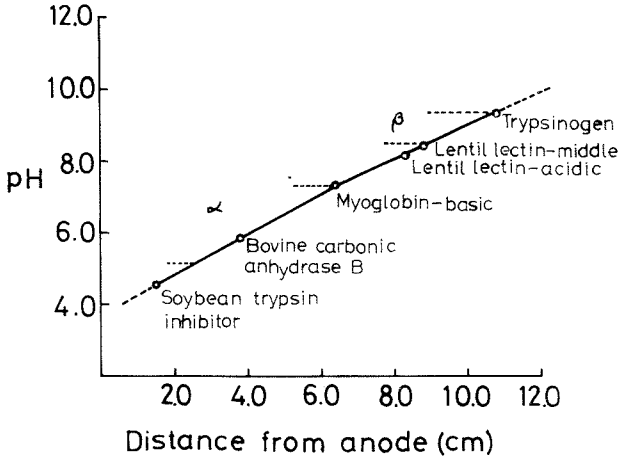

Fig. 13. Standard curve for isoelectric point determination by IEF slab gel electrophoresis (pH 3.5-10).

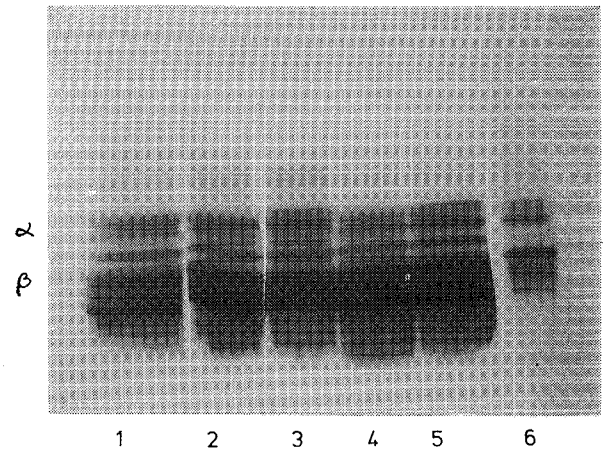

Fig. 14. SDS-polyacrylamide gel electrophoresis of seed proteins extracted from different species of rice*. Lanes are designated as follows: (1) Usen, (2) Zenith, (3) Sha-tiao-tsao, (4) Taichung Native 1, (5) Tainung 67, and (6) antigen.
*The rice seeds had been stored at 4°C for two years.

reported by Wen and Luthe (1985) in which the acidic subunits have pI 6.5-7.5 and the basic subunits have pI 9.4 to 10.3. The discrepancy may be due to the different rice varieties. Another possibility is that different degrees of *in vivo* post-translational modification might occur (Takaiwa *et al.*, 1987).

Preparation of Antisera

The fractions 30-52 of peak II obtained from the DEAE-fractogel column (Fig. 2) were used as the antigen. Titers of four antisera analyzed by dot immuno-blot assay revealed that the dilution factor was: YHN, 1×10^5; YN, 2.5×10^4; YT, 5.0×10^4; and YH, 7.5×10^4.

The polypeptide patterns of total seed proteins from different rice species (Usen, Zenith, Sha-tiao-tsao, Taichung Native 1, and Tainung 67) were analyzed by SDS-PAGE. The overall polypeptide compositions in the five species were similar (Fig. 14). Western-blot analysis indicated that the antiserum of cv. Tainung 67 glutelin crossreacted with all of the acidic and basic subunits of the 5 rice species (Fig. 15), indicating that there was high degree of homology among the primary structures of different rice glutelins. The weaker crossreaction of the acidic subunits with the antiserum (Fig. 15) reflected the much less content of acidic subunits than the basic subunits in the samples (Fig. 14).

Biosynthesis of Rice Storage Proteins

In order to examine the time course of the formation of rice storage proteins, rice panicles were harvested at desired times after flowering, total seed proteins were extracted and analyzed by SDS-PAGE. Gels stained with Coomassie Brilliant Blue R-250 indicated that seed proteins appeared as early as 3 days after flowering (DAF) and the largest accumulation of polypeptides occurred after 9 DAF (Fig. 16). It was noted that the polypeptide patterns in Fig. 16 are different from those in Fig. 14. The former had more high molecular weight polypeptides than the latter. One explanation for such difference in polypeptide patterns is that high molecular weight polypeptides could be gradually degraded into smaller ones during prolonged storage of rice seeds. In fact, the seeds used for the analyzes shown in Fig. 14 had been stored at 5-10°C for about 2 years, while those used for the analyzes shown in Fig. 16 had been stored at −70°C for 8 months.

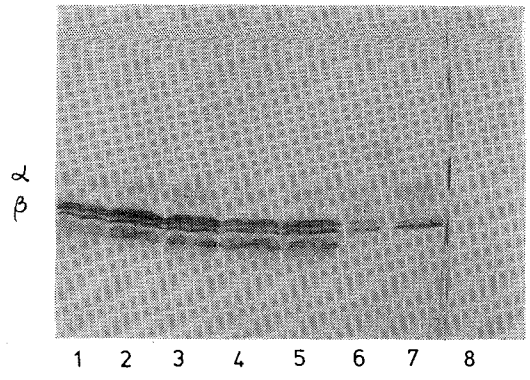

Fig. 15. Western-blot analysis of glutelin from seeds of different rice species. Lanes are designated as follows: (1) Usen, (2) Zenith, (3) Sha-tiao-tsao, (4) Taichung Native 1, (5) Tainung 67, (6) antigen, (7) crude glutelin, (8) negative control. 10^4 dilution of anti-glutelin serum was used.

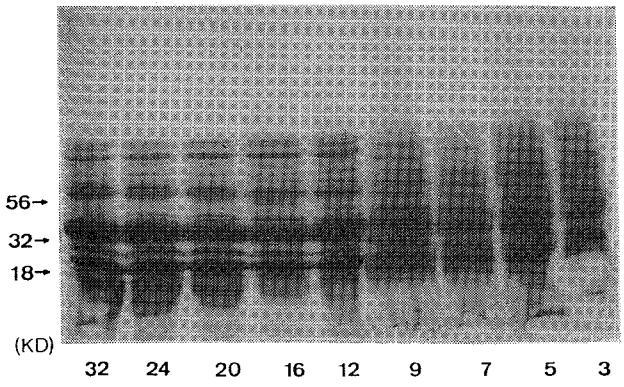

Fig. 16. SDS-polyacrylamide gel electrophoresis of proteins extracted from developing rice seeds*. The numbers on the abscissa indicate days after flowering. The numbers on the ordinate indicate M_r. *The rice grains had been stored at -70°C for eight months.

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粳稻貯存性蛋白質的純化與性質

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水稻臺農67號之種子蛋白質含量以乾重計算約9.6%。其中白蛋白（albumin），球蛋白（globulin），醇溶蛋白（prolamin）及穀蛋白（glutelin）的含量百分比是 1.6：9.6：5.5：83.3。穀蛋白是由一羣微酸性多肽（亦稱爲 α 多肽）及一羣鹼性多肽（亦稱爲 β 多肽）所組成。利用 Sephadex G-150 膠體過濾法及 DEAE 陰離子管柱層析法可將兩多肽分離與純化。以 DEAE 陰離子管柱層析純化的穀蛋白爲抗原，已製備抗體。酸性多肽之分子量爲 28,500～35,000，等電點在 5.4～7.3 之間；鹼性多肽的分子量爲 17,800～19,200，等電點在 8.5～9.3 之間。酸性和鹼性多肽，都在水稻開花後第三天即開始合成，而在開花後 9～12 天之間爲蛋白質合成最旺盛期。