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BIOCHEMICAL AND IMMUNOLOGICAL PROPERTIES OF GLUTELIN FROM *INDICA* RICE[1,2]

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Abstract

The major storage protein glutelin has been purified into two subunit groups 33-35 kDa (acidic) and 21-22 kDa (basic) from ***indica*** rice cv. Taichung Sen 3. Their heterogeneities in charge and molecular weight were analyzed by nonequilibrium pH gradient electrophoresis and two-dimensional gel electrophoresis. The amino acid compositions of both subunits were determined. The immunochemical relationships between rice glutelin and seed proteins extracted from cereals (oat, wheat, barley, maize, sorghum, and millet) and legumes (pea, soybean, mungbean) were investigated by Western blot analysis using the specific antibodies directed against the two denatured rice glutelin subunits. The results suggest high degree of structural similarity in rice glutelin and oat storage globulin. However, there is lack of obvious antigenic homology between rice glutelin and other seed proteins from the tested cereals and legumes.

Key words: Rice glutelin; seed proteins; immunochemical crossreactivity.

Introduction

Rice glutelin, the major storage protein in the starchy endosperm, consists of disulfide-linked basic (19-22 kDa) and acidic (30-36 kDa) subunit groups (Zhao *et al.*, 1983; Wen and Luthe, 1985; Robert *et al.*, 1985). Comparison of N-terminal partial sequences between oat 12S globulin β-subunit and rice glutelin basic subunit suggests 60% of homology (Robert *et al.*, 1985). A cDNA clone of rice glutelin precursor (56.2 kDa) has been obtained recently by Takaiwa *et al.* (1986), which showed 37-38% of sequence homology to those of pea legumin and soybean glycinin.

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However antibodies raised against pea legumin has been reported not able to react with rice glutelin (Zhao *et al.*, 1983). Immunological crossreactivity was observed between oat, wheat and pea globulins (Fabijanski *et al.*, 1985).

Recently, we have purified both subunit groups from *indica* rice and the antisera against the two subunits have been raised successfully in rabbits (Chen and Cheng, 1986). In order to elucidate the immunological relationships between rice glutelin, cereal and legume seed proteins, we utilized the antibodies against rice glutelin subunits to investigate their crossreactions with total proteins extracted from mature seeds of cereals and legumes by Western blot analysis. The chemical properties including charge and molecular weight heterogeneities of glutelin subunits were also characterized by nonequilibrium pH gradient electrophoresis (NEPHGE), isoelectrofocusing (IEF), and two-dimensional gel electrophoresis in this report.

Materials and Methods

Mature dry seeds of cereals including rice (*Oryza sativa* cv. Taichung Sen 3), oat (*Avena sativa*, Taita selected 1), millet (*Setaria italica*, Taitung selected 5), sorghum (*Sorghum vulgare*, Taichung 5), maize (*Zea mays*, Tainan 11), wheat (*Triticum aestivum*, Taichung 31), barley (*Hordeum vulgare*, Nongyuan 2) and mature seeds of legumes including soybean (*Glycine max*, Hualein 1), pea (*Pisum sativum*, Japanese USUI) and mungbean (*Vigna radiata*, VC 1628 A) were used. Goat anti-rabbit IgG conjugated to horseradish peroxidase, nitrocellulose membrane, peroxidase color development reagent and carrier ampholytes (pH 3-10, 5-7, 6-8, 7-9, 8-10) were purchased from Bio-Rad Laboratories, USA. Acrylamide, N, N'-methylene-bis-acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), Nonidet P-40 (NP-40), urea, 2-mercaptoethanol and Coomassie Blue R-250 were obtained from Sigma Chemical Co., USA.

Purification of Rice Glutelin Subunits and Preparation of Antibodies

Glutelin of rice cultivar Taichung Sen 3 was purified into two polypeptide groups of 21-22 kDa (basic) and 33-35 kDa (acidic) by Sephadex G-150 gel filtration as previously described (Chen and Cheng, 1986). The corresponding antisera against glutelin subunits were raised separately in rabbits by four monthly injections of fully denatured glutelin subunits mixed in Freund's adjuvant. The antibodies were purified by two ammonium sulfate precipitations at 33% saturation.

Analysis of Amino Acid Composition

The samples of rice glutelin subunits were hydrolyzed in 6N HCl at 110°C for

24 h. The hydrolysates were then analyzed with Beckman 6300 High Performance Amino Acid Analyzer.

Extraction of Seed Proteins

The dehulled cereal grains and legume seeds were milled into flour and defatted by extraction with cold acetone. The total proteins in the defatted flour were extracted with 50 mM Tris-HCl (pH 7.5) containing 2% SDS, 4 M urea, 0.6% 2-mercaptoethanol and 1 mM EDTA for two hours at room temperature. The protein extracts were collected by centrifugation at 15,000 xg for 20 min. The extraction was repeated three times.

For preparation of oat globulin, the defatted oat flour was extracted with 50 mM Tris-HCl (pH 8.0) to remove albumin. The residue was then extracted with 1 N NaCl in 50 mM Tris-HCl (pH 8.0) to obtain globulin. The extract was exhaustively dialyzed against distilled water. The resulting precipitate of globulin was collected by centrifugation and redissolved in 1N NaCl in 50 mM Tris-HCl (pH 8.0).

Gel Electrophoresis and Western Blot Analysis

SDS electrophoresis in 12% acrylamide gels was performed according to the method of Laemmli (1970) under reducing conditions. Two-dimensional gel electrophoresis was carried out with IEF or NEPHGE as the first dimension and SDS-PAGE (14% acrylamide) as the second dimension (O'Farrell, 1975; O'Farrell, *et al.*, 1977). For IEF, 2% of ampholyte and 7500 volt hours of running time were employed. For NEPHGE, much shorter running time (2,500 volt hours) was used. Because rice protein samples contained high concentration of SDS, NP-40 was always included in the sample lysis buffer (9.95 M urea, 8% NP-40, 5% 2-mercaptoethanol, 2% ampholyte) and sample overlay solution (4 M urea, 5% NP-40, 2% ampholyte). SDS came off the proteins and formed mixed micelles with NP-40 which migrated to the acidic end of the tube gel. The slab gels after second dimensional electrophoresis were soaked in 50% ethanol and 7% acetic acid overnight to remove ampholytes and stained with Coomassie Blue R-250. For Western blot analysis, the slab gels were electrophoretically transfered to nitrocellulose sheets according to the method of Towbin *et al.* (1979). The blots were incubated separately with antibodies against rice glutelin 21-22 kDa and 33-35 kDa subunits. Goat anti-rabbit IgG conjugated to horseradish peroxidase was used to detect the immunocomplexes.

Results and Discussion

Rice glutelin was purified into two subunit groups (21-22 kDa and 33-35 kDa)

from *indica* rice and the purity was checked by SDS-PAGE shown in Fig. 1. The amino acid compositions of purified subunits were listed in Table 1. The most abundant amino acids in this protein are glutamic acid/glutamine, aspartic acid/asparagine, glycine, alanine and leucine. The data are essentially similar to those from the literature (Padhye and Salunkhe, 1979; Wen and Luthe, 1985) with some variations due to different rice varieties analyzed.

The charge heterogeneities of both subunits were investigated by NEPHGE

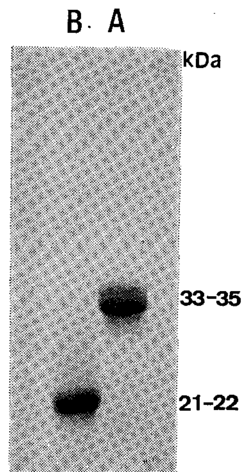

Fig. 1. SDS-PAGE of the purified rice glutelin subunits. The sample containing 30 μg protein was analyzed in each lane.
A: 33-35 kDa acidic subunit.
B: 21-22 kDa basic subunit.

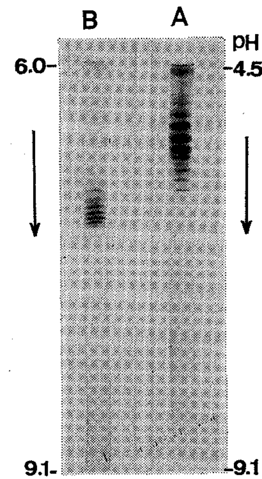

Fig. 2. Nonequilibrium pH gradient isoelectrofocusing of the rice glutelin subunits. A: 33-35 kDa subunit, B: 21-22 kDa subunit. The final pH range after electrophoresis for 2,500 volt hours were indicated on the margins of both tube gels.

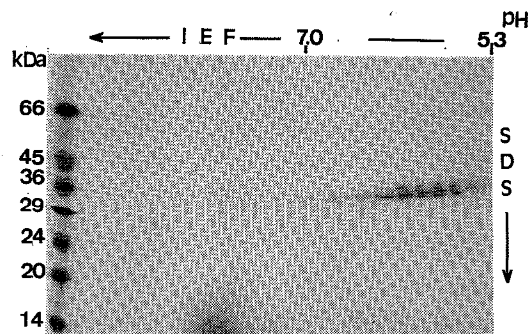

Fig. 3. Two-dimensional gel pattern of rice glutelin 33-35 kDa subunit. The first dimension was IEF, run from right (acidic) to left (basic). The second dimension was SDS-PAGE.

Table 1. *Amino acid composition of glutelin from indica rice Taichung Sen 3*

Amino acid	Acidic subunit (mol %)	Basic subunit (mol %)
Lys	2.9	4.0
His	2.1	2.1
Arg	7.4	7.6
Asx	9.1	11.0
Thr	4.0	4.1
Ser	7.1	6.3
Glx	19.7	13.9
Pro	3.8	3.8
Gly	9.3	8.3
Ala	7.2	9.3
½Cys	nd*	nd
Val	6.7	7.3
Met	1.0	0.9
Ile	3.5	4.9
Leu	8.0	8.2
Tyr	3.0	3.4
Phe	5.2	5.0
Trp	nd	nd

* nd: not determined.

shown in Fig. 2. The subunit group 33-35 kDa was separated into approximately 15 bands in the pH gradient from 4.5 to 9.1. The subunit group 21-22 kDa was separated into approximately 10 bands in the pH gradient from 6 to 9.1. The glutelin subunits revealed more charge and molecular weight heterogeneities by two-dimensional gel electrophoresis. Figure 3 shows that more than 20 spots of polypeptides were resolved from 33-35 kDa subunit.

The antibodies directed against purified rice glutelin 21-22 kDa and 33-35 kDa have been prepared and the titers were determined by enzyme-linked immunosorbent assays giving dilution values around 1/8,000-1/10,000. The specificities of the antibodies were examined. The total protein extracted from rice grains was subjected to two-dimensional gel electrophoresis under reducing conditions followed by Western blot analysis. NEPHGE was employed as the first dimension instead of IEF because NEPHGE gave better resolution of polypeptides in basic subunit group (O'Farrell, 1977). Figure 4 indicates anti 33-35 kDa antibodies reacted most strongly with polypeptides at acidic 33-35 kDa region, and slightly with polypeptides at 55-57 kDa and 15-18 kDa regions. In a similar fashion, anti 21-22 kDa antibodies reacted most strongly with polypeptides at basic 21-22 kDa region and

very slightly with polypeptides at 55-57 kDa and 33-35 kDa regions. The polypeptides at 55-57 kDa region represent the uncleaved precursors of glutelin (Yamagata *et al*, 1982).

To investigate the immunological relations between rice glutelin and other cereal proteins, the cereal proteins were separated by SDS-PAGE, blotted and treated with anti rice 33-35 kDa and anti rice 21-22 kDa antibodies. Figure 5 demonstrates that anti rice 33-35 kDa and anti 21-22 kDa crossreacted very strongly with oat 35-39 kDa and 22-24 kDa which could be the two subunits (α, β) of 12 S storage globulin (Walburg and Larkins, 1983; Brinegar and Peterson, 1982). Among other tested cereals, only millet proteins at 22.5 kDa, 14 kDa and wheat proteins at 57 kDa were weakly immunostained. A similar experiment was

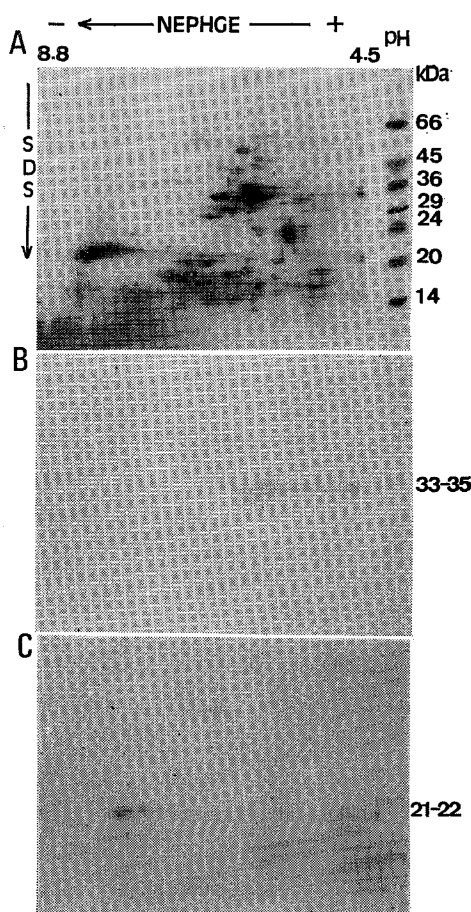

Fig. 4. Two-dimensional gel electrophoresis and Western blot analysis of total protein extracted from rice grains. A: direct staining of the gel with Coomassie Blue. B and C: corresponding blots treated with anti rice 33-35 kDa and anti rice 21-22 kDa respectively.

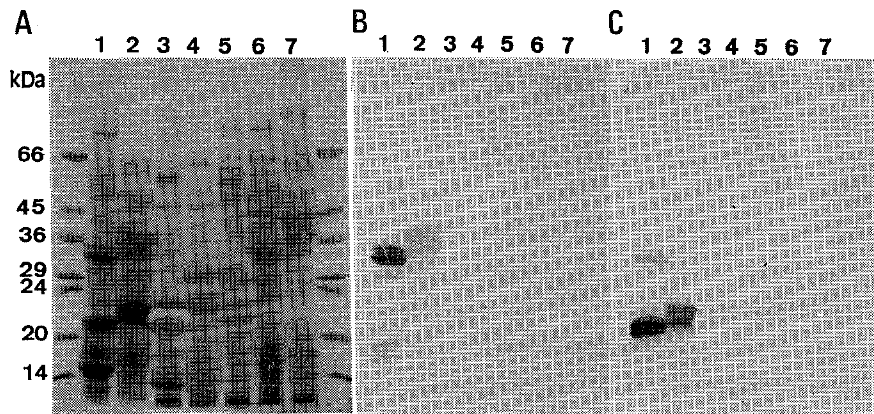

Fig. 5. SDS-PAGE of total proteins extracted from cereal grains and Western blot analysis. A: direct staining of the gel with Coomassie Blue. B and C: corresponding blots treated with anti rice 33-35 kDa and anti 21-22 kDa respectively. Protein of 20-30 μg was analyzed in each lane. Lane 1: rice, 2: oat, 3: millet, 4: sorghum, 5: maize, 6: wheat, 7: barley.

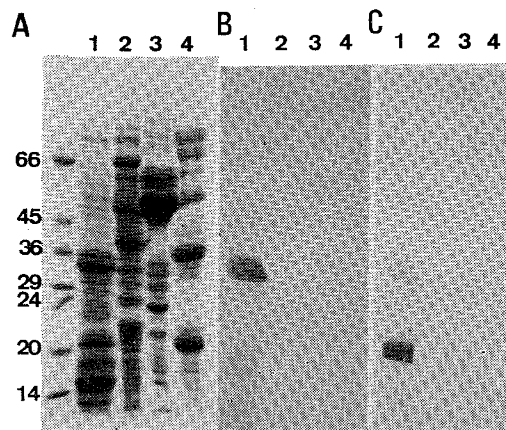

Fig. 6. SDS-PAGE of legume seed proteins and Western blot analysis. A: direct staining of the gel with Coomassie Blue. B and C: corresponding blots treated with anti rice 33-35 kDa and anti 21-22 kDa respectively. Protein of 30-35 μg was analyzed in each lane. Lane 1: rice, 2: pea, 3: mungbean, 4: soybean.

performed to examine the reactions of anti rice antibodies with total proteins from legume seeds. No significant crossreaction was detected as shown in Fig. 6.

Since anti rice glutelin antibodies strongly crossreacted with oat proteins. it is important to determine if anti rice antibodies recognize oat α and β specifically. Figure 7A is the two-dimensional gel electrophoregram of reduced oat globulin showing the heterogeneities within α and β subunits. The 60-kDa polypeptides are presumably the uncleaved precursors (Walburg and Larkins, 1983).

It is clearly demonstrated in Fig. 7B and 7C that anti rice 33–35 kDa and anti 21-22 kDa antibodies reacted specifically with nearly every polypeptide of α and β subunits revealed on the Coomassie Blue-stained gel. Both antibodies also reacted strongly with 60 kDa precursors.

The above studies of immunochemical crossreactions suggest very high degree of homology between the primary structures of rice glutelin and oat storage globulin. The precursor of rice glutelin which consists of acidic and basic subunits linked by disulfide bond has homologous counterpart in oat globulin precursor. It is of interest to have so conserved sequence in rice and oat which belong to different subfamilies of Gramineae—Oryzoideae and Festucoideae, respectively. While it was reported by Fabijanski *et al.* (1985) that antigenic homologies existed

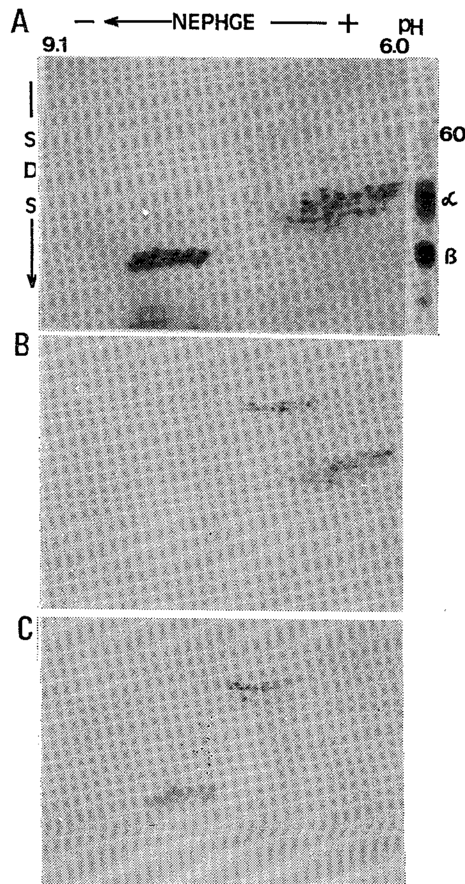

Fig. 7. Two-dimensional gel electrophoresis of oat globulin and Western blot analysis. A: direct staining of 2-D gel and 1-D gel (SDS-PAGE, shown along the right) with Coomassie Blue. B and C: corresponding blots treated with anti rice 33-35 kDa and anti 21-22 kDa respectively.

between oat and wheat globulins because antibodies against oat globulin cross-reacted strongly with wheat globulin, only very poor immunochemical crossreaction was detected in wheat proteins with anti rice antibodies shown in Fig. 5. Possibly the antigenic determinants recognized by anti oat antibodies were quite different from that recognized by anti rice antibodies. Leguminous 11S globulin was shown to be partially homologous to rice glutelin from the sequence data (Zhao *et al*, 1983). However, it also does not possess the antigenic determinant recognized by anti rice glutelin antibodies. Our results thus suggest that there is lack of obvious antigenic homology between rice glutelin and seed proteins from other members in Festucoideae sub-family including wheat and barley, another sub-family Panicoideae (maize, sorghum, and millet), as well as family Leguminoseae (pea, soybean and mungbean).

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秈稻 Glutelin 之生化及免疫性質

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秈稻臺中秈三號之主要貯藏蛋白 Glutelin 已被純化成兩個單元羣 21-22 kDa（鹼性）及 33-35 kDa（酸性）。這兩個單元羣所帶電荷及分子量的複雜性，可用非平衡式 pH 梯度電泳以及雙向電泳來分析。它們的氨基酸組成亦已決定。利用 Western 電泳轉移免疫分析法以及二個 Glutelin 單元羣之特異抗體，分析稻米 Glutelin 與其他穀類（燕麥、大麥、小麥、玉米、小米、高粱）及豆類（豌豆、大豆、綠豆）種子蛋白之免疫學相關性。所得結果指出稻米 Glutelin 與燕麥之主要貯藏蛋白 Globulin 有極高程度的構造相似性。但是稻米 Glutelin 與其他所測試之穀類，豆類種子蛋白，皆缺乏明顯的抗原相似性。