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Purification and characterization of rice prolamins

Lie-Fen Shyur[2], Tuan-Nan Wen[1] and Ching-San Chen[1,2,3]

Institute of Botany[1], Academia Sinica, Taipei, Taiwan, Republic of China

Department of Agricultural Chemistry[2], National Taiwan University, Taipei, Taiwan, Republic of China

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Abstract. Rice prolamins consist of two major groups of polypeptides, with molecular masses of 15.5 and 14.2 kDa. After separation of these groups of prolamins by preparative sodium dodecylsulfate-polyacrylamide gel electrophoresis, each was resolved into at least five polypeptides by two-dimensional electrophoresis. The isoelectric points of the 15.5 and 14.2 kDa prolamins were in the range of 6.6–8.1 and 6.0–8.1, respectively. The two groups of prolamins had similar amino acid composition: high levels of glutamic acid or glutamine, alanine, glycine, and arginine; and low levels of lysine and histidine. Three polypeptides from maize seeds and one each from sorghum and barley cross-reacted with the rice antiprolamin serum. The 15.5 and 14.2 kDa prolamins were almost completely degraded within 14 days after germination.

Keywords: Amino acid composition; Immunological reaction; *Oryza sativa*; Prolamin; Purification.

Introduction

Prolamin is a major storage protein in the endosperm of most cereal seeds. A recent analysis, using 55% n-propanol as the extraction solvent, showed the contents of prolamins in protein body I account for approximately 20% of the total proteins in *japonica* rice, confirming that prolamins are a major storage seed protein in rice (Sugimoto et al.,1986). Studies of the prolamin proteins of maize, wheat, barley, and rye have accumulated a large body of useful information on the structure and evolution of these proteins (Shewry and Tatham, 1990). Recently, the biochemical and molecular characteristics of rice storage proteins, particularly glutelins and prolamins, have been extensively studied.

Rice prolamins consist of heterogeneous polypeptides with molecular masses of 12–17 kDa, and are similar to other cereal prolamins in having high contents of glutamine and low levels of lysine, histidine, cysteine, and methionine (Mandac and Juliano, 1978; Padhye and Salunkhe, 1979). They accumulate within protein bodies which are formed by direct dilation of the endoplasmic reticulum membrane (Krishnan et al., 1986; Yamagata and Tanaka, 1986). Analysis of poly $(A)^+$ RNA directed, in vitro-translated products suggests that rice prolamins are synthesized as a precursor which is cleaved co-translationally to form the mature polypeptide (Masumura et al., 1990). It has been suggested that signal peptides containing 19 amino acids (Masumura et al., 1990) and 18 amino acids (Shyur et al., 1992) are removed from the precursor prolamins to form the 14-kDa prolamins.

Analysis of rice prolamins by isoelectric focusing revealed heterogeneity of prolamin polypeptides (Hibino et al., 1989). This substantiates genomic and cDNA research indicating that the rice prolamins are encoded by a complex multigene family (Kim and Okita, 1988). Structural studies of rice prolamins, like those of rice glutelins and other cereal prolamins, have been hampered by difficulties in obtaining individual polypeptides in the homogeneous state, due to their insolubility in water or dilute salt solutions when in the native state and to the microheterogeneity feature of the polypeptide chains. Nucleotide sequence analysis of the prolamin genomic and cDNA clones provides an alternate approach (Kim and Okita, 1988; Masumura et al., 1989; Masumura et al., 1990; Shyur and Chen, 1990; Yamagata et al., 1992). Although the molecular approach has revealed some of the polypeptide complexity of rice prolamins, relatively little is known about biochemical characteristics of rice prolamins that might help us understand their molecular structure. This report describes the purification process, two-dimensional electrophoretic patterns, and some properties of the two major groups of rice prolamins.

[3]Corresponding author: Ching-San Chen, Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan 115, R.O.C.

Materials and Methods

Plant Material

Oryza sativa L. *japonica* cv. Tainung 67 was grown at the experimental farm of the Institute of Botany, Academia Sinica, located in Taipei. Developing seeds were harvested at the desired times after flowering and stored at -70 °C until use.

Extraction of Rice Prolamins

Rice seeds were dehulled, and ground into 80-mesh flour with a blender. The lipids were extracted with two volumes of ether. After sequential extraction of albumin and globulin using the method described by Luthe (1983), the defatted residual flour was suspended in three volumes of 70% ethanol in 50 mM Tris-HCl (pH 7.5) and 1 mM phenylmethylsulfonylfluoride (PMSF), and stirred for 1 h at room temperature. The suspension was centrifuged at 20,000 x *g* for 30 min and the supernatant liquid was saved. The pellets were re-extracted twice and centrifuged. The supernatant liquids were combined, two volumes of acetone were added, and the mixture stood at 4 °C overnight. The precipitated crude prolamins were dialyzed against distilled water and lyophilized.

Purification of Prolamins

The crude prolamins were dissolved in an equilibration buffer (0.5% sodium dodecylsulfate (SDS), 50 mM Tris-HCl (pH 8.5), and 50 mM β-mercaptoethanol) and centrifuged at 10,000 x *g* for 30 min. The supernatant liquid was applied to a Sephadex G-150 column (26 x 96 cm) previously equilibrated with the equilibration buffer, and eluted with the same buffer solution. The flow rate was 9.2 ml per h and the effluent was collected in 5 ml fractions. A_{280} was measured and an aliquot of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Prolamins were found in the first peak eluted from the Sephadex G-150 column. These were further purified by preparative 14% SDS-PAGE using a modification of the method of Laemmli (1970). After electrophoresis the gel was sliced into 0.75-mm sections. Each slice was put into an eppendorf tube and a buffer consisting of 6 M urea and 50 mM Tris-HCl (pH 8.9) was added. Prolamins were extracted from the gel slices by vigorously shaking the mixture overnight at room temperature. Recovery of prolamins from the gel was approximately 90%.

Isoelectric Focusing

Isoelectric focusing (IEF) was performed by 5% polyacrylamide gel vertical tube electrophoresis. The gel consisted of 9 M urea, 5% (w/v) acrylamide, and 2.28% LKB Ampholine (pH 3.5–10, 1.8%; pH 9–11, 0.2%; pH 5–8, 0.14%; and pH 4–6.5, 0.14%), and 2% NP-40. Before loading samples, the gels were overlaid with overlayer solution (O'Farrell, 1975) and prerun for half an hour at each of 100 V, 200 V, and 300 V, using 10 mM H_3PO_4 as the upper (anode) electrolyte and 20 mM NaOH as the lower (cathode) electrolyte. Lyophilized prolamin samples or pI marker proteins were dissolved in lysis buffer (9.5 M urea, 2% NP-40, 1.6% Ampholine pH 5–8, 0.4% Ampholine pH 3.5–10, and 5% β-mercaptoethanol) and electrophoresed for 7,200 Vh. A control tube without samples was electrophoresed under the same conditions, and was used to determine the pH gradient. The pI of prolamin was obtained by extrapolation from the standard curve of pH gradient vs mobility of pI marker.

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis was performed using a slight modification of the method of O'Farrell (1975). NEPHGE vertical tube gel (described in "Isoelectric Focusing"), was run at 400 V for 5 h as the first dimension. After equilibrating in sample buffer (Laemmli, 1970), 1% agarose was used to attach the tube gel and stacking gel to a 5 to 20% polyacrylamide gradient slab gel. The second dimension of electrophoresis was carried out with the slab gel. The polypeptides on the slab gel were made visible by silver staining (Juang and Su, 1987).

Amino Acid Analysis

The electrophoretically purified, 15.5 kDa or 14.2 kDa prolamin was hydrolyzed and derivatized according to the instruction manual of the PICO-TAG Workstation (Waters). The resulting PTH-amino acids were dissolved in proper amounts of sample diluent (Waters) and an aliquot was analyzed for amino acid composition by HPLC (Waters). Amino acid standard H (Pierce) was used as the control.

Preparation of Antiserum

Two New Zealand white rabbits were used for raising antibodies against the purified prolamins (Fig.1, lane 2) by a previously described method (Shyur et al., 1988). The antigen prolamins were purified by SDS-PAGE as described in "Purification of prolamins". The purified prolamins were shown to contain only the 15.5 and 14.2 kDa polypeptides.

Western Blot Analysis

Western blot analysis was performed as previously described (Shyur et al., 1988). Antiprolamin serum (10^4 dilution) and antirabbit IgG-HRP conjugate (3 x 10^3 dilution, Bio-Rad) were used as the first and second antibodies, respectively. The Ag-1st Ab-2nd Ab complex was made visible by reaction with 0.003% H_2O_2

Table 1. Amino acid composition of prolamins from *japonica* rice Tainung 67. Standard deviations obtained from four determinations.

Amino Acid	Prolamins (mole %)	15.5 kDa polypeptides (mole %)	14.2 kDa polypeptides (mole %)
Asx	7.25 ± 0.25	6.75 ± 0.69	7.26 ± 0.23
Glx	11.17 ± 1.15	12.93 ± 0.34	11.26 ± 0.21
Ala	11.35 ± 0.22	10.64 ± 0.58	11.54 ± 0.63
Val	6.37 ± 0.23	5.96 ± 0.27	6.31 ± 0.21
Leu	7.20 ± 0.32	6.95 ± 0.34	7.21 ± 0.33
Ile	2.80 ± 0.27	3.12 ± 0.35	2.92 ± 0.35
Pro	7.50 ± 0.42	7.75 ± 0.71	7.06 ± 0.57
Met	2.98 ± 0.37	2.46 ± 0.44	2.89 ± 0.29
Phe	2.34 ± 0.15	2.35 ± 0.04	2.38 ± 0.23
Gly	12.50 ± 0.26	12.47 ± 0.53	13.11 ± 0.76
Ser	4.56 ± 0.08	4.24 ± 0.42	4.33 ± 0.47
Thr	2.58 ± 0.08	2.83 ± 0.22	2.45 ± 0.19
Cys	4.14 ± 0.10	4.00 ± 0.37	4.22 ± 0.90
Tyr	3.71 ± 0.15	3.49 ± 0.27	3.81 ± 0.12
Arg	9.95 ± 0.04	10.09 ± 0.24	10.10 ± 0.25
His	1.72 ± 0.09	2.33 ± 0.06	1.70 ± 0.20
Lys	1.50 ± 0.22	1.39 ± 0.18	1.45 ± 0.10

substrate and 5% diaminobenzidine chromogen. The reaction was terminated by washing with water for 10 min.

Results

Purification

The crude prolamins obtained from conventional Osborne fractionation (Luthe, 1983) were shown to consist of two major protein components and several minor proteins (Fig. 1). Chromatography of the crude prolamins on Sephadex G-150 showed two major A_{280} peaks. Aliquots of the peak fractions were separated by SDS-PAGE. The first peak consisted of two major prolamins and lower molecular weight proteins, whereas the second peak was an unknown nonproteineous material which did not bind Coomassie brilliant blue. The two major prolamins had molecular weights of 15.5 and 14.2 kDa, determined by SDS-PAGE (data not shown). They were further purified by preparative SDS-PAGE. This method resolved the 15.5 and 14.2 kDa prolamins; each of them was shown to be homogeneous (Fig. 1).

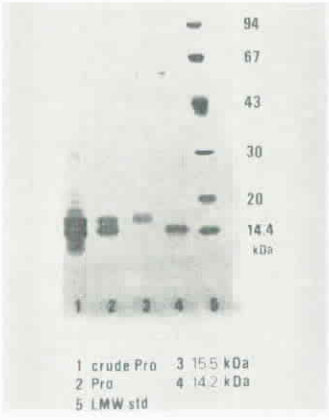

Fig.1. SDS-PAGE of purified rice prolamins.

The 15.5 and 14.2 kDa polypeptides of rice prolamins were isolated by preparative 14% SDS-PAGE. Equal amounts of these prolamins were analyzed by 5–20% SDS-PAGE. Protein bands were detected by silver staining. **1**) Crude prolamins. **2**) Purified prolamins. **3**) 15.5 kDa prolamin. **4**) 14.2 kDa prolamin. **5**) LMW standard.

Two-Dimensional Electrophoresis

Two-dimensional electrophoretograms of the crude prolamins and of the purified 15.5 and 14.2 kDa prola-

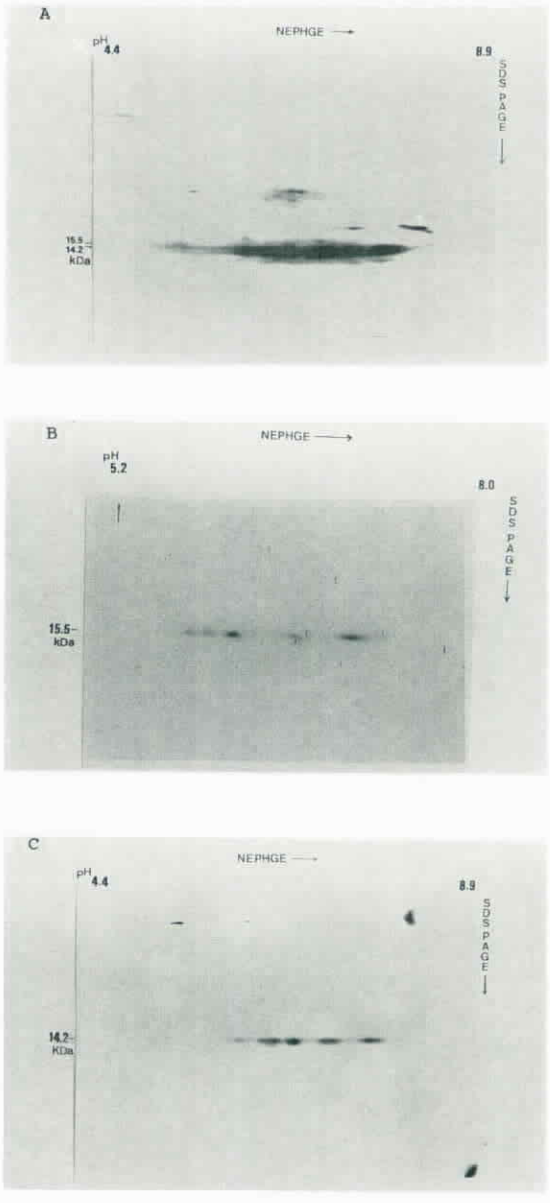

Fig.2. Two-dimensional gel electrophoresis.

A) Crude prolamins extracted from rice flour. **B**) The 15.5 kDa and **C**) the14.2 kDa prolamins, purified to apparent homogeneity by SDS-PAGE.

mins are shown in Figures 2A, 2B, and 2C, respectively. The crude prolamins consist of two major groups of polypeptides having a very narrow range of molecular masses (14–16 kDa), but a rather wide range of isoelectric points (pH 5–8). Two minor groups of polypeptides with molecular masses of approximately 18 and 32 kDa were also found (Fig. 1). Both the15.5 and14.2 kDa prolamins were separated into at least 5 polypeptides showing similar molecular masses and different pI.

By isoelectric focusing, the 15.5 kDa prolamins were separated into nine polypeptides with pIs in the range of 6.6 to 8.1; the 14.2 kDa prolamins were resolved into seven polypeptides with pIs in the range of 6.0 to 8.1. Four polypeptides in the 15.5 and 14.2 kDa prolamins appeared to have similar pI: pH 6.6, 7.2, 7.7, and 8.1. (data not shown).

Amino Acid Compositions

Amino acid compositions of the purified prolamins and of the 15.5 and 14.2 kDa polypeptides, are shown in Table 1. These three prolamin fractions had similar amino acid compositions: high contents of glutamic acid / glutamine, alanine, glycine, and arginine; and low levels of lysine and histidine. Hydrophobic amino acids were the most abundant (39–41%), followed by uncharged polar (27–28%), acidic (18–20%), and basic (13–14%) amino acids. The high contents of hydrophobic amino acids might account for the insolubility of prolamin polypeptides.

Immunological Relationships Between Rice Prolamins and Other Cereal Proteins

Antiserum raised against the purified prolamins containing 15.5 and 14.2 kDa polypeptides (Fig. 1, lane 2) was used for Western blot analysis. Prolamins were isolated from 14 varieties of rice seeds, separated by SDS-PAGE, and subjected to Western blot analysis. From all rice varieties, two polypeptides corresponding to the 15.5 and 14.2 kDa prolamins showed cross-reactivity. In addition, one or two polypeptides around 28 kDa were also reactive (data not shown). Total proteins from seeds of maize, oat, sorghum, wheat, and barley were also prepared and analyzed with the antiserum. Interestingly, three polypeptides from maize seeds (14–28 kDa) and one each from sorghum (~ 28 kDa) and barley (~ 45 kDa) cross-reacted weakly with the rice antiprolamin serum. No detectable reactivity was exhibited by wheat and oat proteins against the rice antiprolamin (Fig. 3). This indicates that rice prolamins share common epitopes with seed proteins from other cereals, such as maize, sorghum, and barley. The structural basis for the cross-reactivity between the rice antiprolamin serum and other cereal prolamins will be described in the "Discussion section".

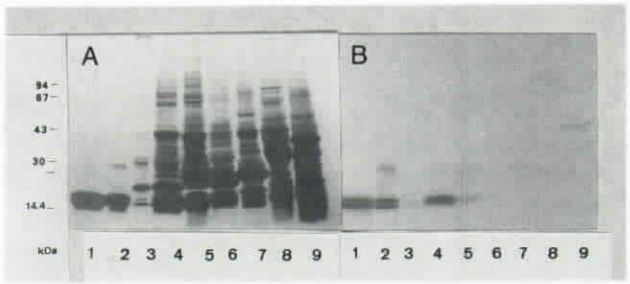

Fig.3. SDS-PAGE and Western blot analysis of rice seed and some cereal proteins.

Cereal seeds were ground in an ice-cold mortar and pestle, and the total proteins were extracted by a buffer solution containing 62.5 mM Tris-HCl (pH 6.8), 4 M urea, 2% SDS, 1 mM EDTA, 5% β-mercaptoethanol and 10% glycerol (Yamagata et al., 1982). The same quantities of total seed protein from different cereals were separated by 5–20% SDS-PAGE and reacted with the rice antiprolamin serum. **1**) Purified rice prolamins. **2**) Crude rice prolamins. **3**) Crude rice glutelins. **4–9**) Total seed proteins of rice, maize, oat, sorghum, wheat, and barley, respectively. **A**) SDS-PAGE, proteins were detected with silver staining. **B**) Western blot analysis.

Synthesis and Degradation of Prolamins

Total proteins were isolated from developing rice seeds at 3-day intervals after flowering, separated by SDS-PAGE, and subjected to both protein staining and Western blot analysis. To analyze the mobilization of prolamins during germination, rice kernels were sterilized and steeped for an hour, and germinated for 1–14 days at 28 °C and 100% humidity in a germination cabinet. Total proteins were isolated from the germinating seeds at 3-day intervals, separated by SDS-PAGE, and made visible by Coomassie brilliant blue staining

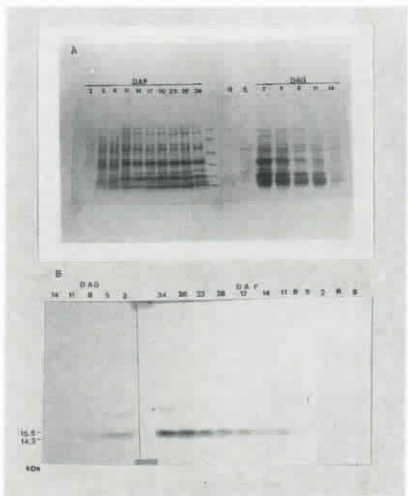

Fig.4. SDS-PAGE and Western blot analysis of rice proteins during seed development and germination.

Experimental details are essentially the same as those described in the legend to Fig.3. **DAF**, days after flowering; **DAG**, days after germination; R, roots; S, shoots. **A**) SDS-PAGE. Proteins were stained with Coomassie brilliant blue. **B**) Western blot analysis.

(Fig. 4A) and Western blotting (Fig.4B). It was found that synthesis of prolamins was first detected between 8–11 days after flowering (DAF) and reached a maximum level around 26 DAF. On the other hand, rice seed proteins were rapidly mobilized during germination. The 15.5 and 14.2 kDa prolamins were almost completely degraded within 14 days after germination. This indicates that prolamins accumulate in the developing rice seeds during seed development and are efficiently metabolized by the rapidly growing young rice plants.

Discussion

Two major components of rice prolamins, the 15.5 and 14.2 kDa polypeptides, were purified by a simple and rapid procedure involving Sephadex G-150 chromatography and preparative SDS-PAGE on 14% gel. Individual polypeptides in each component were further separated by two-dimensional electrophoresis (Figures 2B and 2C). One of the purified polypeptides was recovered from the 2-D gel and was successfully used in the analysis of N-terminal amino acid sequence (Shyur et al., 1992), indicating that the prolamin polypeptides were sufficiently pure for structural study of rice prolamins. High performance liquid chromatography with a reverse-phase column has been employed for the fractionation of rice prolamins (Masumura et al., 1990). The fractionated major prolamin peak seemed to be a mixture of several polypeptides. On the other hand, the molecular masses of individual polypeptides in either the 15.5 or the 14.2 kDa prolamins are so similar that fractionation of the crude prolamins by SDS-PAGE could only distinguish the two prolamins from each other (Fig.1). Further separation of individual polypeptides by SDS-PAGE is difficult. Most of these polypeptides were nicely resolved in the 2-D electrophoresis (Figures 2B and 2C) due to their different pIs.

The amino acid compositions reported in this paper are similar to those from previous analyses (Mandac and Juliano, 1978; Padhye and Salunkhe, 1979; Hibino et al., 1989). There are differences in some amino acids, especially leucine and arginine. Our results indicated lower leucine and higher arginine values than reported in the literature. The difference might be attributable to such factors as the use of different rice varieties, the different methods employed in the preparation of prolamin samples, and the different polypeptide compositions in the prolamins analyzed. As we did, Mandac and Julinao (1978) and Padhye and Salunkhe (1979) used the Osborne fractionation scheme (Luthe, 1983) to isolate prolamins, whereas Hibino et al. (1989) used 55% n-propanol as the extraction solvent (Sugimoto et al., 1986) instead of the 70% ethanol that is generally used in the Osborne method. In addition, complete extraction of prolamins from cereals is difficult (Shewry and Tatham, 1990). This would affect the analytical data for certain prolamin fractions, such as those consisting of sulfur-rich prolamins (Shewry and Tatham, 1990; Tecson et al., 1970; Juliano and Boulter, 1976). In the prolamin fraction analyzed in this report, several minor alcohol-soluble polypeptides were excluded by the preparative SDS-PAGE; this might also be a cause of the difference in amino acid compositions.

Western blot analysis indicated that rice antiprolamin serum cross-reacted with maize proteins at 14–28 kDa, with barley protein at about 45 kDa, and with sorghum protein at about 28 kDa (Fig. 3). The sulfur-rich B1-hordein and γ-hordein have molecular masses in the range of 36–44 kDa. β-, α-, and γ-zeins consist of 14 and 16 kDa, 19 and 22 kDa, and 28 kDa polypeptides, respectively (Shewry and Tatham, 1990). The unreduced prolamin fraction isolated from sorghum exhibited polypeptide bands at about 28–30 kDa on SDS-PAGE (Okita et al., 1988). Based on this information, it is likely that the proteins reacting with the rice antiprolamin serum are prolamins with different molecular masses.

Previous studies showing sequence homology between rice and other cereal prolamins provide a structural basis for the cross-reaction between rice antiprolamin serum and the prolamins of the other cereals mentioned. Rice 10 kDa prolamin (Masumura et al., 1989), 13 kDa prolamin (λRM7) (Masumura et al., 1990), and 16 kDa prolamin (pS18) (Shyur et al., 1992) share a unique octapeptide, Gln-Gln-Gln-Cys-Cys-Gln-Gln-Leu, with other cereal prolamins such as 15 and 27 kDa zein, B- and γ-hordein, α/β- and γ-gliadin, and γ-secalin (Masumura et al., 1990). In addition, high homology (~ 50%) was observed among rice prolamins, barley B1- and γ-hordein (Forde et al., 1985; Cameron-Mills and Brandt, 1988), wheat α/β- and γ-gliadin (Okita et al., 1985), and rye γ-secalin (Kreis et al., 1985). These regions correspond to the signal peptide, the residues 30–54, and the residues 92–113 of pS18 (Shyur and Chen, 1993). The results also suggest that these domains have a common evolutionary origin.

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水稻醇溶蛋白的純化與鑑定

徐麗芬[2] 溫端南[1] 陳慶三[1,2]

[1]中央研究院植物研究所
[2]國立台灣大學農業化學系

水稻醇溶蛋白主要由 15.5 kDa 及 14.2 kDa 兩群多肽所組成。這兩群多肽以製備式電泳分別純化後，再用雙向電泳分析，每一群都可分離出至少 5 種多肽。 15.5 kDa 和 14.2 kDa 醇溶蛋白的等電點分別在 6.6-8.1 和 6.0-8.1 範圍。這兩群多肽的胺基酸組成很接近，都含有多量的麩胺酸 / 麩胺醯胺，丙胺酸，甘胺酸及精胺酸。但是離胺酸和組織胺酸的含量則很低。水稻醇溶蛋白抗血清與玉米種子的三種蛋白質、高粱及大麥種子的某一種蛋白質具交叉反應性。水稻發芽後約十四天，這兩群醇溶蛋白幾乎已完全被分解。

關鍵詞：胺基酸組成；免疫反應；水稻；醇溶蛋白；純化。