NOTE

Discrimination of Rice Cultivars by Polyacrylamide Gel Electrophoresis and High-Performance Liquid Chromatography[1]

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ABSTRACT

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Two methods, polyacrylamide gel electrophoresis and reversed-phase high-performance liquid chromatography, were developed to discriminate 10 different cultivars and an advanced line of rice (Oryza sativa L.) on the basis of genotype-specific protein patterns. The proteins that were analyzed were extracted from ground brown rice with 5M acetic acid solution.

In the context of plant breeder's rights and plant patents, registration of rice genotypes for proprietary purposes requires an accurate system of cultivar discrimination. Because rice cultivars are generally traded as seed, it would be convenient to be able to discriminate cultivars on the basis of analysis of seed rather than phenotypic characters of growing plant or reaction of the plant to certain diseases.

A number of procedures based on cultivar-specific biochemical and chemical markers including electrophoresis and high-performance liquid chromatography have been applied to rice with varying degrees of success (Aliaga-Morell et al 1987, Glaszmann 1987, Kim and Jo 1983, Ladizinsky and Hymowitz 1979, Lookhart et al 1987, Oka 1958, Park and Stegemann 1979, Sarkar and Bose 1984). This article describes two methods, polyacrylamide gel electrophoresis (PAGE) and reversed-phase high-performance liquid chromatography (RP-HPLC), which appear to have the potential of becoming routine methods for rice cultivar discrimination and identification.

MATERIALS AND METHODS

Rice Samples

Cultivars and breeder's lines of rice, i.e., IR 36, IR 42, Basmati 370, Mahsuri, Suweon 358, Khao Dawk Mali 105, Kataktara, Fujisaka 5, Azucena, Moroberekan, and Pankhari 203, designated as samples 1–11, respectively, were used in this study. For analysis, about 10 g of dehulled brown rice was ground on a Udy cyclone sample mill (Udy Corporation, Boulder, CO) to pass a 1-mm sieve.

Chemicals and Reagents

All chemicals used for electrophoresis were of reagent grade or better. Acetic acid, ascorbic acid, ferrous sulfate, lactic acid, and sucrose were obtained from Fisher Scientific (Winnipeg, MB). Acrylamide and bisacrylamide were obtained from Bio-Rad Laboratories (Richmond, LA). Aluminum lactate was purchased from Fluka Chemicals (Hauppauge, NY) and hydrogen peroxide (3%, practical grade) from a local pharmacy.

All chemicals used for HPLC were of HPLC grade, as described by Scanlon et al (1989).

PAGE

Several extraction procedures were investigated. In the successful procedure, 0.5 g of brown rice meal was extracted in 2 ml of 5M acetic acid containing 1% sucrose. The mixture was vortexed occasionally and allowed to stand for at least 2 hr at room temperature. The slurry was centrifuged at 8,850 × g for 10 min at room temperature, and the clear supernatant was freeze-dried. This supernatant contained components of the albumins, globulins, prolamins, and glutelins separated according to Osborne fractionation (results not shown). The extraction procedure, however, was relatively less laborious than the classical Osborne fractionation, and the extract was free of components that cause tailing and shadows in the electrophoregram. For analysis, all of the dry material was dissolved in 150 µl of aluminum lactate buffer (8.5 mM, pH 3.1) and 25 µl of dye solution (0.6% methyl green in aluminum lactate buffer). Ten microliters of this solution was used in each lane.

Electrophoresis was carried out in a locally constructed vertical electrophoresis unit (Bushuk et al 1987). Gel mixture contained acrylamide, 10 g; bisacrylamide, 0.5 g: ascorbic acid, 0.1 g: ferrous sulfate, 0.0015 g, glycerol, 20 ml; and aluminum lactate (8.5 mM) + lactic acid to pH 3.1, 85 ml. This mixture was filtered, degassed, and cooled to 10–12°C before polymerization by 100 µl of 3% hydrogen peroxide. Electrophoretic conditions used were essentially the same as described for lentil (Hussain et al 1989) except that the running current was maintained at 10 mA instead of 8 mA. Electrophoresis was terminated when the first dye band had reached the lower end of the gel. The gel slab was stained overnight in 12% trichloroacetic acid (100 ml) containing 4 ml of 1% Coomassie Blue R solution (Sapirstein and Bushuk 1985). The stained gel was rinsed with soapy water in order to remove precipitated stain particles from the surface of the gel (Hussain et al 1989). The gel was later destained with 12% trichloroacetic acid, and photographed.

HPLC

The seed meal (0.3 g) was extracted with 5M acetic acid (0.5 ml) for 2 hr at room temperature with occasional vortexing. The mixture was centrifuged at 8,850 × g for 10 min at room temperature and the clear supernatant decanted and recentrifuged for a further 10 min at the same speed. The supernatant from the second centrifugation was filtered through a 0.45-µm filter (Millipore Ltd., Mississauga, ON) into a disposable HPLC microvial (Hewlett-Packard Co., Palo Alto, CA) and sealed.

HPLC analysis was carried out according to Scanlon et al (1989), with modifications as described below. An initial separation of all 11 cultivars was performed on a wide-pore SynChropak (SynChrom Inc., Lafayette, IN) C18 column (25 cm × 4.6 mm, i.d.), which showed that reasonable separations might be achieved. For the results reported, a wide-pore Supelcosil (Supelco Canada Ltd., Oakville, ON) C8 column (25 cm × 4.6 mm, i.d.) was used with a 5-cm guard column of the same packing

material. In comparison with the separation of wheat prolamins, a small increase in acetonitrile concentration led to rapid elution of a large number of rice proteins. Shallower acetonitrile gradients gave poor resolution. Partial separation of the first main group of proteins and almost complete separation of the smaller group of later-eluting proteins was achieved at a flow rate of 1.5 ml/min and the gradient conditions of Table I. The data sampling rate for monitoring protein elution at 210 nm was one point per 640 msec.

In the first set of analyses, a sample injection of 20 μl was used. One week later, during which time other wheat protein separations were carried out on the column, a second set of extracts was prepared and 10-μl aliquots were injected. The decrease in sample aliquot allowed a replicate analysis to be performed and provided a check on whether peaks were still present at smaller sample injections. For example, in the case of merged peaks, a peak present at larger concentrations might be obscured by larger peaks near it at decreased sample concentrations.

RESULTS AND DISCUSSION

Electrophoresis Results

Each of the 11 rices selected for this study had a unique electrophoregram (Fig. 1). The variation in the slow migrating region (23–27 mm) divided the 11 rices into six distinct groups. Cultivars represented by electrophoregrams in lanes 8, 9, and 10 showed presence of a band at 55 mm. This band, however, is not found in other samples. Protein patterns of IR 36 and IR 42 (lanes 1 and 2) were almost identical with intensity

TABLE I
Elution Solvents for High-Performance Liquid Chromatographic Separation of Protein Extracts of Rice Cultivars

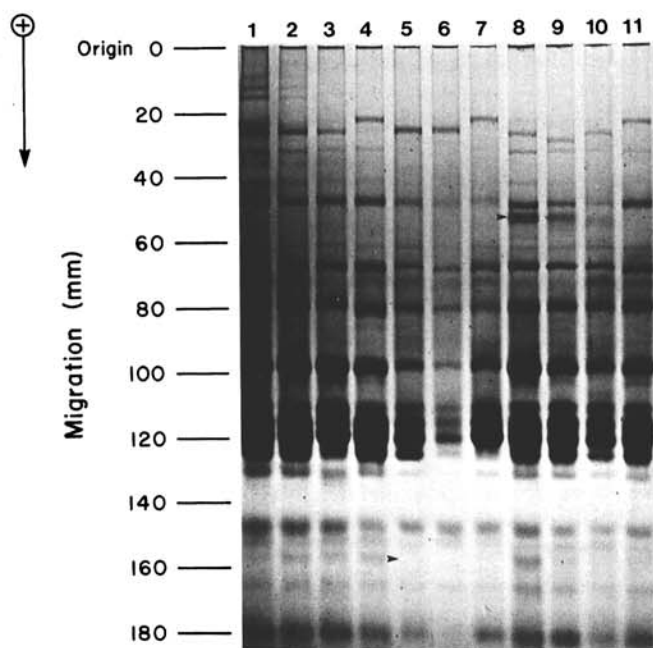| Elution Time (min)	Acetonitrile/Water (both containing 0.1% trifluoroacetic acid)
0	22:78
15	22:78
18	28:72
32	50:50
35	50:50

Fig. 1. Polyacrylamide gel electrophoretic patterns of acetic acid extractable seed proteins of various rice cultivars. From left the cultivars are IR 36, IR 42, Basmati 370, Mahsuri, Suweon 358, Khao Dawk Mali 105, Kataktara, Fujisaka 5, Azucena, Moroberekan, and Pankhari 203.

differences in the region of 0–23 mm. The similarity between the two electrophoregrams could be due to the fact that IR 36 and IR 42 are sister-line varieties, derived from IR 2071. Suweon 358 and Khao Dawk Mali 105 (lanes 5 and 6) gave similar patterns that contained only minor differences in the high mobility region. Three cultivars, namely, Suweon 358, Khao Dawk Mali 105, and Kataktara (lanes 5, 6, and 7), lack a fast moving band at 156 mm that is present in the other cultivars. The patterns shown in Figure 1 were reproduced exactly in a second complete analysis beginning with ground seed. Furthermore, storage of the extracts in a refrigerator for up to four weeks did not affect the patterns.

HPLC Results

For purposes of cultivar discrimination, the chromatograms were split into two regions, 18–26 min (Fig. 2) and 25.5–32 min (Fig. 3). Apart from the components that eluted with the extracting
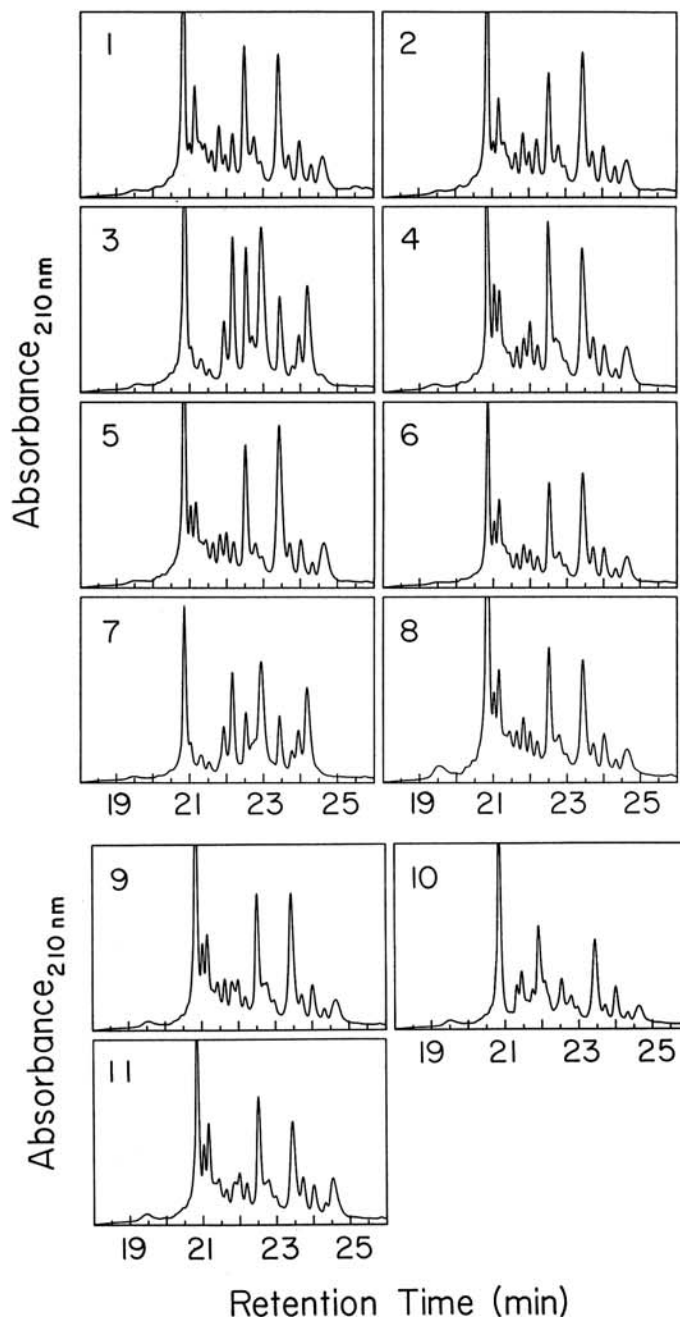

Fig. 2. High-performance liquid chromatographic patterns in the 18–26 min region. Full scale of figure = absorbance of 350 mAU (350 mV). Rice cultivars 1–11: IR 36, IR 42, Basmati 370, Mahsuri, Suweon 358, Khao Dawk Mali 105, Kataktara, Fujisaka 5, Azucena, Moroberekan, and Pankhari 203.
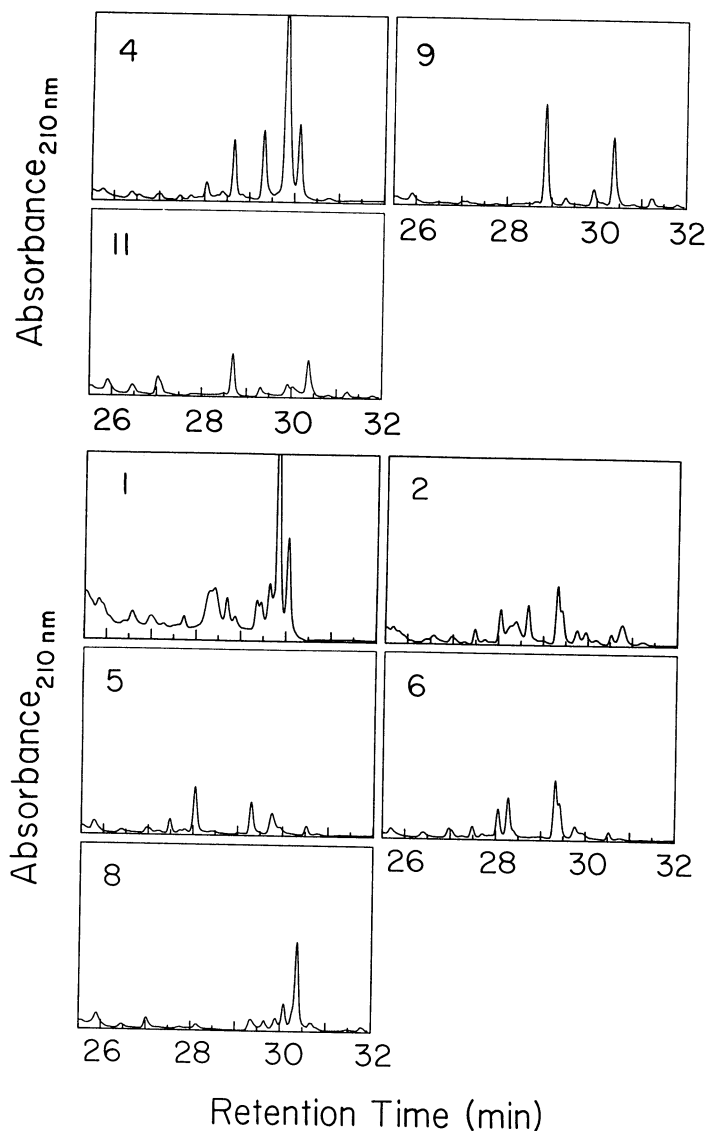
Fig. 3. High-performance liquid chromatographic patterns in the 25.5–32.0 min region. Full scale of figure = absorbance of 100 mAU (100 mV). Upper chromatograms, rice cultivars classified as subgroup AA: Mahsuri, Azucena, and Pankhari 203. Lower chromatograms, rice cultivars classified as subgroup AB: IR 36, IR 42, Suweon 358, Khao Dawk Mali 105, and Fujisaka 5.

solvent, no peaks were present in other parts of the chromatogram. Since peak retention times are unaffected by environmental factors (Huebner and Bietz 1988, Lookhart and Pomeranz 1985), the cultivars were distinguished on the basis of presence or absence of peaks in the chromatogram. Only those peaks that were present in chromatograms of both the 20- and 10-μl injections were used as the basis for qualitative distinction. Reproducibility between the two extractions, however, was not a problem. The chromatograph's HP 79994A analytical software objectively integrated the same peaks as small as 4 mAU (mV) total height in both the 10- and the 20-μl extracts, with the exception of one peak towards the end of the chromatogram of cultivar 1, which appeared 41 sec later in the 10-μl extract than in the 20-μl extract. Quantitative differences were not used to discriminate rice cultivars, since a peak in one cultivar and a shoulder in another cultivar might be two peaks or two shoulders when those cultivars were grown in different locations (Huebner and Bietz 1988).

The basis for the distinctions made below is subjective, so that alternative classifications may be made. However, it does illustrate that using qualitative peak differences, the rice cultivars can be grouped, and thus ultimately discriminated from each other (e.g., Williams 1976). Cursory visual examination of the main group of peaks (Fig. 2) showed that cultivars 3, 7, and 10 were very

different from the other cultivars, and that 10 was also very different from 3 and 7, so that three groups could be formed: group A, cultivars 1, 2, 4, 5, 6, 8, 9, and 11; group B, cultivars 3 and 7; and group C, cultivar 10. On the basis of a more detailed examination, group A was split into two subgroups, AA comprising cultivars 4, 9, and 11, and subgroup AB comprising 1, 2, 5, 6, and 8. The appearance of an extra peak at 22.7 min in the chromatograms of cultivars in subgroup AA was the basis for this classification. Although 11 appears to be similar to 8, the presence of a small peak at 22.7 min in the chromatogram of cultivar 11 distinguishes it from cultivar 8. Likewise, an additional peak in the chromatogram of cultivar 3 at 22.7 min was used to differentiate 3 from 7.

Discrimination of the remaining eight cultivars of subgroups AA and AB was achieved by comparing peaks in the 25.5–32 min region (Fig. 3). In subgroup AA, cultivar 4 can be distinguished from 9 and 11 on the basis of 4 having a peak at 28.0 min. In subgroup AB, cultivars 2, 5, and 6 were deemed different from 1 and 8 due to the presence of a peak at 28.0 min in the former. Cultivars 1 and 8 were then distinguished by a very prominent peak appearing in the former at 29.7 min.

The two remaining cultivars in subgroup AA were distinguished by the presence of a relatively large peak at 28.8 min in cultivar 9 that was absent from 11. The chromatograms of the remaining three cultivars in subgroup AB were sufficiently different from each other; a unique peak at 28.7 min distinguished cultivar 2, and an extra peak at 28.3 min distinguished 6 from 5.

The advantage of the present HPLC procedure is that a 10-μl injection gives large-scale absorbances. Small aliquots confer three advantages: less nonprotein materials extracted by the solvent are injected onto the column, so that less damage is caused to the column (Wehr 1987); less acetic acid is injected onto the column, so that there is less chance of stationary phase destruction (Glajch et al 1987); and, less proteins will be lost with the solvent peak (Marchylo and Kruger 1988).

CONCLUSION

The foregoing results confirm the potential distinctive power of both PAGE and HPLC procedures for discriminating cultivars of rice. Availability of the two identification procedures offers the flexibility of choice depending upon availability of equipment. A sufficient number of proteins was extracted so that the HPLC procedures described herein distinguished cultivars solely on a qualitative basis. The two procedures can be used with advantage not only for genotype discrimination but also in crop improvement in cases where certain agronomic traits are linked to electrophoretic bands or HPLC peaks.

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