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Study of Two Main Approaches-Electrophoresis and Chromatography as Varietal Identification Methods in Rice

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Abstract: Rice is a major worldwide crop that cultivated in the most areas of the north of Iran (Mazandaran and Gillan Province). An increase in the assortment of rice varieties is making it progressively more difficult to distinguish between the many cultivars by traditional visual identification methods. The more advanced identification techniques of electrophoresis and chromatography offer an effective solution to this emerging identification dilemma. This paper reviews the application of these two evaluation techniques. An Electrophoresis analysis includes gel electrophoresis and capillary electrophoresis and compares them with a popular chromatography technique, namely reversed-phase, size exclusion anion-exchange high performance liquid chromatography (HPLC). This paper will also include an interpretation of the results.

Key words: Rice, variety identification, electrophoresis, chromatography

INTRODUCTION

Rice, in terms of total production and value, is the most important cereal in the world. As a crop, it is especially important to developing countries (Juliano, 1995). There are hundreds of rice varieties. The quality of these different varieties varies considerably and as a result, proper identification of each variety has become a crucial matter (Juliano, 1995; Lookhart and Wrigley, 1995). When countries export rice, a declaration of variety is often used as the basis for defining quality type. Specialty varieties of rice include rice varieties such as Italian Arborio, Thai Jasmine, Indian Basmati, Japanese Short Grain, Japanese Mochi, Wild Rice, Red Rice, Black Rice and others. Some of these varieties are now grown in the United States and some are still imported. The founder and president of the company was the Senior VP of Marketing for the largest rice milling and exporting company in the U.S. for 6 years in the late 1980's. During this time period, he spent most of his time traveling the world buying and selling rice and studying the varieties grown in different countries. Sage V Foods takes advantage of this knowledge to help customers source specialty varieties. As the number of rice varieties continues to increase, visual methods for identification become increasingly unreliable (Juliano, 1995). An arising practice of developing rice with unusual quality characteristics has further heightened the necessity of establishing a set of requirements for identification (Juliano, 1995; Cooke, 1995). Many studies have reported various methods of identifying rice based on protein composition. Polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC) are among today's laboratory techniques of choice. Gel electrophoresis methods, acid (A-PAGE) or sodium dodeeyl sultfate (SDS-PAGE), are established techniques for separation of proteins. However, they have several drawbacks that include the use of toxic reagents, long analysis times and data that are difficult to quantify or interpret (Bietz and Schmaizreid, 1992). Recently Bean and Lookhart (2000) reported that rice cultivars were consistently differentiated in less than 15 minutes by capillary electrophoresis (CE). Capillary electrophoresis (CE) is a comparatively modem method of identification with the potential to distinguish between cereal varieties. It has been used to characterize cereal proteins, demonstrating excellent resolution and reproducibility. Additionally, CE is a very fast method and does not necessarily require extensive skilled manpower (Lookhart and Bean, 1995).

Separation of rice proteins: Provided efficient methods of protein analysis can be developed, a determination of the grain-protein composition using this analysis shows promise as a reliable identification procedure. Various other methods of analysis have been evaluated; most of

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these have involved either chromatographic or electrophoretic techniques (Wrigley *et al*., 1982; Bietz and Simpson, 1992).

The protein of rice varies from 7 to 15% in brown rice and from 6 to 13% in rough and milled rice (dry basis). Milled rice protein is 15% salt soluble (albumin and globulin), 20% ethanol soluble (prolamin) and 65% alkali soluble (glutelin) (Ogawa *et al*., 1987) Separation of these rice protein can be performed by either traditional (Osborne solubility) methods or modern methods.

Traditional method (Osborne solubility): According to the Osborne solubility method cereal proteins are classified into five groups on the basis of their solubility in a series of solvents. However, the Osborne system does not provide accurate quantitative data and many researchers have criticized its weak points (Shewry and Mifflin, 1985). These detractors emphasize that the extractability of the proteins is strongly dependent on many factors. These include flour particle size, the composition of the extraction solvents, the conditions of extraction (such as the fineness of milling or the vigor of shaking and stirring), the number and sequence of extraction steps, the temperature and also the type of mixing. Additionally, they point out that cross-contamination of fractions can occur, as can be seen from the wide ranging results of many different workers. However, the classic Osborne procedure is still widely used and is reserved as the first step of the purification process.

Modern techniques of protein separation: As discussed earlier, the separation of protein by the Osborne system is suitable only as the first step for a purification process. To have more precise preparative procedures for the preparation and identification of protein fractions, other techniques such as electrophoresis or chromatography should be employed. Chromatographic and electrophoretic techniques have long been used for protein identification. Chromatography is an analytical tool which selectively partitions components between two phases, while in electrophoresis, charged species are separated, based on their electrophoretic mobilities.

Chromatographic techniques: Chromatographic techniques have long been used for cereal protein isolation and characterization. These include size-exclusion high performance liquid chromatography (SE-HPLC), ion exchange chromatography (IE-HPLC) and reversed phase chromatography (RP-HPLC) (Bietz, 1986). SE-HPLC separates proteins primarily by size IE-HPLC fractionates them by charge differences (Huebner and Wall, 1966) and RP-HPLC separates them by differences in hydrophobicity.

Size-exclusion chromatography (SE-HPLC): Size-exclusion chromatography, also called gel permeation or gel filtration, achieves fractionation with a gel column. It separates molecules according to their size (Autran, 1994). Size-exclusion high performance chromatography (SE-HPLC) has been applied to cereal proteins to relate the quantity of protein fractions to breadmaking characteristics (Huebner and Bietz, 1985; Dachkevitch and Autran, 1989; Huebner and Bietz, 1993). It has commonly been used to provide information about protein aggregates and for further studies into their structures and interactions between the protein components (Autran, 1994).

Ion-Exchange chromatography (IEC): Ion-exchange fractionates proteins by differences in charge since, a charged compound in solution is attracted to and binds electrostatically to a support bearing a group with the opposite charge. However, it has not become a common technique in cereal protein separation due to the limited applications of various methods of ion-exchange HPLC to cereal proteins. In contrast, it has been widely used for biological and medical purposes (Batey, 1994; Bietz, 1986).

Reversed phase chromatography: Reversed-phase high performance liquid chromatography (RP-HPLC) separates proteins on the basis of differences in surface hydrophobicity (Unger *et al*., 1991). It has been used to identify rice proteins by several researchers (Huebner *et al*., 1991; Hussain *et al*., 1989; Lookhart *et al*., 1987) who have shown that rice varieties could be identified by RP-HPLC. The method demonstrated that eleven brown rice varieties were successfully differentiated, but the method was less effective on sister-line IR rice, excepting IR 36 and IR 42. However, various bonded phases from different manufacturers differ significantly in selectivity, which causes elution patterns to vary. Solvent gradient modification can also significantly affect resolution (Huebner *et al*., 1991).

Electrophoresis techniques: Although chromatographic techniques have been gaining acceptance for protein separation, electrophoresis has remained the most popular procedure for analysis of cereal storage proteins (Lookhart and Wrigley, 1995). Various electrophoretic techniques have been reported such as polyacrylamide gel electrophoresis (PAGE) at pH 3 and pH 7.6-8.9 gradient, sodium dodecyi sulfate (SDS) and isofocusing

(IEF) (Huebner *et al*., 1991; Guo *et al*., 1986). Starch gel electrophoresis was reported to separate rice proteins from 14 rice varieties (Li and Worland, 1993) Capillary electrophoresis has been shown to offer excellent capabilities for rice identification in recent years (Lookhart and Bean, 1995; Bean and Lookhart, 2000).

Gel electrophoresis: Gel electrophoresis has long been used for protein separations. Eiton and Ewart (1960) were first to describe the use of starch gel electrophoresis to separate cereal proteins. Other studies have reported the improvement of this procedure for varietal identification of wheat. However, starch gel electrophoresis may have poor reproducibility due to variable starch quality. Polyacrylamide gel electrophoresis (PAGE) using a synthetic polymer of acrylamide monomer, usually as acid-PAGE or with sodium dodecyi sulphate (SDS-PAGE), provides reproducible results. On the other hand, the main drawbacks of this method are that the preparation of gel usually requires a long time, the toxicity of acrylamide and data are difficult to quantify and interpret (Lookhart and Bean, 1995; Hames and Rickwood, 1981).

The ability to correctly differentiate and identify cultivars of cereal grains is an important aspect of cereal science. Polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC) are the laboratory methods of choice. Gel electrophoresis methods, usually acid (A)-PAGE or sodium dodecyl sulfate (SDS)-PAGE, are the established techniques for separation of proteins. However, they have several drawbacks that include the use of toxic reagents, long analysis times and data that are difficult to quantify and interpret. The electrophoretic methods and extraction conditions used to differentiate cultivars of all major cereal crops were recently reviewed by Lookhart (1990). Lookhart and Wrigley (1995) reviewed electrophoretic methods for varietal identification. The methods varied ideally in extraction procedures, in proteins analyzed and in the type of electrophoresis. Analysis times ranged from 1 to 12 h (Lookhart, 1990; Lookhart and Wrigley, 1995).

Jahani *et al*. (2002) studied the Variation of glutelin seed storage protein in Bangladesh rice cultivars. based on their work Glutelin, a major storage protein in rice, is synthesized as 57 kD proglutelin on the rER, transported to the vacuole via the Golgi apparatus and formed by proteolysis of the proglutelin through post-translational cleavage into acidic (alpha) and basic (beta) subunits in the vacuole (Yamagata *et al*., 1982; Takaiwa *et al*., 1986; Masumura *et al*., 1989). Uemura *et al*. (1996) reported that sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) gel electrophoresis are useful to detect the variation of glutelin seed storage protein. Kagawa *et al.* (1988) found the variation in glutelins of local rice cultivars by SDS-PAGE analysis. Satoh *et al.* (1990) reported that a large variation exists in glutelin polypeptides in rice collected in Tanzania. This report deals with the variation of glutelin polypeptides in rice cultivars of Bangladesh, an area considered to be one of the centers of origin of cultivated rice (Chatterjee, 1951).

Five hundred and seventy six Bangladesh rice cultivars preserved at the Laboratory of Plant Genetic Resources, Kyushu University, Japan, were used in this study. Extracted proteins were separated by SDS-PAGE using the discontinuous buffer system of Laemmli (1970) on a slab gel containing a linear of 15 to 25% acrylamide, 0.05 to 0.67% BIS concentration gradient. Rice glutelin was composed of alpha and beta subunits, which were separated into alpha-1 (39 kD), alpha-2 (38 kD), alpha-3 (37.5 and 37 kD) and alpha-4 (34 and 33 kD) for alpha subunit and beta-1 (23 kD), beta-2 (22.5 kD) and beta-3 (22 kD) bands for beta subunit, respectively. Uemura *et al*. (1996) reported that the alpha-3 band of Japanese rice cultivar Kinmaze is smaller in molecular size than that of rice cultivar IR36 developed at IRRI, while the alpha-4 (34 kD) band of Kinmaze is larger than that of IR36 (33 kD). Bangladesh rice cultivars varied significantly in SDS-PAGE profiles of glutelin storage protein. In addition to 'Kinmaze' (type 1) and 'IR36' (type 5) types, mutant types with decreased alpha-2 band and with two alpha-3 bands were observed. One mutant was characterized by decreased intensity of alpha-2 band, as alpha-2 deficient mutants induced by N-methyl-N-nitrosourea (MNU) treatments (Satoh *et al*., 1997). The other mutant possessed two alpha-3 bands with different molecular mass (type 2). The decreased intensity of alpha-2 band was accompanied by an increased intensity of alpha-1 band, suggesting that the total glutelin content remained unchanged. On the other hand, the cultivars having decreased intensity of alpha-2 bands (types 3 and 4) differed in SDS-PAGE pattern of alpha-3 band, indicating that alpha-2 and alpha-3 bands were controlled by different genes. This is the first report on spontaneous glutelin mutants detected by SDS-PAGE from Bangladesh rice cultivars, suggesting that rice genetic resources of Bangladesh provide a rich source of genetic diversity.

In Bangladesh, rice is grown in three seasons, aus in summer, aman in autumn and boro in winter. They showed that ecotypic distribution for glutelin variation. In all of the ecotypes, type 5 was most frequent, indicating that selection preference was higher for this type. Types 2, 3 and 4 were confined to T. Aman ecotype, even if their collection places were different. SDS-PAGE separates proteins based on molecular masses, while IEF separates

them depending on electric charges. SDS-PAGE analysis does not elucidate the genetic traits of the polypeptides because each of the SDS-PAGE bands consists of several polypeptides by IEF (Wen and Luthe, 1985). After extraction by 1% lactic acid, the glutelins of 74 cultivars were analyzed by the horizontal slab gel IEF system. At least 13 types were detected among the cultivars studied for IEF. Types 1 and 5 of SDS-PAGE were separated to types 4 to 10 and types 14 to 16 of IEF respectively, suggesting that both SDS-PAGE and IEF analyses were important for detecting glutelin variation in rice. The alpha-2 band deficient mutants lacked in pI 6.80 band for IEF, while all the cultivars having alpha-2 band, such as IR36, possessed pI 6.80 band, suggesting that pI 6.80 band was the major polypeptide component of alpha-2 subunit. Similarly, increased intensity of pI 6.59 band and the presence of pI 6.30 band in types 12 and 13 in common suggested that they were the polypeptide components of alpha-1 subunit. These results suggest that the mutated subunits were controlled by structural genes. Meanwhile, IEF profile of cultivars having two alpha-3 bands showed increased intensity of pI 7.52 band and reduced intensity of pI 7.19 band, a pattern similar to IR24 by IEF. Rice seed stores most of the proteins as dilute acid/alkali soluble glutelin (about 75% of total protein) which is superior in quality due to its easy digestibility and the presence of high amount of first limited amino acid, lysine (Huebner *et al.*, 1990). The glutelin variation observed in Bangladesh rice cultivars may serve as useful materials to improve rice grain quality.

Capillary electrophoresis: Capillary electrophoresis (CE) is a modem analytical technique. It has proved to be rapid, sensitive and can be automated, providing high resolution, separation and reproducibility (Lookhart and Bean, 1995). Furthermore, the availability of advanced commercial instrumentation of CE has led to its use for the analysis of various compounds such as proteins, sugars, oligosaccharides, anions and soluble vitamins (Cancalon, 1995). Capillary zone electrophoresis (CZE) of endosperm storage proteins was used to differentiate cultivars of both oats and rice in less than 12 min (Lookhart and Bean, 1995). This is the first study that proteins of these two cereals have been separated by CZE. Cultivars were chosen for the difficulty of different-were no differences tiating them by other means, electrophoretic or chromatographic. Ethanol (70%) extracts of the oat samples were separated on a 20-yrm i.d. untreated fused-silica capillary, whereas rice samples were extracted with 60% 1-propanol and the solubilized proteins were separated on a 50-,um i.d. untreated fused-silica capillary. The CZE separation buffer was 0.1M phosphate, pH 2.5, containing 0.05% hydroxypropylmethylcellulose (HPMC). Most cultivars were differentiated quickly and easily. Only the patterns of two rice cultivars, IR36 and IR50, were nearly identical. There were no differences between IR36 and IR50 extracts by high-performance liquid chromatography (HPLC) or acid (A)-polyacrylamide gel electrophoresis (PAGE). CZE is a faster method of separating endosperm storage proteins than A-PAGE and separates as least as well and better in most cases than either A-PAGE or reversed phase (RP)-HPLC.

The use of CE for rice varietal identification has been reported over the past decade (Lookhart and Bean, 1995; Bean and Lookhart, 2000). At present, the use of CE for variety identification has so far involved the use of sophisticated equipment that has been of considerable size and cost, warranting a place in a central laboratory. In the future, the approach of CE would offer the prospect of small, portable equipment that would offer the ideal combination of convenience, speed of analysis and good resolution, together with portability and consequently on-site use (Wrigley and Bekes, 2002).

Capillary Zone Electrophoresis was successfully used for the first time to differentiate rice and oat cultivars (Although RP-HPLC was able to distinguish oat cultivars with identical A-PAGE patterns, the different separation modes enabled more differences to be detected with CZE than with RP-HPLC. In addition, CZE analysis required considerably less time (24 min) to analyze the storage proteins than A-PAGE (2-4 h) and were consistently shorter than RP-HPLC analysis times (30-60 min), even when equilibration was considered. CZE is complementary to RPHPLC, where it has the advantage of speed and resolution with the similarity of ease of automation and differs in mode of separation, charge versus hydrophobicity. Oat avenins were separated in less than 6 min by free-zone capillary electrophoresis using a low pH phosphate buffer. Rice cultivars, both from the U.S. and from the IRRI, were consistently differentiated by CZE in less than 12 min. U.S. long-grain cultivars L202 and Newbonnet, which had identical HPLC patterns, were also readily differentiated by CZE. Cultivars with close genetic relationships may exhibit similar or identical prolamin patterns by CZE, as they do by A-PAGE or RP-HPLC. The fast speed, high resolution and complementary nature of CZE make it well suited to quickly differentiate rice cultivars, even those with similar or identical A-PAGE and RPHPLC patterns (Lookhart and Wrigley, 1995).

Variety identification of rice: Identification of rice varieties is very important because of the differences in quality associated with different varieties (Juliano, 1995). The declaration of variety for rice deliveries is used in

many rice producing countries as a basis for defining quality type (Lookhart and Wrigley, 1995). As the number of rice varieties increase over the years, visual methods for identification of these varieties have become insufficient. Because there is a need to keep pace with the growing number of quality characteristics, the requirements for varietal identification are under continuous development (Lookhart and Wrigley, 1994).

In summary, the routine methods can be compared as in Table 1 (Wrigley and Bekes, 2002). CE and RP-HPLC have advantages with respect to speed of analysis, instant data interpretation and low labor cost. In contrast, gel electrophoresis has potential for greater through-put of samples with relatively low capital costs for the extra equipment needed. Health risks are lower in CE and pre-cast gel than in RP-HPLC and conventional PAGE.

Use of storage protein for variety identification: Storage proteins (tertiary sementides) especially prolamin have long been used for varietal identification because of the consistency of prolamin (gliadin) composition. This despite a wide range of variations in growth and treatment conditions. This aspect of phenotype for the genetic material is extremely stable under variable environmental conditions, such as growth location, growth season and soil conditions. Lookhart *et al.* (1987). Huebner and Bietz (1985) also studied the effects of growth locations and climactic conditions on gliadin composition, using RP-HPLC. He found that total amounts of specific gliadins vary with both location and conditions, but the qualitative aspects of the chromatograms of gliadin were not altered. Recently, Anjum *et al.* (2000) reinforced previous reports that gliadin composition obtained from A-PAGE bands was not affected by growth locations and crop years, with only minor differences in band densities being observed (Bietz, 1986; Hames and Rickwood, 1981).

Summary: The difficulties in identifying different rice varieties are compounded by the use of insufficient, ineffective and subjective visual identification methods. Two main techniques have shown improved abilities to better separate rice proteins and hence provide more effective and reliable variety identification. However, it has been found that the two techniques share a frequently encountered problem, being that a particular variety can be polymorphic for protein composition depending on the electrophoretic or chromatographic method used; that is, analysis of individual grains of the same variety gives more than one electrophoretic or chromatographic pattern due to the presence of multiple biotypes within the variety. A solution to this problem may lie in analysis of whole meal samples, which account for the main biotypes to define the variety. Alternatively, the use of a less discriminating method may reduce the risk of distinguishing between biotypes.

Table 1: The relative effectiveness of routine methods of variety identification based on protein analysis (Wrigley and Bekes, 2002)

Factors	PAGE	Pre-cast Gel	RP-HPLC	CE
Time to set gel or regenerate column	60 min	10 min	10 min	2 min
Sample extraction	20 min	20 min	20 min	20 min
Sample run time	240 min	10-90 min	30 min	10 min
Protein visualisation	Overnight	20 min or overnight	Instant	Instant
Data interpretation	10 min	10 min	Instant	Instant
Through-put in 24 h	20 gel	10 gel	30	100
Health risk to Operators	Moderate	Low	Low-Moderate	Low
Costs-equipment	Low	Low	High	High
Cost-consumables	Low	Medium	Medium	Medium
Costs-labour	High	Moderate	Low	Low

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