Studies of Rice Proteins by Crossed Immunoelectrophoresis, Gel Electrophoresis, and Isoelectric Focusing

A. I. SHADI[1] and R. DJURTOFT, Department of Biochemistry and Nutrition, Technical University of Denmark, DK 2800 Lyngby, Denmark

Cereal Chem. 56(5):402-406

ABSTRACT

Soluble proteins (albumins and globulins) of two rice varieties were investigated by crossed immunoelectrophoresis. We could distinguish 28 or 29 individual components in a salt extract of the embryo and endosperm of the rice grain. The concentration of some proteins seemed to be higher in the embryo than in the endosperm; some were characteristic of only one variety. After seven days of germination in the dark, extracts of variety IR 1561-228 roots and shoots were subjected to immunoelectrophoretic study of proteins. Most proteins were identified in the embryo. All soluble proteins in the whole grain also were found in endosperm extracts. By specific staining methods, three phosphatases, one esterase, and one aminopeptidase were identified in the rice extract. By sodium dodecyl sulfatepolyacrylamide gel electrophoresis, the two rice varieties were shown to have two major subunits with molecular weights of 12,000 and 20,000 and relatively minor subunits with molecular weights of about 31,000, 56,000, and 90,000. After germination, the 20,000 mol wt band nearly disappeared from the extractions of roots, shoots, embryo, and endosperm. The soluble rice proteins were studied further by isoelectric focusing. Twenty-one protein bands with different pI from 4.0 to 8.0 were found, among which were the three phosphatases.

Protein content varies greatly in mature rice grains (Juliano et al 1968). In brown rice it ranges from 5 to 17% (Juliano 1966, Juliano et al 1964, 1968). The protein content of milled rice averages 7% (Athwal 1975).

Water-soluble and salt-soluble proteins (albumins and globulins) account for a minor part of the total proteins of milled rice. In general, the globulins are 7–11% of the total proteins and albumins 0.5–6% (Houston and Mohammad 1970). Each of these classes of proteins is a mixture of components. The albumins and globulins are present mainly in the protein from the outer layers of the endosperm (Houston et al 1968).

Iwasaki et al, (1972), using starch gel electrophoresis at pH 8.9, found 15 albumin bands and six globulin bands. Gel chromatography showed four albumin peaks and three globulin peaks. Narasubhai et al (1974) reported that electrophoretic analysis of the protein fractions of rice germ showed eight protein moieties of the three protein fractions (albumin, globulin, and prolamin). Perdon and Juliano's (1978) gel filtration study of the α-globulin at pH 6.5 showed two proteins with 20,000 and 98,000 mol wt, respectively. Sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis of α-globulin revealed one subunit with 18,000 mol wt.

Interest has recently increased in the use of immunochemical methods to analyze plant (seed) proteins (Catsimpoolas and Meyer 1968, Djurtoft 1969, Djurtoft and Hill 1966, Hejgaard 1978, Hejgaard and Bøg-Hansen 1974). The purpose of this work is to describe some applications of crossed immunoelectrophoresis as a most useful quantitative technique for studying soluble proteins, including some enzymes, in diffferent types of rice grains. For comparison, proteins were also separated by other techniques, including SDS-polyacrylamide gel electrophoresis and isoelectric focusing.

[1]Permanent address: Department of Agricultural Biochemistry, Faculty of Agriculture, Ain-Shams University, Cairo, Egypt.

0009-0352/79/000063$3.00/0

MATERIALS AND METHODS

Materials

Mature rice grains (IR-1561-228 and Giza 159) were taken from the 1977 crop at the Rice Research Institute, Agricultural Research Center, Cairo. The dry grains were brought to Denmark and stored at 4°C until use. The samples were finely ground in a cyclone sample mill (Tecator/Udy S-1700).

Germination

Rice grains (variety IR-1561-228) were sterilized in 0.1% aqueous chlorohexidine for 30 sec, washed twice with sterile water, soaked in ethanol for 30 sec, washed twice, and finally steeped in sterile water for about 5 hr at 4°C. After steeping, the seeds were placed on moist filter paper in covered glass dishes and kept at room temperature (20°C) in the dark. After one week of germination, the roots were 4–5 cm long and the shoots were about 3 cm long. The roots, shoots, embryo, and endosperm were separated and frozen at −20°C until used for protein extraction.

Extraction of Proteins

Soluble proteins (albumins and globulins together) were extracted according to the method of Djurtoft (1969). Milled rice (2.5 g) was extracted with 10 ml of 0.1 *M* NaCl at room temperature for 1 hr. After centrifugation at 10,000 × *g* for 15 min, the supernatant was used for protein analysis.

Immunochemical Methods

Crossed immunoelectrophoresis was performed as described by Hejgaard and Bøg-Hansen (1974), using essentially the equipment, materials, and techniques of Axelsen et al (1973). Antibodies used were purified immunoglobulin fractions (Axelsen et al 1973), made from concentrated pooled rabbit antisera toward 0.1*M* NaCl extract of milled rice (IR-8 rice from IRRI, Philippines).

SDS Gel Electrophoresis

The soluble proteins were subjected to SDS gel electrophoresis to determine the molecular weights of their subunits, according to the method of Weber and Osborn (1969).

Isoelectric Focusing

The technique described by Wrigley (1968) was used for fractionation and characterization of rice protein extracts.

Staining Methods

Protein precipitates on the washed and dried immunoplates were stained with Coomassie's brilliant blue R-250 (Serva, Heidelberg, W. Germany), as described by Axelsen et al (1973). Enzyme activities were traced immediately on the wet immunoplates by using specific histochemical staining methods (Hejgaard and Bøg-Hansen 1974, Uriel 1971). Phosphatases were identified with β-naphthylphosphate (Na-salt) as substrate in 0.05*M* acetate buffer, pH 5.0. Esterase was identified with α-naphthyl acetate as substrate in 0.05*M* phosphate buffer, pH 6.4. Aminopeptidase was identified with leucine β-naphthyl amide in 0.05*M* phosphate buffer, pH 7.4. In each case, the immunoplates were incubated in a solution of approximately 10 mg of the substrate and 20 mg of Fast Garnet GBC diazonium salt (Serva, Heidelberg, W. Germany) in 30 ml of the appropriate buffer. The red color was developed during incubation for 30–60 min at 30° C. The plates were washed, dried, and stored.

RESULTS AND DISCUSSION

Immunoelectrophoresis has evolved through different stages. In the original version (Grabar and Williams 1953), electrophoresis of proteins in one direction in a gel plate was followed by free diffusion of the separated proteins toward a trough containing the antibodies. Specific precipitin lines appeared for the individual proteins when they met their respective antibodies. In more recent applications (crossed immunoelectrophoresis as used here), the proteins are first allowed to separate by electrophoresis in one direction. The gel slab is then placed next to another gel slab containing the antibodies, and electrophoresis toward the antibodies is repeated in a perpendicular direction. The experiment is performed at pH 8.6, where the antibodies are nearly stationary (isoelectric point); most other proteins move toward the anode placed at the other side of the gel that contains antibodies. In this way, proteins meet the antibodies, and precipitin lines develop. The area enclosed by the precipitate is proportional to the amount of the protein and inversely proportional to the corresponding antibody concentration (Clarke and Freeman 1967). The localization and the characteristic morphology of some precipitates (1 and 15 in Fig. 1B), obtained by crossed immunoelectrophoresis of rice extracts, make these precipitates suitable as reference points for standardization and further localization and identification of peaks.

Fig. 1A and B from the two rice varieties show that, by crossed immunoelectrophoresis, rice extract is resolved into a series of components. Detailed analysis permitted us to distinguish 28–29 immunochemically different components[2].

By comparison, starch gel electrophoresis showed 15 albumin bands and six globulin bands (Iwasaki et al 1972).

A series of experiments similar to those in Fig. 1A and B showed that the peaks indicated by an arrow in Fig. 1C are characteristic for the IR rice variety. This indicates that the protein content of different rice varieties may vary qualitatively.

Immunoelectrophoretic experiments also were performed on NaCl extracts of separate fractions of the grain (IR-1561-228). Extracts from the hull fraction gave no precipitin lines. The embryo could not be completely separated manually from the endosperm. We therefore cut the rice grains in two parts with the cutting line as near the embryo as possible. The embryo fraction was 27% of the total weight of the dehulled rice grain (average for 100 grains). The extract of the embryo end and the endosperm end of rice grain gave the same qualitative picture, but we found quantitative differences in some of the precipitates. The areas of the components corresponding to the peaks 1, 2, 3, and 15 (Fig. 1C) were definitely greater (factor 2) in the pattern from the embyo part than in that from the endosperm part. Apparently, the concentration of these

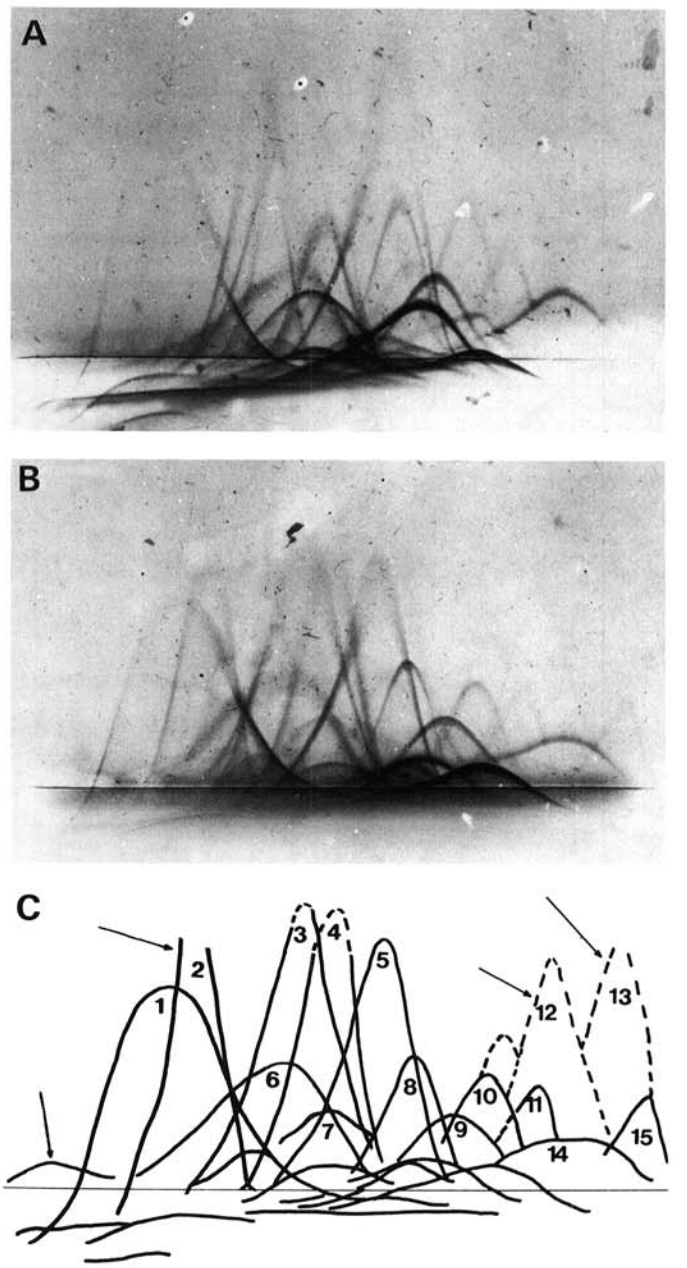

Fig. 1. Crossed immunoelectrophoresis of protein extracts from rice grains. Proteins are first electrophoretically separated in the horizontal direction shown in the picture, then moved in the vertical direction in a gel containing antibodies. **A,** Giza 159 (Egyptian rice variety); **B,** IR-1561-228 (rice variety developed at the International Rice Research Institute, Manila). **C,** Diagram drawn from Fig. 1B. Components are numbered, staring with the slowest migrating component.

[2] Using the same immunoelectrophoretic technique and reversing the current so that the electrophoresis was toward the cathode, we could see two precipitates in the gel. These cathodically migrating antigens were not studied further.

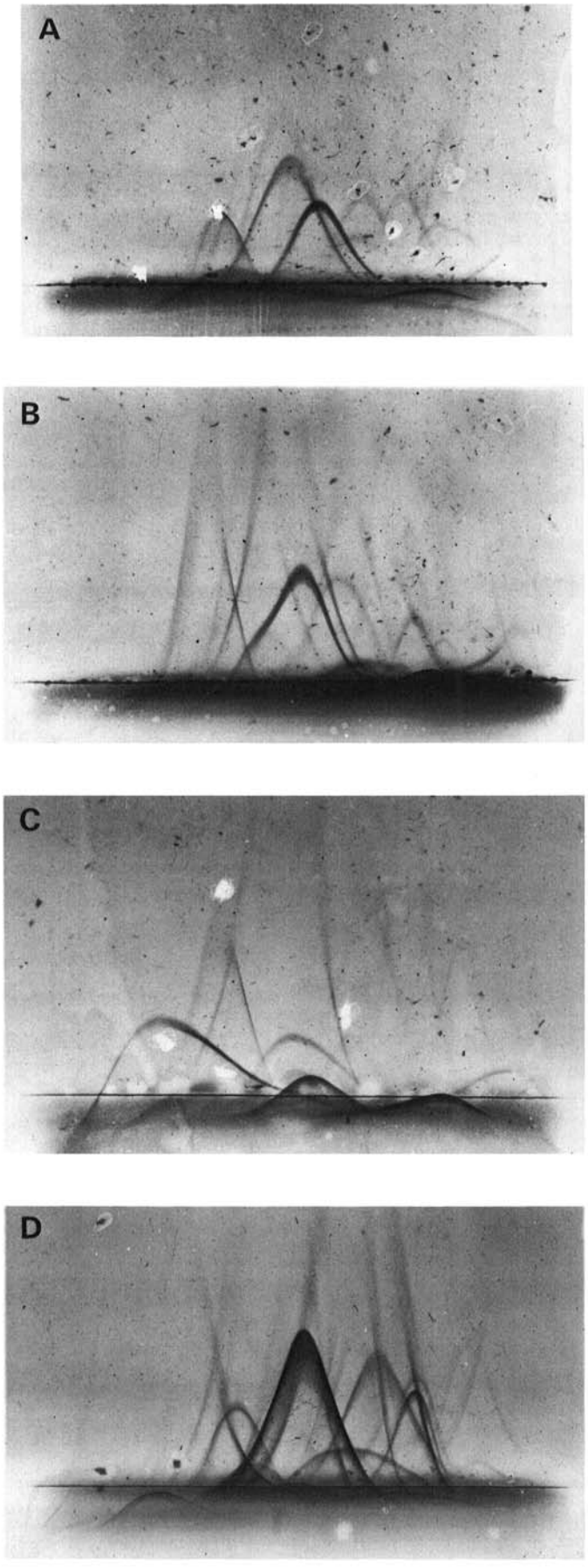

Fig. 2. Crossed immunoelectrophoresis of rice protein extracts (IR-1561-228) after seven days of germination in the dark. **A,** roots; **B,** shoots; **C,** embryo; **D,** endosperm.

proteins was higher in the embryo than in the endosperm.

To analyze in more detail the background of the composite picture of the 28–29 components in Fig. 1A and B, we designed some experiments involving germination and analysis of separated tissues of the seeds.

Extracts of roots, shoots, embryo, and endosperm are compared in Fig. 2A, B, C, and D. We found 12, 17, 14, and 28 proteins in roots, shoots, embryo, and endosperm respectively. Most other studies on protein changes in rice grain during germination have been related to the endosperm (Matsushita 1958, Palmiano and Juliano 1972, 1973). Progressive decrease in total endosperm nitrogen has been noticed during germination (Matsushita 1958). The crude protein started to decrease after two days of germination, indicating breakdown and possibly translocation to the embryonic tissue. Soluble protein increased during the first six days of germination. Protease activity increased five to six times during germination (Palmiano and Juliano 1972). Figure 2A and B also shows that shoots and roots qualitatively contain nearly the same soluble protein. The "root proteins" (similar to peaks 2–6, 10, and 14 in Fig. 1C) were also found in the shoot extracts. We could identify most of the root and shoot proteins in the embryo extract (Fig. 2C). This may indicate translocation of embryo proteins to roots and shoots or new synthesis of these proteins. The endosperm extract (Fig. 2D) contains all the protein components present in the extract of the whole ungerminated grain (Fig. 1B). These results on endosperm agree with those obtained by Houston et al (1968), who found that soluble protein (albumin and globulin) is concentrated in the outer layer of the endosperm.

To obtain more information on the proteins found in our immunoelectrophoretic analysis, we made some tentative studies on their possible enzymatic activities. Fig. 3A,B, and C shows the specific staining for phosphatase, esterase, and aminopeptidase activities of the total extract from IR-1561-228. Three phosphatases (peak 10 and small curves below peaks 8 and 9), one esterase (peak 12), and one aminopeptidase (peak 13) were identified among the 28 protein components identified in Fig. 1C. More detailed study is under way on some of these enzymes in rice extracts.

SDS-polyacrylamide gel electrophoresis can be used to determine the molecular weight of polypeptide chains with an accuracy of at least ±10% (Weber and Osborn 1969). We used this technique to determine the molecular weight of the main protein groups in NaCl extracts of the two types of ungerminated rice (IR-1561-228 and Giza 159). Fig. 4A and B shows that the two preparations have nearly identical disc electrophoretic patterns containing about 13 bands. We find two major fast migrating bands corresponding to about 12,000 and 20,000 mol wt and three relatively slowly migrating bands with molecular weight of about 31,000, 56,000, and 90,000. The electrophoreograms of roots, shoots, embryo, and endosperm extracts are shown in Fig. 4C, D, E, and F. Compared with the total extract in Fig. 4B, they all indicate the disappearance of the band with 20,000 mol wt. We observed a relative decrease in the root extract in the band with 12,000 mol wt. The band with molecular weight of 31,000 or higher remained in all four cases. These results agree with our results on crossed immunoelectrophoresis, where some of the proteins that exist in the whole grain do not appear in roots, shoots, and embryo extracts.

Isoelectric focusing involves the migration of protein molecules to the regions of their isoelectric point in a pH gradient (Svensson 1961, Vesterberg and Svensson 1966). Haglund (1967) described in detail the principle of isoelectric focusing fractionation of a protein sample using the sucrose density column. The main modification in gel electrofocusing is that a polyacrylamide gel is used instead of a sucrose density gradient (Wrigley 1968). In gel electrophoresis, protein molecules are fractionated according to charge and size. Spreading of zones due to difusion continues throughout the run. In the gel electrofocusing method, molecules are moved from any part of the gel and concentrated at the position in the pH gradient corresponding to the isoelectric point. Spreading due to diffusion is minimized (Wrigley 1968).

During our investigation, rice proteins extracted with 0.1*N*

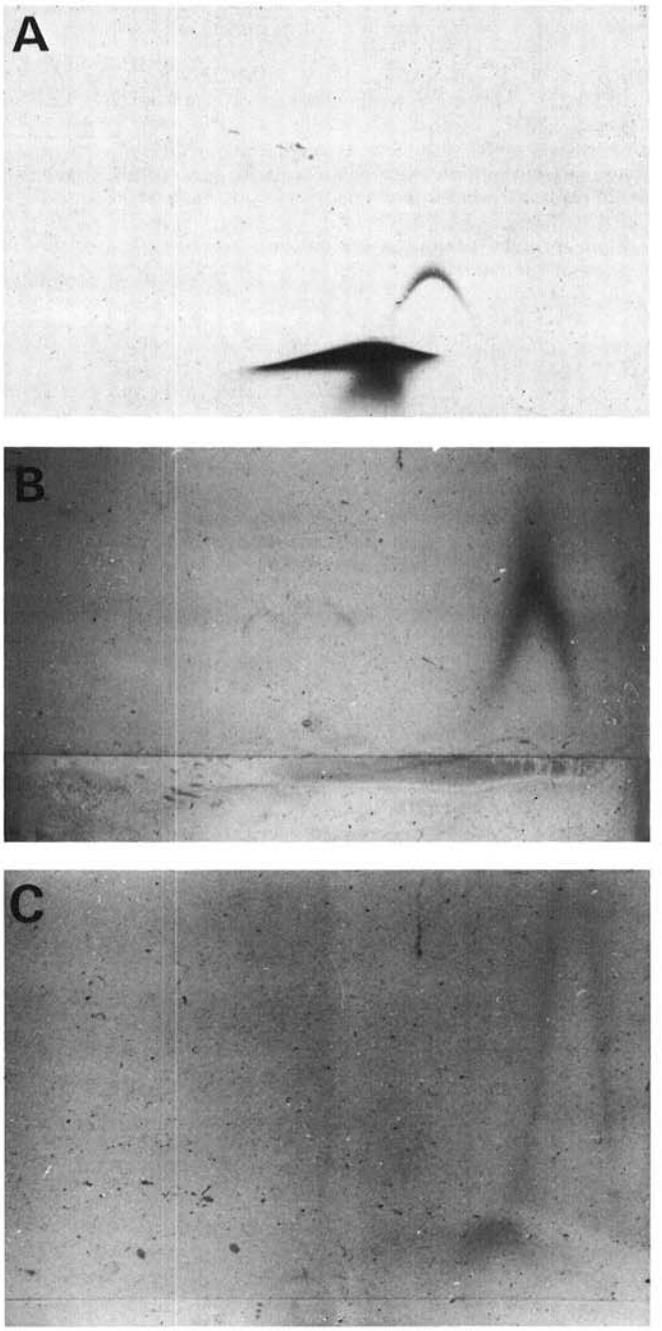

Fig. 3. Crossed immunoelectrophoresis of ungerminated rice grain (IR-1561-228) extract. Plates were stained for phosphatase (**A**), esterase (**B**), and aminopeptidase (**C**) activities, respectively.

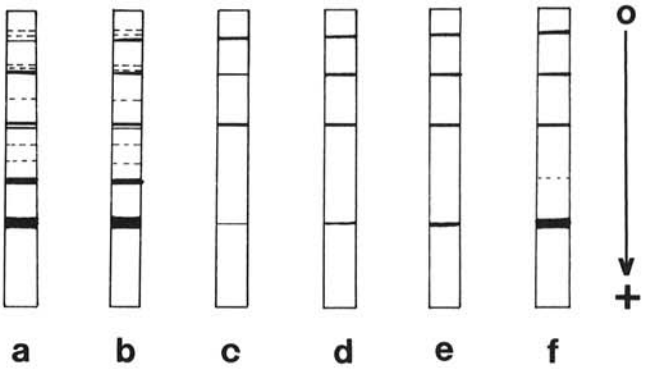

Fig. 4. Polyacrylamide gel electrophoresis (electrophoreograms) of extracts from: **A,** ungerminated rice grains (IR-1561-228); **B,** ungerminated rice grains (Giza 159); **C, D, E,** and **F,** roots, shoots, embryo, and endosperm, respectively, of rice grains (IR-1561-228) germinated seven days.

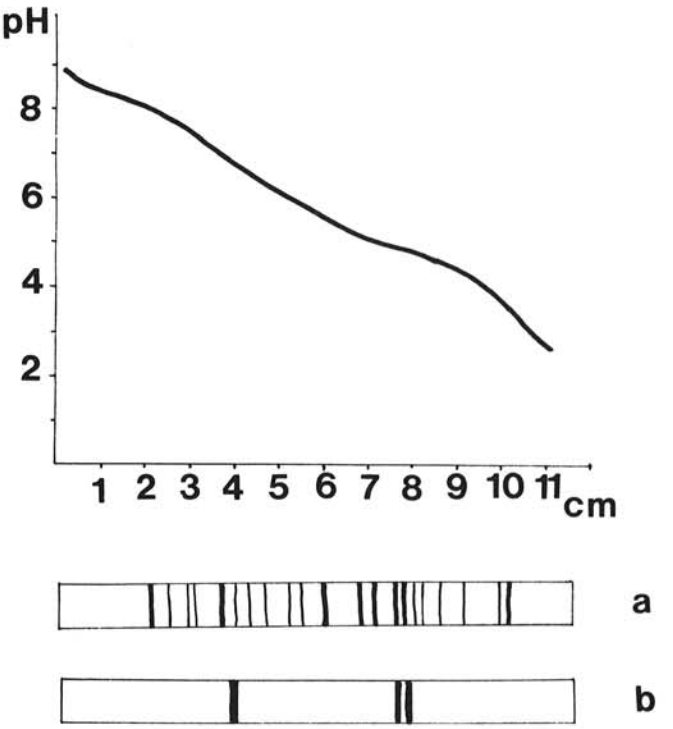

Fig. 5. Plot of pH gradient of gel electrofocusing in the separation of rice proteins (NaCl extraction). pI values shown in diagrams **A** and **B** were read from the pH gradient. **A,** gel stained for proteins; **B,** gel stained for phosphatase activity.

NaCl were subjected to isoelectric focusing using Ampholine (pH 3–10). Isoelectric focusing showed 21 bands at different pHs from 4 to 8 as indicated in Fig. 5. These results illustrate that isoelectric focusing can be used for fractionation of rice protein extracts. The method gave 21 bands and the pI values of these proteins.

Specific staining methods for enzymes and the isoelectric focusing technique can be used to determine the pI of these enzymes and to isolate them in separate and sharp bands. Fig. 5B shows the separation and identification of three phosphatases (identified immunochemically in Fig. 3B). The pI of these phosphatases are 4.85, 4.95, and 6.7, respectively.

In comparison, SDS-gel electrophoresis gave only 13 bands, but crossed immunoelectrophoresis yielded 28 components. This can be explained by the fact that SDS gel electrophoresis determines mainly the molecular weights of protein subunits. Isoelectric focusing shows 21 sharp bands from a rice protein extract, and at the same time, the pI values of these proteins are determined. We conclude that crossed immunoelectrophoresis supplemented by these other techniques is a most powerful way of studying rice proteins. Moreover, specific staining methods with both immunoelectrophoresis and isoelectric focusing can be used to identify enzyme activity in these proteins.

ACKNOWLEDGMENT

We thank our colleague, Dr. Jørn Hejgaard, for advice and stimulating discussions during the work.

LITERATURE CITED

ATHWAL, D. S. 1975. Present and future research on rice. Cereal Foods World 20:354.

AXELSEN, N. H., KRØLL, J., and WEEKE, B. (eds.). 1973. A Manual of

Quantitative Immunoelectrophoresis. Universitetsforlaget:Oslo.
CATSIMPOOLAS, N., and MEYER, E. W. 1968. Studies on the characterization of soybean proteins by immunoelectrophoresis. Arch. Biochem. Biophys. 127:338.
CLARKE, H. G. M., and FREEMAN, T. 1967. In: PEETERS, H. (ed.). Protides of Biological Fluids, p. 503. Elsevier: Amsterdam.
DJURTOFT, R. 1969. Immunochemical and immunoelectrophoretic identification studies on proteins from cereals. New approaches to breeding for improved plant protein. pp. 137-150. Intern. Atomic Energy Agency: Vienna.
DJURTOFT, R., and HILL, R. J. 1966. Immunoelectrophoretic studies of proteins in barley, malt, beer and beer haze preparations. pp. 137-146. Proc. Europ. Brew. Conv. Elsevier: Amsterdam.
GRABAR, P., and WILLIAMS, C. A. 1953. Méthode permettant l'étude conjuguée des propriétés électrophorétiques et immunochimiques d'un melange de protéins. Application au sérum sanguin. Biochim. Biophys. Acta 10:193.
HAGLUND, H. 1967. Isoelectric focusing in natural pH gradients. A technique of growing importance for fractionation and characterization of proteins. Sci. Tools 14:17.
HEJGAARD, J. 1978. "Free" and "bound" β-amylases during malting of barley. Characterization by two-dimensional immunoelectrophoresis. J. Inst. Brew. 84:43.
HEJGAARD, J., and BØG-HANSEN, T. C. 1974. Quantitative immunoelectrophoresis of barley and malt proteins. J. Inst. Brew. 80:436.
HOUSTON, D. F., IWASAKI, T., MOHAMMAD, A., and CHEN, L. 1968. Radial distribution of protein by solubility classes in the milled rice kernel. J. Agric. Food Chem. 16:720.
HOUSTON, D. F., AND MOHAMMAD, A. 1970. Purification and partial characterization of a major globulin from rice endosperm. Cereal Chem. 47:5.
IWASAKI, T., SHIBUYA, N., and CHIKUBU, S. 1972. Rice protein. I. Albumin and globulin in rice endosperm. Their heterogeneity and the difference in subunit composition among stored, heated and fresh rice. Nippon Shokuhin Kogyo Gakkai-Shi 19:70.
JULIANO, B. O. 1966. Physicochemical data on the rice grain. Int. Rice Res. Inst. Tech. Bull. 6.
JULIANO, B. O., ALBANO, E. L., and CAGAMPANG, G. B. 1964. Variability in protein content, amylose content and alkali digestibility of rice varieties in Asia. Philipp. Agr. 48:234.
JULIANO, B. O., IGNACIO, C. C., PANGANIBAN, V. M., and PEREZ, C. M. 1968. Screening for high protein varieties. Cereal Sci. Today 13:299.
MATSUSHITA, S. 1958. Studies on the nucleic acids in plants. III. Changes of the nucleic acid content during germination stage of the rice plant. Mem. Res. Inst. Food Sci. Kyoto Univ. 14:30.
NARASUBHAI, A. V., RAJULU, G. S. and GEORGE, C. J. 1974. Electrophoretic analysis of the protein fractions extracted from the germ of the Ginsali variety of paddy. J. Anim. Morphol. Physiol. 20:128.
PALMIANO, E. P., and JULIANO, B. O. 1972. Biochemical changes in the rice grain during germination. Plant Physiol. 49:751.
PALMIANO, E. P., and JULIANO, B. O. 1973. Changes in the activity of some hydrolases, peroxidase, and catalase in the rice seed during germination. Plant Physiol. 52:274.
PERDON, A. A., and JULIANO, B. O. 1978. Properties of a major α-globulin of rice endosperm. Phytochemistry 17:351.
SVENSSON, H. 1961. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradient. I. The differential equation of solute concentrations at a steady state and its solution for simple cases. Acta Chem. Scand. 15:325.
URIEL. J. 1971. In: WILLIAMS, C. A., and CHASE, M. W. (eds.). Methods of Immunology and Immunochemistry. Vol. III, 294 pp. Academic Press: New York.
VESTERBERG, O., and SVENSSON, H. 1966. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in connection with separation of myoglobins. Acta Chem. Scand. 20:820.
WEBER, K., and OSBORN, M. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406.
WRIGLEY, C. 1968. Gel electrofocusing. A technique for analysis of multiple protein samples by isoelectric focusing. Sci. Tools 15:17.

[Received October 2, 1978. Accepted March 11, 1979]