

# Purification and characterization of ten new rice NaCl-soluble proteins: identification of four protein-synthesis inhibitors and two immunoglobulin-binding proteins

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**Abstract.** Ten new proteins from rice (*Oryza sativa* L. cv. Bahia) including four protein-synthesis inhibitors and two immunoglobulin E (IgE)-binding proteins have been isolated and characterized. These proteins as well as one previously known component,  $\alpha$ -globulin, were purified from a 0.5 M NaCl extract of rice endosperm by a new, apparently non-denaturing, isolation procedure developed for rice proteins. The method is based on extractions of this complex protein mixture with a diluted volatile salt solution and an aqueous solution of ethanol. This preliminary step results in an improvement in the separation of these proteins, thus facilitating their subsequent purification by reversed-phased high-performance liquid chromatography. These new proteins have similar relative molecular masses ( $M_r$ s) from 11 000 to 17 000. The purity of the proteins was analyzed by micro two-dimensional gel electrophoresis. Four of these components were found to be in-vitro protein-synthesis inhibitors in a cell-free system from rat brain. The  $NH_2$ -terminal amino-acid sequences of these four inhibitors were determined from 12 to 26 cycles after direct blotting of the separated proteins from electrophoresis gels. Three of these proteins with  $M_r$ s between 16 000 and 17 000 showed a high degree of homology ranging from 57% to 75% but seem to be unrelated to the fourth inhibitor. In addition, the  $\alpha$ -globulin and one of the new low-molecular-weight proteins of  $M_r$  12 500 seemed to show allergenic properties since they bound IgE antibodies from the sera of hypersensitive patients. Both proteins have blocked  $NH_2$ -terminal amino acids.

**Key words:** Allergenic protein – *Oryza* (proteins) – Protein (NaCl-soluble) – Protein synthesis inhibitor – Sodium chloride-soluble protein

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**Abbreviations:** HMW = high molecular weight; IgE = immunoglobulin E; LMW = low molecular weight;  $M_r$  = relative molecular mass; PAGE = polyacrylamide gel electrophoresis; RP-HPLC = reversed-phase high-performance liquid chromatography; SDS = sodium dodecyl sulphate

## Introduction

Rice has been one of the most important world-wide nutritive staple cereal since ancient times. The protein composition of rice grain is 80% glutenins, 10% globulins, 5% albumins and 5% prolamins (Guo et al. 1986). The protein components of the rice kernel have been less studied compared with those from other cereals such as wheat (Larkins 1980; Golenkov 1983) or barley (Rahman et al. 1982), the globulin fraction being the most studied in rice endosperm (Perdon and Juliano 1978; Juliano 1985; Matsuda et al. 1988). Four different globulins,  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -containing fractions, were originally reported (Morita and Yoshida 1968) and further individual rice globulins have been found (Houston and Mohammad 1980; Takahashi et al. 1973; Perdon and Juliano 1978; Matsuda et al. 1988; Pan and Reeck 1988). Analyses by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of NaCl-soluble fractions from rice grain show the presence of two main polypeptide groups (Houston and Mohammad 1970; Cagampang et al. 1976; Yamagata et al. 1982; Pan and Reeck 1988). The high-molecular-weight (HMW) band from relative molecular mass ( $M_r$ ) 26 000 to 28 000 includes the major globulin,  $\alpha$ -globulin (Perdon and Juliano 1978; Yamagata et al. 1982; Pan and Reeck 1988). The low-molecular-weight band (LMW), which has been called “the most soluble globulin fraction” (Houston et al. 1964) contains an undetermined number of small polypeptide chains with  $M_r$ s from 10 000 to 18 000, including some prolamine components (Yamagata et al. 1982) and a protein of  $M_r$  16 000 with allergenic properties (Matsuda et al. 1988). Nonetheless, information concerning the precise number of LMW polypeptide chains in this fraction is still limited.

The technique of reversed-phase high-performance liquid chromatography (RP-HPLC) has the potential for the isolation of a number of cereal proteins (Bietz 1985; Jones and Lookhart 1985; Lázaro et al. 1985). We recently reported RP-HPLC as a unique technique for the

isolation in a single step of a large number of NaCl-soluble proteins from barley endosperm (Barber et al. 1988). However, RP-HPLC has never been used for the purification of rice proteins.

In this paper, we describe a new strategy for the purification of the NaCl-soluble proteins of rice endosperm based on simple extractions with volatile solutions followed by RP-HPLC. This has allowed us to isolate ten new rice proteins, some of which possess biological properties. To our knowledge, this is the first report describing protein-synthesis inhibitors from rice. It is remarkable that the biological activity found in some of these isolated rice proteins seems to be stable under the conditions used in the purification procedure described.

## Material and methods

Seeds of rice (*Oryza sativa* L. cv. Bahia, purchased from Instituto Nacional de Semillas y Plantas de Vivero, Spain) were used in this study. Acetonitrile was purchased from Scharlau (Barcelona, Spain). Ultrapure water, for HPLC, generated by a Milli-RO4 coupled to a Milli-Q water purification system (Millipore, Bedford, Mass., USA) was used in the preparation of all buffers. Acrylamide and N,N'-methylene-bis-acrylamide were obtained from Eastman Kodak (Rochester, NY, USA). All reagents and solvents for the amino-acid analyzer were from Beckman Instruments (Palo Alto, Calif., USA). Other reagents not specified were purchased from Merck (Darmstadt, FRG).

**Protein extraction.** Rice endosperm was obtained by hand dissection as described by Lázaro et al. (1985). Lipids were removed from the ground endosperm using ten volumes (1 h, room temperature) of light petroleum (b.p. 40–60° C) and the residue dried under vacuum. The flour was extracted with chloroform/ethanol (2:1, v/v) as described by Lázaro et al. (1985). The dried residual flour was extracted with 0.5 M NaCl (2 × 10 volumes, 1 h, 4° C) and the soluble proteins were precipitated by adding trichloroacetic acid to a final concentration of 15%. After centrifugation the precipitate was washed twice with cold acetone and dried under vacuum.

**Fractionation of the NaCl extract with volatile solvents.** The schematic fractionation procedure is indicated in Fig. 1. The NaCl-soluble protein extract (200 mg dry weight) was extracted with 20 ml of 0.05 M ammonium bicarbonate (B) by stirring overnight at room temperature. The pellet (p) was separated from the supernatant (s) by centrifugation at 17000 · g for 10 min. The pellet was washed twice with 10 ml of the same ammonium-bicarbonate solution. The pellet and the resulting supernatants, Bp and Bs, respectively, were lyophilized (see Fig. 1). These two dry fractions were extracted with 20 ml of 70% (v/v) ethanol (E), stirred overnight at room temperature and then centrifuged as above. The two pellets, after washing twice with 10 ml 70% (v/v) ethanol, and their corresponding supernatants were dried under vacuum and lyophilized. These four soluble and insoluble bicarbonate-ethanol fractions were denoted as BsEs, BsEp, BpEs, BpEp, respectively (see Fig. 1).

**High-performance liquid chromatography.** The four partially NaCl-soluble fractions and the total NaCl extract were fractionated by HPLC system using the following equipment: two Waters M6000A pumps (Waters Associates, Milford, Mass., USA), a Waters 680 automated gradient controller and a Waters 990 photodiode array detector with a dynamic range from ultraviolet to the visible region (190 nm to 600 nm), based on an NEC APC III (NEC Information Systems, Boxborough, Mass., USA) personal computer. Samples were injected using a Waters U6K Universal injector. Separations

were by RP-HPLC on a Nucleosil C<sub>4</sub> silica column (particle size, 5 µm; pore size 300 Å; 250 mm long, 8.0 mm i.d.; support from Machery Nagel, Düren, FRG and the steel column from Knauer, Berlin, West Germany) fitted with a guard column (25 mm long, 4.6 mm i.d.) packed with the same support. Columns were filled and packed using a Shandon apparatus (Runcorn, Cheshire, UK) as described by Reinbolt et al. (1983). The column was eluted with a linear acetonitrile gradient from 28% to 34% containing 0.1% trifluoroacetic acid for 330 min and operated at room temperature at a flow rate of 1.0 ml · min<sup>-1</sup>. The effluent was monitored by absorbance at 220 nm. The overall yield of protein recovered from a typical HPLC run was 90%. Occasionally a size-exclusion HPLC column TSK 3000 SW6, 300 mm long; 21.5 mm i.d.; Toyo Soda, Tokyo, Japan) fitted with a TSK 3000 SW6 guard column, was also used and isocratically eluted with 0.1 M ammonium-acetate buffer, pH 5.0. The column was operated at room temperature at a flow rate of 0.5 ml · min<sup>-1</sup>. Protein determinations were made by amino-acid analyses. The protein-containing fractions were lyophilized and subjected to gel electrophoresis and amino-acid analysis.

**One-dimensional polyacrylamide gel electrophoresis.** Analysis by SDS-PAGE was done under the same conditions as described by Barber et al. (1988).

**Micro two-dimensional electrophoresis.** Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), (30 × 38 × 0.5 mm<sup>3</sup>) was done as described by Barber et al. (1988).

**Electroblotting and sequence analysis of blotted proteins.** After one-dimensional gel electrophoresis, proteins were blotted and transferred, using a Bio-Rad Mini Trans-Blot electrophoretic transfer cell, to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore), as described by Walsh et al. (1988), in 25 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl buffer, pH 8.4 containing 0.5 mM dithiothreitol. Blotting was done at 100 V for 120 min at 4° C. Blotted proteins were detected by staining the PVDF membrane with 0.1% Amido Black briefly for 5–10 s and then rinsing in distilled water. The proteins were then sequenced in a new Knauer modular liquid-phase protein sequencer (model 810; equipped on line with a Knauer phenylthiohydantoin (PTH)-amino acid analyzer (Fischer et al. 1989). The PTH-amino acids were identified and quantified on an RP-HPLC system based upon a C-18 column (Knauer) and gradient elution with 85% 6.5 mM sodium acetate, 15% acetonitrile, 0.175% 1.2 dichloroethane adjusted to pH 4.77 as buffer A and 100% acetonitrile as B. Sequences were determined in the absence of polybrene, employing the wet-filter technique of Wittmann-Liebold (1988) in a new flow-through reactor (Fischer and Wittmann-Liebold 1987).

**Immunoblotting.** After one-dimensional gel electrophoresis, proteins were electrophoretically transferred using the same transfer system as above but to nitrocellulose sheet, as described by Towbin et al. (1979). The blot was done on duplicate gels at the same time. The two nitrocellulose sheets were incubated in 20 ml of Tris-buffered saline (TBS), a buffer composed of 20 mM Tris, 500 mM NaCl, pH 7.5, containing 0.5% bovine serum albumin (BSA; Sigma, St. Louis, Mo., USA) and 0.5% Triton X-100 (T; Sigma) for 2 h. The two nitrocellulose sheets were then incubated and shaken overnight at 4° C with a solution containing 1 ml of the pooled sera from four patients with suspected cereal hypersensitivity (Phadebas RAST classes 2–4 to cereals) and 9 ml of TBS-BSA-T. After incubation the nitrocellulose sheets were washed four times in TBS-T buffer for 30 min. One of the nitrocellulose sheets was incubated and shaken for 3 h at room temperature with 10 ml of a 1:4000 dilution of anti-human IgE labeled with alkaline phosphatase (Tago Lab., Burlingame, Calif., USA) followed by four washes for 10–15 min each in TBS-T. For development of the color reaction, the nitrocellulose sheet was incubated for 30 min at room temperature with BCIP (5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt) and NBT (*p*-nitro blue tetrazolium chloride; Bio

Red, Richmond, Calif., USA), according to the manufacturer's instructions. At the same time, the other nitrocellulose sheet was incubated with 10 ml of isotope-labelled antibodies  $^{125}\text{I}$ -anti-IgE (Phadebas RAST) diluted 1:5 in TBS-BSA-T buffer for 10–18 h and then washed with TBS-BSA as above. The dried nitrocellulose was placed on X-ray film (X-OMAT; Kodak) and autoradiographed for 10–15 d.

**Analysis of amino acids.** Proteins were hydrolyzed as described by Barber et al. (1988).

**Oxidation of performic acid.** Appropriate aliquots of native proteins were oxidized with performic acid as described by Moore (1963).

**Preparation of postmitochondrial supernatant.** Brains from suckling rats (4–10 d old) were removed rapidly and homogenized 1:2 (w/v) in ice-cold buffer (0.32 M sucrose containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (Hepes)-KOH, pH 7.6; 140 mM potassium acetate; 4 mM magnesium acetate; 2.5 mM dithiothreitol) using five up and down strokes of a glass-teflon homogenizer. The homogenate was centrifuged at  $17000 \cdot g$  for 10 min at  $4^\circ\text{C}$ . The resulting postmitochondrial supernatant (PMS) was kept in aliquots at  $80^\circ\text{C}$  until used.

**Assay of cell-free protein synthesis.** The cell-free protein-synthesis system employed was based on previously described methods (Cosgrove and Brown 1981; Fando et al. 1985; Calés et al. 1986). Several modifications were introduced in order to improve the initiation and elongation stages of protein synthesis: suckling instead of adult rats (Calés et al. 1986); higher amino-acid concentrations; increased concentrations of ATP, guanosine 5'-triphosphate (GTP) and energy-regenerating systems; and lower incubation temperature. The complete reaction mixture in a final volume of 50  $\mu\text{l}$  consisted of: 10  $\mu\text{l}$  of PMS, 75–85  $\mu\text{g}$  as determined by the method of Bradford (1976); 0.32 M sucrose; 50 mM Hepes-KOH, pH 7.6; 140 mM potassium acetate; 4 mM magnesium acetate; 2.5 mM dithiothreitol; 1 mM ATP; 0.75 mM GTP; creatine phosphokinase at  $100 \mu\text{g} \cdot \text{ml}^{-1}$ ; 5 mM creatine phosphate; 20 unlabeled amino acids each at 25  $\mu\text{M}$ ; and  $[\text{^3H}]$ leucine at  $3.77 \cdot 10^6 \text{ Bq} \cdot \text{ml}^{-1}$  (5  $\mu\text{l}$  of  $37.0 \text{ MBq} \cdot \text{ml}^{-1} \text{ L-[4,5-^3H]leucine}$ ,  $2222.0 \text{ GBq} \cdot \text{mmol}^{-1}$ ). Duplicate aliquots of 20  $\mu\text{l}$  after 45 min incubation at  $30^\circ\text{C}$ , or 10- $\mu\text{l}$  aliquots after 5, 10, 30 and 45 min incubation were removed and processed as previously described (Cosgrove and Brown 1981). Known inhibitors of protein synthesis and crude rice extract, as well as their purified components, were added at the start or after 5 min of incubation as specified in the legends of Fig. 4. The endogenous leucine concentration in the PMS was determined after deproteinization with sulfosalicylic acid in a Beckman (Palo Alto, USA) amino-acid analyzer.  $[\text{^3H}]$ Leucine incorporation after 45 min incubation was 514.267 cpm per mg PMS, which corresponds to 105 pmol of leucine incorporated per mg PMS.

## Results

**Analysis of the NaCl-soluble proteins from rice endosperm by SDS-PAGE.** After SDS-PAGE, the NaCl-soluble proteins from rice endosperm separated into two major polypeptides groups, corresponding to HMW and LMW groups, with  $M_r$ s from 26000 to 28000 and from 10000 to 17000, respectively, and representing more than 95% of the total protein in this extract (Fig. 1). Densitometric analysis from SDS-PAGE showed the ratio of HMW to LMW groups to be 30:70 (data not shown). In order to fractionate these components we first treated the NaCl-soluble proteins with water to obtain soluble (albumins) and insoluble (globulins) fractions (Morita and Yoshida 1968; Cagampang et al. 1976; Pascual et al.

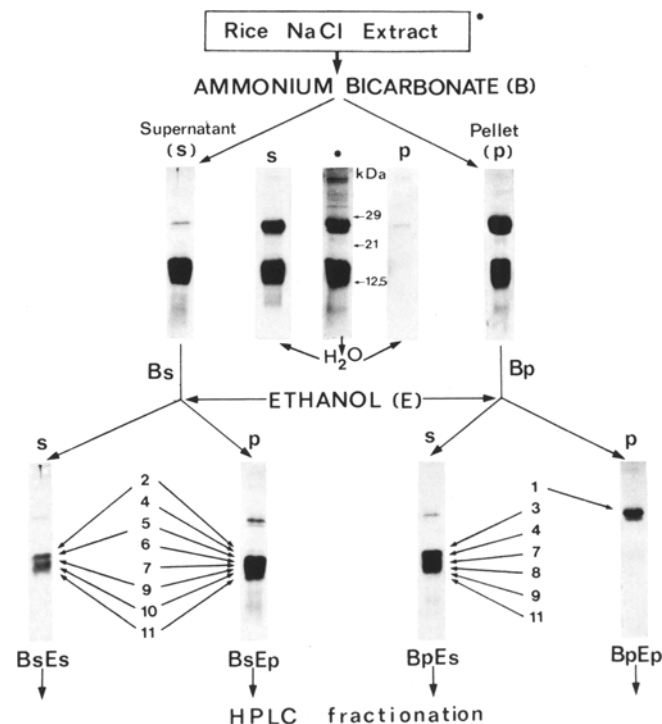


**Fig. 1.** Schematic representation of the fractionation of NaCl-extracted rice endosperm by volatile solvents prior to RP-HPLC. Numbers indicate the distribution of the isolated proteins in the four bicarbonate-ethanol fractions (BsEs, BsEp, BpEs, BpEp). The SDS-PAGE of the rice NaCl-extract is indicated by an asterisk; the mobilities of standard proteins are also indicated

1981). However, as can be seen in Fig. 1 (top, center), SDS-PAGE of the two fractions obtained shows that all of the proteins were in the water-soluble (s) fraction, while only traces were detected in the insoluble (p) fraction, indicating the inefficiency of this extraction procedure, at least for the NaCl-soluble proteins from rice endosperm preparations.

**Fractionation of the total NaCl-soluble proteins from rice endosperm by RP-HPLC.** The total NaCl-soluble protein fraction was dissolved in 10% (v/v) acetonitrile in water containing 0.1% (v/v) trifluoroacetic acid and applied to a semipreparative Nucleosil C4 column. After separation, a large number of peaks could be detected (Fig. 2). The homogeneity of the eluted proteins was analyzed by SDS-PAGE of portions, from 10% to 40%, of each peak as shown in Fig. 2. As can be seen from the SDS-PAGE analysis, the HMW group comprises only a few components while a surprisingly high number of about 20–30 polypeptides, exhibiting  $M_r$ s of 10000–17000, are present in the LMW group. Owing to the complexity of these numerous and apparently similar LMW components, which were distributed in practically all fractions, RP-HPLC itself was not suitable for the direct purification of these polypeptides since all of the fractions contained at least two or three proteins (see gels in Fig. 2). Consequently, we had to employ additional fractionation techniques to obtain simple fractions of protein mix-

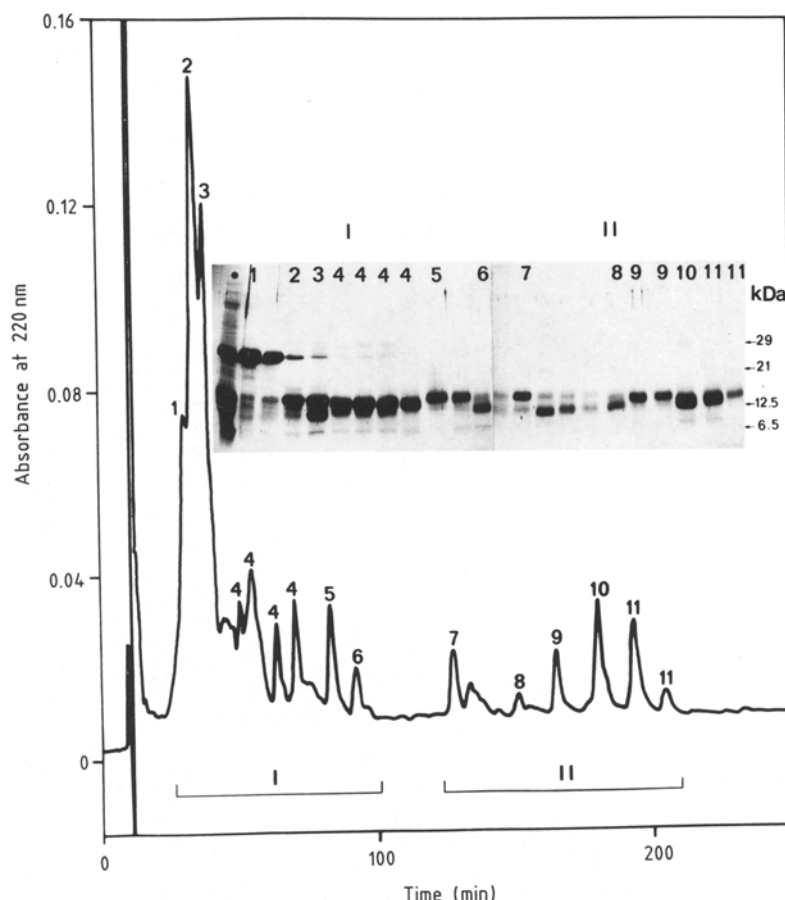


**Fig. 2.** Fractionation by RP-HPLC of the total NaCl extract from rice endosperm. A solution of proteins (1.8 mg in 1000  $\mu$ l of 10% (v/v) acetonitrile containing 0.1% trifluoroacetic acid was injected into a Nucleosil column and eluted with a gradient of acetonitrile as indicated in the *Material and methods*. Inserts show the SDS-PAGE of fractions corresponding to zones I and II of the column profile. The lane marked with an asterisk corresponds to the total protein extract. The mobilities of the standard proteins are indicated

tures, thus facilitating their subsequent purification by RP-HPLC.

*Partial fractionation of the NaCl-soluble rice proteins by extraction with volatile solutions prior RP-HPLC.* A novel fractionation procedure, consisting of a first extraction using diluted ammonium-bicarbonate solution followed by a second extraction with aqueous solution of ethanol was used. The fractionation is shown schematically in Fig. 1 and details are given in the *Material and methods*. Analysis of the four final bicarbonate-ethanol extracts by SDS-PAGE shows that the LMW polypeptides are distributed in three of them, BsEs, BsEp and BpEs, while the HMW polypeptides are mainly concentrated in the fourth extract, BpEp (Fig. 1). After the elimination of the volatile solutions by lyophilization and under vacuum the four fractions were subjected to RP-HPLC.

*Fractionation by RP-HPLC of the bicarbonate-ethanol partial extracts of the NaCl-soluble proteins from rice endosperm.* Aliquots of the four bicarbonate-ethanol extracts, BsEs, BsEp, BpEs and BpEp (Fig. 1) were subjected to RP-HPLC under the same chromatographic conditions as the total NaCl extract shown in Fig. 2. The resulting chromatograms are shown in Fig. 3. Analysis by SDS-PAGE demonstrates that most of the eluted peaks, from all chromatograms, show a high degree of protein homogeneity compared with those shown in

Fig. 2. However, although many of these peaks apparently contain a single protein when analysed by one-dimensional SDS-PAGE, some of them show the presence of contaminants of identical molecular masses when they are analyzed by micro two-dimensional gel electrophoresis (data not shown). Only peak 3 from BpEs and peak 1 from BpEp were rechromatographed by size-exclusion HPLC and RP-HPLC, respectively.

A total of twenty proteins obtained from the four chromatograms (see Fig. 3) was further characterized in this study. Each of these proteins showed a single band upon one-dimensional electrophoresis and a single spot by micro two-dimensional electrophoresis (data not shown).

*Characterization of the purified rice proteins.* When the twenty pure proteins mentioned above were compared, some of them seemed to be identical. In fact, peaks containing proteins Ri-2, Ri-5, Ri-9, Ri-10 and Ri-11 were found in the chromatograms of different extracts (Figs. 1, 3). On the other hand, we also found that certain proteins (Ri-2, Ri-4, Ri-5, Ri-7 and Ri-11) migrate as two peaks on the same chromatogram (Figs. 2, 3). In accordance with their amino-acid compositions and electrophoretic mobilities in SDS-PAGE, as well as their amino-acid sequences and biological activities (see below for protein-synthesis inhibition), the twenty pure proteins originally isolated were found to consist of only eleven different proteins. We have tentatively designated

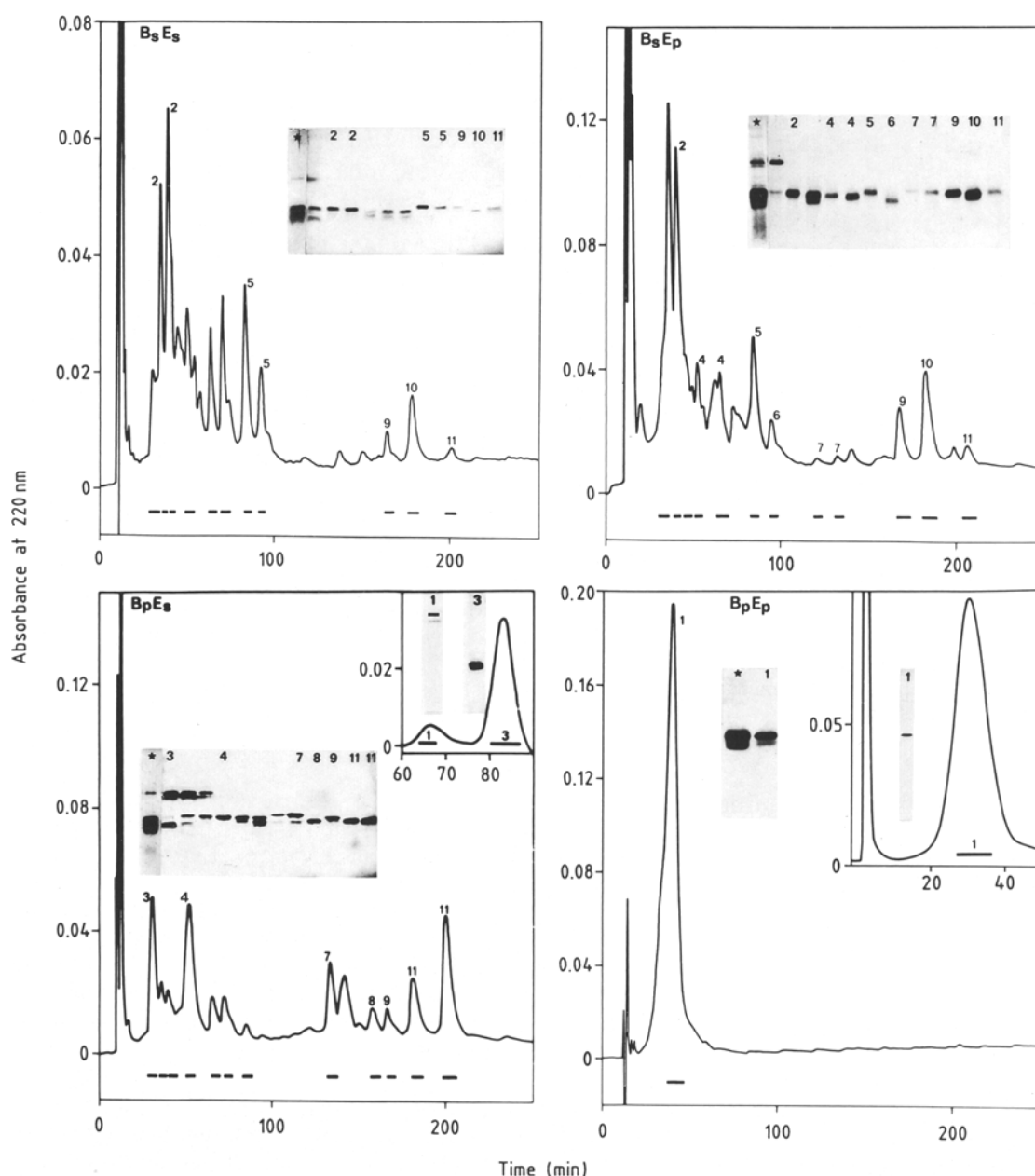


**Fig. 3.** Fractionation by RP-HPLC of the bicarbonate-ethanol rice extracts *BsEs* (1.3 mg), *BsEp* (1.1 mg), *BpEs* (1.5 mg) and *BpEp* (0.8 mg), chromatographed as in Fig. 2. *Inserts* show the SDS-PAGE of the fractions indicated by bars on the column profile. The lanes marked with a star corresponds to the total extract. The repurification of peak 3 from *BpEs* and peak 1 from *BpEp* by size-exclusion HPLC and RP-HPLC (under the same chromatographic conditions), and the SDS-PAGE of the repurified proteins are also shown in the *inserts*

these purified NaCl-soluble proteins as Ri (Rice) followed by a number corresponding to their elution order from the RP-HPLC column (Ri-1 to Ri-11). The locations of the eleven rice proteins in the chromatograms from the total and the partial extracts are indicated by numbers (Fig. 2, 3), and their partial distributions in the four bicarbonate-ethanol extracts are shown in

Fig. 1. Protein R-1, with an  $M_r$  of 28000, which has been characterized by its amino-acid composition as the major  $\alpha$ -globulin (Houston and Mohammad 1970; Perdon and Juliano 1978; Pan and Reeck 1988), was the only component isolated in the HMW polypeptide group, while proteins Ri-2 to Ri-11, exhibiting  $M_r$ s from 11000 to 17000, were isolated from the LMW group. According to their amino-acid compositions and molecular sizes, some of these LMW components are similar but not identical to a few NaCl-soluble proteins already isolated from rice endosperm (Houston and Mohammad 1964; Matsuda et al. 1988). The amino-acid compositions as well as  $M_r$ s of the eleven purified rice proteins are shown in Table 1.

*Effect of rice NaCl-soluble extract and isolated rice proteins on protein synthesis.* In order to study the effect of the rice NaCl extract and its component proteins on

**Table 1.** Amino acid composition of NaCl-soluble proteins purified from rice endosperm

| Amino <sup>a</sup><br>acid | Protein number |             |             |             |             |             |             |             |             |             |             |  |
|----------------------------|----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|--|
|                            | Ri-1           | Ri-2        | Ri-3        | Ri-4        | Ri-5        | Ri-6        | Ri-7        | Ri-8        | Ri-9        | Ri-10       | Ri-11       |  |
| Cys <sup>b</sup>           | 8.3 (3.3)      | 11.0 (6.9)  | 10.3 (8.7)  | 7.4 (4.8)   | 10.1 (5.9)  | 8.9 (7.9)   | 3.2 (1.9)   | 7.8 (6.2)   | 8.8 (5.0)   | 12.8 (8.1)  | 8.4 (5.3)   |  |
| Asp                        | 10.4 (4.1)     | 14.0 (8.8)  | 8.6 (7.2)   | 13.4 (8.8)  | 13.7 (8.0)  | 9.7 (7.7)   | 12.9 (7.7)  | 8.4 (6.7)   | 14.1 (8.0)  | 12.6 (8.0)  | 11.9 (7.5)  |  |
| Thr <sup>c</sup>           | 8.0 (3.2)      | 5.0 (3.1)   | 3.5 (2.9)   | 4.8 (3.1)   | 8.0 (4.7)   | 4.8 (3.8)   | 7.0 (4.2)   | 2.4 (1.9)   | 4.3 (2.4)   | 2.7 (1.7)   | 3.0 (1.9)   |  |
| Ser <sup>c</sup>           | 24.1 (9.6)     | 6.0 (3.8)   | 5.8 (4.9)   | 5.2 (3.4)   | 11.3 (6.6)  | 5.4 (4.3)   | 9.7 (5.8)   | 5.1 (4.1)   | 10.2 (5.8)  | 6.5 (4.1)   | 9.3 (5.9)   |  |
| Glu                        | 67.4 (26.8)    | 17.5 (11.0) | 10.7 (9.0)  | 15.2 (9.9)  | 19.8 (11.6) | 14.9 (11.8) | 18.8 (11.3) | 11.5 (9.2)  | 17.8 (10.1) | 15.1 (9.6)  | 16.6 (10.5) |  |
| Pro                        | 12.3 (4.9)     | 11.9 (7.5)  | 7.3 (6.2)   | 11.7 (7.7)  | 12.7 (7.4)  | 10.8 (8.6)  | 11.4 (6.8)  | 8.2 (6.6)   | 14.5 (8.2)  | 13.3 (8.4)  | 12.6 (7.9)  |  |
| Gly                        | 29.2 (11.6)    | 21.5 (13.5) | 17.9 (15.1) | 20.4 (13.4) | 22.1 (12.9) | 15.6 (12.4) | 28.9 (17.3) | 19.5 (15.6) | 30.1 (17.2) | 22.8 (14.4) | 19.4 (12.2) |  |
| Ala                        | 17.4 (6.9)     | 13.7 (8.6)  | 14.4 (12.1) | 14.2 (9.3)  | 18.2 (10.6) | 13.8 (11.0) | 17.3 (10.4) | 12.9 (10.3) | 18.7 (10.7) | 18.2 (11.5) | 16.2 (10.2) |  |
| Val                        | 9.0 (3.6)      | 12.5 (7.8)  | 9.1 (7.7)   | 12.7 (8.3)  | 10.1 (5.9)  | 7.6 (6.9)   | 8.1 (4.9)   | 9.1 (7.3)   | 11.9 (6.8)  | 10.5 (6.6)  | 10.1 (6.4)  |  |
| Met                        | 5.6 (2.2)      | 1.5 (0.9)   | 1.8 (1.5)   | 2.6 (1.7)   | 1.0 (0.6)   | 0.9 (0.7)   | 2.5 (1.5)   | 2.9 (2.3)   | 0.9 (0.5)   | 1.2 (0.8)   | 2.8 (1.8)   |  |
| Ile                        | 4.9 (1.9)      | 4.0 (2.5)   | 2.9 (2.4)   | 3.6 (2.4)   | 4.2 (2.4)   | 3.5 (2.8)   | 4.4 (2.6)   | 4.4 (3.5)   | 4.8 (2.7)   | 5.0 (3.2)   | 5.8 (3.7)   |  |
| Leu                        | 15.7 (6.2)     | 10.7 (6.7)  | 6.8 (5.7)   | 10.5 (6.9)  | 10.4 (6.1)  | 8.4 (6.7)   | 10.4 (6.2)  | 8.1 (6.5)   | 10.8 (6.2)  | 10.5 (6.6)  | 11.1 (7.0)  |  |
| Tyr                        | 2.9 (1.1)      | 5.3 (3.3)   | 3.9 (3.3)   | 6.1 (4.0)   | 6.3 (3.7)   | 5.0 (4.0)   | 6.3 (3.8)   | 4.9 (3.9)   | 5.6 (3.2)   | 4.0 (2.5)   | 5.9 (3.7)   |  |
| Phe                        | 6.7 (2.7)      | 3.0 (1.9)   | 2.6 (2.2)   | 2.7 (1.8)   | 2.9 (1.7)   | 2.3 (1.8)   | 3.8 (2.3)   | 2.2 (1.8)   | 3.7 (2.1)   | 3.7 (2.3)   | 3.8 (2.4)   |  |
| Lys                        | 1.0 (0.4)      | 3.4 (2.1)   | 1.9 (1.6)   | 2.5 (1.6)   | 1.5 (0.9)   | 0.5 (0.4)   | 6.0 (3.6)   | 3.0 (2.4)   | 5.7 (3.2)   | 5.4 (3.4)   | 4.5 (2.8)   |  |
| His                        | 2.0 (0.8)      | 4.3 (2.7)   | 1.7 (1.4)   | 5.7 (3.7)   | 6.0 (3.5)   | 3.9 (3.1)   | 3.9 (2.3)   | 3.1 (2.5)   | 3.5 (2.0)   | 2.2 (1.4)   | 2.7 (1.7)   |  |
| Arg                        | 26.2 (10.4)    | 13.4 (8.4)  | 9.2 (7.8)   | 13.7 (9.0)  | 12.9 (7.5)  | 9.7 (7.7)   | 12.0 (7.2)  | 11.3 (9.0)  | 9.6 (5.5)   | 11.2 (7.1)  | 13.9 (8.8)  |  |
| MW                         | 28000          | 16000       | 11000       | 15000       | 17000       | 12500       | 17000       | 12500       | 16500       | 15000       | 16000       |  |

<sup>a</sup> Compositions are expressed in residues per mole of protein based on the molecular weight (MW). Percentage compositions are given in ( ). Each value represents the average from 24, 48 and 72 h hydrolysis

<sup>b</sup> Cys was determined as cysteic acid. Trp was not determined

<sup>c</sup> Values extrapolated to zero-time hydrolysis

protein synthesis, we used a cell-free system from the brains of suckling rats. This system yields a high rate of protein synthesis and is sensitive to known inhibitors of both the initiation step, aurintricarboxylic acid and edeine, and the elongation step, puromycin and cycloheximide (Fig. 4a). Rice extract ( $300 \mu\text{g} \cdot \text{ml}^{-1}$ ) produced an inhibition of protein synthesis comparable to that obtained by aurintricarboxylic acid or cycloheximide, indicating the presence of one or more inhibitors in the extract (Fig. 4a). To determine whether the possible inhibitor of protein synthesis corresponded to some of the eleven isolated protein components, each of them was tested at different concentrations on the cell-free system (Fig. 4b). As can be seen, three of these components (Ri-1, Ri-10 and Ri-11) did not inhibit protein synthesis at any of the concentrations used. Nevertheless, four of the components (Ri-2, Ri-3, Ri-5 and Ri-7) produced a clear inhibition of protein synthesis at all the concentrations tested. The other four isolated rice components (Ri-4, Ri-6, Ri-8 and Ri-9) produced only a slight inhibition of protein synthesis at the same concentrations as the other components tested (data not shown). It is interesting that proteins Ri-5, Ri-2 and Ri-7, with  $M_r$ s within the range 16000 to 17000 and similar amino-acid compositions (Table 1), could be considered a family group of protein-synthesis inhibitors since they show a high degree of homology, between 57% to 75%, in their  $\text{NH}_2$ -terminal regions (Fig. 5). However the fourth inhibitor Ri-3 cannot be grouped in the same family since it did not show any homology with these three inhibitors (Fig. 5). The proteins Ri-2, Ri-5 and Ri-7 migrate as two peaks in the same chromatogram (Figs. 2, 3), probably because of the presence

of denatured and native forms, as has been described for certain proteins isolated at low pH by RP-HPLC (Sadler et al. 1984; Katzenstein et al. 1986). However, when Ri-2, Ri-5 and Ri-7 are reconstituted in the buffer used in the protein-synthesis assay, after removing the RP-HPLC solvent, the two forms of these proteins inhibit protein synthesis to the same extent at the same concentrations (data not shown). This biological activity has been used as an additional criterion for grouping some of these proteins as one.

*Detection by immunoblotting of specific IgEs to rice proteins.* Specific IgE detection by immunoblotting of the NaCl-soluble rice extract indicated that two main IgE-binding activities were located in the HMW and LMW groups (Fig. 6b, c; lanes with an asterisk). The corresponding immunoblotting of the eleven isolated proteins was done. Only two proteins (Ri-1 and Ri-8) bound specific IgE (Fig. 6b; lanes 1 and 8) while the other tests were negative (data not shown). To exclude any lectin-like activity or any unspecific interaction between these proteins, the human sera and the alkaline-phosphatase-labeled anti-human IgE antiserum, the immunoblotting was done using a  $^{125}\text{I}$ -labeled anti-human IgE (Fig. 6c; lanes 1 and 8). Both immunoblottings showed the same detection profile. The NaCl-soluble rice extract, Ri-1 and Ri-8 did not show unspecific reactions, and the control using sera from non-allergic patients was negative (data now shown).

Attempts to sequence proteins Ri-1 and Ri-8 were unsuccessful and no amino-acid derivatives were released from the  $\text{NH}_2$ -terminus after automatic degradations. The apparent blocking of these two rice proteins

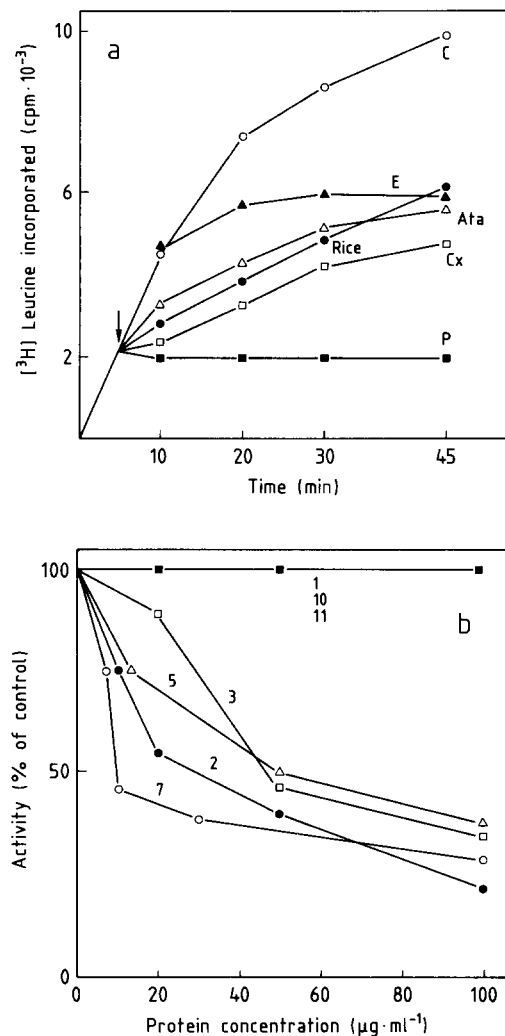


**Fig. 4.** **a** Comparison of the effects of known protein-synthesis inhibitors and NaCl-soluble rice extract on protein synthesis as measured in a cell-free system from the brains of suckling rats (see *Material and methods*). Inhibitors were added after 5 min incubation and radioactivity present in 10-µl aliquots was determined at the indicated times. ○—○, no inhibitor (*C*); □—□, 20 µM cycloheximide (*Cx*); ■—■, 25 µM puromycin (*P*); ▲—▲, 75 µM edeine (*E*); △—△, 75 µM aurintricarboxylic acid (*ATA*); ●—●, 300 µg · ml<sup>-1</sup> rice extract (*RICE*). **b** Percentage inhibition of protein synthesis in the rat brain system caused by different purified components (NaCl-soluble proteins 1–3, 5, 7, 10, 11) from rice endosperm. The purified proteins, at the concentrations indicated, were added at the beginning of the incubation. Radioactivity was determined in the total volume (50 µl) after 45 min of incubation

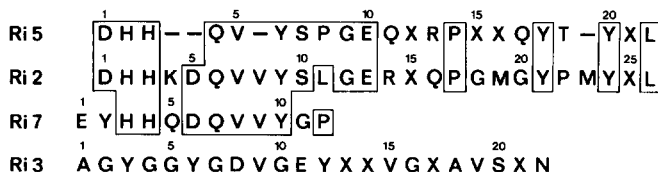


**Fig. 5.** NH<sub>2</sub>-terminal amino-acid sequence of four protein-synthesis inhibitors, Ri-2, Ri-3, Ri-5 and Ri-7. Invariant positions are shown in boxes. Gaps are included to achieve maximal homology. Single-letter designations for amino acids are used. Unidentified positions are indicated by an X

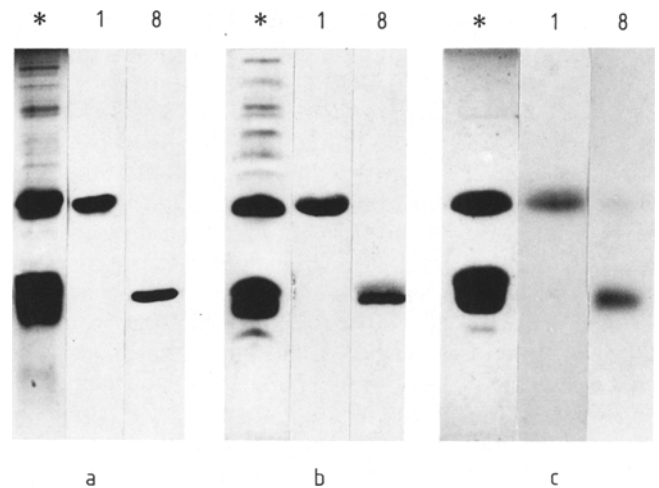


**Fig. 6a–c.** Analysis by SDS-PAGE of total NaCl-soluble proteins from rice endosperm and of two of the isolated proteins, Ri-1 and Ri-8. **a** Staining with Coomassie-Blue. **b, c** The gels were blotted onto nitrocellulose sheet and visualized by developing with anti-human IgE labeled with alkaline phosphatase (**b**) and by autoradiography of the nitrocellulose sheet incubated with <sup>125</sup>I-labelled anti-human IgE (**c**). Lanes with asterisk: total NaCl-soluble proteins from rice endosperm. Lanes 1 and 8: isolated Ri-1 and Ri-8, respectively

has not been investigated in this paper. Partial NH<sub>2</sub> sequences of the remaining isolated proteins were not determined.

## Discussion

It is evident from previous studies that there is little information concerning the composition of NaCl-soluble proteins from rice endosperm (Padhye and Salunkhe 1979; Pascual et al. 1981; Yamagata et al. 1982). In fact, at the present time only a few rice NaCl-soluble proteins have been isolated in pure form and characterized (Houston and Mohammad 1970; Matsuda et al. 1988; Pan and Reeck 1988). Although our first attempt to isolate NaCl-soluble proteins from rice endosperm by using RP-HPLC directly was unsuccessful (Fig. 2), the results highlighted the great complexity of the LMW group of proteins from this extract (Fig. 2). It is worthwhile noting not only the surprisingly large number of components, about 20–30, but also the fact that the majority of them are similar in size, with a narrow range of *M<sub>r</sub>*s from 10000 to 17000 (Fig. 2). In an attempt to improve the separation of such a complex mixture, we introduced a useful and novel ammonium-bicarbonate extraction step, giving rise to two different fractions which were then subjected to an additional 70% ethanol extraction step. The four resulting simple mixtures contain fractions which represent a partial distribution of the proteins in each of them, as well as a selective enrichment of some of these rice proteins. A total of eleven different proteins from the NaCl-soluble fraction of rice endosperm have been purified by this method.

The protein Ri-1, which is the main component of the HMW group of the NaCl extract, has been identified

as the previously described major  $\alpha$ -globulin on the basis of molecular size and amino-acid composition (Houston and Mohammad 1970; Perdon and Juliano 1978; Pan and Reeck 1988). This protein, as well as Ri-8, is antigenic to IgE antibodies from individuals allergic to rice protein (Fig. 6). Very recently, an NaCl-soluble, and also water-soluble, protein isolated from rice endosperm has been reported to be the first pure allergen described in rice (Matsuda et al. 1988). This allergenic protein with an  $M_r$  of 16000 is not only clearly different from Ri-1 (28000) and Ri-8 (12500) but is also different from the other Ri-proteins described in this paper that have similar  $M_r$ s in the range 15000–17000 (based on comparisons of amino-acid compositions; Table 1). Further investigations are in progress in our laboratory using these two new IgE-binding rice proteins from Bahia rice endosperm to determine if these components actually cause hypersensitive reactions on atopic patients. Additional in-vitro and in-vivo studies such as skin tests and histamine-release assays are necessary before the IgE-binding proteins Ri-1 and Ri-8 can be considered to be true allergens.

Purified proteins capable of inhibiting translation in animal cell systems have been found in a number of plant species (Stirpe and Barbieri 1986) and also in grains of wheat, barley, oats, rye, corn and triticale (Coleman and Roberts 1982) but never have been described in rice. Most of these translational inhibitors are active in cell-free systems from several sources, reticulocytes (Asano et al. 1986a), cultured cells (Jones and Lookhart 1985), and animal tissues (Carrasco et al. 1981), while they are less active in cell-free systems from plants (Stirpe and Barbieri 1986). The effect of an NaCl extract of rice on protein synthesis has only been described in a cell-free system from cultured Erlich ascites cells (Coleman and Roberts 1982) but no significant inhibition was found. However, such effects were not assayed with pure rice proteins. To our knowledge, this is the first time that protein-synthesis inhibitors have been purified from rice. Our results demonstrate that some of the isolated proteins, Ri-2, Ri-3, Ri-5 and Ri-7, are protein-synthesis inhibitors in a cell system from rat brain. These four proteins represent two new groups of protein-synthesis inhibitors since their  $\text{NH}_2$ -terminal regions did not show homology with other protein-synthesis inhibitors described in cereals (Ozaki et al. 1980; Asano et al. 1986b; Stirpe and Barbieri 1986).

The physiological significance of the presence of inhibitors of proteins synthesis in plants and in grains still remains obscure. Work is now in progress to find out the exact mechanism by which and the level to which Ri-2, Ri-3, Ri-5 and Ri-7 inhibit protein synthesis.

According to the present results, ten newly characterized proteins have been isolated and purified by a procedure especially developed for rice-endosperm proteins. The method, in which the proteins are subjected to the action of organic solvents (ethanol, acetonitrile) or maintained in solutions with different ranges of pH from 8.5 (ammonium bicarbonate) to 2.0 (trifluoroacetic acid), seems to have no affect on the biological properties of at least some of the isolated proteins.

It is generally believed that RP-HPLC of proteins gives rise to a denatured form and that the protein conformation is altered by the action of the low pH, by the organically modified solvents used (Sadler et al. 1984) or by interactions with the alkyl-bonded phases (Katzenstein et al. 1986). Such alterations seem to occur to proteins Ri-2, Ri-5, Ri-7, Ri-9 and Ri-11 which migrate as two peaks on the same chromatogram (Figs. 2, 3) due to the presence of denatured and native forms. However, at least some of these purified proteins in the denatured state were renatured simply by elimination of the RP-HPLC solvents and by reconstitution in a more physiological buffer. In fact, by using the described purification procedure, we have isolated other cereal proteins which also keep their capacity to inhibit protein synthesis or their antigenic properties (data not shown). In principle the system seems to be suitable for the complete isolation of all components of the NaCl-soluble proteins from rice endosperm as well as for other complex cereal mixtures.

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