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New discoveries concerning rice trypsin inhibitors

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Abstract. Seeds of rice (*Oryza sativa* L.) contained, mainly in the embryo, similar trypsin inhibitor (TI) activity levels in 6 cultivars, excluding Taichung Native 1. Using Tainung 67 seeds, imbibition at about 27°C in the dark for 3 days before germination (30±1°C) changed the levels and time course of TI activity development when compared to imbibition at 37±1°C in the dark for 1 day, which was the normal procedure. Seedlings contained TI activity mainly in the shoots. A temporary increase in TI activity was observed in the early stage of germination. When seedlings were submerged, the TI activity of shoots decreased more than that of the control group. Activity staining on gels showed that TI with M_r 21400 was the major band from rice seeds. Three major TIs, with M_r 33600, 21400, and 12800, were found in shoots. Submergence speeded up the decline of TI33600 and TI21400. These results indicate new biological functions of TI in rice, which have not been reported in the literature.

Keywords: Activity staining; Polyacrylamide gel electrophoresis; Protein staining; Shoots; Submergence.

Abbreviations: **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **TI**, trypsin inhibitor; **TIA**, trypsin inhibitor activity. *Rice cultivars*: **HC56**, Hsinchu 56; **TNG67**, Tainung 67; **TN1**, Taichung Native 1; **TNS14**, Tainung Sen 14; **TNS20**, Tainung Sen 20.

Introduction

The protease inhibitor in rice embryo and rice bran was found to reduce the activity of rice seed protease itself (Horiguchi and Kitagishi, 1971). Later, a trypsin inhibitor was partially purified from rice bran (Tashiro and Maki,1978). In the following years, stability, specificity (Tashiro and Maki, 1986), and the complete amino acid sequence (Tashiro et al., 1987) of rice bran trypsin inhibitor were published. Influence of dietary rice bran trypsin inhibitor on the growth and pancreatic weights of rats (Maki and Tashiro, 1983; Tashiro and Maki, 1990) have also been published. In this paper we report some new discoveries concerning rice trypsin inhibitors.

Materials and Methods

Chemicals and chromatographic materials

Acrylamide, ammonium persulphate, and N,N'-methylene bisacrylamide were products of Bio-Rad (Richmond, CA, USA). Calibration kits for electrophoresis were obtained from Pharmacia (Uppsala, Sweden). Polyvinylpyrrolidone-40 (mol.wt. 40000), ascorbic acid, and other chemicals were obtained from the Sigma Chemical Company (St. Louis, USA).

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Plant material

Fresh rice (*Oryza sativa* L.) seeds of 5 cultivars were obtained from the experimental field of the Institute of Botany, Academia Sinica, Taipei or other research institutes. Seeds of one unknown cultivar were purchased from a local market.

Rice seeds were sterilized with 5% sodium hypochlorite for 10 min and then washed several times with distilled water as described previously (Lee et al., 1993). The sterilized seeds were put in a 250 ml beaker containing 200 ml distilled water (water depth was 8 cm) either at 37±1°C in the dark for 1 day or at about 27°C in the dark for 3 days (this process is called imbibition), and then transferred to Petri dishes and incubated at 30±1°C in darkness. Under aerobic conditions, seeds were placed in a Petri dish (8 cm width, 1 cm depth) containing 8 ml distilled water such that seeds or seedlings were not submerged. For submerged treatment, sixty seedlings were put in a 250 ml beaker containing 200 ml distilled water (water depth was 8 cm). After treatment as above for various time intervals, materials were weighed and fixed in liquid nitrogen, then stored in a -70°C freezer.

Preparation of crude extract

Plant materials were homogenized in liquid nitrogen. Ten-millimolar phosphate buffer (pH 7.8) containing 1% PVP, 1% ascorbic acid, 1 mM potassium chloride, 10

mM magnesium chloride, and 50 mM EDTA was added in the ratio of 1/3 (gram fresh weight/ml of extraction buffer). After centrifuging at 12000 *g* for 20 min at 4°C, the supernatant was collected as samples (crude extract). The freshly extracted samples was immediately subjected to TIA determination by the inhibition of trypsin-catalyzed casein hydrolysis (Lin, 1989) or Bz-L-Arg-4-NA (Erlanger et al., 1961) hydrolysis (Bergmeyer, 1984).

Protein estimation

Protein was determined by dye-binding (Bradford, 1976) using crystalline bovine albumin as the standard.

Non-denaturing PAGE

This was done according to Hames (1981).

SDS-PAGE

This was done according to Weber and Osborn (1969). Gradient gels of 12.5–20% were used. It is already known that rice TI is heat labile (Horiguchi and Kitagishi, 1971), so the boiling step was avoided in preparing samples for activity staining of TI on 12.5–20% SDS-PAGE gels. A 75 μl sample was immediately mixed with 25 μl 4X sample buffer (which contained 0.25 M Tris pH 6.8, 4% (w/v) SDS, 40% (v/v) glycerol, and 0.002% bromophenol blue) and incubated at 37°C in darkness for 18 h. Our preliminery experiments showed that 18 h incubation was enough for sufficient binding of SDS to TIs.

Post-electrophoresis protein staining

Protein were detected with Coomassie Brilliant Blue G (Neuhoff et al., 1988).

Post-electrophoresis TIA staining

This was carried out according to Chan and deLumex (1982). For SDS-PAGE, SDS must be removed before TIA staining (Lin, 1990). Clear bands were obtained for TIA against a red or violet background.

Estimation of molecular weight

The molecular weight of rice TI was estimated by SDS-PAGE (Weber and Osborn, 1969) with α-lactalbumin (M_r 14400), soybean trypsin inhibitor (20100), carbonic anhydrase (30000), ovalbumin (43000), bovine serum albumin (67000), and phosphorylase b (94000) as standards.

Significance test of data

The significance of TIA difference of both Japonica and Indica rice seeds was examined by the Student's test (1908).

Results

TIA of seeds of various rice cultivars

Tables 1 and 2 show TIA of seeds of three cultivars each of Japonica and Indica, respectively. Table 3 summarizes the results of both Tables. Although mean value

Table 1. Trypsin inhibitor activity of rice seeds of three Indica cultivars.

Cultivar	Total TIA[a]	Total water soluble protein [b] (mg)	Specific TIA[c]
TN1	135.1±0.68	8440±40.5	0.0160±0.00013
TNS14	215.2±1.05	6760±32.5	0.0318±0.00025
TNS20	217.4±1.07	6830±34.2	0.0318±0.00024

[a] Total trypsin inhibited (mg) per 1000 g fresh weight.
[b] Based on 1000 g fresh weight.
[c] Mg trypsin inhibited per mg protein.

Table 2. Trypsin inhibitor activity of rice seeds of three Japonica cultivars.

Cultivar	Total TIA[a]	Total water soluble protein [b] (mg)	Specific TIA[c]
TNG67	229.4±1.14	8190±65.5	0.0280±0.00014
HC56	219.2±1.07	7930±62.6	0.0276±0.00013
Unknown	193.8±1.06	6670±53.3	0.0291±0.00014

[a] Total trypsin inhibited (mg) per 1000 g fresh weight.
[b] Based on 1000 g fresh weight.
[c] Mg trypsin inhibited per mg protein.

of Japonica is slightly higher than that of Indica, no significant difference was found by the Student's test. This is due to the extremely low TIA of TN1.

Distribution of TIA within rice seeds of a Japonica cultivar TNG67

This is shown in Table 4. TIA was found in whole seeds, dehulled seeds, embryoes; but not in endosperm. Pretreatment of samples with liquid nitrogen did not cause significant changes of TIA, but we include pretreatment of liquid nitrogen in our routine process of samples to ensure reproducibility of experiments.

TIA of rice seedlings of TNG67

Table 5 shows TIA of TNG67 seedlings (imbibition was at about 27°C in the dark for 3 days). TIA reached the peak value 5 days after imbibition and was found only in newly formed organs, i.e. shoots (including coleoptiles and unexpanded leaves) and roots, 6 days after imbibition. No TIA could be detected in any tissues 10 days after imbibition.

Details were studied (Table 6) using TNG67 seedlings with imbibition at 37±1°C for 1 day (a normal procedure). Among new tissues, TIA was found mainly

Table 3. Summary of trypsin inhibitor activity of rice seeds.

Cultivar	Total TIA[a]	Specific TIA[b]
Indica	189.2±38.29 (n=3)	0.0265±0.00745
Japonica	214.1±14.97 (n=3)	0.0282±0.00634

[a] Total trypsin inhibited (mg) per 1000 g fresh weight.
[b] Mg trypsin inhibited per mg protein.

Table 4. Distribution of trypsin inhibitor activity within rice seeds of a Japonica cultivar Tainung 67.

Experiment	Whole seeds	Dehulled seeds	Embryo	Endosperm
I[a]	191.4±2.24[b] (0.0477±0.00027)[c]	174.3±1.56 (0.0496±0.00029)	23500±940.3 (0.652±0.00673)	0
II[d]	182.6±2.05 (0.0460±0.00025)	190.0±2.21 (0.0510±0.00030)	23200±938.4 (0.747±0.00695)	0

[a] Without liquid N_2 treatment.
[b] Total trypsin inhibited (mg) per 1000 g fresh weight.
[c] Mg trypsin inhibited per mg protein.
[d] With liquid N_2 treatment.

Table 5. Trypsin inhibitor activity of rice seedlings[a] of a Japonica cultivar Tainung 67[b].

Days after imbibition			
4	5	6[c]	10
125.0±1.67[d]	149.0±1.84	103.0±1.37	0
(0.0270±0.00012)[e]	(0.0306±0.00018)	(0.0194±0.00011)	0

[a] Imbibition was carried out in distilled water at about 27°C in the dark for 3 days before germination (30±1°C). Samples were treated with liquid nitrogen before TIA assays.
[b] Total TIA and specific TIA of the whole seeds with hull were 182.6±2.05 mg trypsin inhibited per 1000 g fresh weight and 0.0460±0.00025 mg trypsin inhibited per mg protein, respectively.
[c] Due to insufficient amounts of TIA, whole seedlings instead of individual tissues were determined 4, 5, or 6 days after imbibition. Tripsin inhibitor activies of residual embryos, endosperms, leaves plus stems and roots were also determined separately 6 days after imbibition and TIA was found only in newly emerged tissues, namely shoots and roots, with 179.0±2.24 mg trypsin inhibited per 1000 g fresh weight and a specific TIA of 0.0697±0.00038 mg trypsin inhibited per mg protein; none was found in residual embryos and endosperms.
[d] Total trypsin inhibited (mg) per 1000 g fresh weight.
[e] Mg trypsin inhibited per mg protein.

Table 6. Trypsin inhibitor activity of rice seedlings[a] of a Japonica cultivar Tainung 67[b].

Days after imbibition								
Whole seedlings			Shoots			Roots		
3 d	5 d	3 d + 2 d under water	3 d	5 d	3 d + 2 d under water	3 d	5 d	3 d + 2 d under water
307.8±8.60[c] (0.0857± 0.00378)[d]	336.5±7.65 (0.1068± 0.00011)	304.9±7.17 (0.1056± 0.00416)	497.1±17.69 (0.0570± 0.00344)	156.3±7.65 (0.0499± 0.00402)	71.2±3.82 (0.0180± 0.00158)	20.0±2.39 (0.0053± 0.00096	18.7±1.91 (0.0110± 0.00186	0

[a] Imbibition was carried out in distilled water at 37±1°C in the dark for 1day before germination (30±1°C). Samples were treated with liquid nitrogen before TIA assays.

[b] Total TIA and specific TIA of the whole seeds with hull were 182.6±2.05 mg trypsin inhibited per 1000 g fresh weight and 0.0460±0.00025 mg trypsin inhibited per mg protein, respectively.

[c] Total trypsin inhibited (mg) per 1000 g fresh weight.

[d] Mg trypsin inhibited per mg protein.

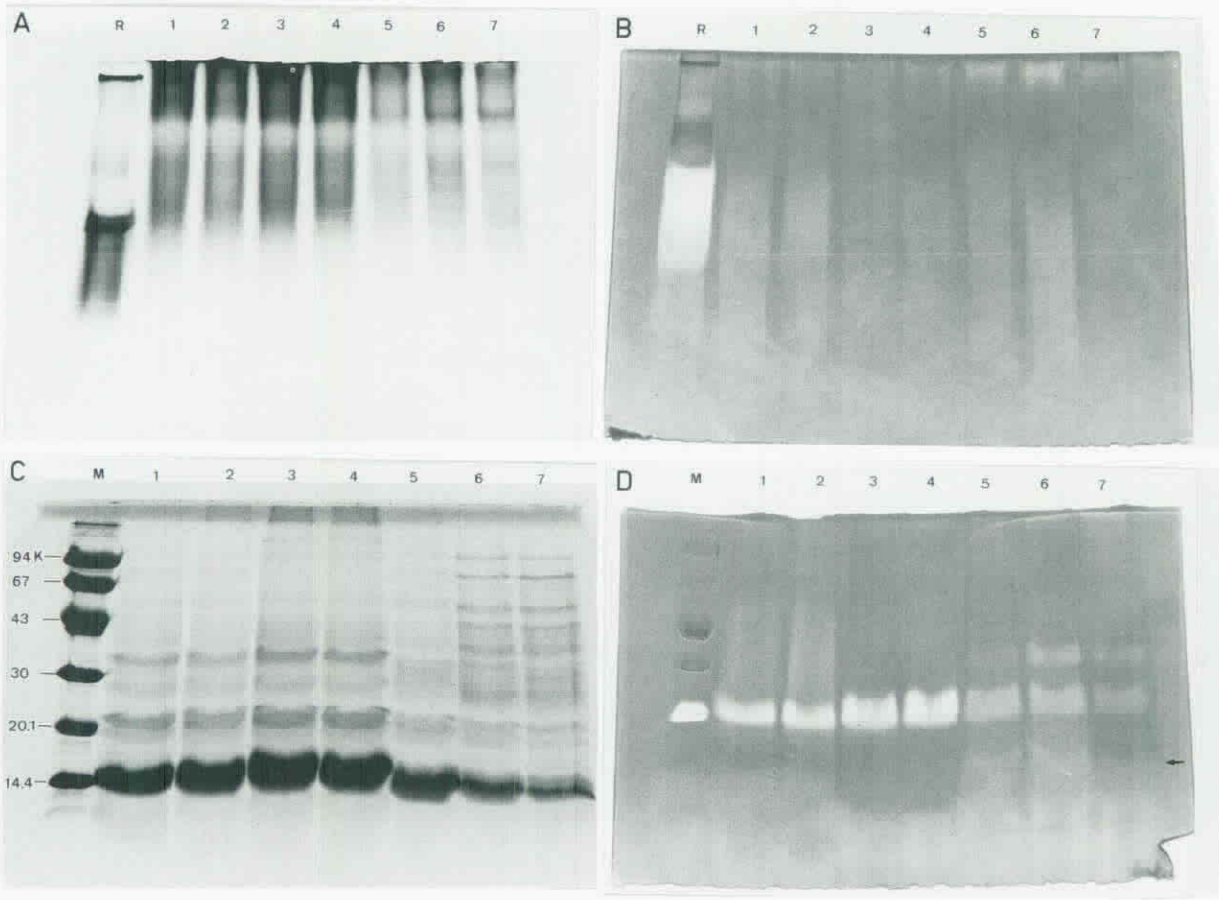

Figure 1. Protein and TIA patterns on both nondenaturing- and SDS-PAGE of crude extract of various parts of seeds and seedlings of rice TNG67. Figure 1A and 1B were 12.5–20% nondenaturing gels; 1C and 1D were 12.5–20% SDS gels. pH of both separating gels was 8.8. 1A and 1C show protein patterns, while 1B and 1D show TIA patterns. Lane R in both 1A and 1B is crude extract of sweet potato Tainong 57 roots. Lane M in both 1C and 1D is a mol. wt. kit (α-lactalbumin, M_r 14400; soybean trypsin inhibitor, M_r 20100; carbonic anhydrase, M_r 30000; ovalbumin, M_r 43000; bovine serum albumin, M_r 67000; and phosphorylase b, M_r 94000). Lanes 1–4 are seeds without liquid-nitrogen-pretreatement; seeds pretreated with liquid nitrogen; dehulled seeds without liquid-nitrogen-pretreatment; dehulled grains pretreated with liquid nitrogen; lanes 5–7 are whole seedlings 4–6 days after imbibition (imbibition was carried out at about 27°C in the dark for 3 days before germination, the same as in Table 5); samples were pretreated with liquid nitrogen. The samples in 1A and 1B, 1C, and 1D are 125, 60, and 100 μg proteins, respectively. Lane R in both 1A and 1B and lane M in both 1C and 1D contained 60 and 30 μg proteins, respectively.

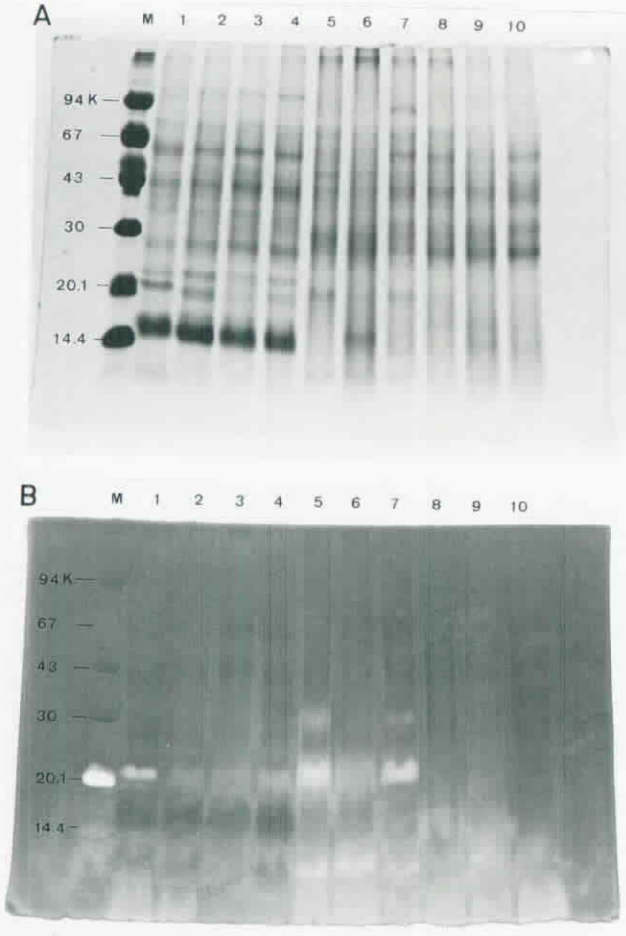

Figure 2. Protein and TIA patterns on SDS-PAGE of crude extract of various parts of seeds and seedlings of rice TNG67. 2A shows protein patterns while 2B shows TIA patterns. Conditions were the same as for Figure 1, except that imbibition was carried out at 37±1°C in the dark for 1 day before germination (the same as in Table 6). Lane M, a mol. wt. kit as in Figure 1. Lane 1, dehulled seeds; lane 2, seedlings 3 days after imbibition; lane 3, seedlings 3 days after imbibition followed by 2-day submergence treatment; lane 4, seedlings 5 days after imbibition. Lanes 5–7, shoots (coleoptiles plus unexpanded leaves) with the same sampling times as for lanes 2–4, respectively. Lanes 8–10, roots also with the same sampling times as for lanes 2–4, respectively. Lane M contained 25 μg proteins, while lanes 1–10 each contained 31.5μg proteins.

in shoots (coleoptiles and unexpanded leaves). Submerging treatment causes TIA of seedlings to decrease compared to that of air-grown ones, and this decrease of TIA also occurred in shoots.

Seed and seedling protein and TIA patterns on nondenaturing PAGE and SDS-PAGE

This is shown in Figure 1. Samples were the same as for Table 5. The strongest TIA band is the one with M_r 21400 (Figure 1D), which is also the only one left if samples are subjected to boiling for 4 min before SDS-PAGE (data not shown) . Comparison between lane 1 plus lane 2, and lane 3 plus lane 4 of Figures 1B and 1D shows that rice hull and bran contained very weak TIA bands, with M_r 33600 and M_r equal to or less than 12800. When seedlings grew, the intensity of proteins with M_r around 20100 and 14400 decreased but the intensity of other proteins increased and new bands appeared. The TIA bands were strongest 5 days after imbibition; this is in agreement with the general trend observed in Table 5.

Further examination of the influence of submerged treatment on TIA bands of shoots by SDS-PAGE

This is shown in Figure 2. Dehulled seeds, seedlings, shoots, and roots have their characteristic protein patterns (Figure 2A) and TIA patterns (Figure 2B).The protein and TIA patterns of all tissues except roots changed after submerged treatment. Two important things were observed. First, three major TIA bands were found only in shoots, which is in agreement with Table 6 and Figure 1. Second, submerged treatment for 2 days decreased the intensity of TI33600 and TI20100 of shoots 5 days after imbibition (lane 5–7 of 2B) while coleoptiles elongated rapidly.

Discussion

Spectrophotometric assays give general levels of TIA and activity staining on gels can show changes of particular TI molecules. Using activity staining, we discovered two new TIs, TI33600 and TI21400, in rice seedlings. As well as major TI bands, we also observed broad bands with M_r less than 12800 (Figures 1D and 2B) in seeds and seedlings.

Since rice seed contains TIA mainly in the embryo, while seedling contain TIA mainly in the shoots (Table 4, Figure 2B), the TIA levels in rice seeds do not necessarily reflect those of seedlings. Thus, although TN1 seeds contain the lowest TIA level among the 6 cultivars examined, seedlings of TN1 do not necessarily contain the lowest TIA.

Horiguchi and Kitagishi (1971) observed the temporary increase in protease inhibitor at an early stage of germination and explained that the inhibitor in the rigid seed was in a bound form and therefore less extractable. We made the same observation, but since we found three major TIA bands only in shoots and also observed changes of TIA bands caused by water-logging, the temporary increase in TIA at an early stage of germination can not be explained solely by differences in the extractability. Trypsin inhibitor, especially TI33600, must have unreported, but important, physiological functions in rice seedlings.

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有關水稻的胰蛋白酵素抑制因子之新發現

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六個品種之水稻（*Oryza sativa* L.）種子，除台中在來 1 號外，均含相似的胰蛋白酵素抑制因子（以下簡稱 TI）活性水準且都存於胚。以台農 67 號蓬萊稻種子爲材料，發現先在攝氏 27°C 黑暗中浸水 3 天然後再於 30°C 發芽，其幼苗之 TI 活性水準及隨時間之變化都和先在 37°C 黑暗中浸水 1 天然後再於 30°C 發芽者（此爲正常過程）有所不同。幼苗之 TI 活性主要存於上胚軸（含葉鞘及未申展的葉）。在發芽早期 TI 活性先升高然後下降。當幼苗轉移到淹水狀態時，TI 活性比對照組者加速下降。在電泳膠片上之 TI 活性染顯示水稻種子的主要 TI 爲分子量 21400 者。而上胚軸含三個主要 TI，即分子量各爲33600，21400 和12800 者。淹水加速 TI33600 及 TI21400 之下降。以上結果指出 TI 在水稻可能具有文獻從未記載之生物功能。

關鍵詞：上胚軸；淹水；電泳；蛋白質染；活性染。