α-Amylase Inhibitors from Rice: Fractionation and Selectivity Toward Insect, Mammalian, and Bacterial α-Amylases[1]

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ABSTRACT

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Rice, a cereal grain in which proteinaceous α-amylase inhibitors have not been well characterized, was found to contain amylase activity and numerous proteinaceous α-amylase inhibitors. Inhibitory activity in crude extracts was best detected after the extracts were heated to inactivate the endogenous amylase activity. A relatively simple procedure was developed to extract α-amylase inhibitors from rice. Rice flour was extracted with 0.15*M* NaCl, and the crude extract was heated at 70°C for 20 min. A portion of the heat-soluble protein was then precipitated by adding ammonium sulfate to a concentration of 1.8*M*. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that the ammonium sulfate precipitate was composed of relatively small proteins with an apparent molecular mass of approximately 14 kDa. With two-dimensional gel electrophoresis, the ammonium sulfate fraction was resolved into at least eight proteins, most with pIs between 8 and 9. Further separation was done by reversed-phase high-performance liquid chromatography (RP-HPLC). More than 17 protein peaks were resolved. Individual HPLC fractions were assayed for inhibitory activities toward a group of insect (rice weevil, red flour beetle, confused flour beetle, and yellow mealworm), mammalian (human salivary and porcine pancreatic), and bacterial (*Bacillus*) α-amylases. The results showed that 13 of the HPLC fractions examined contained inhibitors of one or more of the α-amylases tested. Several fractions were selective toward insect α-amylases. Some inhibited only one of the four insect α-amylases, whereas others inhibited both insect and mammalian α-amylases. None of the fractions inhibited the bacterial α-amylase.

Proteinaceous inhibitors of α-amylases and proteases are widely distributed in cereals, legumes, and other plants. Because of the possible importance of these inhibitors in plant physiology and animal nutrition, extensive research has been conducted on their properties and biological effects (Garcia-Olmedo et al 1987, Silano 1987). So far, α-amylase inhibitory activity has been detected in most cereals, including wheat, barley, rye, sorghum, maize, oats, pearl millet, setaria, ragi, triticale, and several other plants. Rice is an exception in that the presence of α-amylase inhibitors has been uncertain. The absence of mammalian α-amylase inhibitory activity in rice extracts has been reported (Kneen and Sandstedt 1946, Granum 1979, Villareal and Juliano 1981). However, Saunders and Yetter (1977) and Baker (1988) detected low levels of inhibitory activity from rice endosperm extracts toward insect α-amylases. To our knowledge, no further isolation and characterization of rice α-amylase inhibitors have been reported. Like most other cereals, rice contains other types of hydrolytic enzyme inhibitors, such as a cysteine protease inhibitor (Abe and Arai 1985; Abe et al 1987a,b) and a serine protease inhibitor (Tashiro and Maki 1978, 1979; Tashiro et al 1987).

We are interested in cereal proteinaceous inhibitors of insect digestive enzymes and their potential use as resistance factors against insect attack on cereal grains. Recently, we reported about the inhibitory activity and specificity of several wheat α-amylase inhibitors (Feng et al, 1991). In this article, we report that, like wheat and other cereals, rice flour contains a group of proteinaceous inhibitors that inhibit not only insect α-amylases but also mammalian α-amylases. The activity of these inhibitors is more apparent after the crude extract is heated to denature the endogenous rice amylases. Fractionation by reversed-phase, high-performance liquid chromatography (RP-HPLC) revealed that rice contains at least 13 α-amylase inhibitory proteins, some of which are selective for insect α-amylases.

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MATERIALS AND METHODS

Plant Material

Rice (*Oryza sativa*, Newbonnet cultivar) was generously provided by Robert Dilday of the USDA Rice Research Center, Stuttgart, AR. Rice was milled, and the flour fraction (68 mesh) was collected and extracted as described below.

Preparation of Rice Albumin Fraction

Protein extraction and ammonium sulfate fractionation procedures were performed according to Villareal and Juliano (1981) with some modification. Rice flour was extracted with 0.15*M* NaCl (about 5 ml of saline solution per gram of flour) at 4°C for 3 hr. After centrifugation at 12,000 × *g* for 30 min, the supernatant (crude extract) was heated at 70°C for 20 min. The heat-treated extract was cooled and centrifuged at 12,000 × *g* for 10 min. The heat-soluble proteins in the supernatant were precipitated by adding solid ammonium sulfate to 1.8*M*. The precipitate was collected by centrifugation at 4,300 × *g* for 10 min, washed with cold acetone, and then vacuum dried. The powder was resuspended in deionized water, dialyzed extensively against water, and centrifuged at 17,000 × *g* for 15 min. The soluble material, called the ammonium sulfate 1.8*M* (AS 1.8*M*) or rice albumin fraction, was used for further studies.

Fractionation of Albumin Fraction by RP-HPLC

Trifluoroacetic acid (TFA) was added to the rice albumin fraction to give a final TFA concentration of 0.1%. RP-HPLC was first conducted on a Vydac 218 TP54 analytical column (25 cm × 0.46 cm) and finally on a Vydac 218 TP 1022 preparative column (25 cm × 2.2 cm, 10 μm particle size, and 300 Å pore size). Twenty milliliters of the rice albumin fraction AS 1.8*M* (A_{280} = 1.36) was applied to the preparative RP-HPLC column equilibrated with eluent A (0.1% TFA in water). After the sample was loaded, the column was eluted with a gradient of acetonitrile that also contained 0.1% TFA at a flow rate of 8 ml/min. The absorbance of the effluent was monitored at 280 nm. Individual peaks were collected, lyophilized, and dissolved in deionized water.

Assay of α-Amylase Inhibitor Activity

α-Amylases of the rice weevil (*Sitophilus oryzae*), confused flour beetle (*Tribolium confusum*), red flour beetle (*T. castaneum*), and yellow mealworm (*Tenebrio molitor*) were purified or partially purified following the procedure of Baker (1987). Human salivary α-amylase (type IX-A), porcine pancreatic α-amylase (type I-A), and bacterial α-amylase from *Bacillus* species (type II-A) were obtained from Sigma Chemical Co. α-Amylase activity was

measured using the method described by Feng et al (1991). For insect α-amylases, the assay was made in 50 m*M* acetate buffer, pH 5.0, containing 20 m*M* NaCl and 0.1 m*M* $CaCl_2$. For α-amylases from other sources, the assays were done in 20 m*M* phosphate buffer, pH 6.9, containing 7 m*M* NaCl. The incubation time of the enzymes with the inhibitor fractions was 10 min at 37°C. The amount of each RP-HPLC fraction used for assay was estimated by absorbance at 280 nm, assuming an extinction coefficient of 1.0 for 0.1% protein concentration. One amylase unit was defined as the amount of enzyme that liberates 1 mg of maltose during the 5-min reaction. Inhibitory activity was expressed as the percentage of inhibited enzyme activity out of the total enzyme activity used in the assay.

Rechromatography on RP-HPLC and Amino Acid Analysis

Several individual peaks collected from the first RP-HPLC run were rechromatographed on the same analytical RP-HPLC column, but with 0.1% heptafluorobutyric acid as the ion-pairing reagent instead of TFA. Major peaks from the second HPLC separation were collected and lyophilized for amino acid analysis. Amino acids were analyzed according to the Pico-Tag procedure (Bidlingmeyer et al 1984). Cysteine was measured after performic acid oxidation.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel (15%) electrophoresis (PAGE) was conducted at a high tris concentration (Fling and Gregerson 1986). Rainbow protein molecular weight markers were from Amersham Life Science Products (Arlington Heights, IL 60005) (insulin A chain 2,350, insulin B chain 3,400, aprotinin 6,500, lysozyme 14,300, trypsin inhibitor 21,500, carbonic anhydrase 30,000, and ovalbumin 46,000). Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). Urea PAGE was done according to Brewer and Ashworth (1969) with basic-urea PAGE at pH 8.8 and acidic-urea PAGE at pH 4.3. Slab isoelectrofocusing (IEF) was performed as described by Robertson et al (1987), with standard proteins from Sigma Co.

RESULTS

Extraction of Rice Flour and Inactivation of Endogenous Amylases

The rice flour crude extract is mainly a mixture of polypeptides of relatively low molecular weight (about 14 kDa), as revealed on SDS-PAGE (Fig. 1, lane I). An inhibition assay of the crude extract was first conducted toward α-amylase from the rice weevil, but no inhibition was detected. Instead, amylase activity greater than that of the rice weevil α-amylase was observed. Since the assay was based on the measurement of reducing end groups of oligosaccharides released from starch by α-amylase, the apparent increase in α-amylase activity could have been caused by the presence of either interfering reducing substances or the products of endogenous rice amylases in the crude extract. A time course of amylase activity in the dialyzed rice crude extract demonstrated the presence of endogenous enzyme(s) in the 0.15*M* NaCl crude rice extract (Fig. 2). Dialysis did not eliminate the activity, but heating the extract at 70°C for 20 min inactivated the endogenous amylases (data not shown). Their activity was also inactivated by treatment with trypsin (data not shown). Evidently, the endogenous amylases in rice prevented us from detecting the α-amylase inhibitory activity in the crude extract. After inactivating the amylase activity by heating, the inhibitory activity could be easily measured.

Most of the relatively high molecular weight proteins were denatured and precipitated by the heat treatment (Fig. 1, lanes I and II). The major proteins precipitated by addition of 1.8*M* ammonium sulfate were of low molecular weight (apparent molecular mass about 14 kDa) (Fig. 1, lanes III and IV). Most of the 23-kDa protein precipitated out during dialysis of the 1.8*M* ammonium sulfate fraction. The AS 1.8*M* fraction from this fraction was resolved into more than eight protein spots by two-dimensional gel electrophoresis at a pH range of 3.5–10 (Fig. 3). The pIs of the major polypeptides were estimated using a slab IEF gel. Most of the proteins in the AS 1.8*M* fraction were basic and had pIs around 8 and 9 (Fig. 3). Overall, these results demonstrated that the AS 1.8*M* fraction of rice is composed of a heterogenous group of polypeptides of about the same molecular weight and that some of them inhibit the α-amylases.

RP-HPLC of Rice Albumin Fraction

When the AS 1.8*M* rice albumin fraction was subjected to

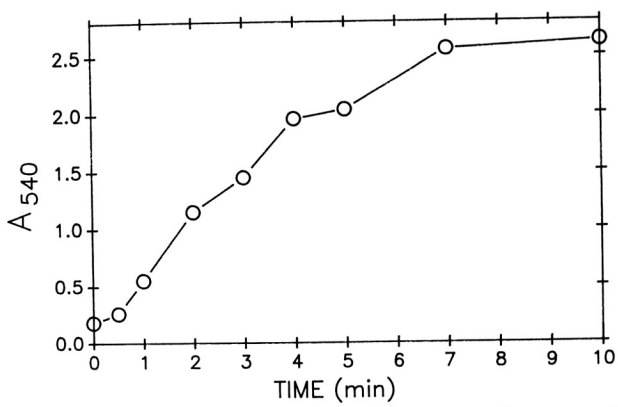

Fig. 2. Time course of amylase activity in 0.15*M* NaCl extract of rice flour. The crude extract was dialyzed against water thoroughly and centrifuged. The supernatant (20 μl) was then used in amylase activity assays.

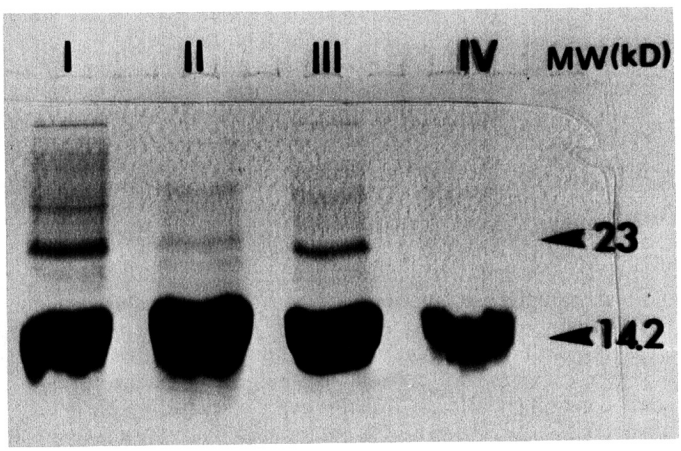

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 0.15*M* NaCl extract of rice flour (lane I), heat-soluble material (lane II), 1.8*M* ammonium sulfate precipitate (lane III), and water-soluble proteins of 1.8*M* ammonium sulfate precipitate (lane IV).

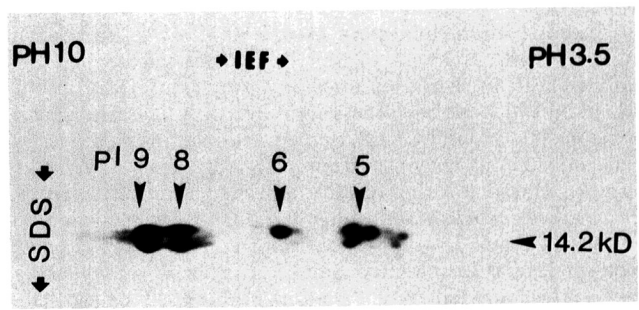

Fig. 3. Two-dimensional polyacrylamide gel electrophoresis (PAGE) of 1.8*M* ammonium sulfate precipitate of heat-treated 0.15*M* NaCl extract of rice flour. The first dimension is isoelectrofocusing (IEF) and the second is sodium dodecyl sulfate (SDS)-PAGE. The arrows indicate the direction of mobility. The pIs were estimated from a slab IEF gel.

TABLE I
Inhibition of Different α-Amylases by Rice High-Performance Liquid Chromatography (HPLC) Fractions[a]

HPLC Fraction	Percent Inhibition of α-Amylases from						
	Sitophilus oryzae (0.86)[b]	*Tribolium*		*Tenebrio molitor* (0.88)	Human Salivary (1.1)	Porcine Pancreatic (0.94)	*Bacillus* (1.0)
		confusum (0.88)	*castaneum* (0.68)				
1	...	...	...	...	23 ± 1	...	...
2	...	82 ± 1	88 ± 3	80 ± 2	96 ± 1	...	...
3	37 ± 1	82 ± 2	91 ± 1	85 ± 1	99 ± 1	32 ± 5	...
4	36 ± 5	41 ± 6	83 ± 1	50 ± 5	29 ± 1	...	...
6	90 ± 4	61 ± 4	63 ± 5	75 ± 1	53 ± 5	...	...
7	88 ± 4	...	...	39 ± 5	...	...	...
8	90 ± 7	53 ± 2	81 ± 3	...	68 ± 1	...	...
9	...	73 ± 1	66 ± 1	...	...	...	...
10	...	74 ± 1	58 ± 3	...	...	...	...
11	...	...	...	...	...	...	...
12	...	85 ± 1	...	...	...	...	...
13	...	87 ± 1	...	...	...	...	...
14	...	82 ± 1	69 ± 2	...	...	...	...
15	...	...	...	67 ± 2	...	...	...
16	...	...	...	...	...	...	...
17	...	...	...	...	...	...	...

[a]About 5 μg of protein of the individual HPLC fractions was used in each assay. Data are mean value (n = 2) ± 0.5 range. Dots mean that less than 20% inhibition was observed.
[b]Number in parentheses is the amylase units (milligrams of maltose released) used in assays.

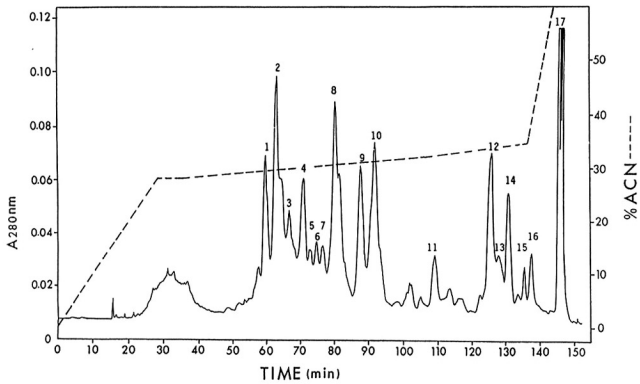

Fig. 4. Reversed-phase high-performance liquid chromatography separation of rice 1.8*M* ammonium sulfate precipitate of heat-treated 0.15*M* NaCl extract of rice flour. The dashed line indicates the gradient of acetonitrile (ACN) applied. Peak 17 is heterogeneous, and the peak height is off scale. It does not contain amylase inhibitory activity.

RP-HPLC, many protein peaks were resolved, and these numbered peaks were collected and further studied (Fig. 4). The first screening of inhibitory activity was conducted by a single assay of each HPLC fraction against seven enzymes. Assays that showed less than 20% inhibition were defined as insignificant in inhibitory activity, and ones that exhibited more than 20% inhibition were confirmed by duplicate tests. The overall results showed that 13 of the 16 fractions tested (HPLC fraction 5 was not tested) contain inhibitors of one or more of the enzymes (Table I). Most fractions exhibited different selectivities. Some inhibited both insect and mammalian α-amylases (HPLC fractions 2–4, 6, and 8), whereas others inhibited only insect α-amylases (fractions 7, 9, 10, and 12–15). Some fractions exhibited selectivity even among the enzymes from different insect species. For example, fraction 7 significantly inhibited α-amylases from *S. oryzae* and *Tenebrio molitor* only; fractions 9, 10, and 14 inhibited α-amylases from *Tribolium confusum* and *T. castaneum* only; fractions 12 and 13 inhibited only *T. confusum* α-amylase; and fraction 15 inhibited only *Tenebrio molitor* α-amylase. Of all the fractions, only fraction 3 had activity toward porcine pancreatic α-amylase, whereas human salivary α-amylase was inhibited by fractions 1–4, 6, and 8. However, none of the HPLC fractions showed inhibitory activity toward *Bacillus* α-amylase. These results revealed that rice, like other cereals, contains a rather large group of proteinaceous inhibitors that inhibit not only insect but also mammalian α-amylases.

Gel Electophoresis of RP-HPLC Fractions

SDS-PAGE of the RP-HPLC fractions showed that all peaks except peak 17 were composed of small polypeptides with apparent molecular weights of approximately 14 kDa (Fig. 5). HPLC fraction 17 was a mixture of relatively higher molecular weight proteins that eluted last from the RP-HPLC column, but none had inhibitory activity toward the enzymes tested. PAGE of the HPLC peaks in the presence of urea revealed heterogeneity. For example, fraction 3 exhibited only one band on an SDS gel, but on a urea gel at pH 8.8, four bands were resolved (Fig. 6, panel I). Major proteins in fractions 8, 12, 14, and 16 were very basic and did not move into the gel, but they were well resolved on a urea gel at pH 4.3 (Fig. 6, panel III). These basic proteins may correspond to the basic proteins observed on two-dimensional gels with pIs about 9 (Fig. 3).

Amino Acid Analysis of Some HPLC Fractions

Table II shows the amino acid compositions of the major components of HPLC fractions 1, 2, and 4 after a second HPLC separation. These three HPLC fractions had similar amino acid compositions. All of them contained a relatively high percentage of Gly, Arg, Ala, and Cys but were low in His, Met, Ile, Phe, and Lys.

Inhibitor Stability

Like wheat α-amylase inhibitors (Silano 1987; Feng et al, 1991), the rice proteinaceous inhibitors were very stable. Exposure to high temperature (70°C) or organic solvent (35% acetonitrile at pH 2.3) did not cause the inhibitors to lose their activities.

DISCUSSION

Our research group is attempting to identify naturally occurring inhibitors that have strong activity against insect digestive enzymes but little or none against mammalian enzymes. We ultimately wish to transfer genes that encode inhibitors selective for insect digestive enzymes into cereals by genetic engineering, with the goal of creating new insect-resistant cereal varieties. In the process of surveying several cereals for potential inhibitors, we examined rice seed, which previously was reported to lack inhibitory activity for α-amylases. We found that heating a 0.15*M* NaCl extract of rice flour facilitated the detection of α-amylase inhibitor activity. Unlike that in wheat and other cereals, the inhibitory

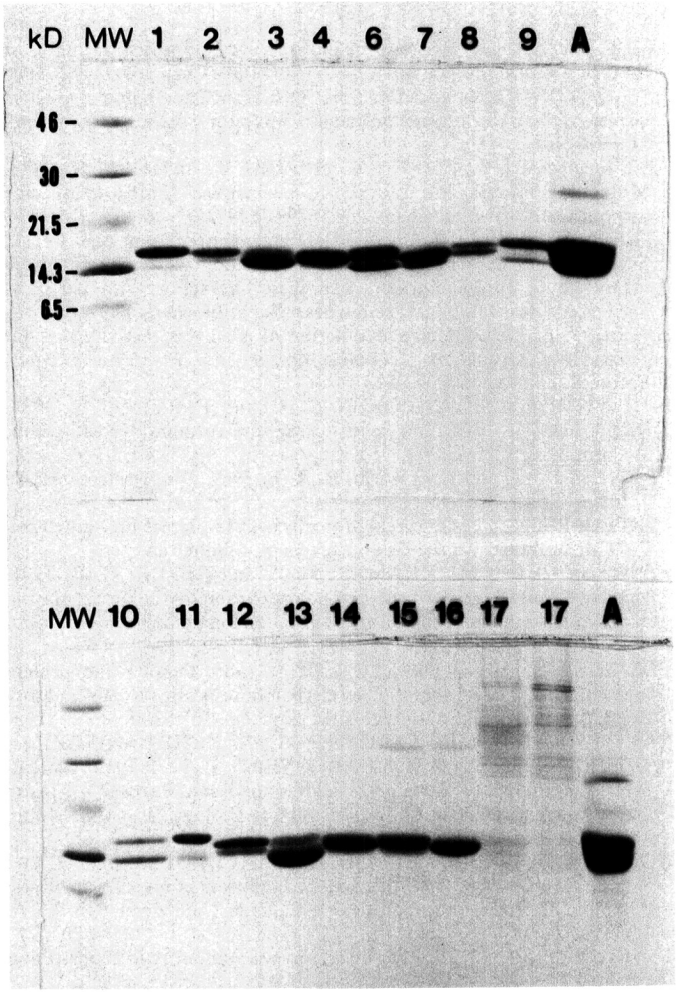

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reversed-phase high-performance liquid chromatographic peak fractions (lanes 1–17). Lane A is the 1.8*M* ammonium sulfate precipitate of heat-treated 0.15*M* NaCl extract of rice flour. Molecular weights (MW) of standard proteins in kilodaltons (kDa): aprotinin, 6.5; lysozyme, 14.3; trypsin inhibitor, 21.5; carbonic anhydrase, 30.0; and ovalbumin, 46.0.

activity in rice flour could hardly be detected in the crude aqueous extract. Heating appeared simply to inactivate the endogenous amylase activity, the presence of which prevented the detection of inhibition of exogenous enzymes. The data from this study are similar to the data reported by other researchers (Kneen and Sandstedt 1946, Villareal and Juliano 1981), who also found the presence of amylase activity in rice extracts but failed to detect inhibitory activity toward human salivary α-amylase in unheated rice extracts. The observation of relatively low amylase inhibitory activity in unheated rice extracts (Saunders and Yetter 1977) was probably due to the presence of little or no amylase activity in the rice variety used for extraction. A low level of inhibitory activity toward rice weevil α-amylases in a heated extract of rice flour was also observed (Baker, 1988).

According to our experience, the endogenous amylase activity in rice flour varies greatly from batch to batch, probably depending

TABLE II
Amino Acid Compositions (mole %) of Some Rice Albumin High-Performance Liquid Chromatography (HPLC) Fractions and Rice Protein-Synthesis Inhibitors (RPSI)

Amino Acids	Rice HPLC Fractions[a]			RPSI[b]		
	1	2	4	Ri-2	Ri-3	Ri-5
Asx	7.2	8.6	7.8	8.8	7.2	8.0
Glx	7.6	8.2	8.3	11.0	9.0	11.6
Ser	4.5	4.0	3.8	3.8	4.9	6.6
Gly	12.6	13.4	13.9	13.5	15.1	12.9
His	4.3	3.7	3.8	2.7	1.4	3.5
Arg	11.4	12.3	11.5	8.4	7.8	7.5
Thr	4.2	3.6	3.0	3.1	2.9	4.7
Ala	9.7	9.2	10.2	8.6	12.1	10.6
Pro	7.2	7.2	7.8	7.5	6.2	7.4
Tyr	3.6	3.9	3.5	3.3	3.3	3.7
Val	6.3	6.4	6.3	7.8	7.7	5.9
Met	1.9	1.9	1.6	0.9	1.5	0.6
Cys	8.5	8.3	8.2	6.9	8.7	5.9
Ile	1.3	0.9	1.2	2.5	2.4	2.4
Leu	5.8	5.8	6.9	6.7	5.7	6.1
Phe	1.6	1.3	1.1	1.9	2.2	1.7
Lys	2.3	1.3	1.2	2.1	1.6	0.9

[a] Amino acid compositions of rice HPLC fractions 1, 2, and 4 were determined by a single analysis.
[b] Amino acid compositions of RPSI are from Limas et al (1990).

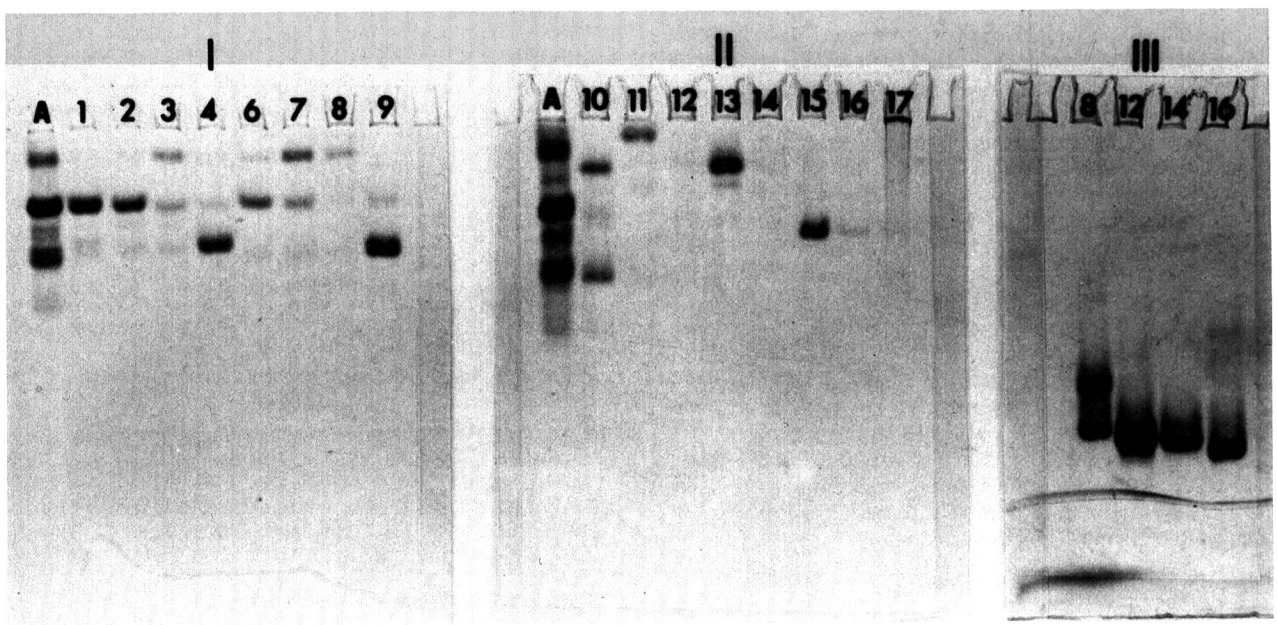

Fig. 6. Urea polyacrylamide gel electrophoresis of reversed-phase high-performance liquid chromatographic peak fractions. Panels I and II are urea-basic pH 8.8 gels, and panel III is urea-acidic pH 4.3 gel. Lane A is the 1.8*M* ammonium sulfate precipitate of heat-treated 0.15*M* NaCl extract of rice flour.

on the conditions under which rice is harvested and stored. Germination of rice seeds greatly increases the amylase activity inside the seeds (Palmiano and Juliano 1973). Growth conditions have been reported to affect the inhibitory activity but not the molecular weight and amino acid composition of α-amylase inhibitors in wheat (Peruanskii et al 1980). We also observed a difference in amylase inhibitory activities between different batches of rice.

The 0.15*M* NaCl extract of rice flour contains two main groups of polypeptides. One of these, α-globulin, is a single polypeptide (Pan and Reeck, 1988) that has an apparent molecular mass of 23 kDa and is encoded by a single gene (Shorrosh 1989). The second major salt-soluble fraction from rice flour is a group of polypeptides with apparent molecular mass around 14 kDa. Heating a 0.15*M* NaCl extract resulted in precipitation of α-globulin and other proteins of relatively high molecular weight. The proteins that remained soluble were for the most part of low molecular weight. The heated extract also contained α-amylase inhibitor activity. Thus, rice is similar to wheat, in which the α-amylase inhibitors also occur in the albumin fraction and have polypeptide molecular weights around 14 kDa (Garcia-Olmedo et al 1987, Silano 1987, Feng et al 1991). Recently, Limas et al (1990) reported that rice contained low molecular weight salt-soluble proteins (11–17 kDa) that inhibit protein synthesis and bind immunoglobulins. Some of the rice protein-synthesis inhibitor fractions had amino acid compositions very similar to those of the HPLC fractions presented in this study (Table II). Whether any of those proteins correspond to the rice α-amylase inhibitors is unknown.

Although both rice and wheat contain a mixture of α-amylase inhibitors, rice generally has a lower level of inhibitory activity in the heat-soluble albumin fraction than that measured in a heat-soluble albumin fraction from wheat. The species selectivity and potency of the inhibitors from these two cereals are clearly different. For example, rice had only one fraction (HPLC fraction 7, a minor one) that selectively inhibited insect α-amylases, including those of the rice weevil, whereas wheat contained many fractions with this type of inhibitor (Feng 1990). Rice RP-HPLC fractions 2 and 3 exhibited high inhibitory activity toward α-amylase from human saliva, *Tribolium confusum*, and *T. castaneum* but low or none toward α-amylases of *S. oryzae*, whereas in a similar study with wheat, all the RP-HPLC fractions that exhibited high inhibition of α-amylases from human saliva and *T. confusum* also strongly inhibited α-amylases from *S. oryzae* (Feng et al 1991). None of individual wheat RP-HPLC fractions inhibited porcine pancreatic α-amylase (Feng et al 1991). However, inhibitory activity toward that enzyme was observed after recombination of certain wheat HPLC fractions, apparently because wheat contains a tetrameric inhibitor with subunits that are resolved by RP-HPLC (Feng et al 1991). A tetrameric α-amylase inhibitor from wheat was previously reported by Gomez et al (1989). In the current study, we found that one of the rice HPLC fractions (fraction 3) weakly inhibits porcine pancreatic α-amylase. Whether the inhibitor in this fraction is monomeric or oligomeric is not known. No inhibitor of bacterial α-amylase was found in rice, a result that is similar to the data obtained with wheat and most other cereals (Silano 1987).

Although RP-HPLC separates rice albumin AS 1.8*M* into more than 17 fractions, most of these fractions are still heterogenous. Individual RP-HPLC fractions can be further fractionated by RP-HPLC using a different ion-pairing agent or by other chromatographic methods (Feng 1990). We will focus our future research on the rice inhibitors that are selective for insect α-amylases, because their levels in cereal grain might be manipulated for insect control purposes. The inhibitors in HPLC fractions 7, 9, 10, and 12–15 are excellent candidates for such a study.

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