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PROPERTIES OF ALBUMINS OF MILLED RICE

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Key Word Index—*Oryza sativa*; Gramineae; milled rice; albumins; isoelectric focusing.

Abstract—Extraction studies on IR36 milled rice showed that albumins solubilized by 0.1–0.15 M $(NH_4)_2SO_4$ consisted of about 20% high (~5%) lysine, fast-migrating proteins on electrophoresis at pH 8.3 and about 80% lower (~2%) lysine proteins of slower mobility. The 2%-lysine albumins were insoluble in 1.8 M $(NH_4)_2SO_4$ while the higher lysine albumins required 4 M $(NH_4)_2SO_4$ to precipitate. The 2%-lysine albumins were not fractionated by gel filtration and gave only one major fraction with MW 19000. SDS-polyacrylamide gel electrophoresis confirmed the major subunit to be of MW 17000. These albumins were separated by DEAE-Sephacel chromatography at pH 8.5 into three fractions of similar aminograms but differing in analytical gel electrophoretic and isoelectric focusing patterns.

INTRODUCTION

Albumins constitute about 5% of milled rice proteins. They are the least studied proteins because of the susceptibility of their aqueous solution to denaturation and the problem of globulin contamination during extraction. Cagampang *et al.* [1] have studied the changes in properties of salt-soluble proteins of developing rice caryopsis. Salt-soluble proteins of milled rice were recently characterized by isoelectric focusing and crossed immunoelectrophoresis [2]. Water-soluble proteins were characterized by 2-dimensional flat gel SDS electrophoresis [3]. Other milled-rice protein fractions—glutelin [4, 5], prolamin [6], and α-globulin [7]—have also been recently characterized. The properties of milled-rice albumins were studied to complete the elucidation of milled-rice proteins. A companion paper reports on the globulins [8].

RESULTS

Extraction and purification

In preliminary studies to find a suitable solvent for extracting albumins from IR36 milled-rice flour, water, 0.25 M NaCl and 0.10–0.15 M $(NH_4)_2SO_4$ gave extracts with identical albumin patterns on analytical disc gel electrophoresis (Fig. 1). However, the 0.25 M NaCl extract already contained some α-globulin contamination. Subsequent extraction of the milled-rice residue with 0.5 M NaCl and 0.8 M NaCl yielded mainly α-globulin. Three consecutive extractions with 0.10–0.15 M $(NH_4)_2SO_4$ solubilized 7–11% of milled-rice protein, in contrast to 1.3–2.2% for water and about 7–8% with 0.25 M NaCl. Thus, $(NH_4)_2SO_4$ was used for the large scale albumin preparation because its further addition to precipitate the protein facilitated purification.

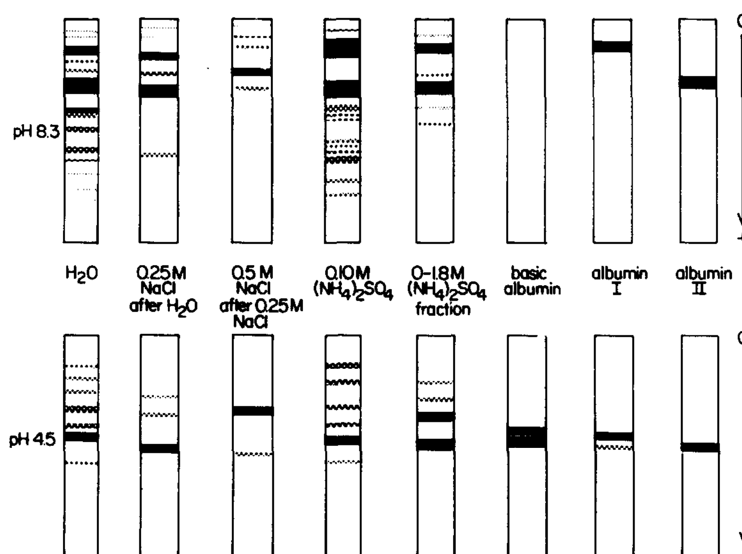

Fig. 1. Analytical disc gel electrophoregrams at pH 8.3 and 4.5 of albumin preparations from IR36 milled rice.

Saturation with $(NH_4)_2SO_4$ to 40% (1.8 M) brought down about 82% of the total solubilized proteins, which subsequent analytical disc gel electrophoresis showed to be the major albumins. Single extraction with dilute (0.1 M) buffers such as sodium acetate at pH 5–6.5 extracted only 3–4% of milled-rice protein.

Some precipitation occurred during storage of aqueous or dilute salt extracts at 0–4° for a few days. Disc gel electrophoresis confirmed the precipitate to be one of the components of the major albumins. In serial extractions with water and NaCl solutions of increasing molarity, addition of DTT up to 1 mM concentration reduced the protein extraction efficiency of H_2O and 0.25 M NaCl but increased those of the subsequent 0.5 M and 0.8 M NaCl solvents. Storage of milled rice above 15° decreased protein extractability with H_2O and the NaCl solutions except for 0.5 M NaCl, as in previous studies [9–11].

Crude albumins extracted with dilute buffers had detectable activities of protease, α-amylase and ribonuclease, as reported earlier [12]. They also showed no inhibitory activity to human salivary α-amylase and bovine pancreatic α-amylase, in agreement with earlier results [13]. An inhibitor of trypsin-like activity, however, was present in the high-MW fraction obtained after Sephadex G-25 gel filtration of the crude extracts. Rice protein and phytate have been found to inhibit trypsin [14, 15].

Identification and purity of the fractions were monitored by analytical disc gel electrophoresis at pH 8.3 and 4.5, by Triton-X- and SDS-polyacrylamide disc gel electrophoreses and by isoelectric focusing. Analytical disc gel electrophoregrams at pH 8.3 of the albumin preparations showed two major groups of protein bands for both the H_2O and 0.10 M $(NH_4)_2SO_4$ extracts (Fig. 1). There were also several other minor but faster moving bands. The 0.25 M NaCl extract, after H_2O extraction, only had the major protein bands of the H_2O extract while the subsequent 0.5 M NaCl and 0.8 M NaCl extracts consisted mainly of a major band between the two groups of slow bands of the earlier albumin extracts. This probably corresponds to α-globulin.

The 0–1.8 M $(NH_4)_2SO_4$ precipitate of the crude extract also showed mainly the two slower migrating bands (Fig. 1). The faster migrating minor proteins were found to be localized in the 1.8–4.0 M $(NH_4)_2SO_4$ fraction. Less α-globulin contamination was obtained with 0.10 M than with 0.15 M $(NH_4)_2SO_4$ as extractant. Ultrafiltration through a PM-30 membrane retained the α-globulin contaminant plus traces of the other albumins. Electrophoresis at pH 4.5 showed only one major albumin band for the H_2O and the 0.25 M NaCl extracts (Fig. 1). The 0.5 and 0.8 M NaCl extracts gave mainly one slower migrating band.

SDS-gel electrophoregrams of the water extract showed a complex pattern with only one major subunit of MW 16 800. The subsequent 0.25 M NaCl extract also contained the MW 16 800 major subunit but had less of the minor high-MW bands. The 0.5 M and 0.8 M NaCl extracts and the 0–1.8 M $(NH_4)_2SO_4$ precipitate of the crude extract also had minor high-MW bands and three major subunits corresponding to MW 22 000, 17 200 and 16 600, respectively. A MW of 16 000 was reported for one of the three major albumin subunits of brown rice [1].

Triton X-gel electrophoresis [16] of the water extract gave essentially the same banding patterns as those shown by pH 8.3 analytical gel electrophoresis. The proteins exhibited a MW range of 20–242 × 10^3 with the major band having a MW of 86 000. The 0.25 M NaCl extract and the 0–1.8 M $(NH_4)_2SO_4$ precipitate of the crude extract gave similar patterns having three major bands with MW (× 10^{-3}) of 242, 126 and 62. The 0.5 M NaCl extract only had one intense polypeptide band with MW 248 000.

Flat-gel isoelectric focusing in the pH gradient 3–10 [17] showed about 16 bands for the water extract, 10–11 of which had pI values of 6.0–7.5 (Fig. 2). There were no major bands. Proteins solubilized by 0.15 M $(NH_4)_2SO_4$ gave a banding pattern similar to that of the water extracts but with a very distinct major protein of pI 5.3 and about four medium-intensity proteins from pH 7.6 to 9.0. The globulins extracted by 0.5 M and 0.8 M NaCl had a major polypeptide with pI 5.3 plus three other minor bands with pI 4.1–4.3.

Amino acid analysis of the various preparations indicated a higher lysine content for the H_2O-extracted albumins than for 0.1 M $(NH_4)_2SO_4$-extracted albumins (Table 1). The 0–1.8 M $(NH_4)_2SO_4$ cut of the crude extract even had lower lysine content than the whole extract. In contrast, the 1.8–4.0 M $(NH_4)_2SO_4$ cut and the H_2O extract had similar aminograms and both were rich in the faster migrating albumin bands, as shown by the analytical gel electrophoregram (Fig. 1).

Fractionation and characterization

Gel filtration of the 0.15 M $(NH_4)_2SO_4$ extract through Sephadex G-25 and G-100 did not sufficiently fractionate

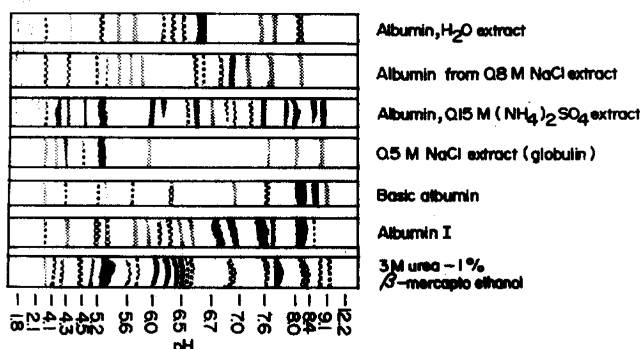

Fig. 2. Flat-gel isoelectric focusing patterns of albumin preparations and IR36 milled-rice protein dissolved in 3 M urea–1% β-mercaptoethanol.

Table 1. Aminograms of defatted IR36 milled rice and various albumin preparations

			0.1 M $(NH_4)_2SO_4$ extract							
					0–1.8 M $(NH_4)_2SO_4$ insol.					
Amino acid	Milled rice	H_2O extract	Whole	1.8–4.0 M $(NH_4)_2SO_4$ insol.	Whole	Basic albumin	Albumin I	Albumin II	Albumin from 0.8 M NaCl extract*	LSD (5%)
Lys	3.6	5.9	3.7	5.8	2.2	2.0	3.1	2.6	2.6	0.6
His	2.4	3.0	3.0	3.1	1.8	2.4	3.0	3.4	3.0	0.4
Amm	2.2	1.8	1.9	2.0	1.6	1.4	2.4	2.9	3.0	0.6
Arg	8.7	8.4	11.2	8.5	13.6	14.7	13.3	12.3	11.4	0.8
Asp	11.4	11.1	9.2	13.1	7.9	8.3	9.0	9.1	8.7	1.0
Thr	3.6	4.0	3.1	4.8	2.4	2.1	2.0	2.6	2.4	0.2
Ser	5.0	4.2	3.8	4.8	4.3	3.0	3.4	3.5	3.2	0.3
Glu	21.5	13.1	13.3	14.7	15.8	9.7	9.9	11.5	9.7	0.7
Pro	4.6	7.5	6.6	7.2	6.5	6.8	6.6	6.2	6.6	0.6
Cys	2.7	3.8	5.8	3.1	5.8	6.4	6.0	2.1†	4.9	0.6
Gly	4.7	6.7	6.7	7.4	6.8	7.6	7.4	6.4	6.8	0.4
Ala	6.4	8.6	7.7	9.9	7.1	7.7	7.1	7.2	7.2	0.3
Val	6.1	6.7	6.4	8.3	5.7	6.2	6.1	5.4	5.8	0.6
Met	3.0	2.2	4.4	7.3	4.0	3.2	2.6	0.1†	2.7	0.3
Ile	4.2	3.4	2.8	4.2	2.4	2.3	1.8	2.1	2.2	0.08
Leu	9.2	8.1	4.5	9.5	7.2	7.7	6.9	6.9	6.7	0.4
Tyr	5.4	4.5	5.4	5.8	6.4	6.0	5.5	5.6	5.4	0.4
Phe	5.9	3.5	3.3	4.8	3.1	2.4	2.2	2.1	2.5	0.2
Trp	1.7	2.1	2.3	1.7	0.4	1.2	1.1	—	0.4	0.6
Wt %‡	100.0	1.3	7.3	1.3	6.0	2.6	1.7	1.3	3.0	

* H_2O-soluble fraction of the 0–1.3 M $(NH_4)_2SO_4$ precipitate.
† Without prior performic oxidation of protein.
‡ Of milled-rice protein.

the albumin proteins. Desalting through Sephadex G-25 gave a minor void-volume protein peak contaminated with amylose (blue colour with iodine), a major protein peak and a third non-protein peak. Subsequent chromatography of both protein fractions on Sephadex G-100 gave a minor void-volume peak and a major but broad peak with a V/V_0 of 1.9–2.1 (MW 18 000–20 000) and a shoulder at V/V_0 1.8–1.9. A similar elution pattern was shown by the semi-purified 0–1.8 M $(NH_4)_2SO_4$ precipitate of the crude extract. The major protein fraction after Sephadex G-100 filtration comprised 61 % of the void volume fraction, 71 % of the major protein fraction from Sephadex G-25, and 82 % of the 0–1.8 M $(NH_4)_2SO_4$ precipitate of the crude albumin extract. The main protein peak from Sephadex G-100 chromatography of albumins from Japanese milled rices also had a V/V_0 of 2 [11].

DEAE-Sephacel chromatography using a 0.02–0.4 M NaCl linear gradient separated the major albumins into an unadsorbed fraction (basic albumins) and three additional peaks (Fig. 3). The analytical gel electrophoregram at pH 8.3 showed differences among the peaks (Fig. 1). There was no migration noted for the basic albumins while the second and fourth peaks gave single bands that were denoted as albumin I and II, respectively. Relative mobilities were 0.15 for albumin I and 0.28 for albumin II. The third peak showed a mixture of albumins I and II. Total protein recovery was 95 %, with the basic albumin accounting for 44 %; albumin I, 21 %; mixture of albumin I and II, 16 %; and albumin II, 14 %. In acid gels, basic albumins showed two intense bands of intermediate mobility; albumin I had one major band and a minor, slightly faster migrating band, while albumin II only had the faster migrating band (Fig. 1).

Flat-gel isoelectric focusing also revealed partial resolution of the proteins by DEAE-Sephacel chromatography (Fig. 2). Basic albumins had major proteins at pI 8.3 and 8.7 but still had minor bands with pI at 7.6 and 7.7 and traces of others with lower pI values. By contrast, albumin I had four major bands with pI 6.8, 7.0, 7.6 and 7.7 plus minor bands at pH 8.3–8.7 and pH 5.2–6.6. Confirmatory disc gel isoelectric focusing showed that albumin II had the most acidic proteins among the three subfractions. Milled-rice albumin has previously been reported to exhibit only one band on IEF with a pI of 6.42 [3]. Also, other workers have found the 0.1 N NaCl extract of milled rice to have 21 components with pI values ranging from 4 to 8 [2].

SDS-gel electrophoresis of the DEAE-Sephacel subfractions gave one broad subunit MW 16 800 for albumin I and two adjacent subunits with MW 16 600 and 17 200 for basic albumin and albumin II. These three albumin fractions were found to have similar amino acid compositions (Table 1).

DISCUSSION

The albumins and globulins of rice cannot be completely distinguished by either water solubility, electrophoretic mobility or amino acid composition. The water-soluble

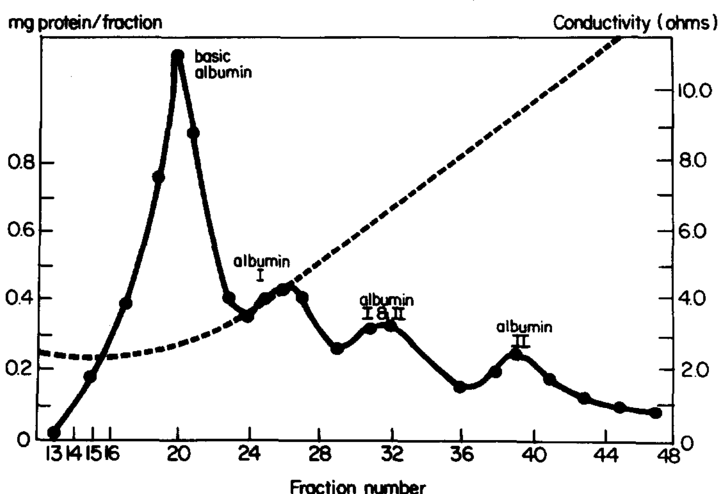

Fig. 3. DEAE-Sephacel column (1.6 × 70 cm) chromatography of the 0–1.8 M $(NH_4)_2SO_4$ fraction of the 0.1 M $(NH_4)_2SO_4$ extract of IR36 milled rice using a 0.02–0.4 M NaCl gradient (6.7 ml/fraction).

albumins could not be exhaustively extracted from milled-rice flour, even after five consecutive extractions. These albumins were present in the H_2O extract but required a higher salt concentration (i.e. 0.1 M $(NH_4)_2SO_4$) for complete solubilization. The requirement of a threshold salt concentration to solubilize all albumin-like proteins has been reported in wheat [18].

The minor, faster migrating components of the 1.8–4.0 M $(NH_4)_2SO_4$ fraction that makes up 1.3 % of milled-rice protein had the typical high lysine content of albumin (5.9 % vs 3.6 % for milled-rice protein) (Table 1). The major albumins had lower lysine content (mean 2.2 %) but both fractions had high cysteine content, which could explain their sensitivity to denaturation during the storage of milled rice or their aqueous extracts.

Isoelectric focusing showed that the unadsorbed protein from DEAE-Sephacel chromatography at pH 8.6 of the 0–1.8 M $(NH_4)_2SO_4$ precipitate of IR36 milled-rice extract were really basic proteins with pI 8.3 and 8.7. These were uncharged at pH 8.3 and hence did not migrate in the basic disc gel. Albumin I contained more neutral proteins (pI 6.8–7.7) while albumin II had the most acidic components. Albumin II had the highest glutamic acid content and basic albumin had the highest arginine content of the three fractions (Table 1).

The purity of the three isolated major albumin subfractions could only be assessed in part. Although albumin I, albumin II and the basic albumin seemed to be homogeneous by electrophoresis on the basic disc gels, they were still quite heterogeneous with overlapping on the IEF gel.

The water-soluble fraction of milled-rice crude globulin and the major albumins had similar aminograms (Table 1). Crude globulin was obtained by repeated precipitation from a 0.8 M NaCl extract with the addition of $(NH_4)_2SO_4$ to 1.3 M concentration. What remained in solution after dialysis of the precipitate against H_2O was termed as non-α-globulin. The major albumins and some milled-rice globulins had some similarities in amino acid composition [7, 8]. However, milled-rice α-globulin had higher glutamic acid and lower lysine, histidine and aspartic acid contents than the non-α-globulin [7, 8] and albumins.

Du Cros *et al.* [17] recently reported the use of 3 M urea–1 % β-mercaptoethanol solvent for the characterization of rice genotypes by gel isoelectric focusing of extracted proteins. Co-operative studies with Dr. Wrigley indicated that the proteins extracted from milled rice by this solvent were mainly albumins and globulins. Prolamin and glutelin were only sparingly soluble in this solvent.

EXPERIMENTAL

Materials. Newly harvested IR36 rice seeds from the IRRI farm were used as a source of defatted milled rice throughout the experiment. Grains were air-dried in the hood at ambient temp. to *ca* 14 % H_2O and subsequently dehulled with a Satake dehuller. Milling to about 11–12 % was done in the Satake TM-05 mill to ensure recovery of pure endosperm material only. Samples were then ground to a 60-mesh powder in a Udy cyclone mill and defatted by stirring 2 × in 3 vol. of petrol at room temp. for 6 hr each.

Protein extraction. Serial extraction with H_2O and NaCl solns of increasing molarity (0.25, 0.5 and 0.8 M) were done to monitor the complete distribution of the albumin–globulin proteins of the rice endosperm. Extraction was done by stirring the defatted rice powder 3 × in 2.5 vol. solvent at 0–4° for 1 hr each. Centrifugation was done at 12 000 g and corresponding supernatants were pooled and subsequently ultrafiltered through PM-10 for further characterization. For bulk preparations, defatted rice powder was extracted 3 × with 0.1–0.15 M $(NH_4)_2SO_4$ by stirring in 3 vol. of solvent at room temp. for 1 hr each. The extracts were pooled, centrifuged at 4000 g for 15 min and the supernatant was passed through Whatman No. 1 to clarify the soln further.

Protein purification. Separate aliquots of the crude bulk extract were processed in two ways—one batch was passed through Sephadex G-25 and the main protein peak was collected for further purification. Ultrafiltration of the major fraction was attempted through a PM-30 Amicon membrane under N_2. Another aliquot was treated with $(NH_4)_2SO_4$ to 40 % satn (1.8 M) and the suspension was aged overnight to being down the protein ppt. The 0–1.8 M $(NH_4)_2SO_4$ ppt. was centrifuged at 12 000 g and stored at 0–4° for further processing.

The 0–1.8 M $(NH_4)_2SO_4$ fraction was purified by chromatography through Sephadex G-100 (2.6 × 70 cm) in 0.05 M phosphate buffer, pH 7.3, with 0.1 M NaCl and 1 mM Na azide. The major components were then separated on DEAE-Sephacel (1.6 × 70 cm) using a linear NaCl gradient (0.02–0.4 M) in 0.05 M Tris–HCl, pH 8.6.

Analytical disc gel electrophoresis. Disc electrophoresis was done following essentially the method of ref. [19] but using 7.36 % acrylamide with a 6.2 % cross-linking. Basic gels were run in Tris–glycine buffer, pH 8.3, while acid gels were run in β-alanine-acetate buffer, pH 4.5 [20].

SDS-disc gel electrophoresis. Subunit compositions of the protein preparations were obtained by running SDS-disc electrophoresis [21] in 12 % acrylamide gels. MW estimates were made using myoglobin, chymotrypsin, ovalbumin and bovine serum albumin as marker proteins.

Triton-X gel electrophoresis. Electrophoresis in the presence of the non-ionic detergent, Triton-X, was made in 6,8 and 10% acrylamide gels to obtain estimates of the MW of the native species in some of the preparations [16]. The standard proteins used were myoglobin, ovalbumin, catalase and ferritin.

Isoelectric focusing. Flat gel isoelectric focusing was done on 7.5% acrylamide gel containing 2 M urea and 2% carrier ampholytes (Pharmacia, pH 3.5–10) [17]. Proteins were initially dissolved in 3 M urea–1 % β-mercaptoethanol. Electrofocusing in cylindrical gels was also done using 5.5 % gels with 4 M urea and 2 % Ampholine (LKB, pH 3–10). Runs were performed using 0.02 M NaOH as anodic buffer and 0.01 M H_3PO_4 as cathodic buffer. Bands were stained by the method of ref. [22].

Protein measurements. Quantitative analyses of protein were done using Folin phenol [23] and Coomassie Brilliant Blue [24]. Column eluates were monitored at A_{280} while gel scans for stained protein bands after disc electrophoresis were obtained at A_{590} using a Gilford gel scanner attached to a Beckman DU spectrophotometer.

Amino acid composition. Acid hydrolysates of the most purified preparations were separated in a Beckman model 120C Amino Acid Analyzer using PA-35 and AA-15 resins [1]. Cysteine was determined as cysteic acid [25] while tryptophan was analysed from $Ba(OH)_2$ hydrolysates [26].

Enzyme assays. Milled-rice powder was extracted 3 × with 2.5 vol. of 0.1 M NaOAc buffer, pH 5.0, with 1 mM dithiothreitol (DTT) by stirring in the cold for 2 hr each. Supernatants were collected after centrifugation at 3000 g for 20 min at 0–4°. These were desalted through Sephadex G-25 and the pooled-protein fractions were analysed for protease, BAPase [27], RNase [28] and α-amylase [29]. Protease was assayed using 0.125% haemoglobin in 2.0 ml soln containing 0.05 mmol phosphate and 0.5 μmol DTT, pH 7.0. Incubation was done at 40° for 90 min and A_{280} was monitored on the supernatant after precipitation with 10 % TCA. BAPase activity (peptidase with trypsin-like activity) was determined by measuring the change in A_{410} of a soln containing 5 ml 1 mM BAPA in 0.05 M Tris–HCl buffer, pH 8.2, with 0.02 M $CaCl_2$, 0.5 ml enzyme extract and 0.5 ml H_2O. The mixture was incubated at 28° for 30 min before the addition of 1.0 ml 30 % HOAc to stop the reaction.

Inhibitors. Dilute buffer extracts of defatted milled-rice powder were also monitored for possible α-amylase and trypsin inhibitors. Aliquots of extracts with 0.05 M Tris–maleate, pH 7.0, as solvent were assayed for inhibitory effect on salivary pancreatic amylase using 1 % soluble starch as substrate. The enzyme was pre-incubated with the extract for 30 min at 37° before the addition of the substrate; incubation was stopped after 10 min by adding an equal vol. of dinitrosalicylic (DNS) acid reagent [30].

Extracts with 0.1 M citrate–phosphate, pH 6.5, containing 1 mM DTT were tested for trypsin inhibitors by adding aliquots to incubation media containing 100 μg trypsin and 500 mg α-*N*-benzoyl-L-arginine ethyl ester · hydrochloride (BAEE · HCl) in 1.5 ml 0.1 M phosphate buffer, pH 7.5. Incubation was done for 30 min at 37°. Controls consisted of incubated trypsin plus BAEE · HCl with the test extract being added after the termination of the reaction with 0.2 ml 30 % HOAc. Absorbance at 253 nm was read after 30 min.

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