Oryzacystatin and Other Proteinase Inhibitors in Rice Grain: Potential Use as a Fish Processing Aid†

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Protease inhibitors specific for cysteine proteases (oryzacystatin), trypsin, chymotrypsin, and subtilisin in 11 major cultivars of rice grown in California are described. The amount of each inhibitor varied considerably with cultivar and was independent of the amount of the other inhibitors. With the exception of subtilisin inhibitor, each of the inhibitors was mainly in the bran fraction of the rice grain. Substrate polyacrylamide gel electrophoresis showed that these protease inhibitors were different low molecular weight proteins ranging in size from 11.5 to 22.0 kDa. Characterization of these inhibitors showed broad pH stability, thermal stability up to 100 °C, and inhibitory action against heat-activated Arrowtooth flounder protease(s).

INTRODUCTION

Increased demand for surimi-based products in the United States has led to interest in the use of underutilized fish, e.g., Pacific whiting and Arrowtooth flounder, for the production of surimi. The gel strength and other functional qualities of surimi from different species is variable. One important reason for the variable quality of surimi gel strength and elasticity is the presence of so-called "modori"-inducing proteases (MIP) which catalyze hydrolysis of key proteins during cooking of the fish meat between 50 and 70 °C (Hamman et al., 1990; Kinoshita et al., 1990). The MIP include a variety of endogenous muscle proteases, notably a family of heat-stable alkaline proteases (Haard, 1990; Hamman et al., 1990; Kinoshita et al., 1990), as well as cysteine proteases associated with infection by myxosporidean parasites, *Kudoa thyrisis* and *Kudoa paniformis* (Chang-Lee et al., 1989; Greene and Babbitt, 1990).

The Food Safety and Inspection Service of the USDA has given approval for two meat products containing surimi as an ingredient (Anonymous, 1988). Finding a means of inhibiting MIP is important for the use of surimi as a versatile food ingredient. Several MIP inhibitors have been reported including egg white (Chang-Lee et al., 1989; Hamman et al., 1990; Wasson et al., 1992a), potato extract (Lanier et al., 1981; Porter et al., 1990), lactoalbumin, and/or beef blood plasma (Hamman et al., 1990; Ueno et al., 1984). Recently, An et al. (1992) reported that good quality surimi products can be produced from Pacific whiting by adding protease inhibitors and practicing proper processing methods. Reppond and Babbitt (1993) found that the protease inhibitors from potato, bovine plasma, and egg white improved the gelling activity of surimi produce from Arrowtooth flounder and from walleye pollock. The specific mechanism of inhibition by these ingredients is not well understood, although it has recently been suggested that the inhibiting agent in egg white and beef blood plasma is an α_2-macroglobulin (Hamman et al., 1990).

It is now clear that proteases with different types of active sites (i.e., serine, metallo, and cysteine) may belong to the family of heat-stable alkaline or neutral proteases found in fish muscle (Haard, 1990; Kinoshita et al., 1990; Wasson et al., 1992).

Proteinaceous cysteine proteinase inhibitors (cystatins) have been identified in a variety of animal tissues, including egg white (Anastasi et al., 1983), as well as in a few plant sources including potato tuber (Rowan et al., 1990), soy bean (Hines et al., 1991), corn (Abe and Whitaker, 1988), and rice (Abe et al., 1987; Liang et al., 1991). It is possible that cystatin is a key component responsible for MIP inhibition reported for effective additives, e.g. egg white and potato extract.

Almost 500 million metric tons/year of rice are produced in the world and more than 6 million metric tons of rice are produced annually in the United States (Chang and Luh, 1991). Rice or a rice byproduct could be a new commercially viable source of protease inhibitor, with cost lower than the inhibitors in current use.

The present study was done to evaluate rice and rice byproducts as a source of additives to prevent the "modori" phenomenon in surimi. In this paper we report the cystatin content of the grain from 11 major cultivars of rice grown in California. The distribution of cystatin in milling fractions of selected cultivars is also reported. We also identified other proteinase inhibitors present in these rice cultivars. In addition, the molecular weight and purity of these inhibitors was determined using electrophoresis and assay for proteinase inhibitor activity.

MATERIALS AND METHODS

Materials. Eleven different cultivars of rice (*Oryza sativa* L.) harvested in 1991 in California were provided by the Department of Agronomy and Range Science, University of California, Davis. These samples consisted of one cultivar of short grain (S-201), four cultivars of medium grain (M-103, M-201, M-202, and M-204), two of long grain (L-202, L-203), two premium quality (M-203 and M-401), and two of specialty rice (Calmochi-101 and A-301).

The whole grain rice was ground to a fine powder using a BelArt Micromill (BelArt Products, Pequannock, NJ). S-201, M-201, L-203, and A-301 rice cultivars were also dehulled and milled into three fractions—hull, bran, and endosperm—by a laboratory-scale mill. The bran fraction included fragments of pericarp,

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tegmen, aleurone layer, and germ. Bran and endosperm were individually pulverized to a fine powder using the Micromill.

Papain, bromelain (from pineapple stem), ficin, trypsin (type III, from bovine pancreas), α-chymotrypsin (type II, from bovine pancreas), subtilisin (type VIII, from *Bacillus licheniformis*), thermolysin (type X, from *Bacillus thermoproteolyticus*), pepsin (from porcine stomach mucosa), cystatin (from egg white), N^{α}-benzoyl-DL-arginine β-naphthylamide (BANA), N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (SAPNA), casein, hemoglobin, egg white, Fast Garnet GBC, and molecular markers for electrophoresis were purchased from Sigma (St. Louis, MO). Bovine plasma (AMP 600N) was a gift from American Meat Protein Corp. (Ames, IA). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) were obtained from Bio-Rad Laboratories (Richmond, CA). All other reagents were of reagent grade.

Isolation and Partial Purification of the Rice Inhibitor. A slight modification of a method described by Abe et al. (1987) was used for the isolation and partial purification of the cystatin from rice. The procedure involved homogenization of powdered rice or rice fraction in 25 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl with a Polytron blender at low setting for 30 min. The homogenate was centrifuged at 7000*g* for 20 min at 4 °C to obtain S_1. The S_1 fraction was heated at 80 °C for 10 min, cooled, and centrifuged again to obtain S_2. After that, S_2 was brought to 30–60% saturation with solid ammonium sulfate. The precipitate (P_1) was suspended in 50 mM acetate buffer (pH 4.9) and dialyzed for 48 h against the same buffer. The nondialyzable fraction obtained was centrifuged at 15000*g* for 5 min, and the extract was freeze-dried. The resulting residue (P_2) was used as partially purified oryzacystatin.

Activity Assays and Estimation of Protein Content. The inhibitory activity against papain was measured by the method of Barret (1972). Solution containing inhibitor (0–100 μL) and an aliquot of papain solution (0.8–1.2 μg) were added to 0.10 M phosphate buffer (pH 6.0) containing 2 mM cysteine and 1 mM EDTA in a final volume of 2.0 mL. After preincubation for 10 min at 40 °C, 50 μL of 40 mg/mL BANA in dimethyl sulfoxide was added to start the reaction. After precisely 10 min of incubation at 40 °C, the reaction was terminated by adding 2 mL of a mixture of 4-(chloromercuri)benzoic acid and Fast Garnet GBC (the stabilized salt of 2-aminoazotoluene). The residual papain activity was measured in terms of the absorbance at 520 nm due to β-naphthylamide liberated and coupled with the Fast Garnet, producing a red azo dye.

Extracts (P_2) were also assayed for inhibitory activity toward (a) trypsin by the method of Smith et al. (1980) using BAPNA as substrate; (b) α-chymotrypsin and subtilisin using SAPNA as substrate (Geiger, 1984); (c) thermolysin with casein according to the method of Matsubara (1970); and (d) pepsin with hemoglobin according to the method of Ryle (1984).

To evaluate the inhibitory activity of the P_2 fraction against a fish proteinase associated with modori , an enzyme extract from 350 g of Arrowtooth flounder flesh was obtained according to the method of Deng (1981). The proteolytic activity of Arrowtooth muscle extract was measured by the method of Wasson et al. (1992), using casein as substrate and an assay temperature of 60 °C.

Protein concentration was determined with Bio-Rad reagent using bovine serum albumin as a standard (Bradford, 1976).

Assay of Inhibitory Activity by Electrophoresis. Proteinase inhibitor activities present in rice extracts were analyzed electrophoretically with SDS–polyacrylamide gels using casein as substrate with a method developed in our laboratory (Garcia-Carreno et al., 1993). Electrophoresis was conducted on a SE 250 Mighty Small II vertical slab gel electrophoresis cell (Hoefer Scientific Instruments, San Francisco, CA) at 10 °C using a constant current of 15 mV per gel. Minigels (8 × 10 × 0.07 cm) of 0.75-mm thickness and 12% acrylamide were used, as describe by Fling and Gregerson (1986) except that 2-mercaptoethanol was omitted. Volumes of 10 μL of sample were applied to the gels.

When the tracking dye front reached the bottom of the resolving gels (after approximately 2.5 h), the gels were incubated with a protease solution in an appropriate buffer (Table 1). The enzyme was allowed to diffuse into the gel at 0 °C for 30 min. Then, the gels were washed with distilled water and incubated again, this time with 1% casein solution (Table 1) for 90 min at 25 °C. After that, gels were washed with distilled water, stained with a staining solution containing 40% methanol, 10% acetic acid, and 0.1% Coomassie brilliant blue, and destained with a mixture of methanol–acetic acid–water. Gels were photographed and dried using a Bio-Rad slab gel dryer (Model 443). This method visualized the bands that contain proteinase inhibitory activity as dark blue against a clear background. The relative migration distance of the inhibitors was compared with standard molecular weights markers to yield an apparent molecular weight (MW) determination.

Table 1. Enzyme and Casein Concentrations and Buffers Employed in the Assay of Inhibitory Activity by Electrophoresis

enzyme concn (mg/mL)	casein concn (%)
papain (0.01 mg/mL) in 0.10 M phosphate buffer + 2 mM cysteine + 1 mM EDTA, pH 6.0[a]	1% in 0.10 M phosphate buffer, pH 6.0
trypsin (0.10 mg/mL) in 0.05 M Tris buffer + 20 mM $CaCl_2$, pH 8.2	1% in 0.05M Tris buffer, pH 8.2
α-chymotrypsin (0.10 mg/mL) in 0.10 M Tris buffer + 20 mM $CaCl_2$ + 0.05% Triton X-100, pH 7.8	1% in 0.10 M Tris buffer, pH 7.8
subtilisin (0.10 mg/mL) in 0.10 M Tris buffer + 20 mM $CaCl_2$ + 0.05% Triton X-100, pH 7.8	1% in 0.10 M Tris buffer, pH 7.8

[a] The papain gels were washed in 2.5% Triton X-100 during 15 min to remove SDS prior to incubation with papain solution.

Effect of pH and Temperature on the Stability of the Proteinase Inhibitors. Solutions of P_2 fraction were kept at 25 °C for 24 h in 10 mM buffers at five different pH values: glycine hydrochloride, pH 2.0; sodium citrate, pH 4.0; sodium phosphate, pH 6.0; Tris-HCl, pH 8.0; and glycine–NaOH, pH 10.0. Aliquots were diluted with appropriate buffer and assayed for inhibitory activity.

To determine thermal stability, the P_2 fraction was incubated in buffer, pH 6.0, at either 40, 60, 80, 100, or 120 °C for 60 min and then quickly cooled in ice bath. Appropriate aliquots were assayed for proteinase inhibitory activity.

RESULTS AND DISCUSSION

The method described by Barret (1972) using BANA to determinate the activity of oryzacystatin was found to be more sensitive than those methods using BAPNA (Barret, 1981) and casein (Arnon, 1970). All data reported are based on assay with BANA as substrate. The inhibition was approximately linear as a function of P_2 fraction up to 70%. At higher inhibitor concentrations, however, the plots deviated from linearity. The inhibitory units (IU) of the sample were always calculated in the range of inhibition between 40% and 60%. If inhibition was $<40\%$ or $>60\%$, the assay was repeated making a suitable dilution of the sample.

The average oryzacystatin and protein contents of the P_2 fractions from three separate extractions of 11 rice cultivars are summarized in Table 2. On the basis of the cystatin activity in the homogenate (or S_1), we calculated that about 22.7 ± 4.9% of the cystatin was recovered in the P_2 fraction, and the increase in specific activity of the inhibitor as a result of the purification was, on average, 3.5 ± 0.9 (Table 3).

Inhibitory activity against papain was detected in all of the rice cultivars studied. The oryzacystatin contents ranged considerably, from 9.4 to 28.6 IU/kg of rice. The long-grain cultivars (L-202 and L-203) and the medium

Table 2. Rice Cysteine Proteinase Inhibitor and Protein Contents of Different Rice Cultivars

cultivar[a]	protein content (mg/kg of rice)	total act. (IU/kg of rice)[b]	sp act. (IU/mg of pt)[c]
S-201	258.2 ± 41.0[d]	20.4 ± 2.5	0.079 ± 0.003
M-103	207.5 ± 11.5	22.0 ± 0.2	0.106 ± 0.005
M-201	206.2 ± 6.5	28.6 ± 2.4	0.138 ± 0.007
M-202	202.7 ± 16.0	22.1 ± 1.0	0.109 ± 0.003
M-204	258.5 ± 6.5	23.1 ± 1.8	0.089 ± 0.005
L-202	159.7 ± 14.0	22.2 ± 0.1	0.139 ± 0.008
L-203	160.6 ± 2.0	22.0 ± 0.3	0.137 ± 0.004
M-203	174.7 ± 31.0	15.9 ± 0.7	0.091 ± 0.012
M-401	188.8 ± 1.5	16.8 ± 0.7	0.089 ± 0.003
Calmochi-101	221.8 ± 3.5	9.4 ± 1.1	0.042 ± 0.005
A-301	233.6 ± 11.5	11.5 ± 1.0	0.049 ± 0.007

[a] The quantity of rice extracted was 200 g. [b] Inhibitory units/kilograms of rice. One inhibitory unit was defined as the amount of inhibitor which suppressed the liberation of 1 μmol of β-naphthylamide per minute by the active papain. [c] Inhibitory units/milligrams of protein. [d] Data are mean values for three preparations of P_2, each analyzed in triplicate.

Table 3. Summary of the Partial Purification of Oryzacystatin from Rice

fraction[a]	protein content (mg/kg)	total act. (IU/kg)	sp act. (IU/mg of pt)	yield (%)	fold
S_1	3154.3 ± 480.5	85.9 ± 21.2	0.02 ± 0.01	100	1
S_2	1435.2 ± 206.8	44.7 ± 10.5	0.03 ± 0.01	52.5 ± 5.6	1.1 ± 0.1
P_1	878.8 ± 69.5	39.3 ± 10.0	0.04 ± 0.01	45.7 ± 3.4	1.6 ± 0.2
P_2	206.6 ± 34.6	19.4 ± 5.6	0.09 ± 0.03	22.7 ± 4.9	3.5 ± 0.9

[a] Fractions were the following: S_1 is homogenate; S_2 extract after heat treatment; P_1 precipitate after addition of ammonium sulfate; P_2 extract after dialysis and freeze-drying.

grain cultivars (M-201) showed the highest inhibitory activity, while the specialty cultivars (Calmochi-101 and A-301) showed the lowest levels of rice cystatin. The characteristics of each different cultivar, the grade of germination, and some environmental factors may influence the proteinase inhibitor contents of cereals (Chang and Tsen, 1979; Boisen, 1983).

Distribution of Rice Cysteine Proteinase Inhibitor. The anatomical distribution of oryzacystatin in one cultivar from each of short- (S-201), medium- (M-201), and long-grain (L-203) and specialty rice (A-301) cultivars is shown in Table 4. In all four samples tested, the oryzacystatin was most concentrated in the bran fraction. The cultivar differences in the total content of oryzacystatin from whole grain appeared to reflect the inhibitor content of the bran fraction. The bran fraction from M-201 and L-203 cultivars showed the highest levels of oryzacystatin.

Specificity of the Oryzacystatin against Other Cysteine Proteases. The partially purified oryzacystatin (P_2 fraction) inhibited papain and ficin nearly 100% but not the cysteine proteinase bromelain (Figure 1). These results agree with those of Abe et al. (1987) and Hines et al. (1991) who demonstrated that the cystatin isolated from rice and soybean, respectively, did not inhibit bromelain.

Influence of the P_2 Fraction on Serine Proteinase, Metalloproteinase, and Aspartyl Proteinase. Considering that other proteinase inhibitors have also been recovered from cereals by similar extraction conditions (Tashiro and Maki, 1979; Ohtsubo and Richardson, 1992) to that employed in this study, we assayed the influence of the P_2 fraction on other proteinases. The proteinases tested were trypsin, α-chymotrypsin, subtilisin, pepsin, and thermolysin. In all cases, we checked the linearity of inhibition, and the IU were always calculated in an inhibitory range between 40% and 60%.

The P_2 fraction from rice did not contain any demonstrable aspartyl proteinase or metalloproteinase inhibitor activity. Pepsin and thermolysin activities were not influenced by rice P_2 fraction ranging from 0.25 to 1.50 mg/mL assay. Boisen (1983) pointed out that inhibitors of pepsin have not been described in cereals. Plant polypeptide inhibitors of carboxypeptidases A and B (both metalloproteinases) were found in potato and tomato (Richardson, 1981; Xavier-Filho and Campos, 1989), but we are not aware of any study of the presence of metalloproteinase inhibitors in cereals.

Among the serine proteinases tested, trypsin and subtilisin were inhibited strongly by the P_2 fraction, while the inhibitory effects on α-chymotrypsin were very weak (Table 5). Only the rice cultivar M-103 showed a relatively high content of α-chymotrypsin inhibitor, about 6 times more than that in the other cultivars tested. The cultivars L-203, L-202, M-103, and Calmochi-101 showed the highest contents of trypsin inhibitor. Trypsin inhibitors have been isolated from the majority of graminae (Boisen, 1983). The most intensively studied cereal trypsin inhibitors are those from barley and wheat.

To ascertain whether the trypsin inhibitor was responsible for the low α-chymotrypsin inhibitory activity detected in the rice, inhibitory activities of the P_2 fraction on trypsin in the presence of concentrations of α-chymotrypsin ranging from 10 to 100 μg were studied. We found that even in the presence of saturating amounts of α-chymotrypsin, the percentage of inhibition against trypsin was always the same. It seemed likely that the rice trypsin inhibitor was not responsible for the inhibitory activity against α-chymotrypsin detected. These findings are also in agreement with the results of Tashiro and Maki (1979) who characterized a "double-headed" rice bran trypsin inhibitor without inhibitory activity against α-chymotrypsin.

The subtilisin inhibitor contents ranged considerably, from 238.4 to 958.8 IU/kg of rice. The highest specific activity was found in a medium-grain rice, while the lowest was in a long-grain cultivar. Ohtsubo and Richardson (1992) isolated a bifunctional subtilisin/α-amylase inhibitor from rice bran, which showed a strong homology with similar bifunctional inhibitors isolated from other cereals and also from other legumes.

In order to know if the concentrations of each inhibitor were related to one another, we calculated the correlation coefficients for the amount of each of four inhibitors in 11 rice cultivars (Table 6). From the results obtained, it can be concluded that inhibitor concentrations are independent of one another.

The anatomical distribution of trypsin and subtilisin inhibitors in rice seed was also studied (Table 7). As observed for the oryzacystatin, the trypsin inhibitor was located mainly in the bran fraction. However, subtilisin inhibitor showed a different distribution, since similar concentrations of this inhibitor was detected in both fractions, bran and endosperm.

Stability of the Proteinase Inhibitors. The effect of pH on the stability of the four inhibitors was measured at 25 °C. As shown in Figure 2, the oryzacystatin and the α-chymotrypsin inhibitor were stable between pH 4 and 8, but there was a significant decrease in activity at pH 10. Subtilisin inhibitor was most stable in a narrower pH range, between 4 and 6. A very marked decrease in its activity was observed below pH 4 and above pH 6. Trypsin inhibitor retained at least 80% of its activity from pH 2 to 7, but at higher pH values, the activity decreased very

Table 4. Distribution of Oryzacystatin in Rice Grain

	rice bran[a]			endosperm[b]		
cultivar[c]	protein content (mg/kg)	total act. (IU/kg)	sp act. (IU/mg of pt)	protein content (mg/kg)	total act. (IU/kg)	sp act. (IU/mg of pt)
S-201	922 ± 108	55.3 ± 0.3	0.06 ± 0.00	115 ± 6	2.3 ± 0.1	0.02 ± 0.00
M-201	797 ± 27	103.6 ± 2.0	0.13 ± 0.01	68 ± 3	2.7 ± 0.1	0.04 ± 0.01
L-203	791 ± 14	118.6 ± 3.1	0.15 ± 0.02	83 ± 2	2.8 ± 0.1	0.03 ± 0.01
A-301	990 ± 43	59.5 ± 4.4	0.05 ± 0.00	105 ± 4	2.5 ± 0.1	0.02 ± 0.00

[a] The quantity of rice bran extracted was 50 g. Rice bran refers here to the total milling fraction composed of pericarp, tegmen, aleurone, and germ. The protein content and the total activity are expressed per kilogram of rice bran. [b] The quantity of endosperm extracted was 100 g. The protein content and the total activity are expressed per kilogram of endosperm. [c] Data are mean values for two preparations of P_2, each assayed in triplicate.

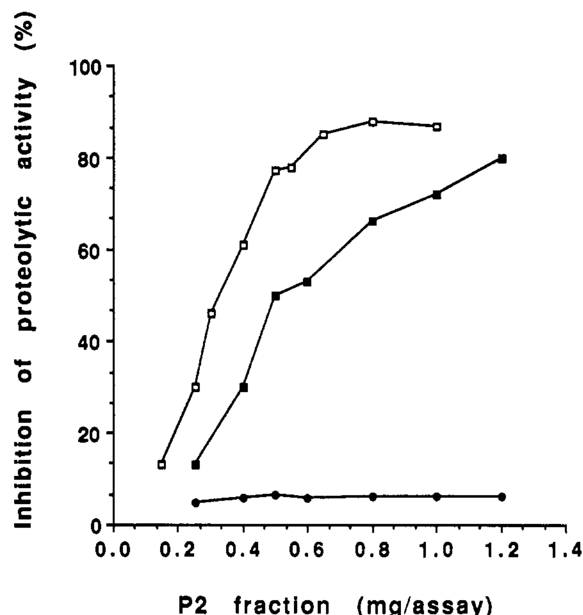

Figure 1. Inhibition of papain (□), ficin (■), and bromelain (●) at different levels of oryzacystatin. Papain (1.2 μg), ficin (6.0 μg), or bromelain (6.0 μg) was incubated at 40 °C for 10 min in reaction buffer with the indicated amount of P_2 fraction (partial purified oryzacystatin). The reaction was initiated by addition of BANA substrate. (Data are the average of three determinations for P_2 fraction from cultivar L-203.)

Table 5. Trypsin, α-Chymotrypsin, and Subtilisin Inhibitor Contents from Different Rice Cultivars

	RTI[a]		RCT[b]		RST[c]	
cultivar	total act.[d]	sp act.[e]	total act.	sp act.	total act.	sp act.
S-201	68.3	0.33	5.2	0.02	769.1	3.70
M-103	82.7	0.40	25.0	0.12	774.2	3.75
M-201	89.1	0.34	5.4	0.02	742.5	2.90
M-202	54.3	0.27	3.2	0.02	958.8	4.75
M-204	62.6	0.24	4.8	0.02	770.4	3.00
L-202	59.3	0.37	2.9	0.02	238.4	1.50
L-203	77.5	0.48	4.3	0.03	601.8	3.75
M-203	54.8	0.31	2.4	0.01	503.0	2.90
M-401	51.7	0.27	1.6	0.01	716.8	3.80
Calm-101	84.1	0.38	14.1	0.06	770.5	3.50
A-301	48.2	0.21	5.4	0.02	586.6	2.50

[a] Rice trypsin inhibitor. One IU was defined as the amount that suppressed the liberation of 1 μmol of p-nitroanilide per minute at 37 °C and pH 8.1 by the active trypsin. [b] Rice α-chymotrypsin inhibitor. One IU was defined as the amount that suppressed the liberation of 1 μmol of p-nitroanilide per minute at 25 °C and pH 7.8 by the active α-chymotrypsin. [c] Rice subilisin inhibitor. One IU was defined as the amount that suppressed the liberation of 1 μmol of p-nitroanilide per min at 25 °C and pH 7.8 by the active subtilisin. [d] IU/kg of rice. [e] IU/mg of protein.

rapidly. Tashiro and Maki (1978) reported that a rice bran trypsin inhibitor was only stable at acidic and neutral pHs.

Table 6. Matrix of Correlation Coefficients between Papain, Trypsin, α-Chymotrypsin, and Subtilisin Inhibitors of Different Rice Cultivars[a]

	papain	trypsin	α-chymotrypsin	subtilisin
papain	1.0000			
trypsin	0.4711*	1.0000		
α-chymotrypsin	−0.0594*	0.4612*	1.0000	
subtilisin	−0.1558*	0.0693*	0.2170*	1.0000

[a] Degree of freedom: 9. Coefficients marked with an asterisk (*) show no statistical significant correlation at the level of $p < 0.05$.

Table 7. Distribution of Trypsin and Subtilisin Inhibitor Activities in Rice Grain

	rice bran				endosperm			
	trypsin		subtilisin		trypsin		subtilisin	
cultivars	TA[a]	SA[b]	TA	SA	TA[c]	SA	TA	SA
S-201	184.7	0.20	1936.2	2.10	1.7	0.01	152.0	1.35
M-201	272.4	0.34	1534.3	1.95	2.7	0.04	118.5	1.75
L-203	316.4	0.40	2020.5	2.55	0.9	0.01	103.7	1.25
A-301	208.4	0.21	1135.5	1.15	2.1	0.02	91.4	0.90

[a] Total activity in IU/kg of rice bran. [b] Specific activity in IU/mg of protein. [c] Total activity in IU/kg of endosperm.

The incubation of the inhibitors at temperatures ranging from 60 to 120 °C for 60 min gave the results shown in Figure 3. All the inhibitor activities were stable between 40 and 80 °C for up to 1 h. Oryzacystatin, α-chymotrypsin, and subtilisin inhibitors were stable up to 100 °C, but their activities decreased when incubated at 120 °C for 1 h. Abe et al. (1987) reported that the activity of oryzacystatin remained stable after incubation at 100 °C for 30 min at pH 6.0; however, about 58% of the inhibitor activity was lost when the incubation temperature was 120 °C.

A feature common to many, but not all, of the proteinases inhibitors is their surprising resistance to denaturation by heat. This peculiar resistance to heat has been attributed to a tightly coiled conformation imposed by the large number of disulfide bonds found in many of these inhibitors (Richardson, 1981; Xavier-Filho and Campos, 1989).

Assay of Inhibitory Proteinase Activity by Electrophoresis. Electrophoresis was found to be useful for detecting the presence of proteinase inhibitors in crude extracts of rice, and it also allowed the assay and partial characterization of the inhibitors to be done without necessitating use of purification methods that could alter their chemical and physical characteristics. We observed that when different amounts of extract were electrophoresed, the width of the stained band gave a semiquantitative measure of the correspondent inhibitor. Moreover, both sensitivity and resolution were very dependent on the amount of extract applied.

We observed that the inhibitory activity against papain was affected by the presence of SDS. For this reason, we washed the gel briefly with a 2.5% Triton X-100 solution

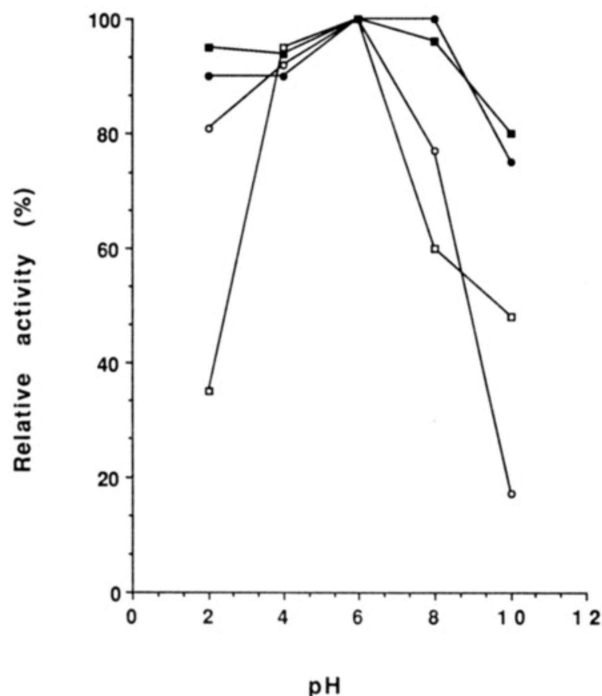

Figure 2. Effect of pH on the stability of the papain (○), trypsin (●), chymotrypsin (■), and subtilisin (□) inhibitors from rice. (Data are the average of two determinations, using P_2 fraction from rice cultivar L-203.)

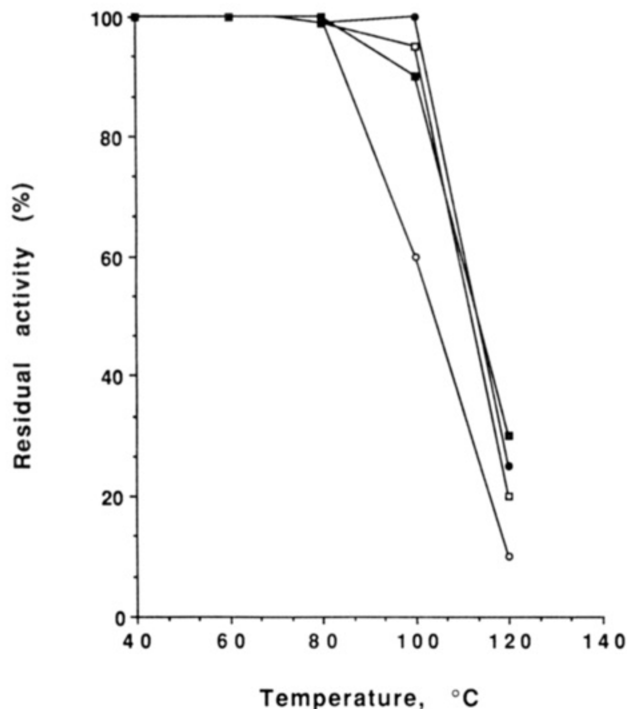

Figure 3. Effect of temperature on the stability of the papain (●), trypsin (○), chymotrypsin (■), and subtilisin (□) inhibitors from rice. (Data are the average of two determinations, using P_2 fraction from rice cultivar L-203.)

to remove SDS before incubation with the papain solution, as suggested by Liang et al. (1991). Better results were obtained when we included 0.05% of Triton X-100 in the incubating subtilisin solution. However, no improvement was observed in the resolution of the trypsin inhibitory activity on the gel by washing with Triton X-100 solution.

The different inhibitory activity gels conducted revealed the presence of different zones of inhibitory activity in the P_2 fraction of rice bran (Figure 4). In this figure, a gel

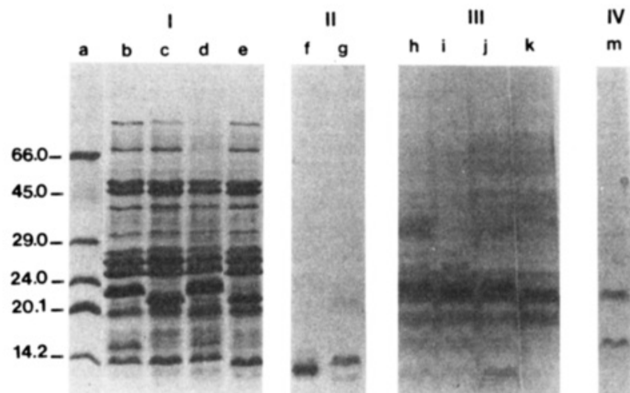

Figure 4. SDS–polyacrylamide gel electrophoresis. Gel I: SDS–PAGE of P_2 fraction isolated from the bran of (lane b) A-301, (lane c) L-203, (lane d) M-201, and (lane e) S-201. Lane a is molecular markers: bovine serum albumin, 66.0 kDa; egg albumin, 45.0 kDa; carbonic anhydrase, 29.0 kDa; trypsinogen, 24.0 kDa; trypsin inhibitor, 20.1 kDa; lactalbumin, 14.2 kDa. Gel II: Papain inhibitory activity gel of (lane f) pure chicken egg cystatin and (lane g) P_2 fraction from the bran of L-203. Gel III: Trypsin inhibitory activity gel of P_2 fraction from the bran fractions of (lane h) A-301, (lane i) L-203, (lane j) M-201, and (lane k) S-201. Gel IV: Subtilisin inhibitory activity gel of P_2 fraction from the bran of (lane m) L-203.

obtained from the same samples and stained with Coomassie blue right after electrophoresis is included to indicate the protein bands in the samples (Figure 4, I, lines from a to e).

No α-chymotrypsin inhibitory activity was detected with this method. One possible explanation could be the fact that the levels of α-chymotrypsin inhibitor in the samples were, in general, very low.

The gel assay for papain inhibition (cystatin) showed two distinct bands and also a minor band at about 20 000 Da (Figure 4, II, g). The major band corresponded to a MW of about 13 000. The second distinct band was less intense than the 13 000-Da component and had a MW of about 11 400. Both distinct bands were always observed in the four samples of rice bran analyzed (data not shown). Abe et al. (1987) reported a MW of about 12 000 for oryzacystatin. Further studies, by the same authors, demonstrated that two forms of oryzacystatin were present in rice (Kondo et al., 1990; Abe et al., 1991). These two forms were named oryzacystatin I with a MW of 11 500 and oryzacystatin II with a MW of 12 000. Kondo et al. (1990) suggested that the reason why two types of cystatins occur in rice seed is that they have different target enzymes, oryzain α and β (two cysteine proteinases of rice).

The estimated MW of the main rice trypsin inhibitor zone was 22 000 (Figure 4, III). Tashiro and Maki (1979) reported a MW, based on gel electrophoresis, of about 14 500 for a rice bran trypsin inhibitor. However, these authors determined, by gel filtration analysis, a MW of 22 000 for the same inhibitor (Maki et al., 1980). They suggested that one possible explanation for this discrepancy was a weak self-association of this molecule with others. In like manner, it has been pointed out that most of the purified and characterized cereal protease inhibitors have molecular weights of 8000–20 000. Many of the reported values higher than 20 000 may be due to adsorption of some inhibitors to other compounds or may reflect the ability of some inhibitors to polymerize (Richardson, 1981; Boisen, 1983; Garcia-Olmedo et al., 1987).

The patterns of the trypsin inhibitor activity gels for the four rice bran samples tested were slightly different (Figure 4, III, h–k). A-301 and M-201 showed similar bands

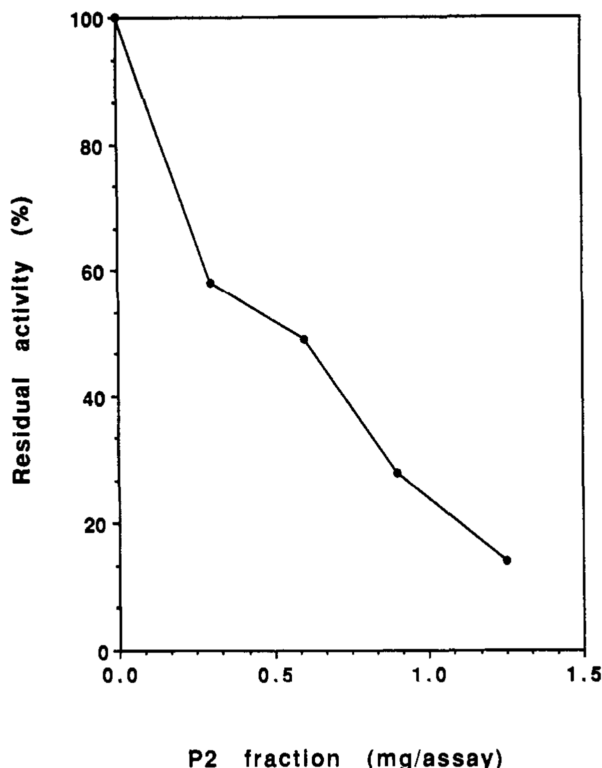

Figure 5. Residual proteolytic activity of a crude extract of Arrowtooth flounder. A 125-μL sample of Arrowtooth muscle extract was incubated first with P_2 fraction from L-203 cultivar for 10 min at 40 °C, and then the reaction was started by addition of BANA as substrate. The percentage of inhibition was calculated by comparing the results of a control reaction with no added P_2 and samples preincubated with P_2. Data are the average of duplicate determinations.

with a MW of about 23 000, while L-203 and S-201 showed bands with a MW of about 22 000. We conducted this assay several times and with different extracts of the same samples, and the same pattern was always observed. Several authors pointed out the frequency in which the proteinase inhibitors from plants exhibited high levels of heterogeneity (Richardson, 1981; Boisen, 1983). We did not observe cultivar differences for the molecular weights of papain or subtilisin inhibitors on gels (data not shown).

As shown in Figure 4 (IV), bands corresponding to a MW of about 15 200 and 22 000 were present in the subtilisin inhibitory gels. The latter subtilisin inhibitor had the same mobility as the trypsin inhibitor band. These results suggested that either two different types of subtilisin inhibitors can be found in rice grain or that the trypsin inhibitor had some activity against subtilisin. Ohtsubo and Richardson (1992) detected a 20 000-Da subtilisin inhibitor in rice bran.

Inhibition of Flounder "Modori"-Inducing Proteinase. Finally, preliminary investigations showed that the P_2 fraction from rice bran was very effective in inhibiting the BANA hydrolysis activity of a heat activated enzyme from Arrowtooth flounder (Figure 5). We observed that 0.5 mg/mL of the P_2 fraction from L-203 (0.14 IU of oryzacystatin/mg of protein) decreased the flounder amidase activity by about 50%.

The inhibitory activities of bovine plasma, egg white, and four different rice P_2 fractions for casein hydrolysis activity by Arrowtooth flounder protease were compared. The P_2 fractions from all rice cultivars tested were more effective than the egg white and bovine plasma in inhibiting casein hydrolysis by the fish extract (Table 8).

Table 8. Influence of Egg White, Bovine Plasma, and Rice P_2 Fraction on the Proteolytic Activity of Arrowtooth Muscle Extract

sample	final concn (mg/assay)	inhibition of proteolytic act. (%)
egg white	5	46.8
bovine plasma	5	61.9
A301	1	76.6
M103	1	90.7
L202	1	93.3
M401	1	88.0

CONCLUSIONS

To our knowledge the present study is the first demonstration that a cereal contains substance(s) that can inhibit a proteinase responsible for "modori" or gel weakening in cooked surimi.

We have identified oryzacystatins (cysteine proteinase inhibitors), as well as serine proteinase inhibitors, in each of the major rice cultivars currently grown in California. Cultivars of long-grain rice, in general, showed the highest concentrations of proteinase inhibitors. Rice cystatin(s) and trypsin inhibitor(s) are concentrated in the bran, while the rice subtilisin inhibitor was present at similar concentrations in both bran and endosperm. These results suggested that rice bran, which is normally used for animal feed, should be further investigated as a source of protease inhibitor(s) against MIP in products made from surimi and fish.

The pH stability and the heat resistance showed by the different inhibitors are in agreement with the data reported in the literature for other (unidentified) rice cultivars.

From electrophoretic results, we can conclude that cystatins, trypsin inhibitor(s), chymotrypsin inhibitor(s), and subtilisin inhibitor(s) are different molecules with molecular weights ranging from 11 000 to 22 000. The molecular weights of the two oryzacystatins agree quite satisfactorily with the MW reported by other authors. For the trypsin inhibitor, our SDS-PAGE data indicate a molecular weight of 22 000, which is half the size of rice trypsin inhibitor previously reported using SDS-PAGE analysis. It is possible that, because we omitted the use of mercaptoethanol in the sample and gel buffers in order to retain inhibitor activity, the active component we have identified is a dimer linked by disulfide linkages.

The observed inhibition of the proteolytic activity from Arrowtooth flounder by the P_2 fraction of rice could have resulted either from the direct action of the rice cystatin or from the combination of both cystatin and rice serine inhibitors. The main "modori"-causing proteinase in Arrowtooth flounder is a cysteine proteinases (Wasson et al., 1992). Also, our results showed that the casein hydrolytic activity of Arrowtooth protease at 60 °C was inhibited 90% by 1 mM iodacetamide, a cysteine protease inhibitor. More studies are underway to study the practical applicability of rice bran extract in preventing modori in surimi made from Arrowtooth flounder and Pacific whiting.

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