# Annual Report
# Comprehensive Research on Rice
# January 1, 1993-December 31, 1993

**PROJECT TITLE:** Proteinase inhibitors in rice

**PROJECT LEADER:** Norman F. Haard, Department of Food Science & Technology, University of California, Davis.

**PRINCIPAL UC INVESTIGATORS:** Norman F. Haard, Professor; Nora Dimes, SRA II; Maria Izquerdo-Pulido, Visiting Scientist; Dr. Houde Han, Visiting Scientist; Ms. Jessie Hung, M.Sc. graduate student; Department of Food Science & Technology, University of California, Davis.

**COOPERATORS:** James Hill, Extension Agronomist

**LEVEL OF 1992 FUNDING:** $23,981

**OBJECTIVES AND EXPERIMENTS CONDUCTED:**

Objective 1: Oryzacystatin Content of Rice Straw.

A. Assay- Samples of rice straw were obtained from James Hill, Extension Agronomist, UCD. The assay method of Barret (1972) was used to determine the oryzacystatin content of rice straw.

B. Isolation and partial purification of oryzacystatin- The method described by Abe *et al.* (1987) was used for the isolation and partial purification of cystatin ($P_2$ fraction) from rice straw.

C. SDS-PAGE electrophoresis- The $P_2$ fraction from rice straw was electrophoresed and assayed for papain-inhibitor activity as described by Garcia-Carreno *et al.*(1993).

Objective 2: Trypsin Inhibitor Content.

A. Isolation of other inhibitors- The $P_2$ fraction was isolated from unhulled rice grain for the 11 major cultivars of rice grown in California. The $P_2$ fraction was also prepared from the bran and endosperm portions of selected cultivars, i.e., S-201, M-201, L-201, and A-301.

B. Assay of proteinase inhibitors - Trypsin inhibitor was assayed by the method of Smith *et al.* (1980) using BAPNA as substrate. Chymotrypsin and subtilisin inhibitors were assayed using SAPNA as substrate (Geiger, 1984). Thermolysin inhibitor was assayed using casein as substrate (Matsubara, 1970). Pepsin inhibition was tested with hemoglobin as substrate according to Ryle (1984).

Objective 3: Properties of Proteinase Inhibitors.

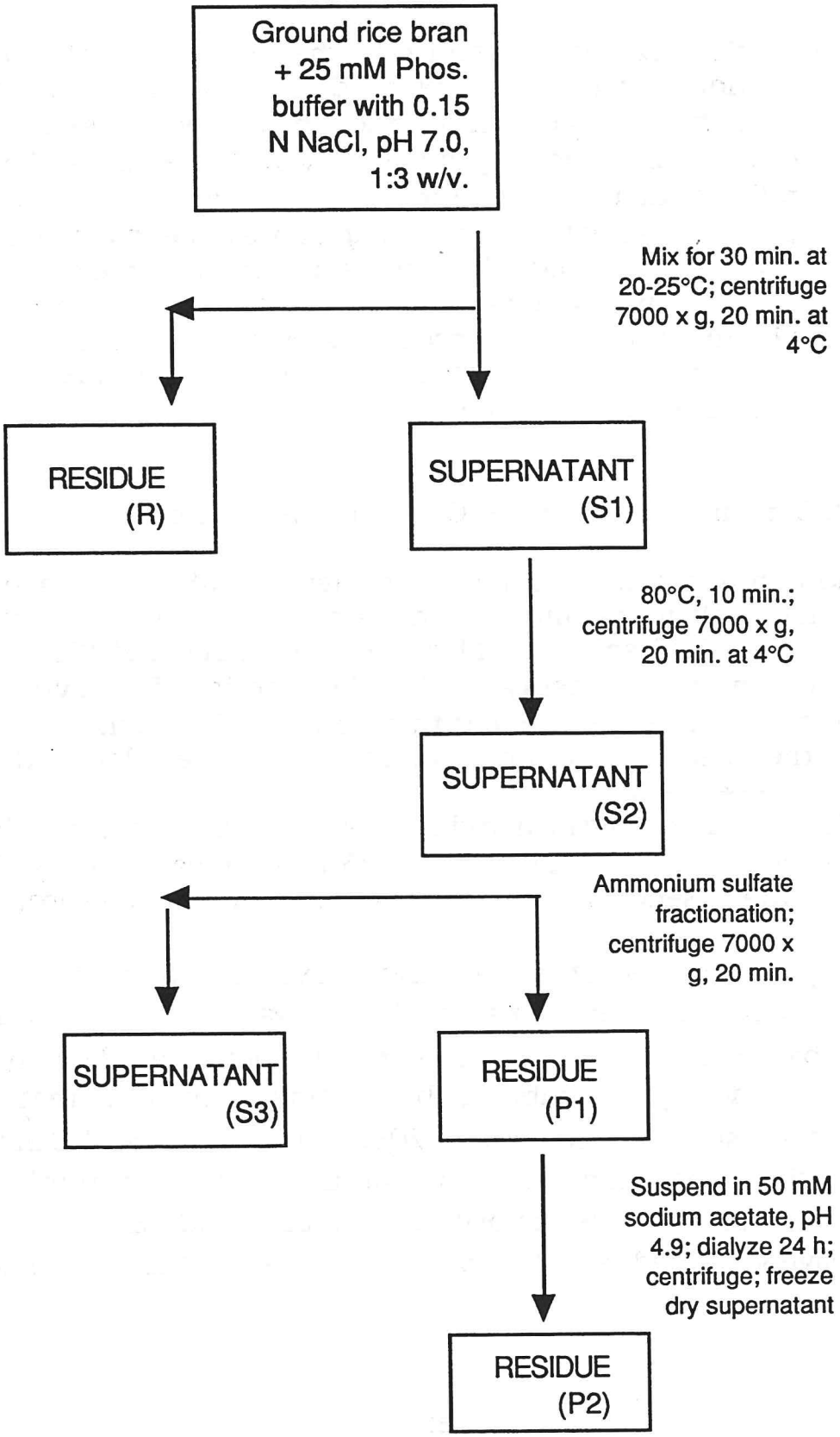
A. Stability- The thermal stability of the inhibitors ($P_2$ fraction) isolated from bran (cultivar L-203) was determined by incubation in water at either 60°C, 80°C or 100°C for 30 min prior to assay for inhibitor units. The pH stability of the same isolate was determined by incubation in buffers ranging from pH 5-11, at room temperature (22-24°C), for 24 h.

B. Molecular Weight and Purity- Oryzacystatin and other inhibitors in the $P_2$ fraction from rice brans were characterized by sodium dodecylsulfate polyacrylamide electrophoresis (SDS-PAGE) using 12% acrylamide with the method of Fling and Gregerson (1986) and Garcia-Carreno *et al.* (1993).

Objective 4. Evaluate Isolation Methodology.

Bran from medium and long grain rice was obtained from the California Rice Growers Association, Woodland, CA. The oryzacystatin extraction procedure of Abe *et al.*(1987), shown in Fig. 1, was modified in various ways including: (A) ratio of extraction buffer to bran, (B) heat treatment time, (C) heat treatment temperature, (D) repeated extraction of bran residue(R), and (E) pre-soaking bran prior to extraction.

Figure 1. Isolation of P2 fraction of oryzacystatin from rice bran



## SUMMARY OF 1993 RESEARCH BY OBJECTIVE:

### Objective 1: Oryzacystatin Content of Rice Straw.

Extracts of rice straw were obtained by the method described in Fig. 1. Marked inhibition of papain was observed in fractions $S_1$ and $S_2$, but no inhibition activity was recovered in fractions $P_1$ and $P_2$. Assay of $S_1$, $S_2$, $P_1$ and $P_2$ for inhibitor activity by SDS-PAGE showed no activity on the gel. This indicates that the inhibitor activity detected in the $S_1$ and $S_2$ fractions by spectrophotometric assay of papain was due to a constituent other than protein. We conclude that the papain inhibition exhibited by fractions $S_1$ and $S_2$ was not caused by oryzacystatin or by other proteinaceous inhibitor. Other evidence indicates the inhibition of papain by the $S_1$ fraction was caused by phenolic compound(s). Accordingly, no further work was done with rice straw.

### Objective 2: Content of Trypsin and Other Proteinase Inhibitors.

In addition to oryzacystatin, other proteinase inhibitors have been isolated from cereals using similar extraction procedures as we used for oryzacystatin (Fig. 1; Tashiro and Maki, 1979; Ohtsubo and Richardson, 1992). Accordingly, we assayed the $P_2$ fraction for trypsin, α-chymotrypsin, subtilisin, pepsin and thermolysin inhibition. In all cases we checked the linearity of inhibition and the IU were calculated in an inhibitory range between 40 and 60%.

Pepsin and thermolysin activities were not inhibited by the $P_2$ fraction at concentrations ranging from 0.25 to 1.50 mg/ml assay. These data indicate the absence of aspartyl-proteinase and metallo-proteinase inhibitors.

Among the serine-proteinases tested, trypsin and subtilisin were inhibited strongly and chymotrypsin inhibition was less strong by the $P_2$ fraction (Table 1). 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

**Table 1. Trypsin, α-chymotrypsin and subtilisin inhibitor contents from different rice cultivars.**

| | RTI[a] | | RCT[b] | | RST[c] | |
|---|---|---|---|---|---|---|
| Cultivars | Total activity [d] | Specific activity [e] | Total activity | Specific activity | Total activity | Specific activity |
| 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 1μmol of p-nitroanilide per min 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 1μmol of p-nitroanilide per min at 25°C and pH 7.8 by the active α-chymotrypsin. [c] Rice Subtilisin Inhibitor. One IU was defined as the amount that suppressed the liberation of 1μmol of p-nitroanilide per min at 25°C and pH 7.8 by the active subtilisin. [d] IU/ Kg rice. [e] IU/ mg protein.

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 per 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 2). From the results obtained, it can be concluded that inhibitor concentrations are independent of one another.

**Table 2. Matrix of correlation coefficients between papain, trypsin, α-chymotrypsin and subtilisin inhibitors of different rice cultivars.**

| | 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 |

The anatomical distribution of trypsin and subtilisin inhibitors in rice seed was also studied (Table 3).

**Table 3. Distribution of trypsin and subtilisin inhibitor activities in rice grain.**

| | RICE BRAN | | | | ENDOSPERM | | | |
|---|---|---|---|---|---|---|---|---|
| | Trypsin | | Subtilisin | | Trypsin | | Subtilisin | |
| Cultivars | T.A.[a] | S.A.[b] | T.A. | S.A. | T.A.[c] | S.A. | T.A. | S.A. |
| 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 rice bran; [b] Specific Activity in IU/ mg protein;

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.

3. Objective 3: Characterization of Proteinase Inhibitors in Rice

A. Stability of the proteinase inhibitors- The effect of pH on the stability of the four inhibitors was measured at 25°C.

As shown in Fig. 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 rapidly. TASHIRO and MAKI (1978) reported that a rice bran trypsin inhibitor was only stable at acidic and neutral pHs.

B. Thermal stability- The incubation of the inhibitors at temperatures ranging from 60°C to 120°C for 60 min gave the results shown in Fig. 3.

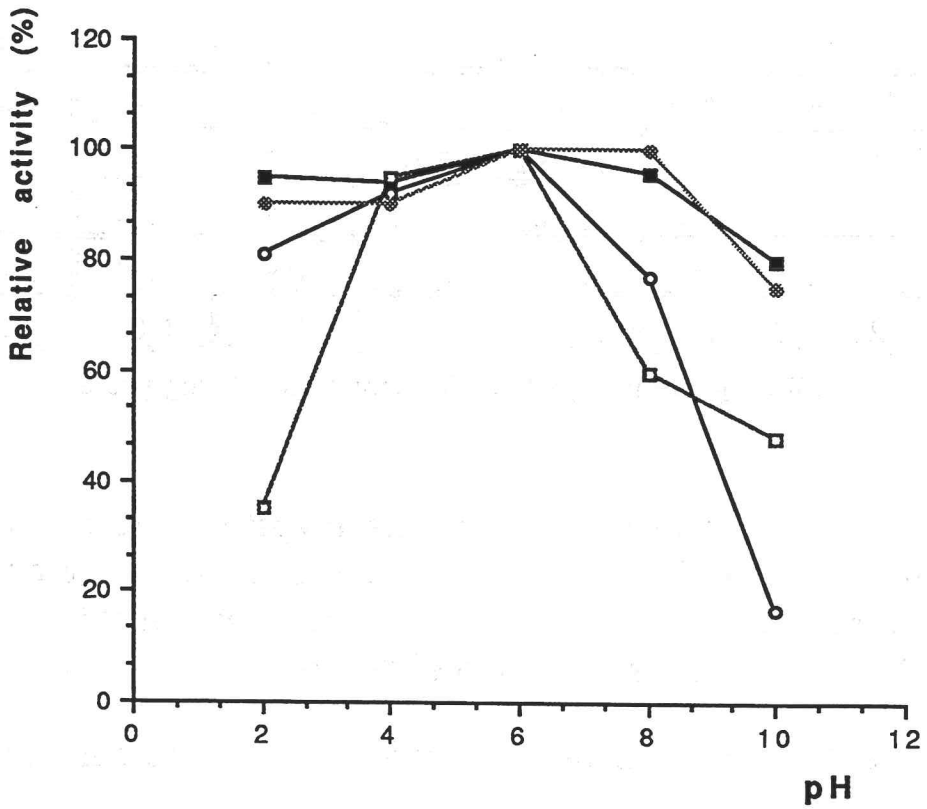



**Figure 2.** Effect of pH on the stability of the papain (o), trypsin (•), chymotrypsin (•) and subtilisin (▯) inhibitors from rice. (Data are average of 2 determinations, using $P_2$ fraction from rice cultivar L-203).

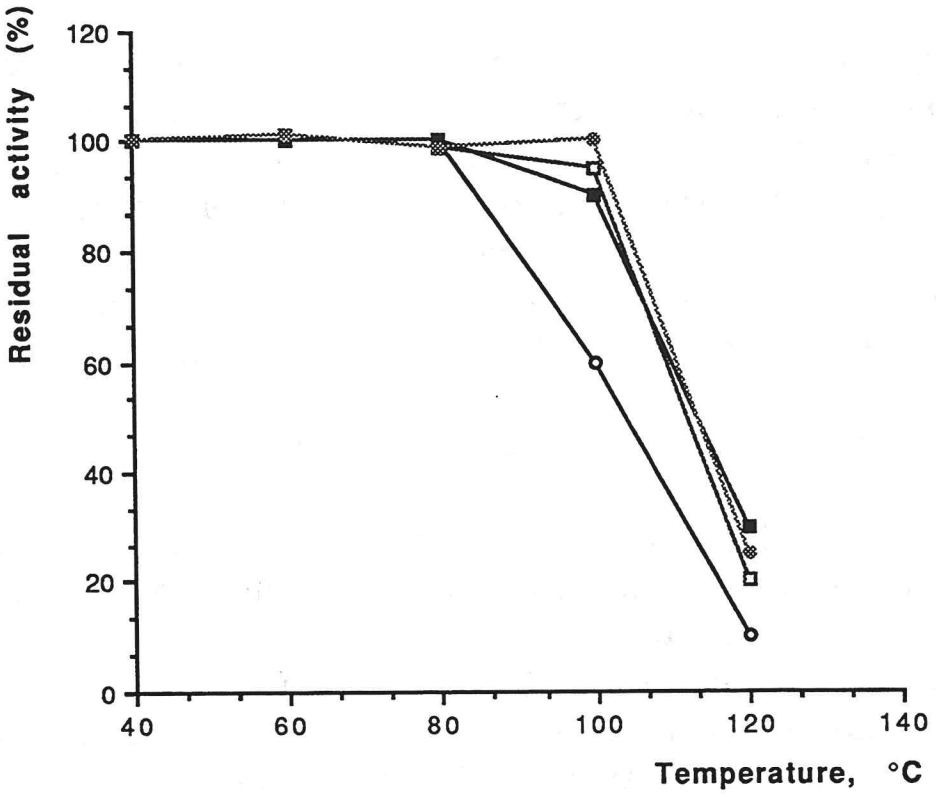



**Figure 3.** Effect of temperature on the stability of the papain (•), trypsin (o), chymotrypsin (•) and subtilisin (▯) inhibitors from rice. (Data are average of 2 determinations, using $P_2$ fraction from rice cultivar L-203).

All the inhibitor activities were stable between 40°C 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 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).

C. 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 semi-quantitative 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 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. 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. The major band corresponded to a MW of about 13,000-Da. The second distinct band was less intense than the 13,000-Da component and had a MW of about 11,400-Da. 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-Da 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-Da and oryzacystatin II with a MW of 12,000-Da. KONDO et al. (1991) 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-Da. TASHIRO and MAKI (1979) reported a MW, based on gel electrophoresis, of about 14,500-Da for a rice bran trypsin inhibitor. However, these authors determined, by gel filtration analysis, a MW of 22,000-Da 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 8,000 - 20,000-Da. Many of the reported values higher than 20,000-Da 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. A-301 and M-201 showed similar bands with a MW of about 23,000-Da, while L-203 and S-201 showed bands with a MW of about 22,000-Da. 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 nor subtilisin inhibitors on gels (data not shown).

Bands corresponding to a MW of about 15,200-Da and 22,000-Da 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.

4. Objective 4. Evaluation of Methodology for Recovering Oryzacystatin.

A. Ratio of extraction buffer to bran- The influence of increasing the ratio of extraction buffer to bran from 2:1 v/w to 6:1 v/w is shown in Fig. 4. For bran from both long and medium grain rice significantly better yields of oryzacystatin were obtained using a buffer to bran ratio greater than 3:1 v/w. The specific activity of oryzacystatin recovered in the $P_2$ fraction tended to decrease as the ratio of extraction buffer was increased, although differences were not significant ($p < 0.05$). considering yields and specific activities, a 4:1 v/w ratio of buffer to bran was selected for process optimization.

B. Multiple extractions of bran- The majority of oryzacystatin was recovered from bran on the first extraction (Fig. 5). For long grain rice bran the yields of oryzacystatin were 208, 32 and 5 IU/ 100 g bran for the first, second and third extractions. Assuming 100% yield, inhibitor recovered was 85%, 13% and 2% for the first through third extractions. For medium grain rice bran, recovery was 182(83%), 23(11%) and 13(6%) IU/ 100 g bran for the first through third extractions. The purity or specific activity tended to increase with each extraction step for both medium and long grain bran (Fig. 5). From these experiments we conclude that a one step extraction with phosphate buffer and 0.15 N NaCl gives satisfactory recovery of oryzacystatin compared to multiple extraction steps.

C. Pre-soaking prior to extraction- Preliminary experiments indicated that overnight soaking of rice or rice bran in water improved the yield of oryzacystatin. Accordingly, we determined the influence of soaking time on the yield and specific activity of oryzacystatin from medium grain rice bran (Fig. 6). Presoaking bran in water for 1 h significantly increased the oryzacystatin yield ($p < 0.05$) and slightly increased the specific activity. We also showed that oryzacystatin is most soluble at about 0.10 N NaCl and is not very soluble in pure water (Fig. 7). Accordingly, presoaking in water may soften the tissue without causing large loss of oryzacystatin due to leaching.

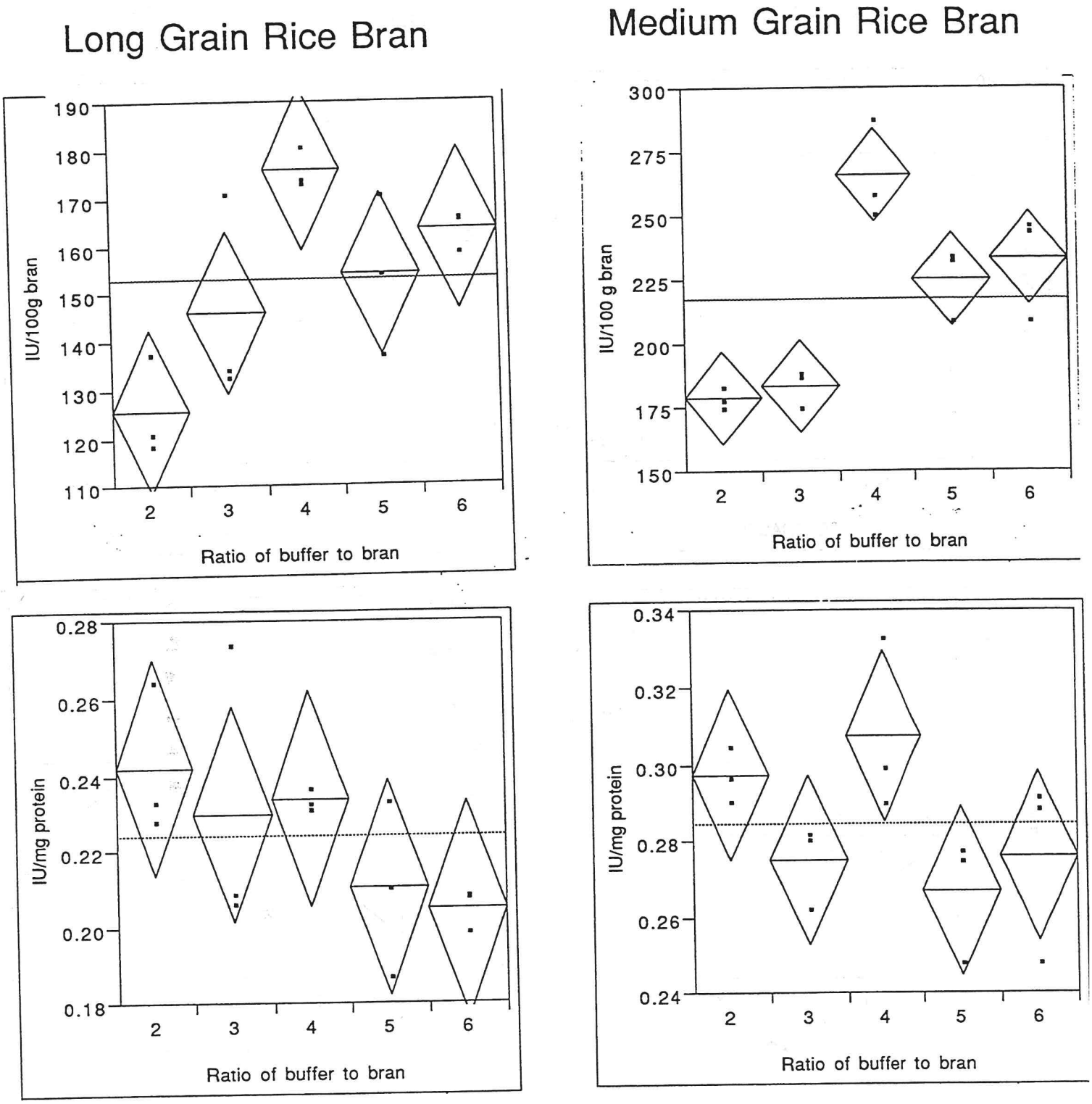



Figure 4. Recovery and specific activity of oryzacystatin ($P_2$) after one extraction with different volumes of buffer. Diamonds show confidence intervals ($\alpha$=0.05)

Medium Grain Rice Bran

Long Grain Rice Bran

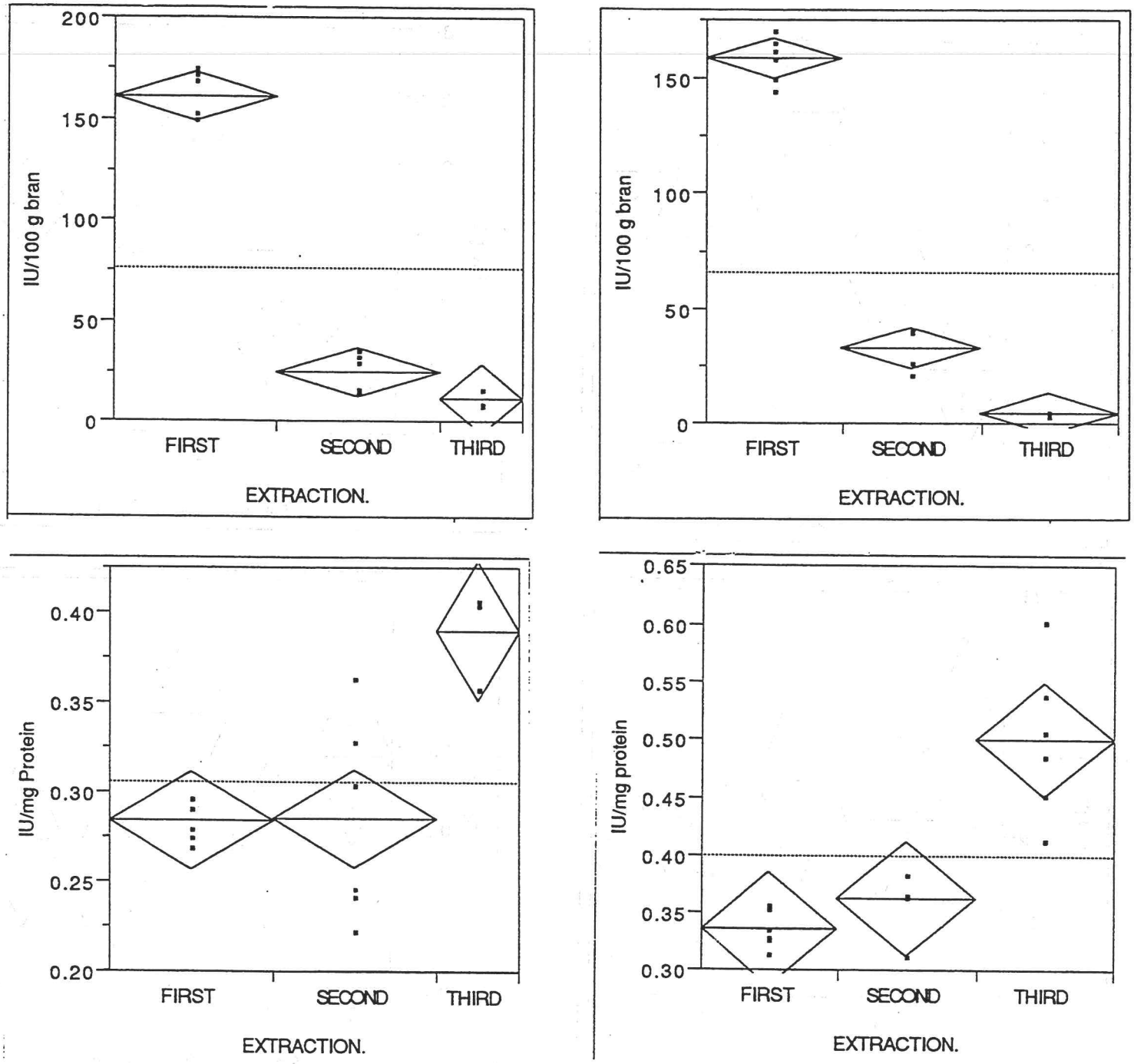



Figure 5. Recovery and specific activity of oryzacystatin ($P_2$) after repeated extraction of rice bran. Diamonds show confidence intervals ($\alpha$=0.05)

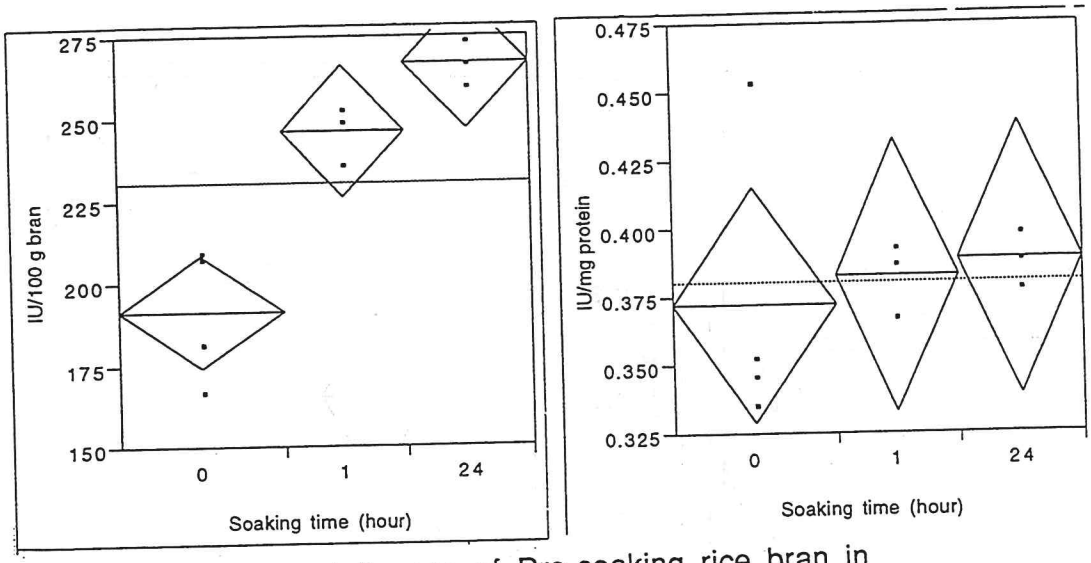



Figure 6. Influence of Pre-soaking rice bran in water on recovery and specific activity of oryzacystatin($P_2$). Buffer: bran ranged from 3-4:1v/w; diamonds are confidence intervals, ($\alpha$=0.05)

Medium Grain Rice Bran

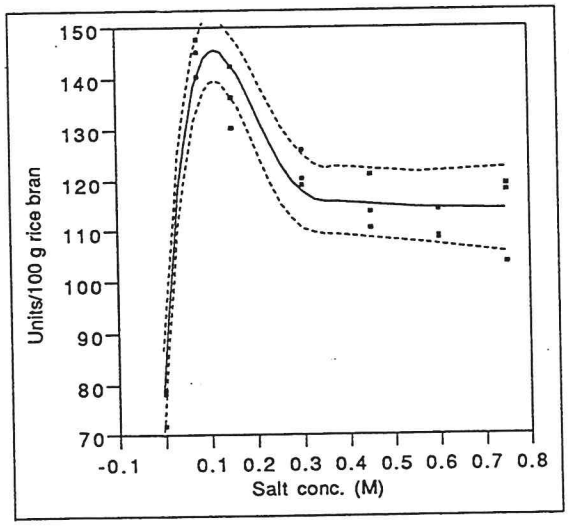



Figure 7. Recovery of oryzacystatin ($S_1$) after extraction with water (100°C) containing different amounts of NaCl. Dotted lines are confidence intervals ($\alpha$=0.05).

# Medium Grain Rice Bran

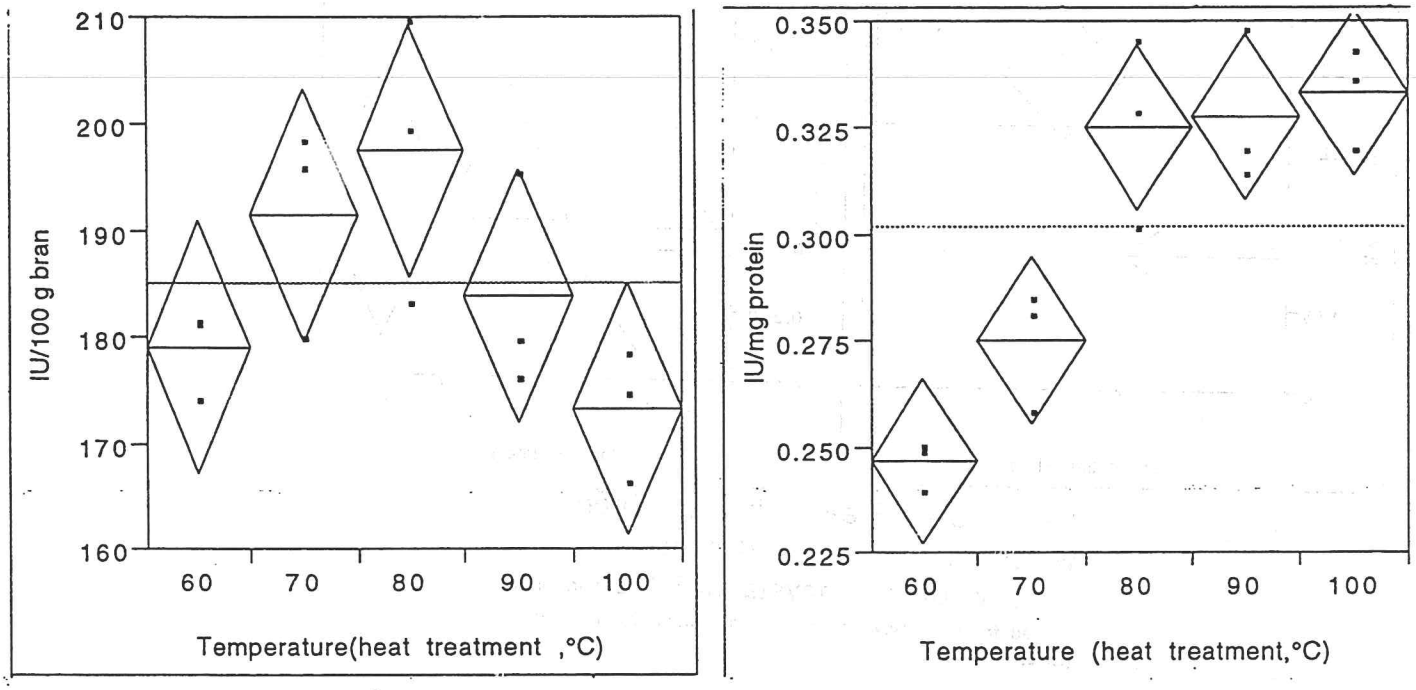



Figure 8. Temperature of heat treatment for 10 minutes vs. the recovery and specific activity of oryzacystatin in the ($P_2$) fraction . Buffer: Bran = 4:1 v/w; 24 hour pre-soak; Diamonds are confidence intervals ($\alpha$=0.05).

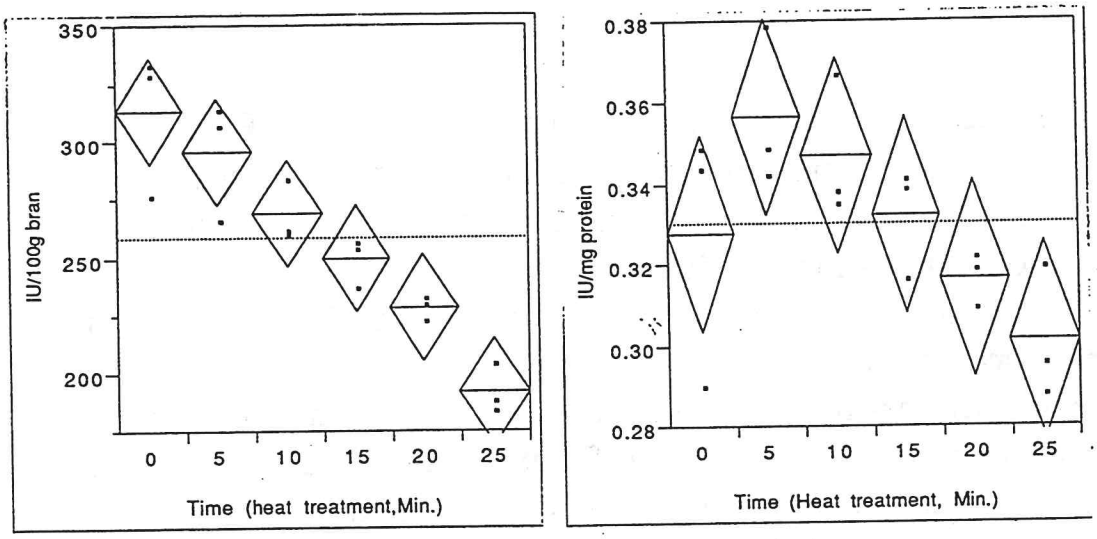



Figure 9. Time of heat treatment at 80°C vs. recovery and specific activity of oryzacystatin in the ($P_2$) fraction.
Buffer: Bran= 4:1 v/w; 24 h pre-soak; Diamonds are confidence intervals ($\alpha$=0.05).

## Medium Grain Rice Bran

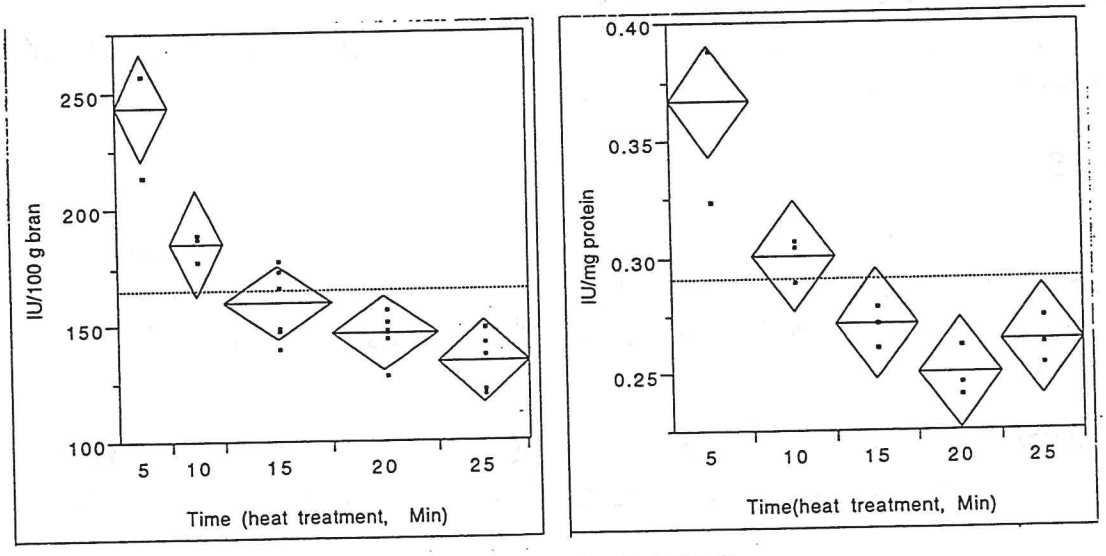



Figure 10. Time of heat treatment at 100°C vs. the recovery and specific activity of oryzacystatin in the ($P_2$) fraction .
Diamonds are confidence intervals ($\alpha$=0.05).

D. Heat Treatment Temperature- The procedure for isolation of $P_2$ (Fig. 1) was modified to vary the temperature of the 10 min. 80°C treatment. The best yield was obtained at 80°C (Fig. 8). A significantly lower yield was obtained with a 100°C treatment. It is not clear why the yield tended to increase as heat treatment temperature increased from 60 to 80°C. It is possible that rice bran contains a heat sensitive substance(s) that causes interference during the assay of oryzacystatin. As expected, the specific activity of oryzacystatin increased significantly ($P < 0.05$) with heat treatment. This is because heat treatment causes the selective coagulation of non-inhibitor proteins and their separation from the soluble fraction containing oryzacystatin.

E. Heat Treatment Time- The influence of heat treatment times at 80°C and 100°C on the yield and specific activity of oryzacystatin are shown in Figs. 9 and 10. Heating at 80°C resulted in decreased yield, but after 5-10 min at 80°C the purity of inhibitor was improved. However, heating at 100°C did not provide any benefits. We also observed that heat treatment serves to prevent interference with the assay of oryzacystatin from turbidity and from endogenous cysteine protease activity. We conclude that a 5-10 min heat treatment at 80°C increases the specific activity of oryzacystatin, but for commercial isolation of inhibitor, the benefits of heat treatment appear to be marginal.

REFERENCES

ABE, K.; KONDO, H.; WATANABE, H.; EMORI, Y.; ARAI, S. Oryzacystatins as the first well-defined cystatins of plant origin and their target proteinases in rice seeds. Biomed. Biochem. Acta, **1991**, 50(4-6), 637-641.

ABE, K.; KONDO, H.; ARAI, S. Purification and characterization of a rice cysteine proteinase inhibitor. Agric. Biol. Chem, **1987**, 51(10), 2763-2768.

ABE, M.; WHITAKER, J. R. Purification and characterization of a cysteine proteinase inhibitor from the endosperm of corn. Agric.Biol.Chem. **1988**, 52(6), 1583-1584.

AN, H.; CHUNG, Y. C.; MORRISSEY, M. T. Proteolysis of Pacific Whiting and its inhibition. In Workshop in Pacific Whiting: Harvesting, Processing, Marketing and Quality Assurance, Newport, Oregon, 1992; pp 36-37.

ANASTASI, A.; BROWN, M. A.; KEMBHAVI, A. A.; NICKLIN, M. J. H.; SAYERS, C. A.; SUNTER, D. C.; BARRET, A. J. Cystatin, a protein inhibitor of cysteine proteinases. Biochem. 1983, 211, 129-138.

ANONYMOUS. Washington News. Food Technol. 1988, 42(9), 42.

ARNON, R. Papain. Methods Enzymol. 1970, XIX, 226-244.

BARRET, A. J. A new assay for cathepsin B1 and other thiol proteinases. Anal. Biochem. 1972, 47, 280-293.

BARRET, A. J. Cystatin, the egg white inhibitor of cysteine proteinases. Methods Enzymol. 1981, 80, 771-775.

BOISEN, S. Protease inhibitors in cereals. Acta Agric. Scandinavia 1983, 33, 369-381.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 1976, 72, 248-254.

CHANG, C. R.; TSEN, C. C. Note on trypsin inhibitor activity in the acetate extract of cereal samples. Cereal Chem. 1979, 56(5), 493-494.

CHANG, T. T.; LUH, B. S. Overview and prospects of the rice production. In Rice; Luh, B.S. Ed.; Van Nostrand Reinhold: New York, 2nd edition, 1991; pp 1-10.

CHANG-LEE, M. V.; PACHECO-AGUILAR, R.; CRAWFORD, D. L.; LAMPILA, L. E. Proteolytic activity of surimi from Pacific Whiting (Merluccius productus) and heat-set gel texture. J. Food Sci. 1989, 54(5), 1116-1119 & 1124.

DENG, J.C. Effect of temperatures on fish alkaline protease, protein interaction and texture quality. J. Food Sci. 1981, 46, 62-65.

FLING, S. P.; GREGERSON, D. S. Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. Anal. Biochem. **1986**, 155, 83-88.

GARCIA-CARRENO, F., DIMES, L.E. AND HAARD, N.F. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. Analytical Biochemistry **1993**, 214, 65-69

GARCIA-OLMEDO, F.; SALCEDO, G.; SANCHEZ-MONGE, R.; GOMEZ, L.; ROYO,J.; CARBONERO, P. Plant proteinaceous inhibitors of proteinases and α-amylase. Oxford Surveys of Molecular & Cell Biology **1987**, 4, 275-334.

GEIGER, R. Chymotrypsin. In Methods of Enzymology; Bergmeyer, H. V. Ed.; Verlag Chemie: Basel, **1984**; pp 99-109.

GREENE, D. H.; BABBITT, J. K. Control of muscle softening and protease-parasite interactions in Arrowtooth flounder, Atheresthes stomias. J. Food Sci. **1990**, 55(2), 579-580.

HAARD, N. F. Enzymes from food myosystems. J. Muscle Foods **1990**, 1, 292-338.

HAMMAN, D. D.; AMATO, P. M.; WU, M. C.; FOEGEDING, E. A. Inhibition of modori (gel weakening) in surimi by plasma hydrolyzate and egg white. J. Food Sci., **1990**, 55(3), 665-669 & 795.

HINES, M. E.; OSUALA, C. I.; NIELSEN, S. S. Isolation and partial characterization of a soybean cystatin cysteine proteinase inhibitor of coleopteran digestive proteolytic activity. J. Agric. Food Chem. **1991**, 39, 1515-1520.

KINOSHITA, M.; TOYOHARA, H.; SHIMIZU, Y. Diverse distribution of four distinct types of modori (gel degradation) inducing proteinases among fish species. Bull. Jap. Soc. Sci. Fish. **1990**, 56(7), 1485-1492.

KONDO, H.; ABE, K.; NISHIMURA, I.; WATANABE, H.; EMORI, Y.; ARAI, S. Two distinct cystatin species in rice seeds with different specificities against cysteine proteinases. J. Biol. Chem. **1990**, 265(26), 15832-15837.

LANIER, T. C.; LIN, T. S.; HAMANN, D. D.; THOMAS, F. B. Effects of alkaline protease in minced fish on texture of heat processed gels. J. Food Sci, **1981**, 46(6), 1643-1645.

LIANG, C.; BROOKHART, G.; FENG, G. H.; REECK, G. R.; KRAMER, K. J. Inhibition of digestive proteinases of stored grain coleoptera by oryzacystatin, a cysteine proteinase inhibitor from rice seed. FEBS Letters **1991**, 278(2), 139-142.

MAKI, Z.; TASHIRO, M.; SUGIHARA, N.; KANAMORI, M. Double-headed nature of a trypsin inhibitor from rice bran. Agric. Biol. Chem. **1980**, 44(4), 953-955.

MATSUBARA, H. Purification and assay of thermolysin. Methods Enzymol.**1970**, XIX, 642-651.

OHTSUBO, K. I.; RICHARDSON, M. The amino acid sequence of a 20kDa bifunctional subtilisin/α-amylase inhibitor from bran of rice (Oryza sativa L.) seeds. FEBS Letters **1992**, 309(1), 68-72.

PORTER, R. W.; KOURY, B. J.; KUDO, G. Method for treating fish meat contaminated with sporoza and potato product for improving heat gelation of fish muscle. U. S. Patent: 4,935,192, 1990.

REPPOND, K. D.; BABBITT, J.K. Protease inhibitors affect physical properties of Arrowtooth Flounder and Walleye Pollock surimi. J. Food Sci. **1993**, 58(1), 96-98.

RICHARDSON, M. Protein inhibitors of enzymes. Food Chem. **1981**, 6, 235-253.

ROWAN, A. D.; BRZIN, J.; BUTTLE, D. J; BARRET, A. J. Inhibition of cysteine proteinases by a protein inhibitor from potato. FEBS Letters, **1990**, 269(2), 328-330.

RYLE, A. (1984). Pepsins, gastricsins and their zymogens. In Methods of Enzymology; Bergmeyer, H. V. Ed.; Verlag Chemie: Basel, 1984; pp 223-238.

SMITH, C.; MEGEN, W. V.; TWAALFHOVEN, L.; HITCHCOCK, C. The determination of trypsin inhibitor levels in foodstuffs. J. Sci. Food Agric. **1980**, 31, 341-350.

TASHIRO, M.; MAKI, Z. Partial purification and some properties of a trypsin inhibitor from rice bran. Agric. Biol. Chem., **1978**, 42(6), 1119-1124.

TASHIRO, M.; MAKI, Z. Purification and characterization of a trypsin inhibitor from rice bran. J. Nutr. Sci. Vitaminol., **1979**, 25, 255-264.

UENO, R.; KANAYAMA, T.; TOMIYASU, K.; FUJIKAMA, A.; NAKASHIMA, T. Processes for production of refrigerated minced fish flesh and fish paste product having improved quality. U.S. Patent: 4,464,404, 1984.

WASSON, D. H.; BABBITT, J. K.; FRENCH, J. S. Characterization of a heat stable protease from Arrowtooth Flounder; Atheresthes stomias. J. Aquatic. Food Product Technol. **1992**, 1(3/4), 167-182.

WASSON, D. H.; REPOND, K.D.; BABBITT, J. K.; FRENCH, J. S. Effects of additives on proteolytic and functional properties of Arrowtooth flounder surimi. J. Aquatic. Food Product Technol. **1992a**, 1(3/4), 147-164.

XAVIER-FILHO, J.; CAMPOS, F. A. P. Proteinases inhibitors. In: Toxicants of plant origin; Checke, P.R. Ed.; CRC- Press: Boca Raton, Florida, 1989; pp 1-27.

**PUBLICATIONS:**

1. Izquirdo-Pulido, M., Haard, T.A., Hung, J., and Haard, N.F. 1993. Oryzacystatin content of California rice and rice milling fractions. Abstract # 348, Institute of Food Technologists Annual Meeting, Chicago, IL, July, 1993.

2. Izquirdo-Pulido, M., Haard, T.A., Hung, J., and Haard, N.F. 1993. Oryzacystatin and other proteinase inhibitors in rice grain: Potential use in preventing proteolysis in surimi and other fish products. J. Agriculture and Food Chemistry (submitted).

3. GARCIA-CARRENO, F., DIMES, L.E. AND HAARD, N.F. 1993 Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. Analytical Biochemistry, 214, 65-69

SUMMARY:

Crude extracts of rice straw inhibited cysteine proteinases, however, the active material was not a protein nor a specific cysteine proteinase inhibitor. Eleven rice cultivars were assayed for inhibitors of trypsin, α-chymotrypsin, subtilisin, aspartyl proteinases, and metalloproteinases. Trypsin, subtilisin and, to a lesser extent, chymotrypsin inhibitors were identified in all cultivars. Trypsin inhibitor was mainly concentrated in the bran, while subtilisin inhibitor was distributed in both bran and endosperm fractions. The pH and thermal stability of proteinase inhibitors from rice was determined. Preliminary studies on the methodology for oryzacystatin isolation showed that the inhibitor is salt soluble, mostly recovered by one step extraction, better recovered from pre-soaked bran, and with respect to a heat treatment loss in yield outweighs gains from improvment in purity.