

## Isolation of Alkaline and Neutral Proteases from *Aspergillus flavus* var. *columnaris*, a Soy Sauce Koji Mold

ATTAWUT IMPOOLSUP, AMARET BHUMIRATANA,\* AND TIMOTHY W. FLEGEL

*Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 4, Thailand*

Received 16 March 1981/Accepted 22 June 1981

Two different extracellular proteases, protease I (P-I), an alkaline protease, and protease II (P-II) a neutral protease, from *Aspergillus flavus* var. *columnaris* were partially purified by using  $(\text{NH}_4)_2\text{SO}_4$  precipitation, diethylaminoethyl-Sephadex A-50 chromatography, carboxymethylcellulose CM-52 chromatography, and Sephadex G-100 gel filtration. The degree of purity was followed using polyacrylamide gel electrophoresis. The activity of P-I was completely inhibited by 0.1 mM phenylmethylsulfonyl fluoride, and that of P-II was completely inhibited by 1 mM ethylenediaminetetraacetate. By using these inhibitors with extracts of wheat bran koji, the proportions of total activity that could be assigned to P-I and P-II were 80 and 20%, respectively. This compared favorably with activities estimated by using polyacrylamide gel electrophoresis slices (82 and 18%, respectively). Extracts from factory-run soybean koji gave comparable results. Both enzymes demonstrated maximum activity at 50 to 55°C and only small changes in activity between pH 6 and 11. For P-I, activity was somewhat higher from pH 8.0 to 11.0, whereas for P-II it was somewhat higher from pH 6 to 9. In the presence of 18% NaCl, the activities of both P-I and P-II dropped by approximately 90 and 85%, respectively. P-I was inferred to possess aminopeptidase activity since it could hydrolyze L-leucyl-p-nitroanilide hydrochloride. P-II was devoid of such activity. The ramifications of the results for factory-produced soy sauce koji are discussed.

The soy sauce fermentation industry in Thailand is typically operated on a domestic factory scale, and manufacturing techniques have been handed down through many generations without much improvement. In a previous report (4), a strain of *Aspergillus* sp. isolated from such factories was used as a controlled inoculum for factory-prepared "koji" which resulted in soy sauce of quality superior to that usually produced. Since the fungus used in this work was *Aspergillus flavus* var. *columnaris*, it was cleared as aflatoxin negative (4) before being recommended for routine use in soy sauce factories in Thailand, and it was of interest to observe the result of its extended use in a factory setting. Although there have been a number of studies (7, 11, 14-21, 22) on the biology of soy sauce fungi and on the purification and characterization of their proteases, most of these investigations have been carried out on the Japanese soy sauce fungus *Aspergillus oryzae*. This study was undertaken as part of a program to evaluate the use of *A. flavus* var. *columnaris* in soy sauce fermentation. It was specifically aimed at the isolation, purification, and characterization of its extracellular proteases, and it included an ex-

amination of the kinetics of enzyme production in factory-prepared koji.

### MATERIALS AND METHODS

#### Microorganism and maintenance conditions.

The strain of *A. flavus* var. *columnaris* used in this study was isolated from a soy sauce koji sample obtained from a factory in Bangkok, Thailand (4), and it has been deposited into the American Type Culture Collection as number 44310. Cultures were stored on Sabouraud dextrose agar (SDA) medium in a refrigerator. They were subcultured on petri dishes with SDA medium and incubated at 30°C for 7 days before the preparation of spore inoculum.

**Chemicals.** Diethylaminoethyl (DEAE)-Sephadex A-50 and Sephadex G-100 were bought from Pharmacia Fine Chemicals (Uppsala, Sweden). Carboxymethyl cellulose (CM-52) was the product of Whatman Ltd. (Maidstone, England). Celite (analytical filter aid) was purchased from Johns-Manville (New York, N.Y.).

Disodium ethylenediaminetetraacetate (EDTA) was acquired from Fisher Scientific Co. (Fair Lawn, N.J.). Ammonium persulfate, phenylmethylsulfonyl fluoride (PMSF), and tris(hydroxymethyl)aminomethane were obtained from Sigma Chemical Co., (St. Louis, Mo.). Crystallized bovine plasma albumin was obtained from Armour Pharmaceutical Company, Ltd.

(Chicago, Ill.). Acrylamide, glycine, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and riboflavin were the products of Eastman Kodak Co. (Rochester, N.Y.). L-Leucyl-*p*-nitroanilide hydrochloride ( $C_{12}H_{17}N_3O_3 \cdot HCl$ ) was bought from Wako Pure Chemical Industries, Ltd., Japan. Amido black (Aminoschwarz 10B), casein (Hammersten), and Folin-Ciocalteu phenol reagent were purchased from E. Merck (Darmstadt, Germany). All chemicals used were of analytical grade or the purest grade available.

**Preparation of spore inoculum. (i) For inoculation of wheat bran koji.** A loopful of spores from a 7-day colony on SDA medium was suspended in a test tube containing melted agar solution (0.7%, wt/vol), and a loopful of this suspension was transferred to each of four 500-ml Erlenmeyer flasks containing 100 ml of SDA medium. The cultures were incubated at 30°C for 7 days. The spores were then harvested by the addition of 80 ml of distilled water containing 0.5% Tween 80 followed by vigorous shaking. The resulting suspensions contained approximately  $10^8$  spores per ml, and 40 ml was used as the inoculum for wheat bran koji.

**(ii) For inoculation of industrial-scale soybean koji.** Spore inoculum for factory koji was prepared by using cooked, broken rice grains as the substrate. Six hundred grams of rice was mixed with 600 ml of water and autoclaved for 15 min at 1 kg/cm<sup>2</sup>. After the rice had cooled to room temperature, it was inoculated with 100 ml of spore suspension prepared as described above. The resulting density of the inoculum was approximately  $10^7$  spores per g (dry weight) of rice. The inoculated rice cultures were then incubated in covered, perforated stainless steel trays (30 by 30 by 15 cm) at 30°C for 48 h. The rice koji was then transferred to a hot-air oven at 60°C for 4 days. Subsequently, the dried rice koji was blended in a Waring blender (Waring Product Division, Dynamics Corporation of America, New Hartford, Conn.). After determination of viable counts, the resulting spore powder was stored in a tightly sealed plastic bag at 4°C until it was used.

**Solid-state fermentations. (i) Wheat bran koji.** In covered, perforated stainless steel trays (30 by 30 by 15 cm), 800 g of wheat bran mixed with 1,000 ml of distilled water was evenly spread. The trays were then sterilized by autoclaving and cooled to room temperature before being inoculated with 40 ml of the spore suspension described above. The culture was incubated at room temperature (30 to 34°C).

**(ii) Soybean koji.** Samples of soybean koji were obtained from a soy sauce factory in the Samut Prakarn. The koji was prepared in a large koji room in the following way. Whole commercial soybeans (1,060 kg) were soaked in excess flowing tap water for 2.5 h. After draining, the beans were cooked under steam for 30 min at 1 kg/cm<sup>2</sup> and then allowed to cool to 50 to 60°C. Next, 350 kg of rice flour and 250 kg of wheat flour were thoroughly mixed in, and the mixture was inoculated with fungal spores at a density of approximately  $10^6$  spores per g (dry weight) of the mixture. To facilitate good distribution, the spores were mixed with 20 kg of flour before addition. The inoculated mixture was spread evenly (15 cm thick) over the

perforated aluminum floor (2.5 by 7 m) of the incubation room (koji room). The temperature in the koji room was held at 30°C by a continual stream of moist filtered air forced up through it from the perforated floor. The koji was turned twice after 17 and 21 h of incubation to facilitate thorough mixing. After an incubation period of 42 h, the koji was transferred to salt brine (moromi stage).

**Preparation of crude enzyme extracts.** Samples from laboratory-prepared wheat bran koji were taken at various time intervals and immediately extracted as described below. The samples from factory soybean koji were packed in ice while being transported to the laboratory for extraction. Crude enzyme was extracted by mixing the wheat bran or soybean cultures with 0.05 M phosphate buffer, pH 7.0, 1:1 (wt/vol). The mixtures were left standing at room temperature with occasional stirring for 1 h. The extract was then filtered through Whatman no. 1 filter paper with the aid of Celite powder. In the same way, the residue was extracted a second time with the same volume of fresh buffer. The second filtrate was combined with the first, and the combination, designated crude enzyme extract, was stored at -20°C until it was processed further.

**Crude enzyme concentrate.** Crude enzyme extract of wheat bran koji was used in this part of the study. Unless stated otherwise, all purification steps were carried out at 0 to 4°C.

First, 2,200 ml of crude extract was concentrated by adding ammonium sulfate to obtain 90% saturation. The precipitate was then collected by centrifuging at 16,000 × *g* for 20 min. The resulting pellet was suspended in 180 ml of 0.05 M phosphate buffer (pH 7.0) and dialyzed overnight in 0.01 M phosphate buffer, pH 7.0. The dialyzed preparation was concentrated to 30 ml by ultrafiltration with a UM-10 Diaflo membrane (Amicon Corp., Lexington, Mass.). This solution was designated crude enzyme concentrate.

**Column chromatography.** Crude enzyme concentrate (30 ml; containing 870 mg of protein) was loaded onto a DEAE-Sephadex A-50 column (2.5 by 45 cm) previously equilibrated with 0.01 M phosphate buffer, pH 7.0. The column was then eluted with 1,200 ml of 0.01 M phosphate buffer (pH 7.0), followed by 2 liters of a 0 to 0.4 M NaCl linear gradient in 0.01 M phosphate buffer, pH 7.0.

Carboxymethyl-cellulose was prepared in a column (2.5 by 45 cm) and equilibrated with 0.1 M acetate buffer, pH 5.0. After sample application, the column was eluted with 720 ml of 0.01 M acetate buffer (pH 5.0) followed by a linear NaCl gradient (0 to 0.4 M in 0.01 M phosphate buffer, pH 7.0).

Sephadex G-100 was prepared in columns (3 by 90 cm) and equilibrated with 0.05 M phosphate buffer, pH 7.0. These columns were eluted with the same buffer.

For all columns, 12-ml fractions were collected. Protein contents were determined by adsorption at 280 nm, and protease activity was measured as described below.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis (PAGE) was performed with a Buchler electrophoresis apparatus by the method of

Davis (5). In all cases, stacking gels and separating gels contained 2.5 and 7% acrylamide, respectively. Samples containing between 50 and 200  $\mu\text{g}$  of protein were layered onto each gel, and electrophoresis was carried out with a current of 2 mA per tube, with 0.005 M tris(hydroxymethyl)aminomethane-0.04 M glycine at pH 8.3 used as the running buffer. A few drops of 0.001% bromophenol blue were added as a tracking dye. By using the tracking dye as an indicator, electrophoresis was stopped at an appropriate time, and gels were removed from their tubes. They were either stained with 1% amido black as described by Davis (5), treated with peptidase activity stain (0.01 M L-leucyl-p-nitroanilide hydrochloride) as described by Strongin et al. (24), or sliced into 2-mm pieces in a macrotome GTS-I gel slicer (Yeda R & D Co., Ltd. Rehovot, Israel) and immersed in 0.05 M phosphate buffer (pH 7.0) for 1 h before proteolytic activity assays were performed. Relative migration distance ( $R_d$ ) was defined as the ratio of the protein or proteolytic activity migration distance to the tracking dye migration distance.

**Assay for protease activity.** Protease activity was assayed by the method of Anson (1), except that 1.5% milk casein was used as the substrate and incubation was at 40°C. One unit of protease activity was defined as the amount of enzyme that liberated 1  $\mu\text{g}$  of tyrosine in a reaction mixture per min at 40°C. In all of these assays, it was confirmed that detectable activity was proportional to the amount of protein added and that the activity increased proportionally to the increase in incubation time.

Protease activity at various temperatures was determined by using a controlled-temperature water bath during the digestion period. To determine heat stability, enzyme preparations were preincubated in a water bath at various temperatures for either 10 or 60 min before being cooled in an ice bath and subsequently assayed.

To determine the effect of NaCl on proteolytic activity, various amounts of NaCl were added to the reaction mixtures to obtain final concentrations of 5, 10, 15, 18, and 20%. To determine the effect of salinity on enzyme stability, 18% NaCl and 0.02%  $\text{NaN}_3$  were added to the crude enzyme extract, and the mixture was left standing at room temperature. Samples were taken at intervals and assayed for the remaining activity.

For protease activity determinations at various pH values, the following buffers were used with 1.5% milk casein: pH 6.0 to 9.0, 0.1 M citrate-phosphate buffer; pH 6.0 to 8.0, 0.1 M phosphate buffer; pH 8.5 to 12.0, 0.1 M glycine-NaOH buffer. Since casein is insoluble at pH 4.0 to 5.5, proteolytic activity in this range was not tested. For an assay at pH 3.0, casein was prepared by the method of Ichishima (6).

**Miscellaneous methods.** Total spore counts were determined directly by using a Bright-line hemacytometer (American Optical Corp. Buffalo, N.Y.). The total viable spore count was determined by the plate count technique, using SDA medium incubated for 3 days at 30°C after inoculation.

Dry weights for koji samples were determined by drying to constant weight at 100°C in a hot-air oven.

Colorimetric protein determinations were carried out by the method of Lowry et al. (9), using crystalline bovine plasma albumin (Armour Pharmaceutical Co.) as the standard.

## RESULTS

**Extracellular proteases production by *A. flavus* var. *columnaris* grown in wheat bran culture.** Figure 1 shows protease activity (expressed as kilounits per gram [dry weight] of koji) in wheat bran koji extracts. Only a very small amount of activity could be detected during the first 20 h after inoculation. Thereafter, the enzyme activity increased rapidly to a maximum level of 1.7 kU/g at 32 h of incubation. After this, the protease activity remained the same for a further incubation of 40 h. It was of interest that during the first 20 h, when protease activity was low, fungus growth (revealed by a whitish mycelial mat on the wheat bran) was very rapid. After 20 h of incubation, fungal sporulation gave the wheat bran a yellow-green color. The color deepened as the quantity and the age of the spores increased. Sporulation appeared to reach a maximum by 36 h of cultivation. Thus, the rapid increase in protease activity coincided with late growth and the formation of spores.

Based on these data, 36-h wheat bran cultures of *A. flavus* var. *columnaris* were used for the preparation of crude enzyme extracts.

**Partial purification of proteases of *A. flavus* var. *columnaris*.** The elution profile of DEAE-Sephadex A-50 chromatography (30 ml of wheat bran crude enzyme concentrate, see above) is shown in Fig. 2. There were two major protein peaks which contained proteolytic activity. The first peak, designated protease I (P-I), came out with the void volume, and the second peak, designated protease II (P-II), was eluted

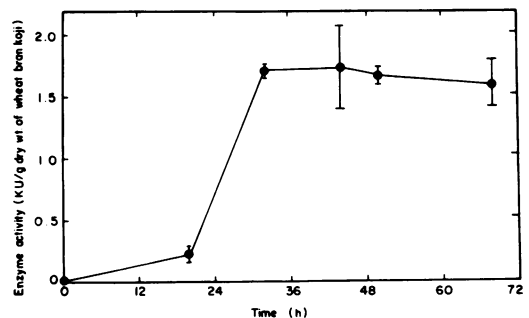


FIG. 1. Time course for the production of protease by *A. flavus* var. *columnaris* grown in wheat bran substrate. Each point represents the mean activity of two 5-g samples, and the bar indicates the range activity obtained. KU, kilounits.

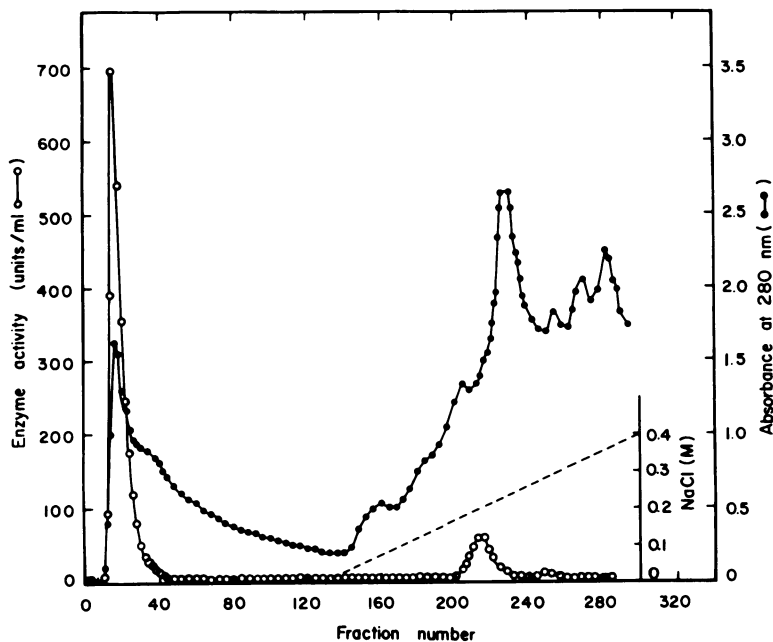


FIG. 2. Separation of protease activities by DEAE-Sephadex A-50 column chromatography. Crude enzyme concentrate (30 ml) containing a total of 870 mg of protein was loaded onto the column and eluted with 1,200 ml of 0.01 M phosphate buffer (pH 7.0), subsequently followed with a gradient (---) from 0 to 0.4 M NaCl in 2 liters of 0.01 M phosphate buffer (pH 7.0) at a flow rate of 35 ml/h. Each 12-ml eluate fraction was analyzed for protein (●) by measuring absorbance at 280 nm and was assayed for proteolytic activity (○) by using casein as a substrate.

with 0.2 M NaCl in 0.01 M phosphate buffer, pH 7.0. One minor peak of protease activity was found just behind the P-II peak. However, since the activity of this peak was very small in comparison with the two major peaks, no further purification was attempted. When the eluted fractions were tested at pH 3.0 (activities shown in Fig. 2 were determined at pH 7.0), no activity was found in any fraction.

**(i) Partial purification of P-I.** The fractions constituting the P-I peak from the DEAE-Sephadex A-50 column (fractions 10 to 35) were pooled and concentrated to 20 ml by ultrafiltration. The concentrate was then dialyzed overnight at 4 liters of 0.01 M acetate buffer, pH 5.0. The dialyzed preparation (18 ml) was then applied to a carboxymethylcellulose column. The fractions with protease activity were pooled, concentrated by ultrafiltration, and stored at  $-20^{\circ}\text{C}$ . A summary of the purification steps is given in Table 1. P-I was purified approximately 360-fold, with a total recovery of 8%.

The amido black-stained PAGE pattern of the concentrated carboxymethylcellulose pooled fractions (partially purified P-I) is shown in Fig. 3A. There were two major protein bands. The first ( $R_d$ , 0.17) was the predominant one. The presence of a second band ( $R_d$ , 0.32) showed that

the P-I preparation was not pure. Nevertheless, on the basis of proteolytic activity measured in slices of parallel-run unstained PAGE gels (Fig. 3B), the first band ( $R_d$ , 0.17) was the only one with proteolytic activity, and it could be identified as P-I. Staining PAGE gels of P-I preparation with L-leucyl-*p*-nitroanilide hydrochloride produced one peak of absorbency coincident with P-I ( $R_d$ , 0.17) (Fig. 4), and this indicated that P-I also possessed peptidase activity.

**(ii) Partial purification of P-II.** The fractions constituting the P-II peak from the DEAE-Sephadex A-50 column (fractions 200 to 225) were pooled (270 ml), concentrated to 4 ml by ultrafiltration, and dialyzed overnight with 0.05 M phosphate buffer, pH 7.0. The dialyzed solution was loaded onto a Sephadex G-100 column. Protease-active fractions were pooled (57 ml), concentrated to 4.5 ml by ultrafiltration, and then applied to a second Sephadex G-100 column. The fractions with protease activity were pooled and stored at  $-20^{\circ}\text{C}$  for further study. A summary of the purification steps is given in Table 2.

This partially purified P-II, after PAGE and staining with amido black, gave the pattern shown in Fig. 5A. There were one major and three minor protein bands located at  $R_d$  values

TABLE 1. Partial purification of P-I

Enzyme fraction	Vol (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%)	Fold purification
Crude extract	2,200	101,120	11,853	9	100	1.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	180	100,962	9,550	11	100	1.2
UM-10 ultrafiltration	30	78,210	873	90	77	10
DEAE-Sephadex A-50	290	68,000	96	708	67	79
Carboxymethyl cellulose	150	8,076	2.5	3,230	8	359

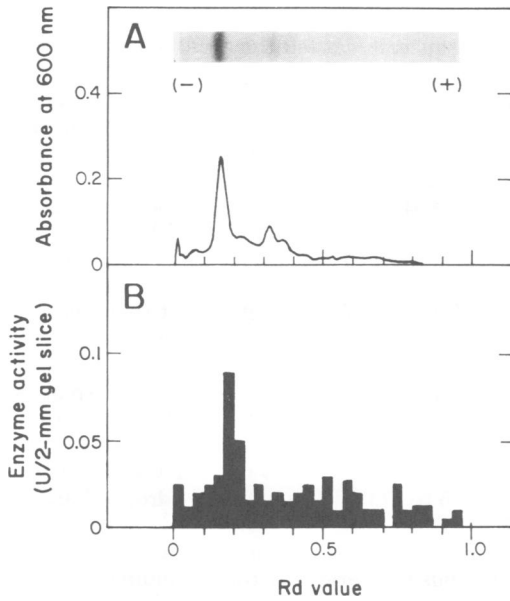


FIG. 3. PAGE pattern of partially purified P-I. Each sample contained 31.25 µg of protein. One gel was stained with amido black and scanned at 600 nm (A); another gel was sliced into 2-mm fractions, and the proteolytic activity of each fraction was determined (B).

of 0.68, 0.46, 0.58, and 0.83, respectively. However, an assay of unstained 2-mm gel slices immediately after electrophoresis showed that proteolytic activity was associated with the major protein band at an *R<sub>d</sub>* of 0.68 (Fig. 5), and this was labeled P-II. Unlike P-I, P-II was found to be devoid of peptidase activity when stained with 0.01 M L-leucyl-p-nitroanilide hydrochloride. However, some peptidase activity was associated with the protein band at an *R<sub>d</sub>* of 0.83 (Fig. 5B).

**Separation of P-I and P-II in crude enzyme extracts by using PAGE.** Data in the previous sections showed that on polyacrylamide gel, P-I and P-II corresponded to protein bands of *R<sub>d</sub>* values of 0.17 and 0.68, respectively. This suggested that two proteases could be unambiguously separated from one another in crude extracts by using PAGE and that such a technique might be used to determine the rela-

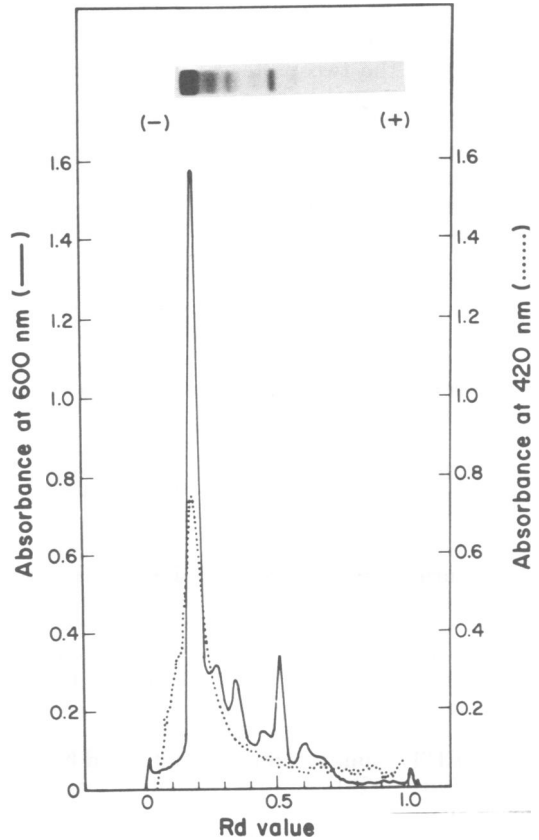


FIG. 4. PAGE pattern and L-leucyl-p-nitroanilide hydrochloride hydrolysis activity of P-I. A sample (240 µg of protein) from the DEAE-Sephadex A-50 column was loaded onto each gel. One gel was stained with amido black and scanned at 600 nm (—). Another identical gel was immersed in 0.01 M L-leucyl-p-nitroanilide-hydrochloride, and, after 15 min of incubation at 30°C, the gel was scanned at 420 nm (---).

tive activity of each protease in the crude extracts. The electrophoretic pattern of crude enzyme extract of *A. flavus* var. *columnaris* grown in wheat bran is shown in Fig. 6A. It shows at least 11 major protein bands. The protein band corresponding to P-I was well separated from other protein bands, but the P-II band was not. In view of the interfering protein bands for P-II,

TABLE 2. *Partial purification of P-II*

Enzyme fraction	Vol (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%)	Fold purification
Crude extract	2,200	19,200	11,853	1.6	100	1.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	180	19,170	9,550	2.0	100	1.3
UM-10 ultrafiltration	30	14,850	873	17	77	11
DEAE-Sephadex A-50	270	10,350	168	62	54	39
1st Sephadex G-100	77	2,015	24	84	11	53
2nd Sephadex G-100	100	1,000	7	143	5	89

it was very difficult to determine the relative amounts of the two proteases on the basis of relative stain uptake. Therefore, unstained gels were sliced into 2-mm pieces that were assayed for protease activity. As predicted, there were two bands of protease activity corresponding to  $R_d$  values of 0.17 and 0.68 (Fig. 6B) and consequently to P-I and P-II, respectively.

Calculations revealed that the relative activities of P-I and P-II were approximately 82 and 18%, respectively. An identical analysis (data not shown), using crude enzyme extracts from soy-bean koji, gave relative activities of P-I and P-II of approximately 77 and 23%, respectively.

#### Determination of relative activities of P-I and P-II by using specific inhibitors.

EDTA is known for its inhibition of metal-chelator-sensitive proteases, and PMSF is known for its inhibition of serine proteases. The effects of these two inhibitors were tested on the partially purified preparations of P-I and P-II (Table 3). It was concluded that 0.1 mM PMSF almost completely inhibited (99%) P-I activity, but had little or no effect on P-II activity (i.e., the decrease in P-II activity in the PMSF treatment could be attributed almost entirely to the isopropanol solvent for PMSF). On other hand, 1 mM EDTA almost completely inhibited (99%) the activity of P-II, but had little effect on P-I (original activity remained). These results suggested that P-I was a serine protease and that P-II was a metal-requiring protease.

The ability of EDTA and PMSF to specifically inhibit different proteases with very minimum cross-reaction presented the possibility of determining the relative activities of P-I and P-II in crude enzyme preparations. When such an experiment was carried out, it showed that the activity in crude enzyme extract of wheat bran culture could be attributed 80% to P-I and 20% to P-II. When crude enzyme extract from soy-bean koji was used, this changed to 70% for P-I and 30% for P-II. These relative activities are comparable to those calculated by using the sliced-gel experiments described above.

**Properties of P-I and P-II.** In view of the well-established role of proteases in the brewing

of soy sauce, it was felt that studies on activities of P-I and P-II at different pH values, temperatures, and salt concentrations might be useful for evaluating brewing procedures in koji and moromi fermentations. Again, selective inhibition by EDTA or PMSF was useful in determining the activity of only one enzyme at a time in mixtures containing both P-I and P-II.

The activity of P-I was found to be highest in the alkaline range (between pH 8.5 and 9.0 in 0.05 M glycine-NaOH buffer), but there was no sharp peak. For P-II, the highest activity was between 7.0 and 7.5 (0.05 M citrate-phosphate buffer), although again there was no sharp peak and the activity was only slightly higher than that from pH 6.0 to 7.0. The activity of P-II decreased slightly when the pH was increased from 7.5 to 11.0 and then rapidly dropped above pH 12.0.

When the activity of the enzymes was assayed at various temperatures, the optimum for casein digestion was between 50 and 55°C for both P-I and P-II; 50% of P-I and 15% of P-II activity was lost when the enzymes were incubated at 50°C for 10 min. Both enzymes were completely inactivated when they were incubated at 70°C for 10 min. The presence of sodium chloride greatly reduced the activity of both enzymes. The respective activities of total protease and of P-I and P-II dropped sharply to 13, 12, and 17% of their original activities immediately after the addition of 15% NaCl. Further increases in NaCl beyond 15% resulted in only slight further reductions in enzyme activities. At 18 and 20% NaCl (i.e., the percent salinities commonly used in the moromi stage of soy sauce production), the respective activities of P-I and P-II remaining were only 10 and 15% of the original. Measurement of the stability of this residual activity for P-I and P-II after 18% NaCl addition and incubation at room temperature in the presence of 0.02% NaN<sub>3</sub> gave the following results. For P-II, the residual activity remained rather constant throughout 45 days of incubation (i.e., at 15% of the original activity). For P-I, it decreased a further 5% to give approximately 5% of the original activity at the end of 45 days.

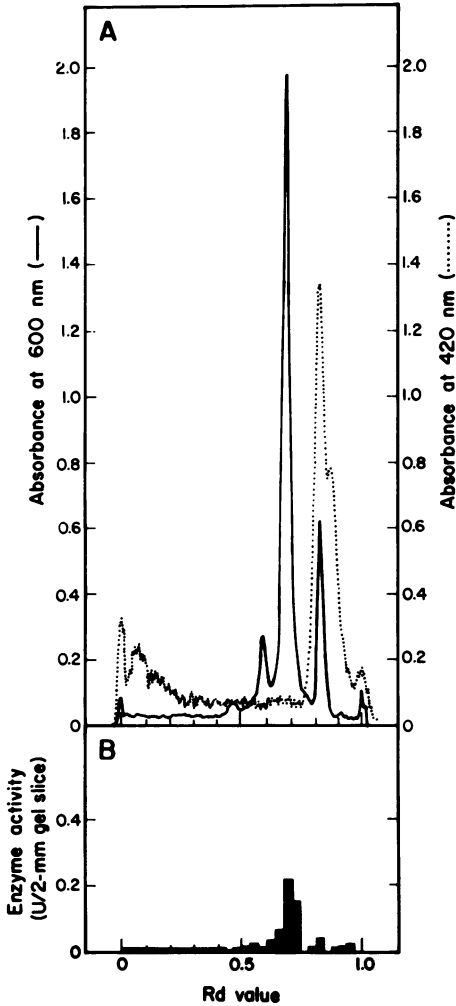


FIG. 5. PAGE pattern of partially purified P-II. A 70- $\mu$ g protein sample was loaded onto each gel. (A) Absorbance pattern of an amido black-stained gel scanned at 600 nm (—) and of an activity-stained gel, using L-leucyl-p-nitroanilide-hydrochloride scanned at 420 nm (.....). (B) Proteolytic activities of each 2-mm fraction of an unstained but similarly prepared gel.

## DISCUSSION

The data presented here indicate that the strain of *A. flavus* var. *columnaris* used in this study produced two major proteases, P-I (an alkaline protease) and P-II (a neutral protease). There was no evidence for the existence of an acid protease in assays of crude enzyme extracts or in fractions from DEAE-Sephadex A-50 column chromatography. Perhaps irreversible denaturation by the conditions used for extraction of crude enzyme (i.e., 0.05 M phosphate buffer

at pH 7.0), rather than nonproduction, could account for this absence. The stability pH of the acid protease of *A. oryzae*, for example, was reported to be between pH 2 and 6 (10). However, since the pH of both the soybean koji and the early moromi are near 7.0, the practical role played by a sensitive acid protease in soy sauce brewing would be minimal. Yong and Wood (27) report that alkaline and neutral proteases play

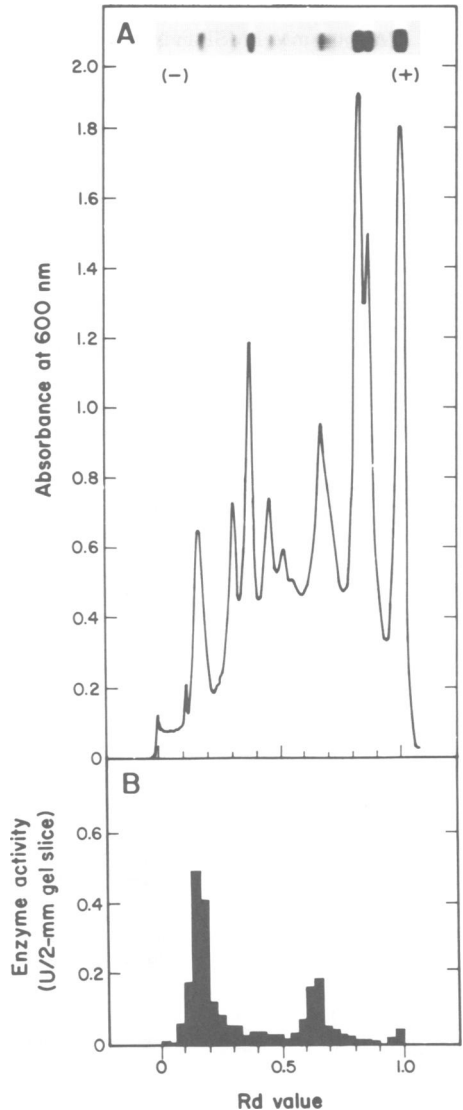


FIG. 6. PAGE pattern of crude enzyme extract from *A. flavus* var. *columnaris* grown in wheat bran culture. A crude extract (0.71 mg of protein) was loaded onto each gel. (A) Absorbance pattern of amido black-stained gel. (B) Proteolytic activity of the sliced gel.

TABLE 3. Effect of EDTA, PMSF, and EDTA + PMSF on the proteolytic activity of partially purified P-I, partially purified P-II, and crude enzyme preparations

Reaction mixture <sup>a</sup>	Proteolytic activity (%)			
	Partially purified <sup>b</sup> P-I	Partially purified <sup>c</sup> P-II	Crude extract <sup>d</sup> preparation	
			Wheat bran koji	Soy sauce koji
No inhibitor added	100	100	100	100
1 mM EDTA	97	1	79	70
0.1 mM PMSF in isopropanol	1	95	15	30
Isopropanol	100	96	98	100
1 mM EDTA + 0.1 mM PMSF in isopropanol	ND <sup>e</sup>	ND	2	6

<sup>a</sup> Reaction mixtures contained 1.0 ml of enzyme preparation with or without inhibitors and 1.0 ml of 1.5% casein. Proteolytic activities were determined as described in the text.

<sup>b</sup> 26  $\mu$ g of P-I per ml was used.

<sup>c</sup> 67  $\mu$ g of P-II per ml was used.

<sup>d</sup> 145.5  $\mu$ g of albumin equivalent per ml for wheat bran koji and 65  $\mu$ g/ml for soy sauce koji.

<sup>e</sup> ND, Not determined.

a significant role in the digestion of soybean protein during soy sauce processing, and most reports dealing with proteases isolated from *A. oryzae* or *A. sojae* (the most common fungi reported in soy sauce manufacture) are usually focused on these proteases (7, 12, 18–20, 21, 22).

The activity profiles of the alkaline P-I and neutral P-II described in this study are similar to those for proteases described from other fungi in the *A. flavus*-*A. oryzae* group. In a manner similar to that of P-I, alkaline proteases of *A. oryzae* (3, 13, 24), *A. sojae* (5a), and *A. flavus* (25) were found to be either inhibited by diisopropyl fluorophosphate or PMSF, and maximum activities fell within the pH range of 8 to 10.5 (3, 7, 11, 13, 24). As with P-II, neutral proteases of *A. oryzae* (12, 19, 20) and *A. flavus* (8), were inhibited by EDTA, and the optimum pH values for activities of neutral proteases of *A. oryzae* (3, 19, 20) and *A. sojae* were in the range of 5.5 to 7.0. The temperature for maximum enzyme activity for P-I and P-II (50 to 55°C) was also the same as that reported for *A. oryzae* (3, 12). However, Nakadai et al. (18) reported that an alkaline protease from *A. oryzae* had neither carboxypeptidase nor aminopeptidase activity, and this contrasts with the results for P-I reported here. The difference may be characteristic or may have resulted because of impurity of the P-I preparation.

The kinetics of total protease production in wheat bran and soy sauce koji showed that the largest increase in protease activity appeared in the late phase of fungal growth. This agrees with a report by Maxwell (11) for gelatinase activity with *A. oryzae* grown in steamed wheat bran. Also, Bergkvist (2) found that *A. oryzae*, when

grown in submerged cultivation on a protein- and carbohydrate-rich medium shows a marked increase in proteolytic enzymes as soon as abundant spore formation occurs. Yamamoto (26) reported a similar result for *A. sojae* strain KS.

Although the kinetics of protease production in wheat bran and soybean koji were found to be similar, the amount of protease produced was not (i.e., 1.70 kU/g [dry weight] of wheat bran and 0.85 kU/g [dry weight] of soybeans). Also, the maximum level of protease was reached much sooner in wheat bran koji (36 h) than in soybean koji (48 h). These differences could have arisen from a number of factors including the type of substrate used, the incubation temperature, the pH of the medium, the moisture content of the medium, etc. Maxwell (11) reported a substrate effect when *A. flavus-oryzae* is used, and Yamamoto (27) reported a temperature effect when *A. oryzae* is used. This study (data not shown) showed that the kinetics of P-I and P-II production in soy sauce koji paralleled those for total protease production. These results led to a recommendation for reducing the length of koji fermentation before transfer to salt brine from the traditional 4 to 7 days to only 2 days. This has meant more than a doubling of koji production capacity without any expansion of production facilities. The inhibitory effect of NaCl on P-I and P-II was similar to that reported by Nakadai et al. (18–20) for the alkaline and neutral proteases of *A. oryzae*. In addition, we have shown here that the residual proteolytic activity of P-I and P-II stored in 18% salt solution for 45 days was rather stable, but that the activity of the neutral protease (P-II) was more stable than that of the alkaline protease (P-I).



This could be an important consideration in the selection of high-protease fungal strains if one wished to increase moromi proteolysis via koji-produced enzymes. However, the most obvious conclusion from these results is that any substantial increase in soybean proteolysis via the koji-produced enzymes must be accomplished before the moromi incubation. With this in mind, together with the data on the temperature for maximum enzyme activity, a process has been devised to increase proteolysis by briefly incubating the koji (1 to 2 h) at 50 to 55°C at the end of the regular 2-day fermentation. This scheme is presently being tested, and it is hoped that it will increase proteolysis and perhaps reduce the time necessary for moromi incubation.

The reason for the difference in the proportional activities of P-I and P-II in wheat bran koji (8:2) and soybean koji (7:3) extracts is not immediately apparent. Perhaps differences in enzyme affinity (binding) for the substrate (22) or the presence of repressor components in the substrate (22) could account for this phenomenon. In any case, one should view the data on the relative activities presented here with some caution, especially when extrapolating them to soy sauce brewing. This is because all enzyme assays were performed using milk casein as the substrate, and therefore the proportion of total enzyme activity attributed to P-I and P-II can be strictly stated only for casein. The point is important, since Hattori and Matsuyama, as cited by Sekine et al. (22), have shown that the neutral protease from *A. sojae* has higher hydrolytic activity on soybean glycinine than does the alkaline protease. Also, the neutral protease has a higher affinity for soybean protein than for casein (23). More tests are necessary with soybean protein as the substrate before the precise activities of P-I and P-II may be given for soybean koji. Molecular weight determinations are necessary before precise quantities of P-I and P-II may be determined.

#### ACKNOWLEDGMENTS

We thank the owner of Nguan Chieng soy sauce factory, Pra-Pa-Dang, Sumut Prakan, Thailand, for cooperation in the preparation of soybean koji.

This work was supported by a research grant from the Association of South East Asian Nations-Australian Economic Cooperation Programme Protein Project and by the National Research Council of Thailand.

#### LITERATURE CITED

1. Anson, M. L. 1938. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* **22**:79-89.
2. Bergkvist, R. 1963. The proteolytic enzymes of *Aspergillus oryzae*. I. Methods for the estimation and isolation of the proteolytic enzymes. *Acta Chem. Scand.* **17**:1521-1540.
3. Bergkvist, R. 1963. The proteolytic enzymes of *Aspergillus oryzae*. II. Properties of the proteolytic enzymes. *Acta Chem. Scand.* **17**:1541-1551.
4. Bhumiratana, A., T. W. Flegel, T. Glinsukon, and W. Somporan. 1980. Isolation and analysis of molds from soy sauce koji in Thailand. *Appl. Environ. Microbiol.* **39**:430-435.
5. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum protein. *Ann. N.Y. Acad. Sci.* **121**:404-427.
- 5a. Hayashi, K., M. Terada, and K. Mogi. 1970. Enzymatic properties of purified alkaline proteinase from *Aspergillus sojae*. *Agric. Biol. Chem.* **34**:627-637.
6. Ichishima, E. 1970. Purification and mode of assay for acid proteinase of *Aspergillus oryzae*. *Methods Enzymol.* **19**:397-406.
7. Kundu, A. K. and S. Manna. 1975. Purification and characterization of extracellular proteinases of *Aspergillus oryzae*. *Appl. Microbiol.* **30**:507-513.
8. Lisenkov, M. V. and S. O. Tsiperovich. 1969. Properties of *Aspergillus flavus* protease. *Ukr. Biokhim Zh.* **41**:157-162.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
10. Matsubara, H., and J. Feder. 1971. Other bacterial, mold, and yeast proteases, p. 721-795. In Boyer, P. D. (ed.), *The enzymes*, 3rd ed., vol. 3. (Hydrolysis peptide bonds). Academic Press, Inc., New York.
11. Maxwell, M. E. 1950. Production of protease by cultivation of *Aspergillus oryzae* on wheat bran. *Aust. J. Appl. Sci.* **1**:348-362.
12. Misaki, T., M. Yamada, T. Okazaki, and J. Sawada. 1970. Studies on the protease constitution of *Aspergillus oryzae*. I. Systematic separation and purification of proteases. *Agric. Biol. Chem.* **34**:1383-1392.
13. Morihara, K., and H. Tsuzaki. 1969. Comparison of the specificities of various serine proteinases from microorganisms. *Arch. Biochem. Biophys.* **129**:620-634.
14. Nakadai, T., and S. Nasuno. 1977. The action of acid proteinase from *Aspergillus oryzae* on soybean proteins. *Agric. Biol. Chem.* **41**:409-410.
15. Nakadai, T., and S. Nasuno. 1977. Hyperproduction of peptidase and proteinase by mutants of *Aspergillus oryzae*. *J. Ferment. Technol.* **55**:273-276.
16. Nakadai, T., S. Nasuno, and N. Iguchi. 1972. The action of peptidases from *Aspergillus oryzae* in digestion of soybean protein. *Agric. Biol. Chem.* **36**:261-268.
17. Nakadai, T., S. Nasuno, and N. Iguchi. 1972. The action of peptidases from *Aspergillus sojae* on soybean proteins. *Agric. Biol. Chem.* **36**:1239-1243.
18. Nakadai, T., S. Nasuno, and N. Iguchi. 1973. Purification and properties of alkaline proteinase from *Aspergillus oryzae*. *Agric. Biol. Chem.* **37**:2685-2694.
19. Nakadai, T., S. Nasuno, and N. Iguchi. 1973. Purification and properties of neutral proteinase I from *Aspergillus oryzae*. *Agric. Biol. Chem.* **37**:2695-2701.
20. Nakadai, T., S. Nasuno, and N. Iguchi. 1973. Purification and properties of neutral proteinase II from *Aspergillus oryzae*. *Agric. Biol. Chem.* **37**:2703-2708.
21. Sekine, H. 1972. Neutral proteinases I and II of *Aspergillus sojae*: isolation in homogenous form. *Agric. Biol. Chem.* **36**:198-206.
22. Sekine, H., S. Nasuno, and N. Iguchi. 1970. Digestion of soybean protein by the proteolytic enzymes of *Aspergillus sojae*. A significant contribution of neutral proteinase. *Agric. Biol. Chem.* **34**:1690-1696.
23. Strongin, A., Ya., N. M. Azarenkova, T. I. Vaganova, E. D. Levin, and V. M. Stepanov. 1976. Visualization

- of leucineaminopeptidase activity after acrylamide gel electrophoresis. *Anal. Biochem.* **74**:597-599.
24. **Subramanian, A. R., and G. Kalnitsky.** 1964. The major alkaline proteinase of *Aspergillus oryzae*, aspergillopeptidase B. I. Isolation in homogenous form. *Biochemistry* **3**:1861-1867.
25. **Turkova, J., O. Mikes, K. Gancev, and M. Boublik.** 1969. Isolation and characterization of alkaline proteinase of *Aspergillus flavus*. *Biochem. Biophys. Acta* **178**:100-111.
26. **Yamamoto, K.** 1957. Koji: effects of cultural temperature on the production of mold protease. *Bull. Agric. Chem. Soc. Jpn.* **21**:319-324.
27. **Yong, F. M., and B. J. B. Wood.** 1974. Microbiology and biochemistry of soy sauce fermentation. *Adv. Appl. Microbiol.* **17**:157-194.