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Screening and characterization of koji molds producing saline-tolerant protease

N-W Su and M-H Lee

Graduate Institute of Agricultural Chemistry, National Taiwan University, Taipei 10617, Taiwan

Three mold strains isolated from soil in the Taipei area of Taiwan were compared with a commercial strain of *Aspergillus oryzae* for their proteolytic activities in an 18% NaCl aqueous solution system. Among these strains, the one subsequently identified and designated as *Aspergillus* sp. FC-10 produced protease with superior saline tolerance. In aflatoxin tests, this strain did not generate detectable aflatoxin after growing on steamed grain polished rice substrate for 24 days. Two types of extracellular proteases were preliminary fractionated by column chromatography on DEAE Sepharose CL-6B. Proteolytic activity of the nonadsorbed protease (P-I) was reduced to 9.4% in the 18% NaCl solution compared to its original activity determined in the buffer solution. However, the adsorbed protease (P-II) was particularly salt tolerant and stable, with 50% proteolytic activity retained throughout the 6-h stability test in 18% NaCl solution. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 230–234.

Keywords: salt tolerance; protease; koji; *Aspergilli*

Introduction

The conventional manufacture of soy sauce, an oriental fermented food, is time-consuming and complicated. The raw material is made from steam-treated soybean and roasted wheat by inoculating it with koji molds, usually *Aspergillus oryzae* or *Aspergillus sojae*, and then harvested as koji to be mixed with a high level of saline to make a mash for the subsequent hydrolysis of soy protein and development of the unique soy sauce aroma. Of these steps, the selection of the koji mold is extremely important because it promotes toxin-free production and ensures good flavour and aroma in the final product [3,34]. Therefore, the work of koji cultivation should concentrate on selection of koji molds producing high protease activity, which promotes the hydrolysis of soy protein and improves the quality of the final soy sauce products [3,5,13,14,18–21]. The methods explored to improve the protease activity of koji molds include traditional induced mutation, protoplast fusion and modern recombinant DNA technology [6,9,15,19–21,24,26,31]. However, the proteolytic activity of koji molds declines significantly when the harvested koji is mixed with concentrated sodium chloride solution to make a mash [10,16,17], resulting in retarded proteolytic hydrolysis of the soy protein and extending the overall maturation time for soy sauce production. Thus, the development of a salt-tolerant protease will contribute to the acceleration of proteolytic hydrolysis and consequently shorten the production time. Many researchers have studied fractionation and purification of proteases from koji molds, classified the purified proteases as alkaline, neutral and acid types according to their optimal pH, and demonstrated their roles in the manufacture of soy sauce [10,16–20]. Nevertheless, there have been no reports of saline-tolerant proteases from koji molds. In addition, the mechanism of protease hydrolysis of soy protein under high NaCl stress remains unclear. Consequently, it is important to isolate koji molds that produce proteases with superior saline tolerance. Further studies on the amino acid sequences and protein engineering of saline-tolerant proteases may help in the construction of more active saline-tolerant proteases.

In the present study, we describe the screening and characterization of mold strains producing salt-tolerant proteases. Aflatoxin formation tests, taxonomical studies, and preliminary fractionation of the proteases are also discussed.

Correspondence: Dr M-H Lee, Department of Agricultural Chemistry, National Taiwan University, Taipei 10617, Taiwan
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Materials and methods

Microorganisms and culture conditions

Soil samples from the Taipei area were mixed with defatted soybean and 10% NaCl as enrichment cultures and incubated at room temperature for several weeks with occasional mixing and moisture adjustment. A specific screening test described by Sekine *et al* [26] was employed with little modification. The protease-producing molds were selected from the clear zones formed through casein digestion by fungi grown on casein agar medium containing 5% or 10% NaCl to evaluate the productivity of the saline-tolerant protease. Colonies exhibiting relatively large clear zones were transferred to potato-dextrose agar medium (PDA; Difco Laboratories, Detroit, MI) containing 10% or 18% NaCl and cultured at 30°C for 2 weeks. Strains exhibiting robust growth were isolated and stored at 4°C and were subcultured on agar plates with 1% skim milk to confirm the ability to produce protease. Identification of the isolates was according to morphogenetic and physiological characteristics exhibited in cultures grown on Czapek's agar (CzA), malt extract agar (MEA), Czapek yeast extract agar (CYA) and Czapek yeast extract agar with 20% sucrose (CY20S) [4,22]. The commercial *A. oryzae* strain used was kindly provided by Dr Li-Yun Lin (Department of Food and Nutrition, Hung Kuang Institute of Technology, Sha-Lu, Taichung, Taiwan) from a factory in Taichung, Taiwan.

Preparation of spore inoculum and koji

To prepare a spore suspension inoculum, 10 ml of sterilized distilled water was added directly to a slant tube (diameter 16 mm) containing the fully grown mycelial mat on the surface of saline PDA medium at 30°C, and the spores were suspended by vigorous stirring. Koji was prepared at a laboratory scale. One hundred grams of steamed defatted soy flakes were mixed with an equal amount of crushed roasted wheat in the stainless steel trays and inoculated with 5 ml of spore suspension. Each tray was loaded with an approximately 3-cm thickness of the fermenting koji and incubated at 30°C and 92% relative humidity for 2 to 3 days. The koji was harvested when a greenish brown or yellow mass appeared as a result of mold growth and sporulation.

Preparation of crude protease extracts

Crude enzyme extracts were prepared according to Hirose's method [8] with little modification. The crude protease was extracted by mixing 100 g of harvested koji with 2 l of distilled water containing 0.9% sodium chloride. The mixture was left standing at ambient temperature with occasional stirring for 4 h and then filtered through Toyo No. 2 filter paper, or centrifuged at 5000×*g* for 20 min, to produce a supernatant containing 5% crude protease.

Crude protease concentration and preliminary fractionation

Five liters of crude protease extract was concentrated to about 1 l using a spiral-wound module ultrafiltration column (MW cut off: 10 kDa; Advanced Biotechnology Laboratories, Taipei, Taiwan), and in sequence, 10 l of distilled water were added into the concentrate to diafiltrate to a final volume of 500 ml. The resulting retentate was centrifuged at 5000×*g* for 20 min to remove the insoluble portion. The supernatant was lyophilized to produce crude protease powder and stored at −20°C. The preliminary fractionation of the crude protease was performed by anion exchange column chromatography. The crude protease powder (650 mg) was dissolved in 20 ml of 0.01 M phosphate buffer (pH 7.0) and loaded onto a DEAE Sepharose CL-6B column (Amersham Pharmacia Biotech, Uppsala, Sweden; 2.6 by 40 cm) previously equilibrated with 0.01 M phosphate buffer (pH 7.0). The column was then eluted with 1200 ml of the same buffer followed by 1500 ml of a linear gradient of 0–1 M NaCl in 0.01 M phosphate buffer (pH 7.0). The eluate was collected in 10-ml fractions and the proteolytic activity and protein content for each fraction were determined. Fractions showing proteolytic activity were merged and concentrated by ultrafiltration (MW cut off: 10 kDa), followed by overnight dialysis against distilled water at 4°C.

Assay for protease activity

Protease activity was assayed according to a method modified from Anson [1]. The reaction mixture for determination of protease activity contained 1 ml of enzyme solution and 3 ml of 1.5% Hammarsten milk casein in 0.1 M phosphate buffer (pH 7.0) and was incubated at 30°C for 20 min before the reaction was halted with 3 ml of 10% trichloroacetic acid (TCA). One unit of protease activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per minute in the reaction mixture at 30°C. To determine the effect of NaCl on proteolytic activity, amounts of NaCl were added to the reaction mixtures to obtain final concentrations of 5%, 10% and 18%. To determine the effect of salinity on enzyme stability, 18% NaCl was added to the enzyme solution, and the mixture was left standing at room temperature. Samples were taken at intervals and assayed for the remaining activities.

Assay for aflatoxins

Aflatoxin was assayed according to a procedure modified from AOAC official methods for analysis [2] and the FDA bacteriological analytical manual [30]. Fifty grams of grain polished rice was mixed with 50 ml of distilled water and autoclaved in a 300-ml Erlenmeyer flask for 15 min at 121°C. The medium was then inoculated with the isolate and incubated at 22–25°C until growth covered the entire surface and the mycelium penetrated to the bottom of the flask. The harvested culture was homogenized, and 50 g of homogenate was mixed with 5 g of NaCl and 100 ml of 80% methanol in a blender, and blended for 2 min at high speed. The homogenous liquid was filtered through a prefolded filter paper, and 10 ml of the filtrate was pipetted into 40 ml of distilled water and then filtered through a glass microfiber paper (Whatman GF/A). The final filtrate was then used as the sample for aflatoxin assay by affinity column chromatography as described in the AOAC official methods of analysis [2].

Results

Screening tests

Fungal strains from soils in the Taipei area were selected based on vigorous growth on saline PDA medium and transferred to the 1% skim milk–agar media containing various concentrations of NaCl. Further selection of the molds with high proteolytic activity was according to the clear zone criteria described above. Three filamentous fungi, designated as 6a, 11a and 13a, revealed notable proteolytic activities when cultivated on 1% skim milk–agar

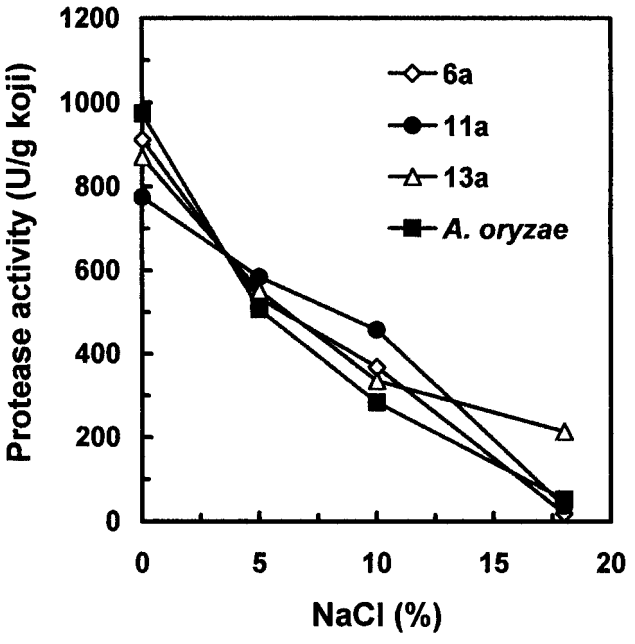

Figure 1 Effect of NaCl on the proteolytic activity of crude protease extracts from isolates 6a, 11a and 13a and an *A. oryzae* strain.

containing 18% NaCl during the 2-week incubation period. These three isolates and an *A. oryzae* strain were cultured to prepare the koji and their proteolytic activities at several NaCl levels were compared. Figure 1 illustrates the effect of various NaCl concentrations on the activities of the crude protease extracts from isolates 6a, 11a, 13a and the *A. oryzae* strain. The results demonstrated that the presence of sodium chloride greatly reduced the activities of all crude protease extracts. The activities of all crude protease extracts, except that from isolate 13a, dropped sharply to less than 10% of their original level in the presence of 18% NaCl. However, the proteolytic activity of the crude protease extract from isolate 13a exhibited a remarkable salt tolerance, retaining around 25% of its original activity in the presence of 18% NaCl.

Taxonomic studies of strain 13a

Isolate 13a was grown on CzA, MEA, CYA and CY20S media in order to observe the morphogenetic and physiological characteristics. Figure 2 presents photos revealing the morphological characteristics of 13a and the appearance of the clear zone on casein medium. The colonies were of white mycelia on CzA, MEA, CYA and CY20S, and grew to 6.5–7.1, 5.8–6.0, 4.8–5.8 and 6.4–7.4 cm diameter, respectively, by the seventh to the tenth day at 25°C. Conidiophores arose directly from the MEA culture and terminated in a swollen vesicle bearing flask-shaped phialides. The conidia are produced in long chains at the ends of the phialides. Conidial heads were radiate and columnar (poorly defined), yellow-green in color and turned to olive green with age (approximately 2 weeks). The conidiophores on MEA were not tightly packed with a size of 4.2–8.4 by 88–570 μm. Vesicles revealed globose to subglobose shapes, 15 to 39 μm in diameter standing on the conidiophores. Sterigmata were mostly on the upper half of the vesicles, uniseriate and 4 to 17 μm in length. Conidia were ball-shaped, 3–6 by 3–7 μm in size, with a smooth or echinulate surface. The isolate was classified as a part of the *Aspergillus flavus* group and is similar to *A. flavus* var. *columnaris* except for the length of some conidiophores and the size of vesicles [22]. We temporarily named this isolate *Aspergillus* sp. FC-10 and have deposited it in our culture collection pending further classification. *Aspergillus* sp. FC-10 was subjected to an assay

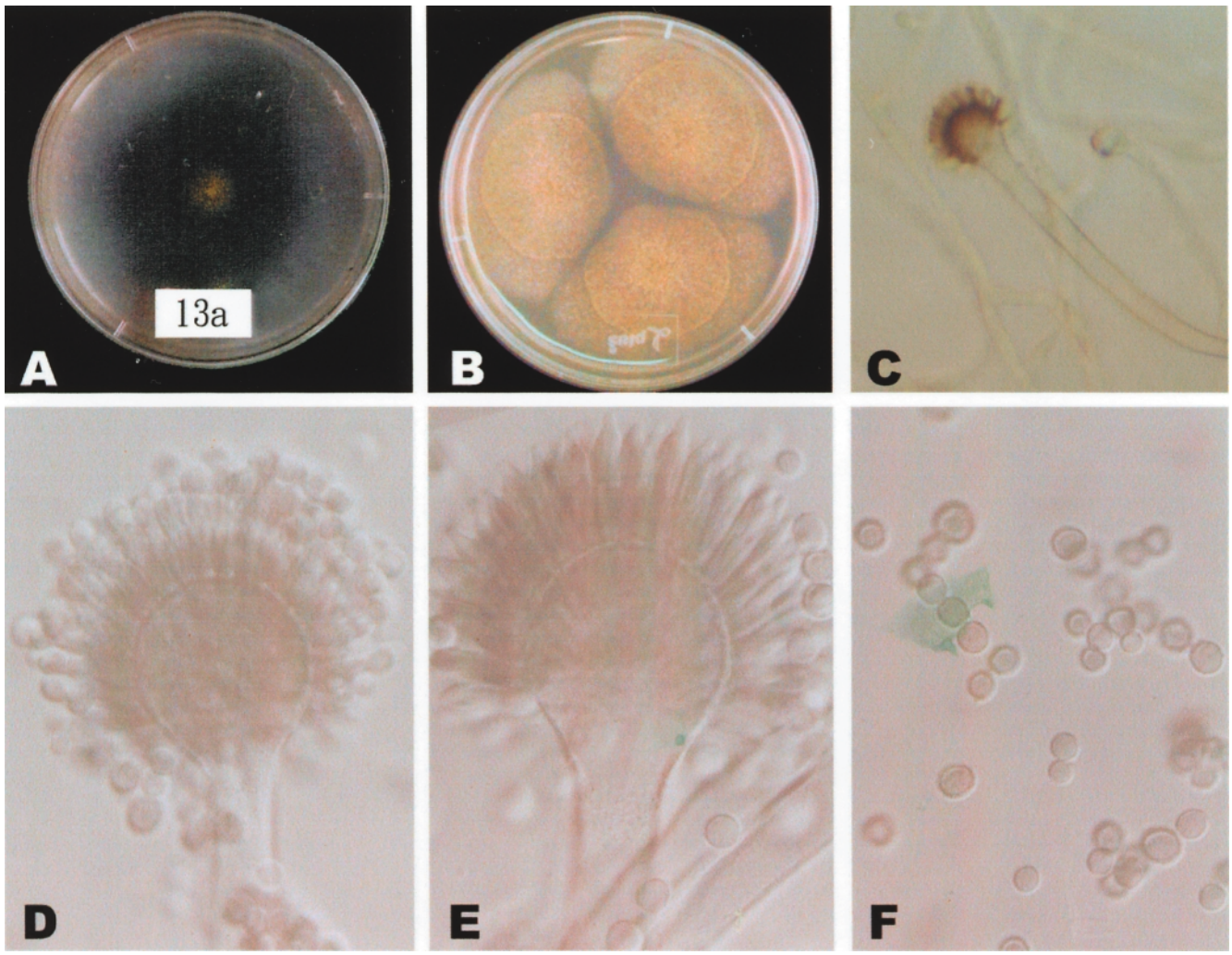

Figure 2 Photographs of isolate 13a designated as *Aspergillus* sp. FC-10. (A) Clear zone formation on casein medium containing 5% NaCl at 30°C for 96 h, (B) appearance of colonies on CzA, (C) young conidiophores arose directly from MEA (200×), (D) young conidiophore showing major uniseriate sterigmata (1000×), (E) young conidiophore showing minor biseriate sterigmata (1000×), (F) mature spores (1000×).

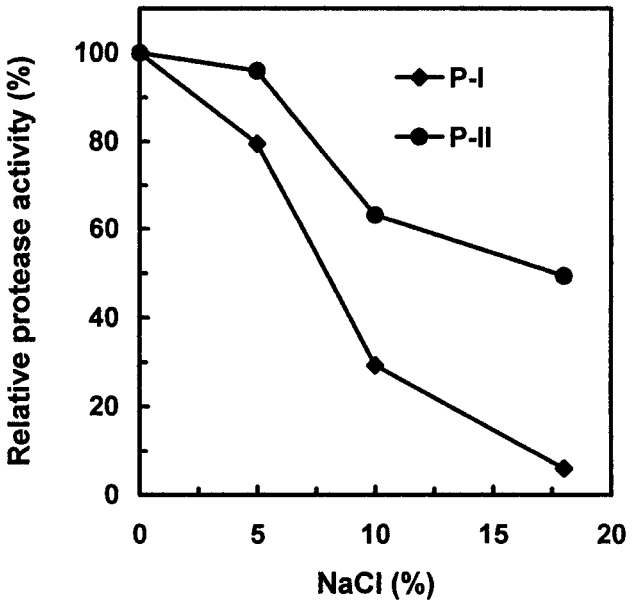

Figure 3 Effect of NaCl on the proteolytic activities of partially purified proteases P-I and P-II from *Aspergillus* sp. FC-10.

for aflatoxin as a prelude to its use in food production, and no aflatoxin was detected in triplicate tests.

Protease profile of crude protease extract from Aspergillus sp. FC-10 and preliminary fractionation

In preliminary fractionation tests, the crude protease powder was dissolved in 0.01 M phosphate buffer (pH 7.0) and fractionated by DEAE Sepharose CL-6B column chromatography. The results demonstrated that the two proteases in the crude extract could be effectively separated into two fractions. One of these was in the nonadsorbed fraction and was referred to as P-I. It contributed around 70% of the original activity in the crude protease. The other was in the adsorbed fractions and was referred to as P-II. This was eluted by NaCl concentrations higher than 0.2 M.

Effect of sodium chloride on the activity of the partially purified protease

Figure 3 illustrates the reduction of activity in the presence of sodium chloride for both partially purified proteases (P-I and P-II). The activities for P-I and P-II were reduced to 8% and 49%, respectively, of original levels in the presence of 18% NaCl. The residual activities of P-I and P-II after incubation in 18% NaCl at room temperature for different durations are shown in Table 1. The residual activity of P-I decreased continuously to near 4% of its original level at the end of a 6-h incubation. For P-II, the activity remained rather constant after a 30-min incubation in 18% NaCl solution, and approximate 46% of the original activity remained after a 6-h incubation.

Discussion

There have been reports of fungi living under environmental stress condition where the fungal morphology *in situ* showed an optimal small surface–volume ratio for the colonies [29,33]. Sterflinger's study of the NaCl tolerance of rock-inhabiting meristematic fungi [28] revealed that all of the isolates grew better without NaCl and their growth rate was decreased by increasing NaCl concentration. *Hortaea werneckii*, however, was an exception, with improved growth in 7% NaCl. *H. werneckii* was shown to be adaptive toward NaCl or a halophile [28]. However, it was reported that salt-tolerant yeasts played an important role in the formation of flavor components such as 4-hydroxy-2 (or 5)-ethyl-5 (or 2)-methyl-3 (2*H*)-furanone (HEMF) during soy sauce manufacture [25]. According to our observations, our isolates all grew more slowly in the presence of increased NaCl concentrations in agar media and sporulated earlier. From the present study, we found that a mold possessing salt-tolerant characteristics could produce extracellular salt-tolerant proteases, but only a few filamentous fungi were able to survive on high NaCl agar. These fungi exhibited remarkably high tolerances against NaCl and could even grow on 18% NaCl agar. In the present study, three isolates (6a, 7a and 13a) were selected based on their proteolytic activities when grown on casein–agar medium with 18% NaCl. Among these isolates, the 13a strain excreted extracellular proteases that displayed a remarkable salt tolerance, with about 25% residual activity retained under 18% NaCl stress. There have been few reports of salt-tolerant proteases except for some from halophiles and archaeobacteria [11,12,23]. To our knowledge, there have been no previous reports of salt-tolerant proteases from filamentous fungi. In our research, we isolated the protease P-II with superior salt tolerance from strain, 13a, and we found that around 50% of its original proteolytic activity was retained in the 18% NaCl reaction system.

This phenomenon is of great interest because most koji proteases retained less than 10% of their original proteolytic activities in the presence of 18% NaCl [10,16,17]; perhaps the active proteases were salted out and denatured decreasing the protease content and/or inactivating the protease in the high-NaCl solutions.

We are investigating the use of *Aspergillus* sp. FC-10 as a koji mold for manufacturing soy sauce. However, the identification of this isolate as an *Aspergillus* sp. causes some concern over the possibility of toxin production, especially the aflatoxins. It was reported that strains of *A. oryzae* were very closely related to *A. flavus* and perhaps should be classified as a species of *A. flavus* Link [32]. Precise identification of the 13a isolate has not been completed, because only relatively limited morphogenetic and physiological criteria are available at this stage. However, we have tested strain 13a for aflatoxin production by the FDA method to evaluate the aflatoxin production by molds [30], and no toxin was

Table 1 Effect of 18% NaCl on stability of the partially purified proteases from *Aspergillus* sp. FC-10

Protease[a]	Original activity (units)	Protease activity (units) after incubation[b] in 18% NaCl for (h)				
		0.5	1	2	4	6
P-I	457 (100)[c]	47 (10)	38 (8)	23 (5)	22 (5)	19 (4)
P-II	212 (100)	102 (48)	98 (46)	101 (48)	101 (48)	97 (46)

[a]P-I and P-II were obtained by DEAE Sepharose CL-6B column chromatography.
[b]Incubated at room temperature.
[c]Numbers in parentheses refer to the percentages of original protease activity.

detected. In addition, it has been reported that soybean is a poor substrate for aflatoxin production [7,27]. Therefore, *Aspergillus* sp. FC-10 could be recommended as a potential koji mold for further improvement of soy sauce manufacture. Further research including elaboration of the activities of other related enzymes, exact identification of the strain and extensive toxicity tests are all still necessary for evaluating the possibility of utilizing *Aspergillus* sp. FC-10 in commercial applications.

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