CHARACTERIZATION OF EXTRACELLULAR PROTEINASES OF THE GENUS *MONASCUS* BY THEIR pH-ACTIVITY PROFILES

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Extracellular proteinase activities produced by 36 strains in the genus *Monascus* were determined at various pHs using casein and Azocoll as substrates. These strains were grouped into three types (A, B, and C) according to the pH-activity profiles of their proteinases.

Type A was characterized by only acid proteinases with an optimal pH around 3 and was found in eight strains of six species of *Monascus* (i.e., *M. araneosus*, *M. fuliginosus*, *M. kaoliang*, *M. pilosus*, *M. pubigerus*, and *M. vitreus*). Type B was characterized by only alkaline proteinases with an optimal pH about 8-9 and was found in 24 strains of nine species and two varieties of *Monascus* (i.e., *M. albidus*, *M. albidus* var. *glaber*, *M. anka*, *M. anka* var. *rubellus*, *M. araneosus*, *M. kaoliang*, *M. major*, *M. purpureus*, *M. ruber*, *M. rubiginosus*, and *M. serorubescens*). Type C was characterized by the two kinds of proteinases observed in types A and B and was found in three strains of one species of *Monascus* (i.e., *M. kaoliang*).

It was revealed in this study that *Monascus* spp. secreted extracellular alkaline proteinases. However, there was not any considerable correlation between the classification system of the genus *Monascus* and the type of proteinase produced.

Monascus spp. have been widely used for manufacturing Oriental fermented foods such as red rice wine, red soy bean cheese, etc. Recently, its pigments have been used in food coloring as natural food additives in place of synthetic coloring agents.

We have been studying the genus *Monascus* from the view points of biochemical, biological, and taxonomical aspects. From the results of morphological and

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physiological studies, IIZUKA and LIN (1) presented a new classification system of the genus *Monascus*, and defined *M. kaoliang* as a new species. Then IIZUKA and MINEKI (2, 3) characterized the enzymatic properties and specificities of extracellular glucoamylases purified from *M. kaoliang* F-1. So far, there have been many reports of extracellular proteinases from various fungi (4, 5), but for *Monascus* spp. little information is now available except for the reports dealing with acid proteinases from limited numbers of species (6, 7) and strains (8).

In the present study, we investigated the extracellular proteinase production by 36 strains of *Monascus* spp. and compared the pH-activity profiles of their proteinases with the classification system. To detect almost all the types of proteolytic activity caused by acid, neutral and alkaline proteinases, casein was adopted as a basal substrate, because it is a typical substrate for screening general proteinases of fungi (5), although it is insoluble in the pH range 3.5–6. In addition, Azocoll (10) was also used as a substrate, because it is usable in a wide pH range especially to characterize neutral and alkaline proteinases. These are particularly interesting, for their existence had not been known.

MATERIALS AND METHODS

Microorganisms. Twenty-two strains in the genus *Monascus* were isolated in Southern East Asia, and the other 14 strains were purchased from IAM and IFO culture collections. These are listed in Table 1. The test strains were maintained on a potato-dextrose-agar slant (pH 6.0) at 28°C and preserved at 7°C.

Cultivation. Ten grams of wheat bran and 12 ml of distilled water in a 200-ml Erlenmeyer flask was autoclaved at 120°C for 20 min, cooled and inoculated with 1 ml of a heavy suspension of spores and mycelia in sterilized distilled water. The solid medium culture was incubated statically at 30°C for 5 days with hand-shaking once a day.

Preparation of crude enzyme solutions. Two hundred milliliters of 10 mM phosphate buffer (pH 7.0) was added to the culture, and filtered through Toyo filter paper No. 2 after gentle stirring for 60 min. One hundred milliliters of the filtrate was saturated to 80% with ammonium sulfate and the resulting precipitate was collected by centrifugation at 7,000 rpm for 10 min. The precipitate was dissolved in 15 ml of 10 mM phosphate buffer (pH 7.0) and dialyzed against 3 l of the same buffer at 5°C. The dialyzed solution was filled up to 50 ml with the same buffer and used as a crude enzyme solution.

Proteolytic activity assay. Casein (Hamersten, Merck) and Azocoll (Sigma Chemical Co.), which is immobilized cowhide protein modified with an azo-dye on porous glass, were used as substrates for determining proteolytic activity.

To assay proteinase activity with casein (9), 1 ml of the properly diluted crude enzyme solution and 1 ml of 1% casein in 0.2 M buffer (pH 2.5–9.0) were mixed and incubated at 30°C for 60 min. The reaction was stopped by adding 3 ml of 5% trichloroacetic acid (TCA) solution. After standing for 30 min at room temperature,

Table 1. Strains of *Monascus* spp. examined and the protein contents of their crude enzyme solutions.

No.	Species and strain ^a		Protein in crude enzyme solution ($\mu\text{g/ml}$) ^b
1	<i>M. anka</i> Sato MR MO-6	IAM 8001	122
2	<i>M. anka</i> Sato VN-1	isolated in Vietnam	249
3	<i>M. anka</i> var. <i>rubellus</i> Sato MR MO-5	IAM 8081	212
4	<i>M. araneosus</i> Sato MR MO-7	IAM 8083	84
5	<i>M. araneosus</i> Sato MR MO HH-2	isolated in Hong-Kong	143
6	<i>M. araneosus</i> Sato K-2	isolated in Kimmon	153
7	<i>M. araneosus</i> Sato LH-3	isolated in Hong-Kong	109
8	<i>M. araneosus</i> Sato VN-3	isolated in Vietnam	82
9	<i>M. kaoliang</i> Iizuka T-1-1	isolated in Thailand	78
10	<i>M. kaoliang</i> Iizuka T-1-2	isolated in Thailand	71
11	<i>M. kaoliang</i> Iizuka T-2	isolated in Thailand	125
12	<i>M. kaoliang</i> Iizuka MR MO-F1	isolated in Taiwan	83
13	<i>M. kaoliang</i> Iizuka MR MO-F2	isolated in Taiwan	195
14	<i>M. kaoliang</i> Iizuka HH-1	isolated in Hong-Kong	112
15	<i>M. kaoliang</i> Iizuka HH-3	isolated in Hong-Kong	230
16	<i>M. kaoliang</i> Iizuka H-D	isolated in Hong-Kong	75
17	<i>M. kaoliang</i> Iizuka H-L	isolated in Hong-Kong	126
18	<i>M. kaoliang</i> Iizuka K-1	isolated in Kimmon	138
19	<i>M. kaoliang</i> Iizuka LH-2	isolated in Hong-Kong	75
20	<i>M. kaoliang</i> Iizuka M-2	isolated in China	57
21	<i>M. kaoliang</i> Iizuka C-1	isolated in Cambodia	184
22	<i>M. kaoliang</i> Iizuka C-2	isolated in Cambodia	82
23	<i>M. kaoliang</i> Iizuka C-3	isolated in Cambodia	236
24	<i>M. kaoliang</i> Iizuka VN-2	isolated in Vietnam	143
25	<i>M. pilosus</i> Sato MR MO-4	IAM 8003	25
26	<i>M. pubigerus</i> Sato MR MO-8	IAM 8004	15
27	<i>M. purpureus</i> Went MR MO-9	IAM 8010	206
28	<i>M. ruber</i> van Tieghem MR MO-10	IAM 8009	187
29	<i>M. ruber</i> van Tieghem MR MO-LH-1	isolated in Hong-Kong	210
30	<i>M. vitreus</i> Sato MR MO-3	IAM 8007	22
31	<i>M. albidus</i> Sato	IFO 4489	182
32	<i>M. albidus</i> var. <i>glaber</i> Sato	IFO 4486	217
33	<i>M. fuliginosus</i> Sato	IFO 4483	224
34	<i>M. major</i> Sato	IFO 4485	212
35	<i>M. rubiginosus</i> Sato	IFO 4484	228
36	<i>M. serorubescens</i> Sato	IFO 4487	218

^a Abbreviations: IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; MR, Institute for Microbial Resources, Tokyo, Japan.

^b Indicated are the values from which the value of an un-inoculated sample is subtracted.

the absorbance of the TCA-soluble fraction was measured at 280 nm by a spectrophotometer. To assay the activity with Azocoll (10), 20 mg of Azocoll suspended in 3 ml of 0.13 M buffer (pH 2.5–9.0) and 1 ml of the diluted enzyme solution were incubated for 60 min at 30°C with gentle shaking. The reaction was stopped by adding 1 ml of 1 M NaOH, and the absorbance of the supernatant was

Fig. 1. pH-Activity profiles of extracellular proteinases produced by *Monascus* spp. Proteolytic activity of each crude enzyme solution was measured using casein (○) and Azocoll (●) as substrates as described in the text. The number set in each box corresponds to that of the strain listed in Table 1.

measured at 520 nm. For convenience, one unit of proteinase activity for each assay was defined as the amount of enzyme which increases 1 OD unit in the absorbance at 280 or 520 nm per 60 min. The buffers used were: citrate-phosphate buffer (pH 2.5–7.0) and Tris-HCl buffer (pH 8.0–9.0).

Determination of protein contents. Protein concentration of the crude enzyme solution was determined by the method of BRADFORD (11) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The amount of protein and proteinase activity in each crude enzyme solution prepared from 36 strains of 13 species and two varieties of *Monascus* grown on the wheat bran medium were determined. The protein contents of the crude enzyme solutions ranged from 15 $\mu\text{g/ml}$ to 249 $\mu\text{g/ml}$, as shown in Table 1. Except for one strain, *M. kaoliang* H-D which showed only slight activity, 35 strains had more or less distinct proteolytic activities against casein or Azocoll. The activity of each enzyme preparation was assayed at various pHs, and expressed in terms of activity unit for 60 min of the reaction per ml of the crude enzyme solution. Figure 1 shows the pH-activity profiles obtained from all the strains examined. Stronger activities were generally seen for samples containing larger amounts of protein. Two main proteinase activities were observed around pH 3 for casein and at pH 8 to 9 for both casein and Azocoll. As Azocoll was not hydrolyzed by pepsin at low pH to any extent, the activity detected near pH 3 was presumed to arise from pepsin-like acid proteinases, as reported earlier (7,8).

According to the optimal pH of the proteinase activity determined in this study, 35 strains in the genus *Monascus* were grouped into 3 types (A, B, and C). Type A strains produced only acid proteinase, type B strains produced only alkaline proteinase, and type C strains produced both acid and alkaline proteinases. The number of strains grouped into each type of proteinase production are summarized in Table 2. The most abundant strains belonged to type B (24 strains of nine species and two varieties of *Monascus*), followed by type A (eight strains of six species of *Monascus*) and type C (three strains of one species of *Monascus*). It was noteworthy that strains in *M. araneosus* and *M. kaoliang* were classified into different types of proteinase production. We have not yet found a reasonable correlation between the type of proteinase production and the classification system offered by IIZUKA and LIN (1). TSAI et al. (7) and YASUDA et al. (8) examined pepsin-like acid proteinases prepared from a strain of *M. kaoliang* and an unidentified strain of *Monascus* sp. grown on a steamed rice medium. Neither group has shown any activity in the alkaline region so far. The obvious proteinase activity in the alkaline region was first detected in this study. To examine the effect of growth conditions, several strains of each type were cultured on a steamed rice medium. All the strains examined showed caseinolytic activity around pH 3 but not in the alkaline region. The protein contents of crude enzyme solutions were also less than those on the wheat bran solid culture.

Table 2. The number of strains of *Monascus* spp. grouped into the three types of proteinase production.

Species	A	B	C	Total
<i>M. albidus</i>	—	1	—	1
<i>M. albidus</i> var. <i>glaber</i>	—	1	—	1
<i>M. anka</i>	—	2	—	2
<i>M. anka</i> var. <i>rubellus</i>	—	1	—	1
<i>M. araneosus</i>	1	4	—	5
<i>M. fuliginosus</i>	1	—	—	1
<i>M. kaoliang</i>	3	9	3	15
<i>M. major</i>	—	1	—	1
<i>M. pilosus</i>	1	—	—	1
<i>M. pubigerus</i>	1	—	—	1
<i>M. purpureus</i>	—	1	—	1
<i>M. vitreus</i>	1	—	—	1
<i>M. ruber</i>	—	2	—	2
<i>M. rubiginosus</i>	—	1	—	1
<i>M. serorubescens</i>	—	1	—	1
Total	8	24	3	35

From these results, it appeared that some growth conditions were responsible for the enzyme production and the type of proteinases, especially for alkaline proteinase, although details of effective factors are not clear at present.

To gain a better understanding of how strains of *Monascus* spp. are characterized and how their enzymes are industrially utilized, it will be important to investigate enzymologically the features and similarity of excreted proteinases. In particular, the finding of alkaline proteinases provides a subject of considerable interest. Therefore, the properties of the alkaline proteinase found in this study are now under investigation.

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