Monascus pilosus の産生する酸性プロティナーゼの酵素化学的性質(生物資源科学科)

Properties of Acid Proteinase from *Monascus pilosus*

Masaaki YASUDA*, Yoshio KATO*, Satoshi ISHIKAWA**,
Shinkichi TAWATA*, Naotada KOBAMOTO*, and Seizen TOYAMA*

Summary

The screening was carried out to find *Monascus* fungus that would produce a high activity of the acid proteinase to be used for making fermented protein foods. *Monascus pilosus* was found to have the highest activity of the enzyme which was formed by solid state culture.

Properties of the enzyme were investigated. The optimum pH was around 2.5 and the optimum temperature was 50 ℃. The enzyme was stable during a 10-min treatment at 50 ℃ in a range of pH 3.0～6.0 and was still stable up to 55 ℃. The enzyme was active on human hemoglobin, milk casein, serum albumin, and isolated soybean protein. However, the enzyme was not active on β-lactogloblin, and gelatin. The enzyme was markedly inhibited by pepstatin.

Introduction

The mold *Monascus* has been used in the fermentation industry not only for preparation of red rice wine, red Shao-Hsing wine, and red sufu in China but also for that of tofuyo (fermented tofu) in Okinawa Prefecture, Japan[10]. Studies on tofuyo-manufacturing have been carried out in our laboratory[10-13,15,16]. The red koji prepared by growing *Monascus* steamed rice is an important material for tofuyo manufacturing. It is a source of enzymes for converting starch into fermentable sugars and proteins into peptides and amino acids. The ripening of this food is always greatly affected by quality of the koji, whose proteinase is thought to be a key enzyme[13]. Although fungal proteinases have been extensively investigated[1-4,6,8,9], information on the enzyme produced by *Monascus* fungus is limited. The authors reported production and properties of the enzyme prepared by the method of submerged cultivation[14].

Recently, vegetable protein food has been recognized as the food which is a cholesterol free food. In order to develop the fermented vegetable protein foods for the health, the microorganism which has a

* Department of Bioscience and Biotechnology, University of the Ryukyus
1, Senbaru, Nishihara-cho, Okinawa 903-01 Japan
** Department of Research and Development, Tropical Technocenter Limited
568, Toyama, Urasoe-city, Okinawa 901-21 Japan
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high proteinase activity in the solid state cultivation is needed.

In this paper, screening of *Monascus* fungus which has a high proteinase activity obtained by the method of solid state culture and some properties of the enzyme are described.

Materials and Methods

Microorganisms : Twelve strains of genus *Monascus* in the type-culture collection preserved in the department of Bioscience and Biotechnology, University of the Ryukyus. Sixteen strains were isolated from red koji and fermentation foods such as sufu, Hong Zhaou, *etc*. in China, Hong Kong, and Taiwan.

Chemicals : Hammarsten milk casein was obtained from Merck, Germany. Isolated soybean protein was presented from Fuji Oil Co. Ltd., Japan. The other chemicals were analytical grade.

Assay methods : Proteinase activity was assayed as stated in our previous paper[14]. The reaction mixture, containing 1.0 ml of 2% Hammarsten milk casein solution, 0.5 ml of 0.4 M lactate buffer (pH 3.0), 0.5 ml of water, and 1.0 ml of enzyme solution, was incubated at 37 ℃ for 10 min. One unit of the enzyme was defined as the amount of enzyme which liberated 1 μmol of tyrosine from the Hammarsten milk casein under the above conditions. The specific activity was expressed as units per mg of protein.

The amount of protein in the enzyme solution was determined by the method of Lowry *et al*.[5] and by using bovine serum albumin as the standard.

Screening of proteinase was carried out as follows; Each microorganism was tested for the abilities of proteinase production on the solid state medium (koji). Polished rice (60 g) was soaked in water overnight. Excess water was drained off. The swollen rice was cooked with steam at 120 ℃ for 20 min. The microorganisms were inoculated in the medium in 500-ml flasks. And then the media were incubated at 32 ℃ for 8 days. The enzyme was extracted as follows; Red koji (10 g) was suspended in 30 ml of 10 mM citrate-20 mM sodium phosphate buffer (pH 5.0) for an hour at room temperature (25 ℃). The insoluble residue was removed by filtration through cloth and centrifugation and the supernatant was used as the crude enzyme.

Results and Discussion

1. Screening of acid proteinase in *Monascus* fungus

The screening was carried out to find the strains in *Monascus* fungus, that would produce a high activity of acid proteinase. As shown in Table 1, a high activity was found in the kojis of *Monascus pilosus*, *Monascus* sp. 4820, *Monascus vitreus*, *Monascus fuliginosus*, and *Monascus* sp. H-1. *Monascus pilosus*, in which acid proteinase occurs most abundantly, was chosen for the purpose of production of the enzyme.

2. Properties of the enzyme

Effect of pH on activity and stability of the enzyme

The effect of pH on activity of the enzyme is shown in Fig.1. The enzyme is most active at around pH 2.5 for milk casein hydrolysis. Being similar to the enzyme of *Corticium rolfsii* (pH 2.5)[6] and of

Table 1. Distribution of Acid Proteinase in Various Strains of *Monascus* Fungus

Strains	Enzyme activity (units/mg)	Strains	Enzyme activity (units/mg)
Monascus anka IFO 4478	0.052	*Monascus* sp. C-1	0.028
Monascus anka IFO 6540	0.044	*Monascus* sp. C-1-1	0.048
Monascus anka var. rubellus IFO 5965	0.028	*Monascus* sp. C-3	0.055
Monascus araneosus IFO 4482	0.103	*Monascus* sp. C-4	0.084
Monascus fuliginosus IFO 4483	0.234	*Monascus* sp. C-6	0.047
Monascus major IFO 4485	0.100	*Monascus* sp. C-11	0.068
Monascus paxii IFO 8201	0.064	*Monascus* sp. H-1	0.178
Monascus pilosus IFO 4480	0.484	*Monascus* sp. H-2	0.021
Monascus pubigerus IFO 4521	0.114	*Monascus* sp. H-3	0.145
Monascus ruber IFO 4492	0.113	*Monascus* sp. HK	0.033
Monascus vitreus IFO 4532	0.348	*Monascus* sp. N	0.107
Monascus vitreus IFO 7537	0.145	*Monascus* sp. T-1	0.057
Monascus sp. 4303	0.043	*Monascus* sp. TSG	0.057
Monascus sp. 4820	0.365		

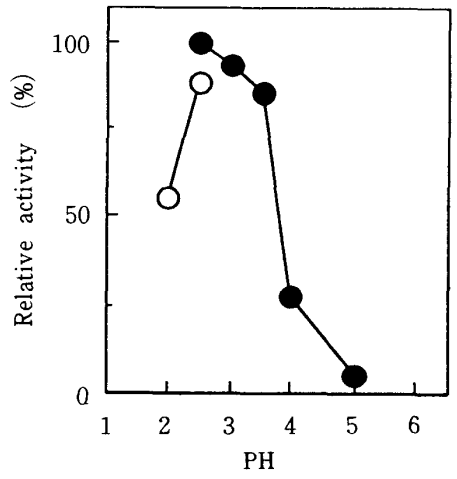

Fig. 1

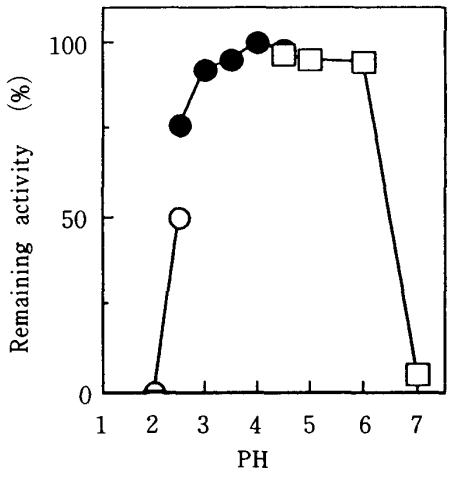

Fig. 2

Fig.1 Effect of pH on the Activity of Acid Proteinase from *Monascus pilosus*
The enzyme activity was assayed at various pHs. Assay conditions are shown in the text.
Buffers : -○- ; HCl-Sodium citrate buffer,
-●- ; Lactate buffer

Fig.2 pH Stability of Acid Proteinase from *Monascus pilosus*
The enzyme was incubated at 50 ℃ for 10 min, and the activity was then measured under the standard assay conditions.
Buffers : -○- ; HCl-Sodium phosphate buffer,
-●- ; Lactate buffer,
-□- ; Citrate-Sodium phosphate buffer

Aspergillus saitoi (pH 2.5～3.0) [3], this value was lower than other fungus proteinase (*Rhizopus* enzyme ; pH 2.9-3.3 [1], *Monascus* enzyme: 3.2[14]). As shown in Fig.2, the enzyme was quite stable for a 10-min treatment in a range of pH 3.0 to 6.0 when it was maintained at 50 ℃ for 10-min, but lost 99% of the initial activity at pH 2.0 and 95% at pH 7.0. The pH stability of the enzyme was almost similar to the case of enzymes in *Aspergillus saitoi* (pH 3.0～5.0) [3], *Aspergillus oryzae* (pH3.6～6.0) [9] and *Rhizopus chinensis* (pH 2.8～6.5) [1].

Effect of temperature and stability of the enzyme

The enzyme reaction was carried out at various temperatures at pH 2.5 for 10 min. The temperature for the maximum enzyme activity was 50 ℃ as shown in Fig.3. This value was similar to that of

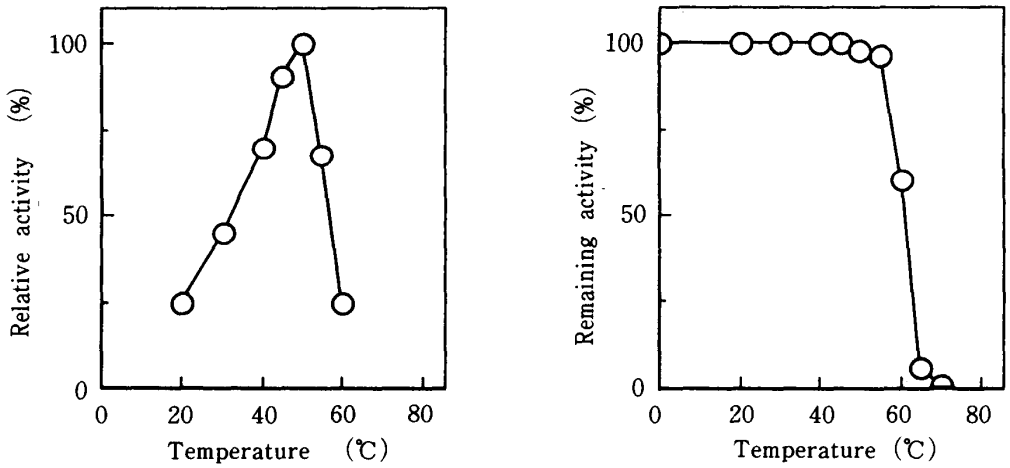

Fig.3 Effect of Temperature on the Activity of Acid Proteinase from *Monascus pilosus*
The enzyme activity was assayed at various temperatures. Assay conditions are shown in the text.

Fig.4 Temperature stability of acid proteinase from *Monascus pilosus*
The enzyme was incubated at various temperatures at pH 2.5 for 10 min, and the residual activity was then measured under the standard conditions.

the enzyme of *Monascus* sp.[14] but was lower than that of *Rhizopus chinensis* (60 ℃) [1]. The enzyme solution was incubated at various temperatures for 10 min at pH 2.5 and the remaining activities were checked under the standard assay conditions. The results indicated that the enzyme was stable up to 55 ℃, 60% of the activity of the enzyme remained at 60 ℃, and no activity remained at 75 ℃ as shown in Fig. 4. This limit temperature for stable activity was higher than that of *Aspergillus oryzae* enzyme [E1 (50 ℃)[9], E2 (40℃)[9]].

Substrate specificity

Substrate specificity of the enzyme was investigated. As shown in Table 2, the enzyme catalyzes the hydrolysis of various proteins, of which human hemoglobin, milk casein, bovine serum albumin, and isolated soybean protein were better substrates than human γ-globulin, egg albumin, and wheat gluten. β-Lactogloblin, gelatin and keratin were not substrates for the enzyme. Relative activity of the enzyme of *Monascus pilosus* for isolated soybean protein as 1.4 times higher than that of *Monascus* sp.[14]. However, relative activity of the former for wheat gluten was lower than that of the latter. Therefore, it is considered that the strain of *Monascus pilosus* is useful for making a novel fermented soybean protein foods.

Table 2. Substrate Specificity of Acid Proteinase from *Monascus pilosus*

Substrates	Relative activity (%)
Hammarsten milk casein	100
Human hemoglobin	145
Human gamma globulin	47
β-Lactogloblin	0
Isolated soybean protein	60
Gluten	20
Bovine serum albumin	89
Egg albumin	32
Keratin	4
Gelatin	0

Inhibitory studies

The effects of proteinase inhibitors on the enzyme activity were investigated. As shown in Table 3, the enzyme was completely inhibited by pepstatin A and was inhibited 41～54% by 1mM Pb^{2+} and Sn^{2+} ions. It was not inhibited by *p*-chloromercuribenzoate, diiospropylfluorophosphate, soybean trypsin inhibitor, ethylenediaminetetraacetate, or *o*-phenanthroline. The enzyme activity was not affected by Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+} and Cu^{2+} ions. The enzyme was markedly inhibited by pepstatin A. It was reported that acid proteinases of other fungi such as *Aspergillus saitoi*[7], *Mucor pusillus*[7], *Penicillium janthinellum*[7], and *Rhizopus chinensis*[7] were also inhibited by pepstatin. It is well known that inactivation of acidic proteinase by pepstatin was elucidated by that carboxylic group of aspartic acid in active center of the enzyme was modified by the chemical. Therefore, it was supposed that the proteinase of *Monascus pilosus* had carboxylic group in the active center.

Table 3. Effect of Chemical Compounds on the Enzyme Activities

Chemical compounds (1 mM)	Relative activity (%)
None	100
$CaCl_2$	105
$MgCl_2$	98
$MnCl_2$	98
$BaCl_2$	99
$SrCl_2$	96
$SnCl_2$	59
$CuSO_4$	103
$ZnSO_4$	90
Pb $(CH_3COO)_2$	46
p-Chloromercuribenzoate	97
Diisopropylfluolophosphate	110
Soybean trypsin inhibitor (1.0mg/ml)	90
Chymostatin (0.1mM)	78
Pepstatin A (0.1mM)	0
Ethylenediaminetetraacetate	106
o-Phenanthroline	95

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Monascus pilosus の産生する酸性プロティナーゼの酵素化学的性質

安田正昭*・加藤義男*・石川達**・多和田真吉*・小波本直忠*・当山清善*

要　約

Monascus 属菌は中国では紅酒、紅腐乳の製造に、沖縄ではとうふようの製造に用いられる産業上重要な麹菌である。*Monascus* 属菌を蒸米に生育させた紅麹はデンプンをグルコースに、たん白質をペプチドやアミノ酸などに変換する酵素源として重要である。特に、同麹菌の産生するプロティナーゼはとうふよう熟成の鍵酵素として重要な役割を担っている。

たん白質を原料とする発酵食品の開発に資する目的で、本研究においては固体培養法により調製した紅麹中のプロティナーゼ活性の高い菌株のスクリーニングを行った。その結果、本酵素活性の高い *Monascus pilosus* を選抜した。選抜菌株の産生する本酵素の反応最適pHは2.5付近、反応最適温度は50℃であった。本酵素は50℃、10分間の処理でpH3.0～6.0の範囲内で安定であった。また、熱安定性は55℃であった。本酵素はヒトヘモグロビン、ミルクカゼイン、牛血清アルブミン及び分離大豆たん白質等によく作用したが、β－ラクトグロブリン及びゼラチンには全く作用しなかった。本酵素反応はペプスタチンにより顕著に阻害された。

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* 琉球大学農学部生物資源科学科

** (株)トロピカルテクノセンター