Monascus sp. No. 3404 による大豆たん白質分解酵素の生産(生物資源科学科)

Production of Enzyme Hydrolyzing Soybean Protein from *Monascus sp.* No. 3404+

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Summary

The production of the enzyme which hydrolyzed soybean protein was investigated with twenty-three strains in *Monascus* fungus (thirteen strains of type culture, and ten strains isolated from red koji). The highest production of the enzyme was found in the culture broth of *Monascus* sp. No. 3404. In order to find the medium conditions required for improvemet of the enzyme production by this strain, the microorganism was grown in the medium containing defatted soybean meal. The best enzyme production was obtained when the organism was grown in the medium (initial pH 3.5) containing defatted soybean meal (0.75%) and sucrose (8%) at 30℃ for 72 hr under aerobic conditions. The enzyme had the maximum reactivity at pH 3.2 and 50℃.

Introduction

The mold *Monascus* has been used for tofuyo in Okinawa Prefecture, Japan. Tofuyo is a vegetable protein food made from tofu by the action of fungal proteinase. Studies on tofuyo-manufacturing have been carried out in our laboratory[9,10,12,13]. Red koji prepared by growing *Monascus* on steamed rice is an important material for tofuyo-manufacturing. The ripening of this food is always greatly affected by the enzymatic quality of the koji and its proteinase is thought to be a key enzyme[13]. Although fungal proteinases have been extensively investigated[1-4,6,8], information on the enzymes produced by *Monascus* fungus was very limited. The authors[6] reported about purification and some properties of the enzyme hydrolyzing milk casein, obtained from red koji of *Monascus* fungus. However, the microorganism which has a high activity of the enzyme hydrolyzing soybean protein is needed for making tofuyo.

In this paper, screening of *Monascus* fungus which has a high activity of the enzyme hydrolyzing soybean protein, the optimum culture conditions, and some properties of the enzyme are described.

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Materials and Methods

Microorganisms : Thirteen strains of genus *Monascus* in the type culture collection preserved in the department of Bioscience and Biotechnology, University of the Ryukyus. Ten strains were isolated from red koji in China, Hong Kong and Taiwan.

Chemicals : Soy protein isolate (SPI) was presented from Fuji Oil Co. Ltd., Japan. Hammarsten milk casein was obtained from Merck, Germany. The other chemicals were analytical grade.

Assay methods: Activity of the enzyme hydrolyzing soybean protein was assayed as reported in our previous paper[11]except SPI. The reaction mixture, containing 1.0mℓ of 1% SPI solution, 1.0mℓ of 0.2 M lactate buffer (pH 3.0), and 1.0 mℓ of enzyme solution, was incubated at 37℃ for 60 min. One unit of the enzyme was defined as the amount of enzyme, which liberated 1 μmol of tyrosine from the SPI 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] using bovine serum albumin as the standard.

Screening of the enzyme hydrolyzing soybean protein was carried out as follows : Each microorganism was tested for abilities of the enzyme production on the defatted soybean meal medium (pH 3.5) composed of 10 g of defatted soybean meal, 50 g of sucrose, 1 g of yeast extract, 1 g of K_2HPO_4 and 0.5g of $MgSO_4 \cdot 7H_2O$ in 1 ℓ of tap water. The microorganism were inoculated in 50 mℓ of the medium in a 500mℓ flask. And the media were incubated at 30℃ for 156 hr on a reciprocal shaker. The centrifuged culture filtrate was used as enzyme solution. Cultivation conditions of the microorganism for production of the enzyme were examined. When effect of carbon source (or nitrogen souce) in the medium on the enzyme production was tested, the varieties of saccharides (or nitrogen compounds) were used instead of sucrose (or soybean meal) described above.

The enzyme was purified by DEAE-cellulose and DEAE-Sephadex A-50 column chromatographies in order to examine the enzymatic properties.

Results and Discussion

1. Screening of the enzyme hydrolyzing soybean protein in *Monascus* fungus

The screening was carried out to find strains in *Monascus* fungus that would produce a high activity of the enzyme hydrolyzing soybean protein. As shown in Table 1, a high activity of the enzyme was found in the culture broth of *Monascus* sp. No. 3404, *Monascus* sp. No. 3403, *Monascus anka* IFO 4478, *Monascus* sp. No. C-101, *Monascus albidus* IFO 4486, and *Monascus pilosus* IFO 4480. *Monascus* sp. No. 3404, in which the enzyme hydrolyzing soybean protein occurs most abundantly, was chosen for the purpose of production of the enzyme.

2. Effect of culture conditions on production of the enzyme

Amount of the enzyme produced in the culture broth of this strain depended on the nitrogen source used for growth. The relative production of the enzyme on various nitrogen sources is shown in Table 2. The greatest amount of enzyme was obtained in the culture broth when defatted soybean meal was used as a nitrogen source. Bran extract had also ability to stimulate

Table 1. Distribution of Enzyme Hydrolyzing Soybean Protein in Various Strains of *Monascus* Fungus.

Strains	Enzyme activity (Units / mg × 10^{-3})	Growth (Mycelia wt., g / 50mℓ)
Monascus albidus Sato var. glaber Sato IFO 4486	16	1.04
Monascus albidus Sato IFO 4489	5	1.16
Monascus anka Nakazawa & Sato IFO 4478	20	1.13
Monascus araneosus Sato IFO 4482	7	1.16
Monascus fuliginosus Sato IFO 4483	14	0.93
Monascus major Sato IFO 4485	6	1.01
Monascus paxii Lingelsheim IFO 8201	4	1.17
Monascus pilosus Sato IFO 4480	16	1.09
Monascus pubigerus Sato IFO 4521	8	1.01
Monascus ruber Van Tieghem IFO 4492	4	1.14
Monascus rubiginosus Sato IFO 4484	5	0.98
Monascus serorubescens Sato IFO 4487	6	1.19
Monascus vitreus Sato IFO 4532	9	1.01
Monascus sp. No. 3403	28	1.18
Monascus sp. No. 3404	37	1.21
Monascus sp. No. C-101	18	1.21

Table 2. Effect of Nitrogen Sources in the Medium on Activity of the Enzyme Hydrolyzing Soybean Protein.

Nitrogen sources (1%)	Enzyme activity (Units / mg × 10^{-3})	Growth (Mycelia wt., g / 50mℓ)
None	0	0.55
$NH_4H_2PO_4$	2	0.99
KNO_3	0	0.83
$NaNO_3$	1	0.80
$(NH_4)_2SO_4$	0	0.91
NH_4NO_3	4	0.85
Peptone	10	1.10
Bran extract	34	1.11
Defatted soybean meal	36	1.13

production of the enzyme. Little enzyme activity was detected in the culture broth when inorganic nitrogen compounds such as ammonium sulfate, potassium nitrate were used. An addition of defatted soybean meal at an initial concentration of 0.75% caused the maximum increase in the activity. Therefore, it was suggested that the enzyme was produced inducibly by an addition of soybean meal in the culture medium. This phenomenon agreed with the case of Black *Aspergilli* [3] and *Corticium rolfsii* [6].

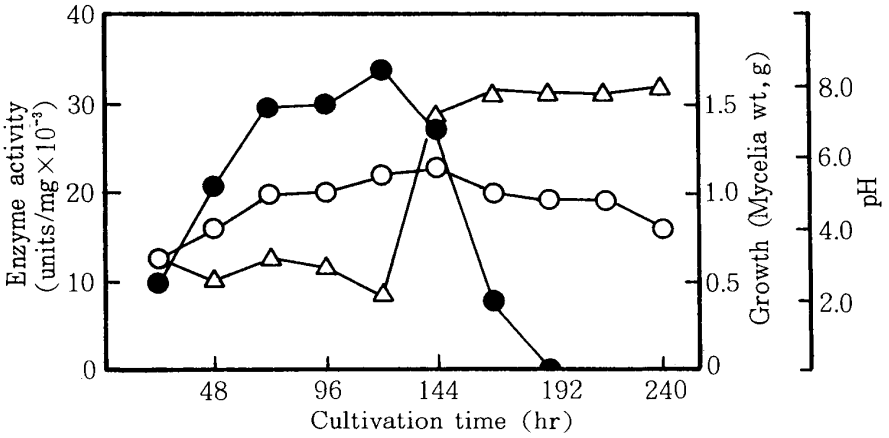

Fig. 1 Effect of Cultivation Time on Production of the Enzyme Hydrolyzing Soybean Protein by *Monascus* sp. No. 3404.
— ● — ; Enzyme activity, — ○ — ; Growth, —△— ; pH

The relative production of the enzyme on various carbon sources (glucose, sucrose, maltose, fructose, galactose, arabinose and xylose) was also investigated. The greatest amount of enzyme was obtained in the culture broth when sucrose was used as a carbon source. An addition of sucrose at an initial concentraction of 8% caused increased maximumly in the activity.

When the initial pH of the medium was adjusted to 3.5, the enzyme production was the maximum : 28% and 17% of the maximum at pHs 3.0, and 4.0, respectively. The initial pH of the medium for production of the enzyme was very limited. Compared with Black *Aspergilli*[3] which showed production of the enzyme at the initial pH 5.5, *Monascus* sp. No. 3404 produced the enzyme in preference to acidic range of the medium.

When strain of *Monascus* sp. No. 3404 was grown at 30℃ for 120 hr in 100 mℓ of flask medium (pH 3.5) in a 500-ml flask under aerobic condition, the amount of enzyme in the culture broth reached to its maximum (Fig.1). Weight of mycelia of the strain in the culture broth also reached to the maximum after incubation at 30℃ for 120 hr. However, relative activity of the enzyme after an incubation at 30℃ for 72 hr was 85% against the maximum activity (an incubation at 30℃ for 120 hr). Although the pH value of the culture broth was maintained at 3.5-4.0 for 120 hr, the value was increased after 120 hr (pH 7.5 for 168 hr).

And thus, culture conditions on the production of the enzyme were determined as follows : the organism was cultured at 30℃ for 72 hr in a medium (pH 3.5), containing 0.75% of soybean meal, 8% of sucrose, 0.1% of yeast extract, 0.1% of K_2HPO_4, and 0.05% of $MgSO_4 \cdot 7H_2O$.

3. Properties of the enzyme

Effect of pH on activity and stability of the enzyme

The effect of pH on activity of the partially purified enzyme is shown in Fig.2. The enzyme is most active around pH 3.2 for soybean protein hydrolysis. This value was similar to other fungus proteinases (*Aspergillus* enzyme; pH 2.5-3.0[4], *Rhizopus* enzyme; pH 2.9-3.3[1] and *Corticium rolfsii* enzyme; pH 2.5[6]. As shown in Fig.3, the enzyme was quite stable for a 5-min treatment in a range of pH 2.5 to 6.0 when it was maintained at 50℃, but lost 90% and 35% of the initial activity at

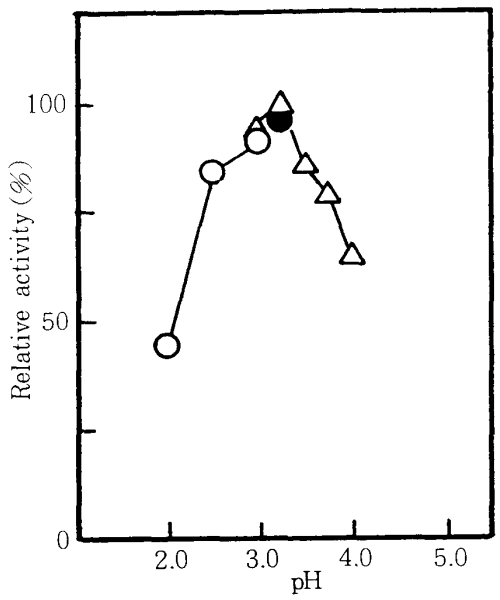

Fig. 2

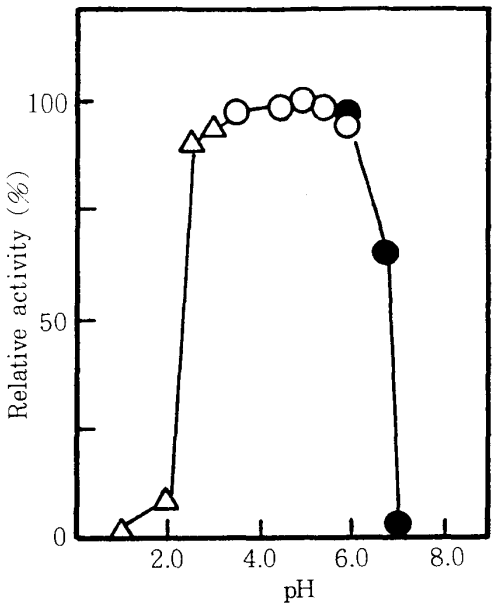

Fig. 3

Fig. 2 Effect of pH on Activity of the Enzyme Hydrolyzing Soybean Protein
The enzyme activity was assayed at various pHs.
—○— ; HCl-Sodium citrate buffer, —△— ; Lactate buffer,
—●— ; HCl Sodium acetate buffer

Fig. 3. Effect of pH on Stability of the Enzyme Hydrolyzing Soybean Protein.
The enzyme was incubated at various pHs and 50 ℃ for 5 min, and the activity was then measured under the standard assay conditions.
—△— ; HCl-Sodium acetate buffer, —○— ; McIlvaine buffer,
—●— ; Potassium phosphate buffer

pHs 2.0 and 6.5, respectively.

Effect of temperature on activity and stability of the enzyme

The enzyme reaction was carried out at various temperatures at pH 3.2 for 60 min, the temperature maximum of the enzyme was observed at 50 ℃ as shown in Fig. 4. The enzyme solution was incubated at various temperatures for 5 min and the remaining activities were checked under the standard assay conditions. The results indicated that the enzyme was stable up to 45℃, about 90% of the activity of the enzyme remained at 50℃, and no activity remained at 60 ℃ as shown in Fig.5.

Further purification and detailed properties of the enzyme are currently under investigations in our laboratory.

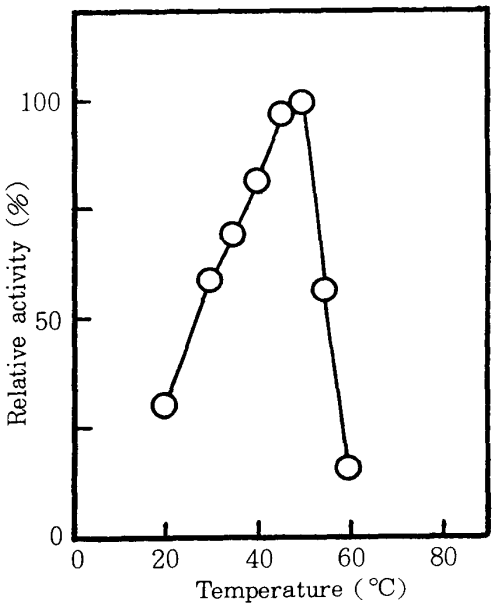

Fig. 4

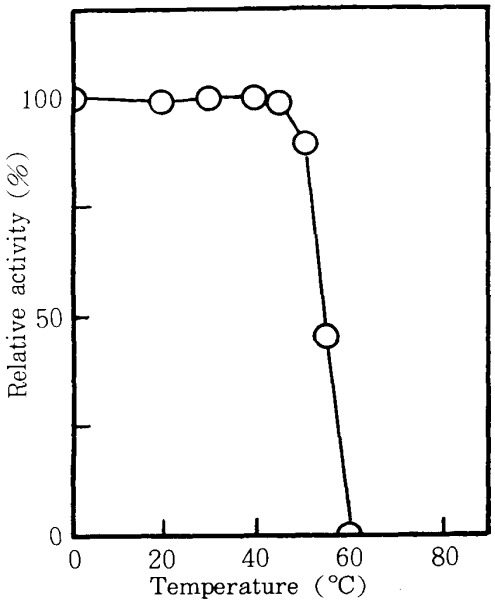

Fig. 5

Fig. 4. Effect of Temperature on Activity of the Enzyme Hydrolyzing Soybean Protein.
The enzyme was assayed at various temperatures.

Fig. 5. Effect of Temperature on Stability of the Enzyme Hydrolyzing Soybean Protein.
The enzyme was incubated at various temperatures at pH 3.0 for 5 min, and the residual activity was then measured under the standard assay conditions.

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Monascus sp. No. 3404による大豆たん白質分解酵素の生産

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要　約

*Monascus*属菌はとうふよう（大豆たん白質の発酵食品）の製造に用いられる麹カビであり、その生産するたん白質分解酵素はとうふようの熟成で重要な役割を担っている。本研究においては、紅麹菌における大豆たん白質分解活性の高い菌株のスクリーングを行い、その培養条件を検討した。その結果、大豆たん白質分解活性の高い*Monascus* sp. No. 3404菌が選抜された。本酵素生産のための供試菌株の最適培養条件は、脱脂大豆粉（0.75%）及び蔗糖（8%）を含む液体培地（pH 3.5）で、30℃、72時間振とう培養することに設定された。本酵素の反応最適pHは3.2で、反応最適温度は50℃であった。

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