好アルカリ性細菌 Bacillus sp. による大豆たん白質分解酵素の生産と二・三の酵素化学的性質(生物資源科学科)

Production and Some Properties of Soybean-Protein-Hydrolyzing Enzyme from Alkalophilic *Bacillus* sp.†

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Summary

The production of the enzyme which hydrolyzed soybean protein was investigated with 18 strains of bacteria in the type culture collection, and 153 strains of bacteria were isolated from varieties of food such as tofu, tofuyo and others. The higest production of the enzyme was found in the culture broth of strain TYO-67. The organism was identified as bacterium belonging to the genus *Bacillus*. In order to find the medium conditions required for improvement of the enzyme production by this strain, the microorganism was grown in the medium containing soy protein isolate (SPI). The best enzyme production was obtained when the organism was grown in the medium (initial pH 11.5-12.5) containing SPI (2%) and glucose (0.2%) at 30℃ for 48 hr under aerobic conditions. The enzyme had the maximum reactivity at 8.0-9.5 and 50℃.

Introduction

Bacterial soybean-protein-hydrolyzing enzyme is considered to play an important role in "prefermentation" which affects the quality of tofuyo by degrading soybean protein to some extent during dehydration of tofu cubes in the room temperature[12]. Tofuyo is the excellent vegetable protein food made from tofu by the action of microoranisms in Okinawa Prefecture, Japan. It is nutriously rich with good protein, fat, and other nutrients. Studies on tofuyo manufacturing have been carried out in our laboratory[9,10,12,13]. The authors have isolated and screened useful microorganisms for establishing effective methods of tofuyo-manufacturing[14,15]. In the previous paper[15], genus *Monascus* which produced the soybean-protein-hydrolyzing enzyme abundantly, was isolated from red koji. Moreover, the bacterium which has a high activities of the soybean-protein-hydrolyzing enzyme is also needed for obtaining the best quality of tofuyo. Although, there are many reports on the

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milk-casein-hydrolyzing enzyme[2,4-8,10,11], the report on the soybean-protein-hydrolyzing enzyme is limited.

In this paper, screening of bacteria which have a high activity of the soybean-protein-hydrolyzing enzyme, culture conditions and some properties of the enzyme are described.

Materials and Methods

Microorganisms: Eighteen strains of bacteria in the type culture collection preserved in Department of Bioscience and Biotechnology, University of the Ryukyus. One hundred and fifty three strains were isolated from soils and varieties of food such as tofu, tofuyo, and others.

Chemicals: soy protein isolate (SPI : Fujipro R) was kindly gifted by Fuji Oil Co. Ltd., Osaka, Japan. All other chemicals were of analytical grades.

Assay methods: The reaction mixture, containing 1.0 mℓ of 1% SPI solution, 1.0 mℓ of 0.2 M $Na_2CO_3-H_3BO_4-KCl$ buffer (pH 9.2) 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.*[3] using bovine serum albumin as the standard.

Screening of the soybean-protein-hydrolyzing enzyme was carried out as follows : Each microorganism was tested for ability in the enzyme production on SPI medium (pH 7.0) composed of 0.5% of SPI, 0.2% of glucose, 0.25% of peptone, 0.1% of yeast extract, 0.2% of KH_2PO_4 and 0.2% K_2HPO_4. The microorganism was inoculated in 50 mℓ of the medium in 500-mℓ flask. And the media were incubated at 30℃ for 72 hr on a reciprocal shaker. The centrifuged culture filtrate was used as an enzyme solution. Cultivation conditions of the microorganism for production of the soybean-protein-hydrolyzing enzyme were examined. When the effect of carbon source (or nitrogen source) in the medium on the enzyme production was tested, the varieties of saccharides (or nitrogen compounds) were used instead of glucose (or SPI) described above.

The enzyme was purified by fractionation of ammonium sulfate (80% saturation), DEAE-cellulose and QAE-Sephadex A-50 column chromatographies from culture broth in order to examine the enzymatic properties.

Results and Discussion

1. Screening of soybean-protein-hydrolyzing enzyme in bacteria

The screening was carried out to find the bacterial strains that would produce a high activity of the enzyme hydrolyzing soybean protein. As shown in Table 1, high activities were found in the culture brothes of TYO-67, TYO-3, TYO-69, which were isolated from dehydrated tofu cubes for tofuyo production, and TF-25, which was isolated from commercial tofu. Characteristics of the isolated bacterium (strain, TYO-67) were examined by the method of Cowan and Steel[1]. Cells grown in the broth containing glucose, yeast

extracts and peptone were gram-positive and rod-shaped. It was also indicated to be spore-forming and motile. The strain grew in aerobically but did not grow anaerobically. Catalase activity of the strain was positive. Oxidative reaction was shown in OF-test (oxidation or fermentation-test) of this strain. It was clear from "Bergey's Manual of Determinative Bacteriology", (8 ed.) that the bacterium belongs to the genus *Bacillus*, and the strain was named as *Bacillus* sp. TYO-67. *Bacillus* sp. TYO-67, in which proteinase occurs most abundantly, was chosen for the purpose of production of the enzyme.

Table 1. Distribution of Soybean-Protein-Hydrolyzing Enzyme in Various Bacteria

Strains	Enzyme activity (10^{-2} units/mg)
Pseudomonas fluorescens IFO 3461	0
Agrobacterium tumefaciens IAM B-26-1	0.30
Achromobacter polymorph ICR 88	0.01
Alcaligenes faecalis IAM B-14-1	0
Flavobacterium arborescens IAM 1100	0.24
Escherichia coli K-12 IFO 3208	0
Aerobacter aerogenes IFO 3320	0.01
Erwinia aeroideae IFO 3830	0
Proteus vulgaris IFO 3045	0.01
Micrococcus flavus IFO 3242	0.02
Staphylococcus aureus IFO 3060	0.02
Sarcina auratiaca IFO 3064	0
Brevibacterium ammoniagenes IFO 12072	0
Corynebacterium sepedonicum IFO 12188	0.02
Bacillus cereus IFO 3001	0.02
Bacillus mesentericus var. flaus IFO 3028	0.09
Bacillus subtilis IFO 3009	0.02
Bacillus sphaericus IFO 3525	0.08
TYO-3	1.70
TYO-67	3.10
TYO-69	0,18
TF-25	0.52

2. Effect of culture conditions on production of the enzyme.

The amount of the enzyme produced in the culture broth of this strain was depended on the nitrogen source used for growth. Relative production of the enzyme on various nitrogen sources is shown in Fig. 1. The greatest amount of the enzyme was obtained in the culture broth when SPI was used as a nitrogen source. Polypeptone and sodium nitrate had a little ability to stimulate production of the enzyme. And thus, it was found that soy protein is best nitrogen source than polypeptone. This phenomenon agreed with the reported case of *Monascus*[15] but differed from that of *Bacillus*[2]. Little additional effect was detected in the culture broth when 0.25% of ammonium sulfate, ammonium carbonate, ammonium acetate, ammonium nitrate and peptone to the SPI (2.0%). An addition of SPI to the medium with

an initial concentration of 2.0% caused an increase in the maximum activity. Therefore, it was suggested that the enzyme was produced inducibly by an addition of SPI to the culture medium.

Relative production of the enzyme on various carbon sources (glucose, fructose, galactose, arabinose, xylose, sucrose, maltose glycerol, and starch) was also investigated. The greatest amount of enzyme was obtained in the culture broth when glucose was used as a carbon source. An addition of glucose to the medium with an initial concentration of 0.2% caused the maximum increase in the activity.

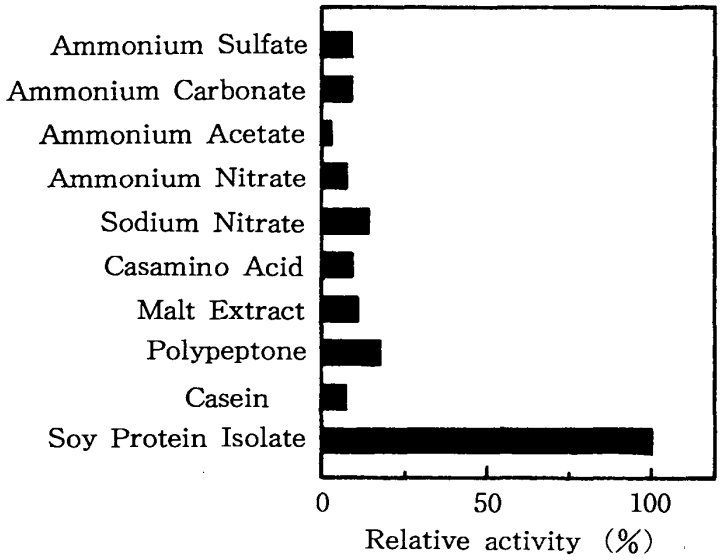

Fig. 1. Effect of Nitrogen Sources in the Medium on Activity of soybean-Protein-Hydrolyzing Enzyme.

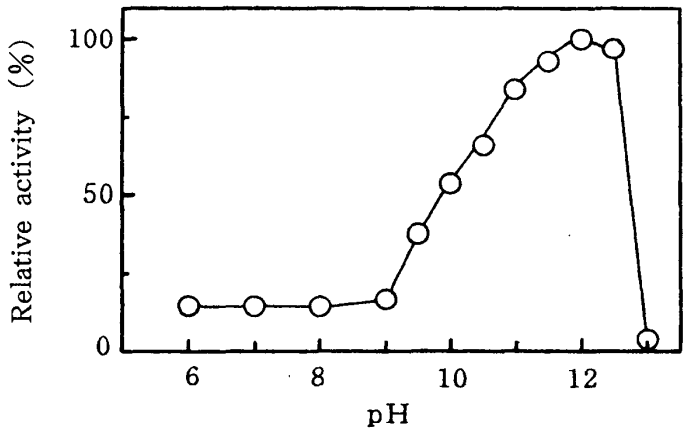

Fig. 2. Effect of Initial pH in the Medium on Activity of Soybean-Protein-Hydrolyzing Enzyme.

When the initial pH of the medium was adjusted to 11.5-12.5, the enzyme production was the maximum. The enzyme production decreased to 66% of the maximum at pH 10.5 and to 4% of that at pH 13.0 (Fig.2). The initial pH of the medium for production of the

enzyme was appropriate in an alkaline region. It was reported that the alkaline proteinase of *Streptomyces*[4] or *Bacillus sp.*[2] was also produced at around pH 10.

When a strain of *Bacillus* sp. TYO-67 was grown at 30℃ for 48-96 hr in 100 mℓ of medium (pH 11.5) in a 500-mℓ flask under an aerobic condition, the amount of the enzyme in the culture broth reached to its maximum.

And thus, culture conditions for production of the enzyme were determined as follows: The organism was cultured at 30℃ for 48 hr in a medium (pH 11.5-12.0), containing 2.0% of SPI, 0.2% of glucose, 0.1% of yeast extract, 0.2% of KH_2PO_4 and 0.2% of K_2HPO_4.

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.3. The enzyme is most active around pH 8.0 for soybean protein hydrolysis. This pH value was relatively similar to that of *Bacillus thermoruber*-enzyme (pH 9.0[5]), but lower than that of other *Bacillus*-proteinases (*Bacillus* sp. ; 10-11[6], *Bacillus* sp. Y; 10.0-12.5[7], *Bacillus* sp. ; 11.5[2], and *Bacillus* sp. AH-101;12-18[8]) for milk casein. As shown in Fig.4, the enzyme was quite stable for a 10-min treatment in a range of pH 7.0 to 9.0 when it was maintained at 50℃, but lost 83% and 68% of the initial activity at pH 4.0 and pH 10.0, respectively.

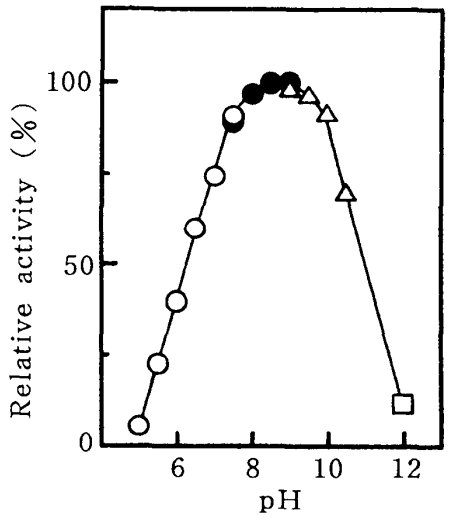

Fig. 3.

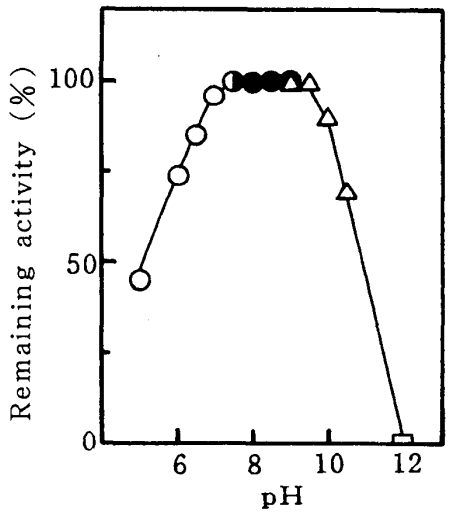

Fig. 4.

Fig. 3. Effect of pH Activity of Soybean-Protein-Hydrolyzing Enzyme.
The enzyme activity was assayed at various pHs.
Buffers: -O- ; McIlvaine buffer, -●- ; KCl-H_3BO_3-NaOH buffer.
-△- ; KCl-H_3BO_3-Na_2CO_3 buffer, -□- ; 0.1N NaOH

Fig. 4. pH Stability of Soybean-Protein-Hydrolyzing Enzyme.
The enzyme was incubated at 37℃ for 30 min. And the activity was then assayed at pH 9.2.
Buffers: -O- ; McIlvaine buffer, -●- ; KCl-H_3BO_3-NaOH buffer,
-△- ; KCl-H_3BO_3-Na_2CO_3 buffer, -□- ; 0.1N NaOH

Effect of temperature on activity and stability of the enzyme

The enzyme reaction was carried out at various temperatures at pH 8.2 for 60 min, the temperature for the maximum enzyme activity was 45℃ as shown in Fig. 5. This value was similar to the *Bacillus thermoruber*[5]. Recently, the alkaline proteinase having maximum activity at 80℃ was found[8]. The enzyme solution was incubated at various temperatures for 10 min at pH 9.0 and the remaining activities were checked under the standard assay conditions. The results indicated that the enzyme was stable up to 45℃, about 35% of the activity of the enzyme remained at 55℃, and no activity remained at 65℃ as shown in Fig. 6. This limit temperature for stable activity was higher than that of another *Bacillus* sp. enzyme (40℃[6]) but lower than that of *Bacillus*-AH101 enzyme (70℃[8]).

The enzyme clotted soybean milk at pH 6.1. Further purification and detailed physicochemical and enzymological properties of the enzyme, such as molecular weight, CD-analysis, substrate specificity and the mode of action on soybean protein, are currently under investigation in our laboratory.

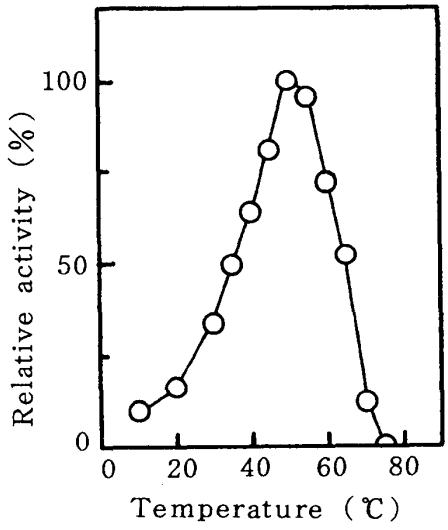

Fig. 5.

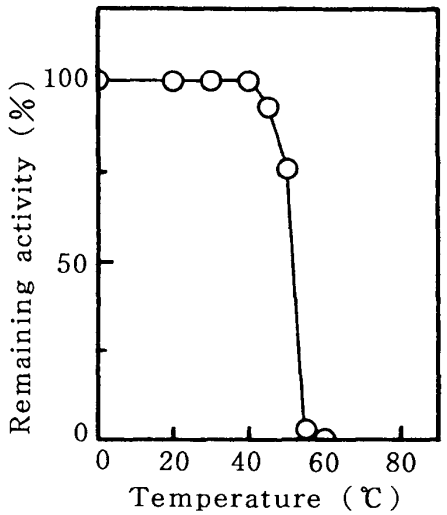

Fig. 6.

Fig. 5. Effect of Temperature on Activity of Sobean-Protein-Hydrolyzing Enzyme.
The enzyme activity was assayed at various temperatures.

Fig. 6. Temperature Stability of Soybean-Protein-Hydrolyzing Enzyme.
The enzyme was incubated at various temperatures and at pHs for 10 min. And the activity was then assayed at pH 9.2.

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好アルカリ性細菌*Bacillus* sp.による大豆たん白質分解酵素の生産と二・三の酵素化学的性質

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要　約

とうふようの製造で、豆腐の乾燥工程は微生物による“前発酵”として意義づけられ、特に、細菌の生産する大豆たん白質分解酵素はその重要な鍵を握っている。本研究においては、各種細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ニングを行い、本酵素活性の高い菌株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細菌TYO-67菌が選抜された。本菌は好アルカリ性の*Bacillus*属細菌であると同定された。本酵素生産のための供試細菌の最適培養条件は、分離大豆たん白質（SPI, 2％）及びグルコース（0.2％）を含む培地（pH 11.5～12.5）で30℃、48時間振とう培養することに設定された。培養ろ液から、硫酸アンモニウム塩析（80％飽和）、DEAE－セルロース及びQAE－セファデックスA－50カラムクロマトグラフィーを行って得られた本精製酵素の反応最適pHは8.0～9.5で、反応最適温度は50℃であった。

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