[J. Ferment. Technol., Vol. 49, No. 6, p. 544~551, 1971]

Formation of Glutamic Acid from Defatted Soybeans by *Aspergillus oryzae* 460

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Abstract

A strain of *Aspergillus* species, which liberates a large amount of glutamic acid from defatted soybeans, was isolated from air. The isolated strain was identified as *Aspergillus oryzae* 460 based on various mycological and physiological properties. This strain never produced aflatoxins in the two different special media for the aflatoxin production. It liberated more than twice as much glutamic acid from defatted soybeans as did ordinary shoyu molds. Among alkaline, neutral, and acid proteinases, carboxypeptidase, aminopeptidase, and leucyl-β-naphthylamidase activities, amino-peptidase activity was found to have a significant correlation with the formation of glutamic acid by the isolated strain and the related *Aspergillus* species. A mutant exhibiting about twice as much activity of aminopeptidase as that of the parent strain was obtained by UV irradiation

Introduction

There are two big targets in shoyu industry to raise the yield and to improve the quality of the product. As to the yield, proteinases from shoyu molds have been believed to play the most active role to increase total nitrogen and many attempts have been carried out to isolate more proteolytic strains or mutants. For example, Iguchi[1,2] succeeded in inducing highly proteolytic mutants of *Aspergillus sojae* by X-ray irradiation and in elevation of the yield of shoyu.

On the other hand, the quality of shoyu depends mainly on taste and flavor. Among the ingredients concerning taste, glutamic acid is considered to be the most important component for good taste. Therefore, it is better to use koji mold which is highly potent to form a large amount of glutamic acid from soybean protein in order to produce high quality of shoyu. However, exact knowledge on the relation between the liberation of glutamic acid from soybean protein and the activities of proteolytic enzymes has not yet been established, although this amino acid is assumed to be derived from the digestion of proteinous raw materials by the action of some of the proteolytic enymes from shoyu molds.

Thus, there has been no systematic approach to obtain valuable strains or mutants which are effective to improve the quality of shoyu. Fortunately, such a potent strain of *Aspergillus* species was, isolated by chance, during the screening

processes of useful strains of shoyu molds.

In this paper, we describe the taxonomic identification of the isolated strain, non-productivity of aflatoxins, and relation between the activities of various proteolytic enzymes and glutamic acid formation from defatted soybeans in the isolated strain and related *Aspergillus* species.

Materials and Methods

1. Taxonomic identification of the isolated strain An isolated fungus was grown on Czapek's agar medium and other specified media at 25°C or 30°C for an appropriate period and identified according to the description by Raper and Fennel[3], the classification by Sakaguchi and Yamada[4], and the reports by Murakami *et al.*[5~8]. Color of colony surface and the other side was described in accordance with Ridgway's "Color Standard and Color Nomenclature".[9]

2. Isolation of mutants After UV irradiation, mutant strains which could produce more proteinase and aminopeptidase were isolated as described previously[12].

3. Digestion of defatted soybean The reaction mixture contained 20 g of defatted soybean, 20 to 40 ml culture extract, 7% (w/v) NaCl, 2% (v/v) ethanol, and 0.05% $NaHSO_3$ in a total volume of 75 ml. After incubation at 30°C for 72 hr, analyses of glutamic acid and total formol nitrogen were carried out.

Concentration of glutamic acid was determined by the modified method of Schales and Schales[10] with glutamic acid decarboxylase from *Escherichia coli* (Kyowa Fermentation Co., Inc., Tokyo). Total and formol nitrogen were measured by the conventional methods used for analyses of shoyu[11].

4. Enzyme assay Alkaline, neutral, and acid proteinases and total peptidase activities were assayed by the methods reported previously[12]. Leucyl-β-naphthylamidasea activity was assayed by the method of Goldbarg and Rutenburg.[13] Carboxypeptidase and aminopeptidase activities were assayed by Rosen's ninhydrin method[14] with 0.5 μmole N-carbobenzoxy-L-alanyl-L-glutamic acid as a substrate in 50 mM acetate buffer at pH 4.0 and with 250 μmoles leucylglycine as a substrate in 50 mM tris-HCl buffer at pH 7.5, respectively.

Except for experiments on mutants, each analytical value or enzyme activity was expressed as a relative value to that of *Aspergillus sojae* strain ×74. In case of mutants each activity of proteinase and aminopeptidase for the parent strain was taken as control (100%).

5. Comparison of UV absorbing products with aflatoxins The isolated strain was incubated in YES[15] and YES-salts[16] media at 25°C for 14 days. Fermentation products from 400 ml of culture medium were extracted with chloroform by the extraction method for aflatoxins and were applied to thin layer chromatography with a plate of Kieselgel-G (0.5 mm) and solvent system of chloroform and methanol (98 : 2)[17]. The UV absorbing spots were scraped off from the glass plate and

extracted with chloroform. Extracts were dried up below 40°C under vacuum and redissolved in 4 ml of ethanol. Absorption spectra from 220 to 400 mμ were recorded by a spectrophotometer. The authentic samples of aflatoxin B1, B2, G1 and G2 were purchased from Makor Chemicals, Israel.

Results and Discussion

1. Superiority of the isolated strain Fig. 1 showed the histograms of the concentrations of glutamic acid, formol and total nitrogen in the digested filtrate of defatted soybeans by 72 strains of shoyu molds which are practically used in various districts of Japan. The isolated strain formed 162% of glutamic acid under the test condition, although this amino acid was also produced by the ordinary shoyu molds in a range from 20 to 100% with the average of 32.5%. Superiority of the isolated strain is now evident in glutamic acid production from defatted soybeans. As for the concentration of formol nitrogen and total nitrogen, the isolated strain showed the values of 145% and 112% of control, while the other 72 strains did 44.0% and 65.7% on an average respectively.

2. Taxonomic identification of the isolated strain Microscopic observation of conidial head of the isolated strain showed a typical head of *Arpergillus* species (Fig. 2). Wall character of conidia was slightly echinulated in an electronmicrograph

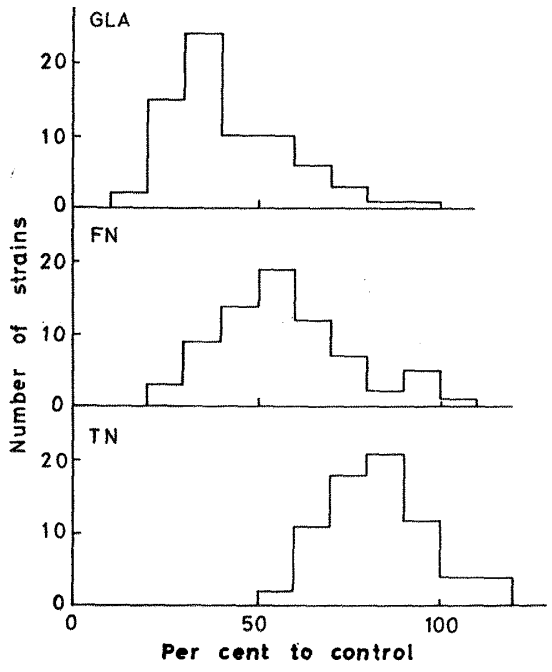

Fig. 1. Histograms of glutamic acid (GLA), formol nitrogen (FN), and total nitrogen (TN) in digested solution of defatted soybean by various strains of *Aspergillus sojae* and *Aspergillus oryzae*.

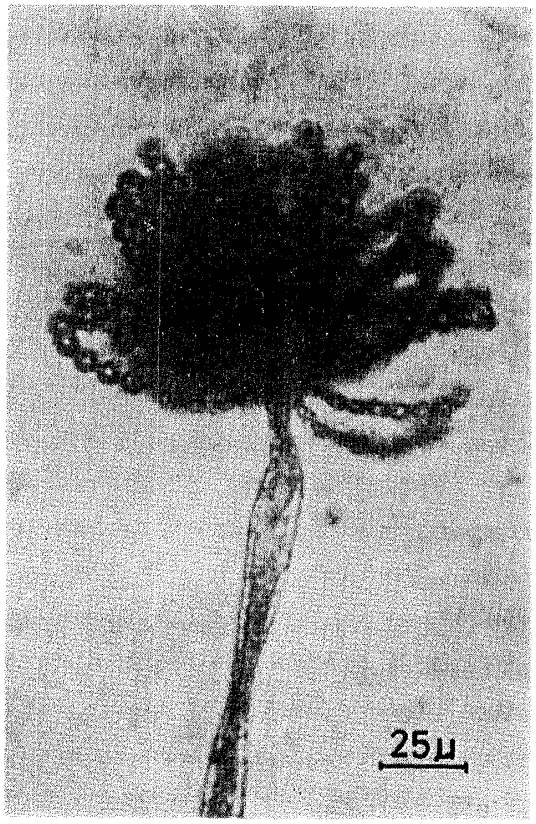

Fig. 2. Conidial head of *Aspergillus oryzae* 460.

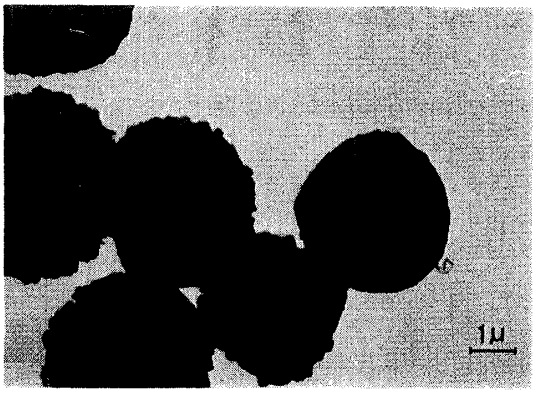

Fig. 3. Electronmicrogrph of conidia of *Aspergillus oryzae* 460.

(Fig. 3). Based on the cultural and morphological characteristics (Tables 1 and 2), the isolated strain was identified and specified as *Aspergillus oryzae* strain 460 according to the classification and description by Raper and Fennel[3], Sakaguchi and Yamada[4], and Murakami *et al.*[5~8].

3. Non-productivity of aflatoxins

Because of highly toxic and carcinogenic nature of aflatoxins to a number of avian and mammalian species[18], numerous reports have been published on aflatoxins which were known to be produced by some strains of *Aspergillus flavus* and *Aspergillus parasiticus*[3]. Fortunately, Japanese industrial strains of *Aspergillus* species do not produce aflatoxins[17,19~23], although some strains were described as probable synonyms of the above two species[3]. However, since the test strain was newly isolated from air, it was necessary to confirm that this strain did not produce aflatoxins before application to practical use in industry.

Isolates of *Aspergillus flavus* were reported to produce 0.8 to 252 mg of aflatoxins B1 and G1 in 400 ml of YES medium[15] and 0.8 to 40 mg of aflatoxins

Table 1. Cultural characteristics of *Aspergillus oryzae* 460 incubated at 30°C for 7 days on Czapek's solution agar.

Colony characteristics:	
Rate of growth	76—80 mm
Texture	velvety
Surface	furrowed
Amount of sporulation	abundunt
Margin	3—5 mm, uncolored
Color and its change	
Central area	Citron Yellow to Olive Ocher
Marginal area	Baryta Yellow to Olive Yellow
Color of colony reverse	
Central area	Warm Buff —no change
Marginal area	Cream color —no change
Other remarks:	
Change of conidial color	dull brown shade in age
Response to anisic acid	negative
Kojic acid production	positive in koji extract culture
No sclerotia and asci formed.	
Conidiophores arose from the substrate.	

Table 2. Microscopic characteristics of *Aspergillus oryzae* 460 incubated at 30°C on Czapek's solution agar.

Heads	
Manner in which borne	radiate
Color	yellow green
Form	globose
Overall dimensions	73—129×120—161 μ
Vescicle	
Shape	subglobose
Dimensions	7.2—23.3 μ
Color	pale yellow green
Sterigmata	
Arrangement	uniseriate
Dimensions	7.2—10.7 ×14.3—21.5 μ
Color	pale yellow green
Conidiophore	
Length	524—1010 μ
Diameter	5.4—8.9 μ
Wall character	rough
Conidia	
Dimensions	5.6—7.6 μ
Wall character	smooth
Color	yellow green

B1 and B2 in 400 ml of YES-salts medium[16]. These amounts were found to correspond to approximately 12.5 to 394 of optical density at 363 mμ under the experimental conditions described above by the calculation from molar extinctions and molecular weights of aflatoxins[23].

On the other hand, in the culture filtrates of YES or YES-salts medium strain 460 produced very small amount of UV absorbing substances. Although some of these compounds showed similar mobility on a thin layer chromatogram to that of aflatoxin B or G (Fig. 4), absorption spectra ranging from 210 to 400 mμ of all the above spots were completely different from those of authentic aflatoxins and the absorption peak around 363 mμ, which is characteristic of aflatoxins, was not detected in the above spectra (Fig. 5 and 6). These results were in favor of conclusion that aflatoxins were not produced by strain 460.

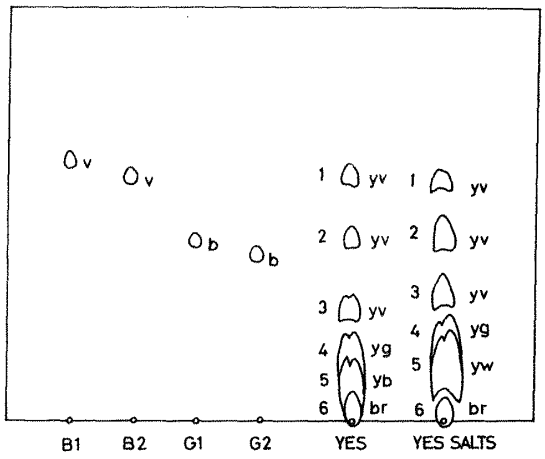

Fig. 4. Thin layer chromatogram of UV absorbing products by *Aspergillus oryzae* 460 in YES and YES-salts media and authentic aflatoxins B1, B2, G1, and G2.
v, violet; b, blue; yv, yellowish violet; yg, yellowish green; yb, yellowish blue; yw, yellowish white; br, brwon.

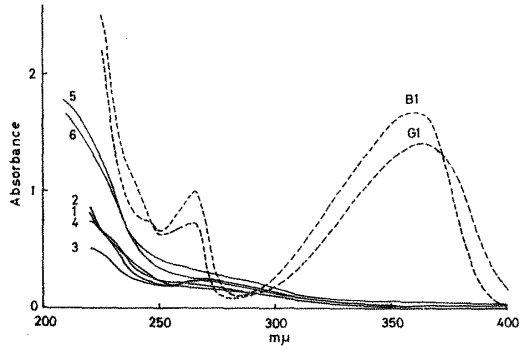

Fig. 5. Absorption spectra of UV absorbing products by *Aspergillus oryzae* 460 in YES medium and authentic aflatoxins B1 and G1. The numerical suffix of curves correspond to the numerical suffix of the spots in Fig. 4.

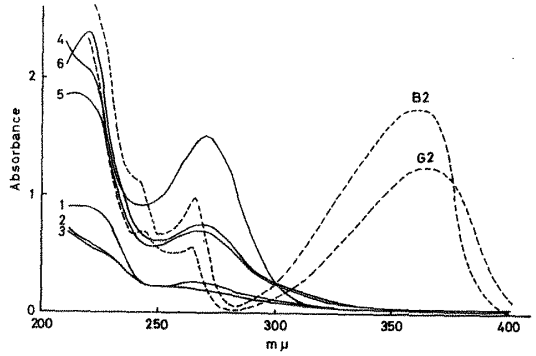

Fig. 6. Absorption spectra of UV absorbing products by *Aspergillus oryzae* 460 in YES-salts medium and authentic aflatoxins B2 and G2. The numerical suffix of curves correspond to the numerical suffix of the spots in Fig. 4.

4. Correlation between glutamic acid formation and various proteolytic enzymes Concentration of glutamic acid and activities of various proteolytic enzymes by some strains of *Aspergillus sojae* and *Aspergillus oryzae* were determined to find which enzyme would play the most important role of glutamic acid formation in digestion of defatted soybean. Fig. 7 showed that glutamic acid formation did not depend on the activities of proteinase at pH 7.3, alkaline proteinase, neutral proteinase or acid proteinase. On the contrary, there seemed to be some correlation between

the amount of glutamic acid produced and the activity of total peptidase, leucyl-β-naphthylamidase, carboxypeptidase or aminopeptidase (Fig. 8). Among these peptidases, aminopeptidase activity exhibited the highest correlation (correlation coefficient 0.69) to liberation of glutamic acid from defatted soybeans.

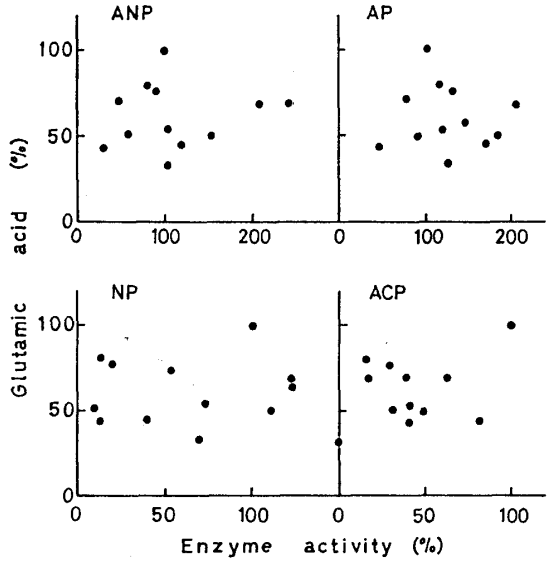

Fig. 7. Glutamic acid formation from defatted soybeans and activities of proteinase at pH 7.3 (ANP), alkaline proteinase (AP), neutral proteinase (NP) and acid proteinase (ACP) from some strains of *Aspergillus sojae* and *Aspergillus oryzae*.

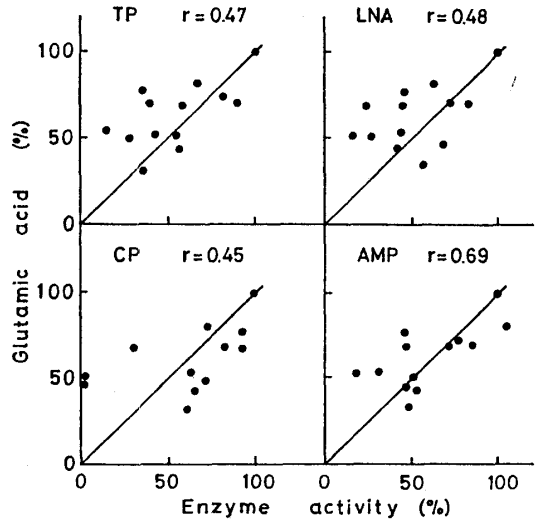

Fig. 8. Correlation of glutamic acid formation from defatted soybeans and activities of total peptidase (TP), leucyl-β-naphthylamidase (L NA), carboxypeptidase (CP) and aminopeptidase (AMP) from some strains of *Aspergillus sojae* and *Aspergillus oryzae*.
r: correlation coefficient

Only a few reports have been published on amino acid formation from soybean protein by proteolytic enzymes in shoyu production. Although, Hattori and Matsuyama[24] reported that neutral proteinase liberated a large amount of glutamic acid from soybean protein, our results did not consist with their observation. Takeda and Nakayama[25] presented the data to suggest that amino acid formation was promoted by total peptidases in shoyu production, but no data was available for correlation of glutamic acid formation and any type of peptidases.

5. Isolation of mutants by UV irradiation Strain 460 was treated with UV irradiation to obtain more potent strains which had higher activities of proteinase at pH 7.3 and aminopeptidase. Fig. 9 illustrated the distribution of proteinase at pH 7.3 and aminopeptidase produced by 122 mutant strains randomly picked up. The highest activities of both enzyme of a mutant strain were each over 160% to those of the parent strain.

Significant correlation (correlation coefficient 0.66) was shown between proteinase activity at pH 7.3 and the ratio of the activities of this enzyme to aminopeptidase (Fig. 10). In other words, it seemed that genetic alternation in the production of proteinase occurs more easily than that of aminopeptidase. From above results, it was

suggested that screening of valuable mutants was quite favorable in shoyu production, since proteinase could play an important role in the increase of total nitrogen and the isolated strain was not the best one in production of this enzyme among the strains examined. Further studies will be necessary to prove that the potent mutants obtained above are practically useful in shoyu brewing for the increase of the yield and improvement of the quality of shoyu.

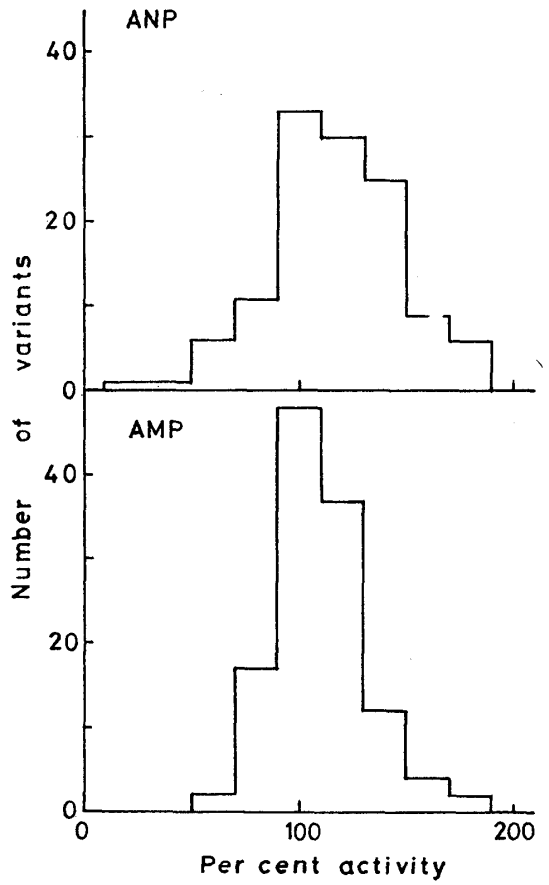

Fig. 9. Distribution of proteinase at pH 7.3 (ANP) and aminopeptidase (AMP) in the UV-induced mutants of *Aspergillus oryzae* 460.

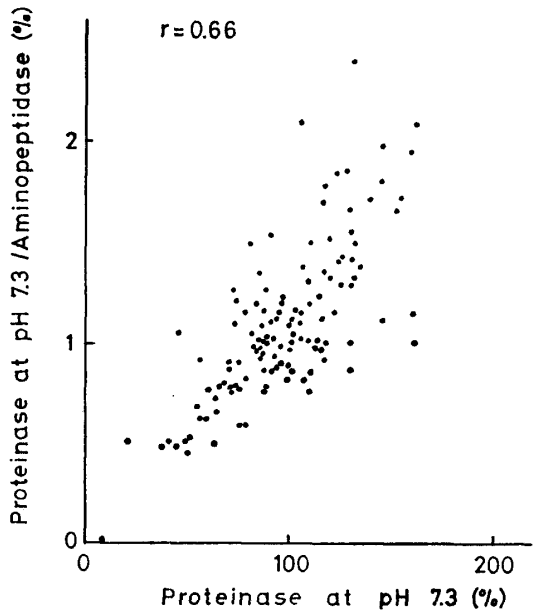

Fig. 10. Correlation between activities of proteinase at pH 7.3 and aminopeptidase in the UV-induced mutants of *Aspergillus oryzae* 460. r: correlation coefficint

Acknowledgement

The authors wish to express their sincere thanks to Profs. K. Arima, Y. Ikeda and T. Asai for their kind guidance and also to Drs. M. Mogi and N. Iguchi for their encouragement. Thanks are also due to Dr. H. Murakami for his valuable discussions and Messrs. M. Sasaki, S. Kitahara and K. Hayashi for their advice and help throughout this work.

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(Received November 25, 1970)