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Antioxidant Production from Several Xerophilous Fungi Used in "*Katsuobushi*" Molding

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The potential ability for producing effective antioxidants was investigated in 5 xerophilous fungi used in "*katsuobushi*" molding. When strains were cultivated with media containing malt extract, yeast extract, pepton and 20% glucose, all strains produced antioxidative substances, which were extracted from the culture media by the combined extraction of methanol, chloroform, and ethyl acetate. *The Eurotium herbariorum* NE-1 strain had the slightly stronger activity as indicators of peroxide value (POV) and tribarbituric acid reactive substances (TBARS), while *Aspergillus ruber* gave excellent results due to its high quantity. A partially purified fraction of the antioxidative substance from *A. repens* indicated the potent activity corresponding to that of DL -α- tocopherol.

1 Introduction

"*Katsuobushi*" is made from skipjack *Katsuwonus pelamis*, and is a valuable ingredient of Japanese soup stock. The commercial preparation of *katsuobushi* has several steps, including boiling, smoke-drying and molding. The molding process is a unique step in the preparation using xerophilous fungi, which contribute to the flavor improvement, soup stock clarification, and water activity reduction[1]. The raw muscle of skipjack utilized for *katsuobushi* includes a 1-3% lipid fraction. Polyunsaturated fatty acids (PUFA) account for equal to or more than 50 % of the fatty acids composition in skipjack muscle[2,3]. PUFA is easily oxidized, particularly when water activity is low. However, molded *katsuobushi* is resistant to lipid oxidation, and is known to remain in good condition over long periods.

As for resistance to lipid oxidation, Suzuki and Motosugi[4] reported that antioxidants occur in smoke-dried skipjack meats. The smoke-drying procedure of skipjack meats was repeated 10-12 times, and the antioxidative effect seemed to stem from phenolic compounds absorbed on *katsuobushi*. However, the molding procedure is, in practice, carried out after material adhered to the surface is shaved off with a grinder.

Kawai *et al.*[5,6] and Oeda *et al.*[7] found that the antioxidative substances produced by *Aspergillus niger* A-12 strain had higher activity than those of some food-borne

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fungi. Several strains of the genus *Aspergillus* are also reported to produce antioxidative substances in fermented sardine meal[8-10]. These results suggest that several xerophilous fungi produce effective antioxidants during *katsuobushi* molding. In this study, the potential ability for producing effective antioxidants was investigated in several xerophilous fungi used in *katsuobushi* molding.

2 Materials and Methods

2. 1 Fungal Strains and Cultivation

The strains used in this study were *Aspergillus repens* (*A. repens*) IFO 8914 and *A. ruber* IFO 7712. *Eurotium herbariorum* (*E. herbariorum*) NU-1, NU-2 and NE-1, which were kindly provided by Ninben Co. Ltd. (Saitama Prefecture), were also used.

Glucose (40 g) and agar (4 g) were added to distilled water (180mℓ), and autoclaved at 121℃ for 20 min. A MYP solution containing malt extract (0.6 g), yeast extract (0.6 g), pepton (1.0 g) and distilled water (20 mℓ) was filtered through a 0.45 μm membrane filter (Millipore Co. Ltd.). The medium was prepared by mixture of MYP and glucose solution. The reason for which the medium has been divided into two parts is to prevent the occurrence of aminocarbonyl reaction between MYP and glucose by autoclaving, since the amino-carbonyl compounds have an antioxidative effect. Strains were inoculated on the medium (20 g, 9cm dish) and cultivated at 25℃ for 50 days.

2. 2 Extraction of Antioxidative Substance

After cultivation, the medium was mixed with sea sand and 100mℓ of methanol, and milled finely in a mortar. The mixture was left to stand at 4℃ overnight, and then filtered through a filter paper (No.1, Advantec Co. Ltd.). The residue was washed with 100 mℓ of methanol again on the filter. After the methanol filtrates were dried with a rotary evaporator to dryness, the residue was partitioned between water and chloroform. The aqueous phase was washed twice with an equal volume of chloroform. The chloroform extracts were evaporated to dryness, and the residue was extracted with ethyl acetate. The ethyl acetate extracts were used for assay of the antioxidative activity, unless otherwise noted.

2. 3 Auto-oxidative Test

The antioxidant fractions obtained were added to 2 g of lipid extracted from fresh sardines. The mixture was spread out into a dish of 9cm - diameter, and maintained at 37 ℃ for 5-10 days. Antioxidative activity was evaluated both by measuring peroxide value (POV) and tribarbituric acid reactive substances (TBARS)[11] of the oxidized sardine lipid. TBARS value is expressed in terms of the malondialdehyde (MDA) equivalent.

3 Results and Discussion

3. 1 Extraction of Antioxidative Substance

The methanol extract from the medium on which *A. repens* was not inoculated was negligible. In the medium in which *A. repens* was cultivated for 50 days, the methanol extract was partitioned between chloroform and water, and of antioxidative activity the fractions concentrated both from the water and chloroform phases were tested. The POV and TBARS values were higher in the water soluble fraction than those in the control. The water soluble fraction accelerated the oxidation of lipid obtained from sardine. When the sardine lipid was maintained in the presence of 10mg of the chloroform fraction (0.5% addition) for 10 days, the POV was about 20 meq/kg, and TBARS value was 80% of sardine lipids in which α-tocopherol was added at concentration of 0.05% (Fig. 1). When being partitioned with ethyl acetate, the fraction gave almost the same POV and TBARS value as chloroform fraction. Since the slightly stronger activity was obtained in the antioxidative substance which was first partitioned by chloroform and then dissolved with ethyl acetate, the combination of chloroform and ethyl acetate were employed to extract antioxidative substance in this experiment.

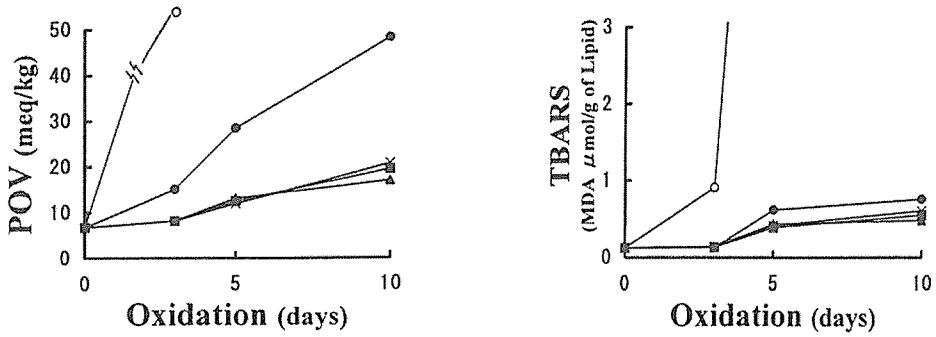

Fig. 1. Effects of various extraction methods on oxidation of sardine lipid.
Control (○), α-tocopherol (●), Chloroform (×), Ethyl acetate (■), Chloroform-ethyl acetate (▲).
Extract from each solvent (0.5%) and α-tocopherol (0.05%) were added to sardine lipid. POV were 221 and 768 (meq/kg) in which sardine lipids without antioxidants were maintained for 5 and 10 days.

3. 2 Relationship between Antioxidative Activity and Cultivation Time

Antioxidative substances were extracted from the media in which *A. repens* was cultivated for 10 - 90 days, and sardine lipids in the presence of 0.5% extracts were oxidized for 5 or 10 days. As shown in Fig. 2, the antioxidative substances were produced during the 10 days cultivation, and the POV and TBARS value were as same as those of the 20 - 90 days cultivation. The weights of antioxidative fractions obtained from 40 g of media were 0.046, 0.083, 0.091 and 0.111 g for 10, 30, 60 and 90 days cultivation, respectively. These results suggest that the components of antioxidative substances were similar among the cultivation stages of 10 - 90 days, though the weights of antioxidative fractions were different in the fungal growth during 10 - 90 days.

3. 3 Antioxidant Production from Five Fungal Strains

Antioxidative substances were extracted from the media in which 5 fungal strains were cultivated for 50 days. The weights of extracts obtained from 40 g of media were 0.083, 3.16, 0.059, 0.080 and 0.235 g for *A. repens*, *A. ruber*, *E. herbariorum* NE-1, NU-1 and NU-2 strains, respectively. Antioxidative activity was found in all strains tested as shown in Fig. 3. The order of activities (POV and TBARS value) was slightly large in NE-1, followed by *A. repens*, NU-2, NU-1 and *A. ruber*. It is suggested that after considering the factors of activities and productive weights, *A. ruber* is the most effective strain for suppressing lipid oxidation. When cultivated for 50 days, fungal color became dark green for *A. repens*, NE-1 and NU-1, and reddish-brown for NU-2 and *A. ruber*. The color of the medium of *A. ruber* cultivation

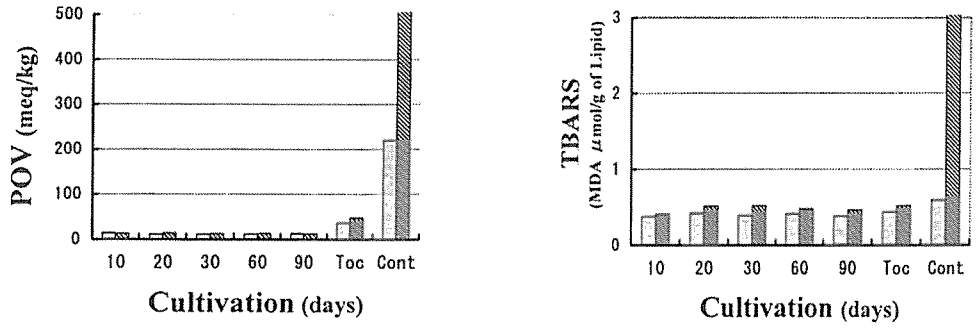

Fig. 2. Antioxidative activity of extracts from media in which *A. repens* was cultivated for 10 - 90 days. Extracts and α-tocopherol (Toc) were added at concentrations of 0.5% and 0.05% to sardine lipid, respectively. Left and right bars indicate oxidation time of 5 days and 10 days, respectively. TBARS values are calculated as shown in Fig. 1.

changed from an initial bright-yellow to dull brown at the end.

Figure 4 displays the thin layer chromatogram (TLC) in which the extracts from the media were developed by using the chloroform and methanol (88 : 12) solvent system and then the bands were detected under ultraviolet light. The band colors (Rf 0.75, 0.62, 0.45 and 0.33) of *A. repens* were different from those of the other fungal strains. In additon, the TLC patterns of NU-1 and NE-1 were similar to that of *A. ruber*. This result suggests that the TLC patterns did not necessarily coincided with their colors of the media.

3. 4 Partial Purification of Antioxidative Substances and their Activity

The antioxidative extract from the medium in which *A. repens* was cultivated for 50 days was developed by using the TLC described above, and fractionated by scraping out the developed bands under ultraviolet light. The partially purified antioxidative fractions were divided into 9 fractions (A - I) as shown in Fig. 5. The sardine lipid containing 0.05% of each fraction was maintained for 5 or 10 days. Fraction B, C, G and I indicated antioxidative activity as shown in the POV and TBARS value (Fig. 6). In

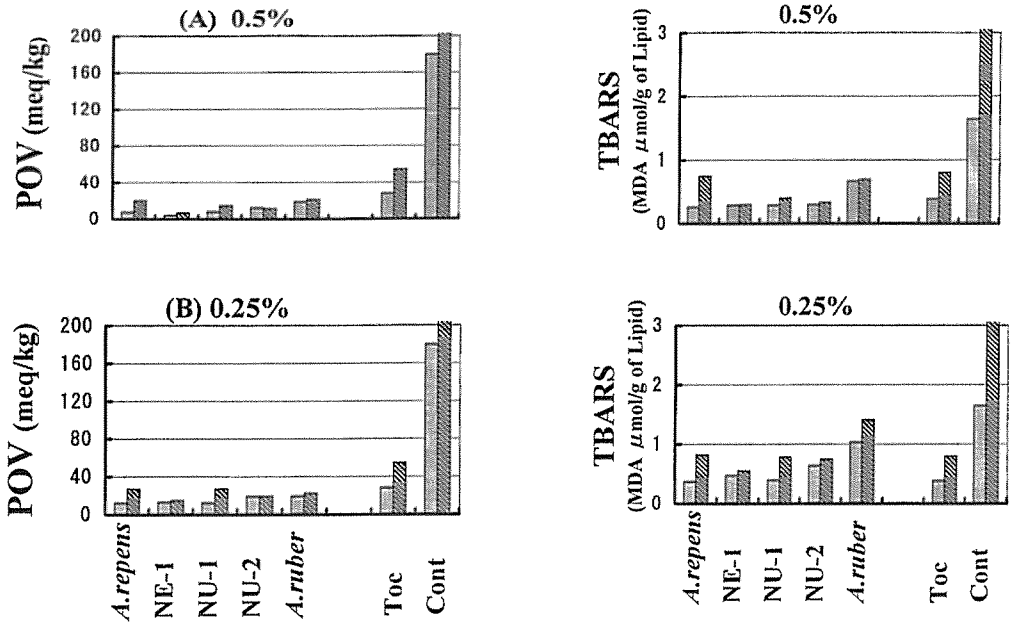

Fig. 3. Effects of extracts produced from 5 xerophilous fungi on oxidation of sardine lipid. The concentration of α-tocopherol and the bars are the same as in Fig. 2.
TBARS values are calculated as shown in Fig. 1.

particular, fraction C had the lowest POV and TBARS value among the fraction obtained.

It was found that the 5 strains of xerophilous fungi utilized in *katsuobushi* molding produced effective antioxidative substances. In addition, we suggest that the multiple substances produced by the fungi participate in the suppression of lipid oxidation.

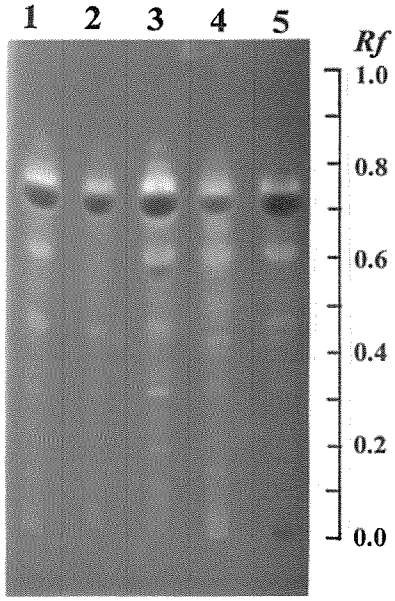

Fig. 4. Thin layer chromatogram of the extracts from 5 xerophilous fungi.
Lane : 1, NE-1; 2, NU-1; 3, NU-2; 4, *A. repens*; 5, *A. ruber*.
TLC was developed using silicic acid (Kiesel Gel 60G, Merck) with a chloroform-methanol (88 : 12, v/v) solvent system, and was detected under ultraviolet light.

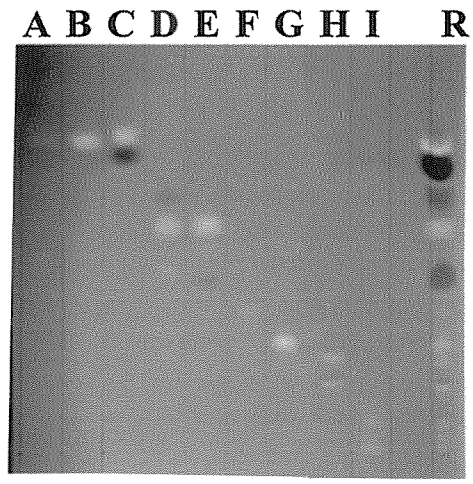

Fig. 5. Thin layer chromatogram of a partially purified fraction from *A. repens* extract.
The fraction was separated as described in Fig. 4, and obtained by scraping out the developed bands under ultraviolet light.

References

1) S. Ohta : "*Katsuobushi*". *New Food Industry*, **23**, 35-41 (1983) (in Japanese).
2) M. Motosugi, S. Doi, T. Suzuki and M. Ishikawa : Studies on the improvement of processes for broil-drying skipjack - IX. Changes of the lipids during molding for broil-drying skipjack. *Shizuoka-ken Kyogyoshiken Center Hokoku*, **25**, 117-122 (1981) (in Japanese).
3) L. Dimici and S. Wada : Lipid changes inbonito meat in the katsuobushi processing and quality assessment of the com mercial product based on lipid composition. *J. Jpn. Oil Chem. Soc.*, **43**, 470-478 (1994).

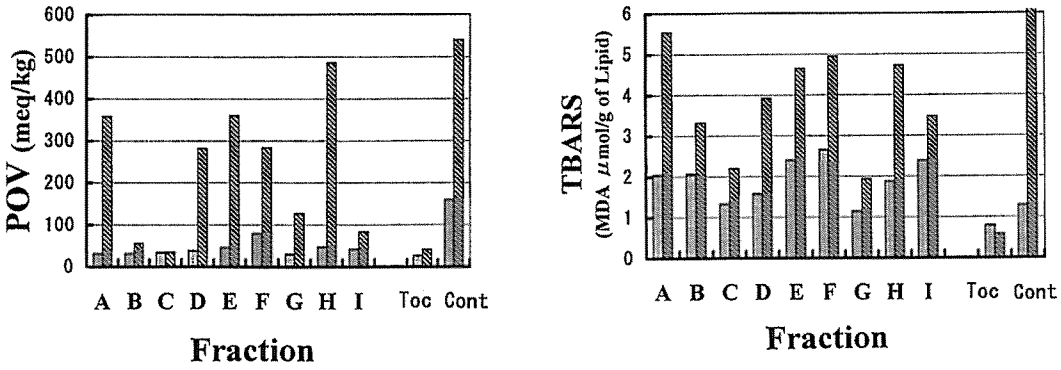

Fig. 6. Effects of partially purified fractions on oxidation of sardine lipid.
Each fraction and α-tocopherol were added at a concentration of 0.05% to sardine lipid.
TBARS values were calculated as shown in Fig. 1.
The bars are defined in Fig. 2.

4) T. Suzuki and M. Motosugi : Changes in volatile flavor components and antioxidant activity of absorbed phenolic compounds of dried bonito stick (Katsuobushi) during smoking process. *Nippon Shokuhin Kagaku Kogaku Kaishi*, **43**, 29-35 (1996) (in Japanese).

5) Y. Kawai, Y. Oeda, M. Otaka, T. Kasakawa, N. Inoue and H. Shinano : Screening and identification of antioxidant-producing strains in food-borne fungi. *Bull. Fac. Fish. Hokkaido Univ.*, **44**, 141-146 (1993).

6) Y. Kawai, M. Otaka, M. Kakio, Y. Oeda, N. Inoue and H. Shinano : Screening of antioxidant-producing fungi in *Aspergillus niger* group for liquid- and solid-state fermentation. *Bull. Fac. Fish. Hokkaido Univ.*, **45**, 26-31 (1994).

7) Y. Oeda, Y. Kawai, N. Inoue, H. Shinano, Y. Nagai, Y. Hidaka and K. Furube : Antioxidative effect of an isolate from a culture filtrate of *Aspergillus niger*. *Biosci. Biotech. Biochem.*, **57**, 1374-1375 (1993).

8) F. Kato, I. Nakazako, T. Shiraishi, K.Hayashi, A. Murata and Y. Yone : Screening for antioxidative activity in microorganisms. *Nippon Nogeikagaku Kaishi*, **59**, 901-907 (1985) (in Japanese).

9) F. Kato, I. Nakazato, A. Murata, S. Okamoto and Y. Yone : Use of waste fish for large scale production of fermented fish meal and its feed efficiency. *Nippon Nogeikagaku Kaishi*, **60**, 287-293 (1986) (in Japanese).

10) M. A. Hossain, M. Furuichi and Y. Yone : POV, TBA, and VBN values, and proximate, amino acid, and fatty acid compositions of scrap meals fermented with fungi. *Nippon Suisan Gakkaishi*, **53**, 1629-1632 (1987).

11) Y. Shinma : Method for lipid extracted from fishy product. (in T. Saito, H. Uchiyama, T. Umemoto, and T. Kawabata (eds.). Suisan Seibutsukagaku - Syokuhingaku Jikkensyo.) Koseisya Koseikaku, Tokyo, 1974, pp. 80-102.

数種のかつお節カビ付け菌による抗酸化物質の産生

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かつお節のカビ付けに用いられている5菌株を対象に，20%グルコースを含むMYP寒天培地での抗酸化物質の生産能力について検討した。その結果，メタノール，クロロホルム，酢酸エチルの組み合わせによる抽出より，5菌株すべての抽出物に抗酸化活性が認められた。過酸化物価（POV），TBARS を指標とした活性では *Eurothium herbariorum* NE-1 が高い値を示したが，生産量を考慮すると *Aspergillus ruber* が優れていた。*A. repens* から部分精製した画分のひとつは，α-トコフェロールと同等の抗酸化活性を有していた。