Note

Studies on Antioxidative Substances of Microbial Origin

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Received March 5, 1984

In our laboratory we have isolated antioxidative substances from microbes and reported the outline of the screening method in a previous paper.¹⁾ Furthermore, in order to isolate effective antioxidants, we have screened organisms using an improved method, which is a combination of the test described in a previous paper¹⁾ and the indophenol (Tillman reagent)²⁾ test.

As a result of our screening, two strains were selected. Strain No. 114, identified as *Streptomyces melanogenes*, produced a substance which showed a positive reaction on the indophenol test. F-114 substance was obtained as colorless crystals by extraction of the culture broth with ethyl acetate and silica gel column chromatography done twice using chloroform-acetone and *n*-hexane-ethyl acetate to elute.³⁾ The physicochemical properties of F-114 substance are shown in Table I. Its mp and IR, UV and MS spectral data agreed well with those previously reported^{4,5,6,7)} for 5-hydroxymaltol. Bacteria such as the genera *Gluconobacter* and *Pseudomonas*⁸⁾ have been re-

ported as 5-hydroxymaltol producers, and this is the first report of 5-hydroxymaltol production by an actinomycete.

Penicillium janthinellum C-268, a producer of curvulic acid as reported previously,⁹⁾ produced some substances which had positive results with both tests, by altering the composition of culture medium for curvulic acid production. The fermentation was carried out in a jar fermentor with a medium containing 20 g of sucrose, 10 g of Polypeptone, 1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 of KCl, 10 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, and 10 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 liter (pH 6). The filtrate was extracted with ethyl acetate. It was ascertained that active substances were present in the acidic extract (I-1) and the neutral extract (I-2, I-3 and I-4) by both tests (Fig. 1). The acidic extract was chromatographed on a silica gel column, followed by a Toyopearl HW-40F column. I-1 was obtained as colorless needles. The neutral extract was chromatographed on a silica gel column and eluted with mixtures of *n*-hexane-ethyl acetate, increasing the ratio of ethyl acetate in steps. The fraction containing I-2 was rechromatographed on a silica gel column. Recrystallization of I-2 from hot *n*-hexane gave colorless crystals. The fraction containing I-3 was then subjected to chromatography on a Sephadex LH-20 column. I-3 was obtained as colorless needles. From 40 liters of culture broth the yields of I-1, I-2, and I-3 were 30 mg, 15 mg, and 40 mg, respectively. However, I-4 could not be isolated because of the low yield. From the data in Table I, I-1 was assumed to be homogentisic acid¹⁰⁾ and I-3 to be 3-methoxytoluhydroquinone.¹¹⁾ I-1 was confirmed by direct comparison with an authentic sample. In spite of being a well-known substance, the fermentation production of homogentisic acid by microorganisms except for the genus *Aspergillus*¹⁰⁾ has not been reported to date. On the other

TABLE I. PHYSICOCHEMICAL PROPERTIES OF F-114, I-1, AND I-3 SUBSTANCES

	F-114	I-1	I-3
mp (°C)	157 (microscope hot plate) 182~184 (sealed tube)*	145~147	123~124
MS	142 ($\text{C}_6\text{H}_6\text{O}_4$) 142 (100), ** 113 (17) 96 (9), 87 (8), 85 (18) 71 (12), 68 (25)	168 ($\text{C}_8\text{H}_8\text{O}_4$)	154 ($\text{C}_8\text{H}_{10}\text{O}_3$)
IR ($\nu_{\text{max}}^{\text{KBr}}$ cm^{-1})	3160, 1645, 1600 1545, 1440, 1400 1300, 1220, 1190	3500, 1695 1505	3500~3100 1605, 1470
^1H NMR δ (ppm)	2.3 (3H, s) 7.9 (1H, d, $J=3$ Hz) 7.93 (1H, d, $J=3$ Hz) 8.0 (1H, s) in dioxane- d_8	3.6 (2H, s) 6.7 (3H, s) 7.6 (3H, s) in acetone- d_6	2.0 (3H, s) 2.6 (2H, s) 3.6 (3H, s) 6.0 (1H, d, $J=3$ Hz) 6.1 (1H, d, $J=3$ Hz) in acetone- d_6

* Reference 5.

** Relative intensity.

FIG. 1. TLC of the Extracts.

TLC was carried out using a Kieselgel 60 GF₂₅₄.

Left: The acidic extract, developed with benzene-acetone-acetic acid (70:30:2).

Right: The neutral extract, developed with *n*-hexane-benzene (1:1).

FIG. 2. Antioxidant Activity of I-1, I-2 and I-3.

—●—, control; —□—, BHT; —△— I-1; —▲—, I-2; —○—, I-3.

hand, I-3 was confirmed by deriving the corresponding quinone from I-3, and the quinone (mp 147~148°C, ¹H-NMR δ_{TMS}^{CDCl₃} ppm: 2.1 (3H, d, *J*=2 Hz), 3.8 (3H, s), 5.8 (1H, d, *J*=2 Hz), 6.5 (1H, m)) agreed well with the data in the literature.¹² I-2 was an interesting compound containing a *t*-butyl group in the structure, and its detailed characteristics will be reported in our following paper.

The antioxidative activity of 5-hydroxymaltol was examined in a water-alcohol system by the measurement of the total carbonyl value.¹¹ The result indicated that 5-hydroxymaltol had an activity the same as α-tocopherol in alkaline conditions. The antioxidative activities of I-1, I-2, and I-3, were examined in an oil system by the measure-

ment of peroxide values. The ethanol solutions (0.6 ml), which contained 1 mg of each test sample in 1 ml, were placed in 50 ml beakers. After the addition of 3 g of linoleic acid to each beaker, the mixtures were placed in a constant temperature oven set at 63°C. The result of comparison with BHT indicated that the activity of I-2 was stronger than BHT and that of I-1 was approximately equal to BHT (Fig. 2). On the other hand, I-3 had a weaker activity than BHT, I-1, and I-2, and its decolorization period of indophenol was appreciably short in comparison with them.

Consequently, 5-hydroxymaltol and homogentisic acid may not be appreciably useful as antioxidants from a practical point of view, whereas they are of interest as fermentation products, and I-2 is of interest because of its unique structure and strong antioxidative activity. Moreover, it is suggested that the screening for antioxidants from microbes would offer not only effective antioxidants but also interesting natural products.

Acknowledgment. The authors wish to thank members of the Fermentation Research Laboratory of Fujisawa Pharmaceutical Co., Ltd., for jar fermentation.

REFERENCES

- 1) T. Aoyama, Y. Nakakita, M. Nakagawa and H. Sakai, *Agric. Biol. Chem.*, **46**, 2369 (1982).
- 2) Y.-T. Chen, F. A. Isherwood and L. W. Mapson, *Biochem. J.*, **55**, 821 (1953).
- 3) T. Aoyama, Master of Agriculture Thesis, Department of Agricultural Chemistry, University of Osaka Prefecture, 1983, p. 57.
- 4) G. R. Jurch and J. H. Tatum, *Carbohydrate Res.*, **15**, 233 (1970).
- 5) F. Shafizadeh, R. H. Furneaux, T. T. Stevenson and T. Cochran, *Carbohydrate Res.*, **67**, 433 (1978).
- 6) Aldo Ferretti and Vincent P. Flangan, *J. Agric. Food Chem.*, **21**, 35 (1973).
- 7) O. Terada, S. Suzuki and S. Kinoshita, *Nippon Nôgeikagaku Kaishi*, **36**, 623 (1962).
- 8) K. Arima, Y. Sakurai and K. Izaki, *Nippon Nôgeikagaku kaishi*, **42**, 103 (1968).
- 9) Y. Nakakita, S. Shima and H. Sakai, *Agric. Biol. Chem.*, **48**, 1899 (1984).
- 10) K. Yuasa, K. Ishizuka and T. Sakasai, *Agric. Biol. Chem.*, **42**, 167 (1978).
- 11) J. Better and S. Gatenbeck, *Acta Chem. Scand., Ser. B*, **B 30**, 368 (1976).
- 12) R. S. Sood, K. Roy, G. C. S. Reddy, J. Reden and B. N. Ganguli, *J. Antibiot.*, **35**, 985 (1982).