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Studies on Antioxidants of Microbial Origin

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微生物이 生産하는 抗酸化物質에 關한 硏究

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Antioxidant, tentatively named PA-29B substance was isolated from the fermentation broth of rare Actinomycetes. It was isolated by means of silica gel column chromatography and obtained as colorless plates, mp 155-157°C. The structure of PA-29B substance was assigned to be α-phenyl acetamide by ^{1}HNMR spectrometer and mass spectrometer.

Hundreds of materials, both synthetic and of natural origin, have been developed[1)] as antioxidants for food preservation, but only tert-butyl-4-hydroxy anisol(BHA) and tert-butyl-4-hydroxytoluene (BHT) as synthetic antioxidants and tocopherol as natural ones are practically used.

However, the most widely used antioxidants, BHA and BHT, are suspected of causing liver damage.

Moreover, synthetic chemicals used as food additives tend to be eliminated. On the other hand, tocopherols are widely used as safe natural antioxidants but they are not so effective as synthetic antioxidants and the manufacturing cost is high.

Recently, the development of natural antioxidants has been researched and expected to replace the synthetic compounds which are widely used at the present time. Some natural antioxidants of microbial origin have already been isolated, but the screening procedures used were indirect and complicated.[2,3)]

Fortunately, a simple and direct method for screening was developed by T. Aoyama *et. al.* They have also tried to isolate these active substances from fungi.[4)]

In this paper, the screening method, fermentation and isolation, identification and evaluation of the isolates from rare Actionomycetes will be described.

Meterials and Methods

Screening method

Isolation of microorganism was carried out using medium A listed in table I. Selected colonies were transferred on medium B (table I) and stored. From stored slants, rare Actinomycetes were used for antioxidant screening. Fermentation was carried out on reciprocating shaker at 30°C for 3-4 days using medium consisted of 1% Glucose, 0.1% Yeast extract, 0.2% Polypeptone and 0.1% Meat extract. The culture filtrate was then subjected to antioxidant screening. This procedure was developed using the method for evaluation of peroxide value, and is shown in scheme I. Two hundred and twenty strains of newly isolated rare Actinomycetes were screened accordiong to this procedure.

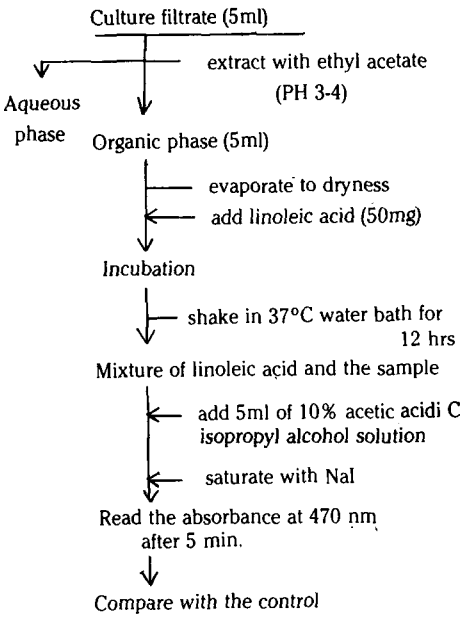
The composition of medium used for seed and main culture was same as screening medium (liquid). Preculture was carried out in reciprocating shaker at 30°C for 48 hrs in 500ml flat bottom flask of 100ml medium. In large scale fermentation, 5% of seed culture was inoculated in 5L elenmeyer flask of 2L medium and cultured on reciprocating shaker at 30°C for 72 hrs.

The cultured broth (ca. 30 liters) was filtered and adjusted to pH 3.5 with HCl. The filtrate was extracted with ethyl acetate (ca. 30 liters), separated and concentrated *in vacuo*.

The concentrate (ca. 2.5g) was charged on a silica gel (Fugi BW-820MH) column with benzene-ethyl acetate as the solvent system.

Table 1. Composition of the Medium for Isolation

A	Soluble starch	1 (%)
	K_2HPO_4	0.05
	HN_4Cl	0.05
	Agar	1.5
B	Glucose	1 (%)
	Meat extract	0.1
	Poly peptone	0.2
	Yeast extract	0.1
	Agar	1.5
	PH 7.5	



Scheme 1. Screening Procedure for Antioxidants

The fractions eluted with benzene-ethyl acetate (3:7v/v) exhibited a very strong antioxidative activity with the method for evaluation of peroxide value, and were combined and subjected to futher silica gel column chromatography. The fractions eluted with benzene-ethyl acetate which exhibited a strong activity in second silica gel column chromatography were pooled. Active substance obtained as crude crystals by concentration and recrystallized from n-hexane-ethyl acetate.

Instruments for structural elucidation;

melting point : Microscope hot plate

UV: Shimadsu UV-200 spectro photometer

IR: JASCO IRA-2 spectrometer

^{1}H-NMR: JASCO JAM-MH-60 spectrometer

Mass spectrum: Hitachi RMU-6M mass spec trometer

TLC was carried out on a silica gel with acidic ethyl acetate. The spots were observed under UV light, scraped off and eluted with acetone. The elutes were subjected to antioxidative activity test.

Results

Result of screening test

Of about 120 strains isolated, 37 strains were positive. From the 37 strains that gave positive results, the most active strain, numbered N-29 was selected for further study.

Thin layer chromatography of extracts from both of N-29 strain;

Detailed investigation of the active components in the broth extract was carried out by use of TLC analysis. As shown in Fig. 1, 9 spots were observed on TLC and each band of the preparative TLC corresponding to these spots was scraped off and extracted with acetone. Each elutes was assayed by the procedure shown in Scheme 1. Significant antioxidative activity was observed for the PA-29A, 29B and PA-29C fractions, which named tentatively

Among the active substances shown on TLC, PA-29B substance which exhibited a very strong antioxidative and ease crystallized, was under taken for further investigation. The Rf value of this substance on TLC in

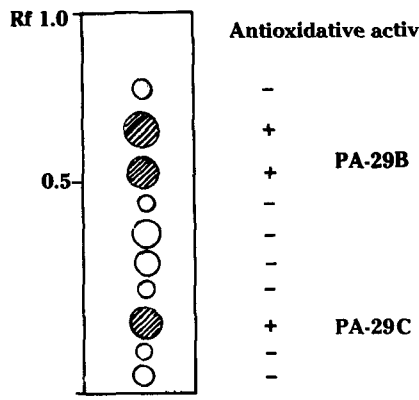


Fig. 1 TLC and Antioxidative activity of extract from fermentation broth. TLC analysis was carried out using a silica gel G(type 60) plate and ethyl acetate-acetic acid (100:2) as the solvent system

ethyl acetate:acetic acid (100:2) as solvent system is 0.5.

Isolation and physicochemical properties of PA-29B substance;

Ninety three mg of pure PA-29B substance was obtained from 30 liters of cultured broth. It was obtained colorless plates, mp 155-157°C. It shows positive color reaction in Indo-phenol, $FeCl_3$, $KMnO_4$, o-tolidin-KI and negative in Dragendorff, ninhydrine, Erlich. It is soluble in methanol, slightly soluble in organic solvent such as chloroform, acetone, ether, benzene and in water.

Fig. 2 shows the ultraviolet absorption spectrum of PA-29B substance in methanol, λ_{max}^{MeOH} = 248, 258, 291 nm. Its infrared absorption spectrum measured in KBr gave peacks at the following frequency as shown in Fig. 3, 3300, 3150, 1620, 1400, 1280, 680$cm^{(-1)}$.

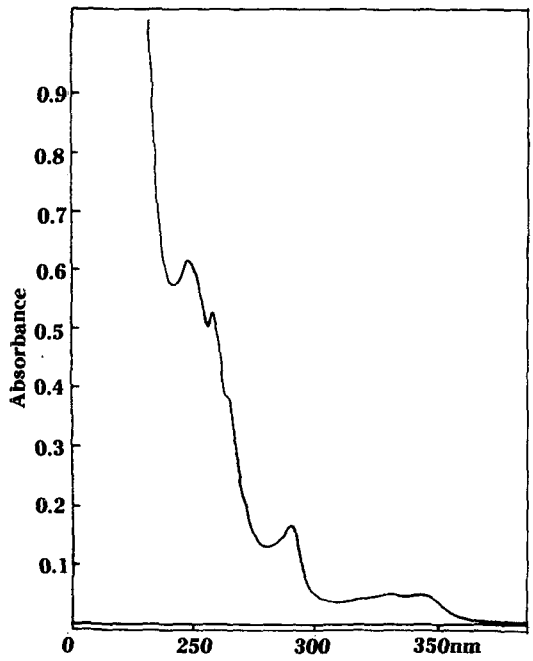


Fig. 2. UV Spectrum of PA-29B Substance

structural analysis of PA-29B substance;

The ^{1}H-NMR spectrum in $CDCl_3$ (Fig. 4) showed signals at δ 3.52ppm (2H, singlet), δ 5.50ppm (2H, broad) and δ 7.33ppm (5H, singlet). In the ^{1}H-NMR spectrum in $CDCl_3$, the signals at δ 5.50ppm were disappeared by adding D_2O. This indicated two protons at δ 5.50ppm were exchangeable. In the mass spectrum of PA-29B substance (Fig. 5), the base peak was due to m/e 92 ion (C_7H_8, Rel.int. 100%) and other prominent peaks were observed at m/e 117 (C_8H_7N, Rel.int. 4%). m/e 135 (C_8H_9ON, Rel. int. 23%) which indicated lose of H_2O ion

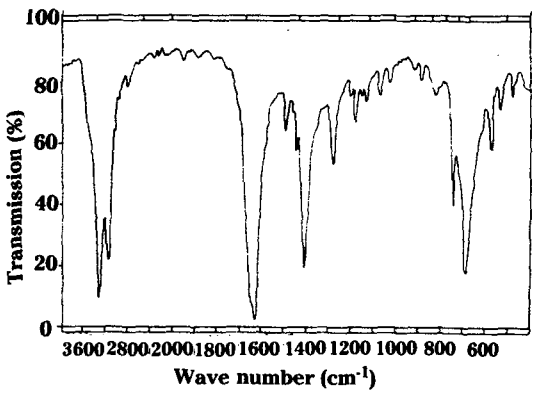


Fig. 3 IR Spectrum of PA-29B Substance (KBr)

from molecular ion and molecular ion respectively. It was suggested that the loss of H_2O molecular from PA-29B substance occured from its enol form shown in Fig. 6.

On the basis of the data so far obtained, structure of PA-29B substance was unequivocally deducecd for the α-phenyl acetamide represented in Fig. 6. (its keto and enol form) The Rf values of PA-29B substance and α-phenyl acetamide was exactly same on TLC in various solvent.

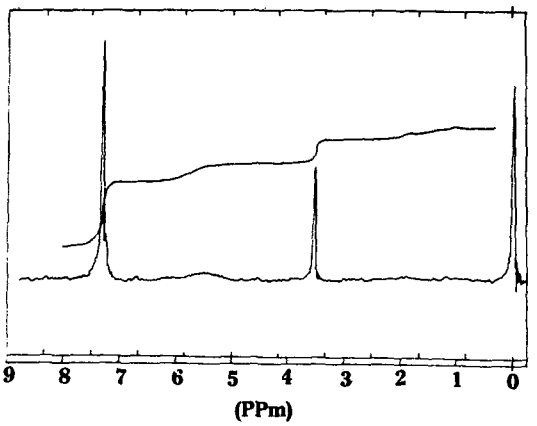


Fig. 4 ^{1}H-NMR Spectrum of PA-29B Substance ($CDCl_3$)

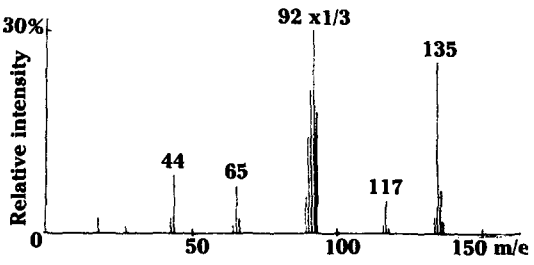


Fig. 5 Mass Spectrum of PA-29B substance

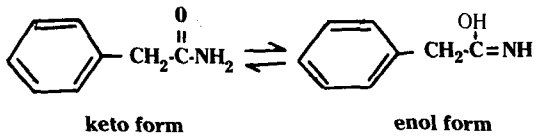


Fig. 6. α-**Phenyl acetamide**

Discussions

Most synthetics and natural antioxidants have phenolic hydroxy group in the structures. However, PA-29B substance (α-phenyl acetamide) is a completely new type of antioxidants which belongs to the amide. α-phenyl acetamide was known as raw material in manufacturing of penicillin G. It is first time that α-phenyl acetamide was produced by rare Actinomycetes. These results indicated that the screening method is effective for obtaining new microbial antioxidants.

PA-29A and PA-29C substances which showed as strong antioxidative activity as PA-29B are under investigation.

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