Purification and Characterization of the Antioxidative Substance Produced by Aspergillus oryzae

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Summary

Antioxidative substance extracellularly produced by Aspergillus oryzae in the culture fluid was purified by different chromatographies. The substance was purified 19-fold with an activity recovery of 26% from culture fluid. Purity of the substance was confirmed with a single peak through reversed phase HPLC as well as a single spot on TLC. The purified substance consisted of equimolar aspartic acid and glycine, indicating that the substance is a peptide. From mass spectral analysis the molecular weight was about 700, but the precise sequence of the amino acids is not clear. The substance was stable at 80°C for 60 min. Besides, the substance was completely stable at pH 4~14. This substance was able to suppress the oxidation of fish oil.

Key words: Aspergillus oryzae, antioxidant, peptide, aspartic acid, glycine.

Introduction

In our previous paper1) we have reported that Aspergillus sojae K and Aspergillus oryzae strongly suppressed the peroxide formation in waste fish meal, indicating that the microorganisms possibly produced antioxidative substance in the environment. On the basis of this assumption molds were cultivated in Czapek medium and an antioxidative activity was found in the culture fluid. Meanwhile we have purified and characterized the antioxidative substance of A. sojae K2) and it was found that the antioxidative substance is a low molecular weight peptide (MW 710) consisted of equimolar ratio of aspartic acid and glycine. Moreover, the antioxidative substance possessed aspartyl-glycyl β-peptide bond and two C-terminal. There were only few reports that some amino acids, peptides or proteins had antioxidative activity.3~7) On the other hand, compared to high molecular weight peptide the antioxidative effect of low molecular weight peptides (<700) were found to be superior.8)

In order to make a clear distinction between the antioxidative substances that were produced by A. sojae K and A. oryzae, purification and characterization of the antioxidative substance produced by A. oryzae were attempted.

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**Materials and Methods**

1. **Microorganism.** *Aspergillus oryzae* was used as an antioxidative substance producer. The microorganism was maintained at 4°C on a slant of commercial potato dextrose agar with 0.2% more agar for subsequent use.

2. **Medium and cultural condition.** Czapek medium consisting of 0.3% NaNO₃, 0.05% KCl, 0.05% MgSO₄·7H₂O, 0.1% K₂HPO₄, 0.001% FeSO₄·7H₂O, and 3% sucrose were dissolved in deionized water. The pH was adjusted to 6 with 1 N HCl and then sterilized at 121°C for 15 min. The microorganism was cultivated in 5-liter flask seeded with the precultured mold. Precultivation was done in a test tube (2.2 x 20 cm) containing 10 ml of Czapek medium inoculated with enough spores for 72 hr, and 3-4 tubes were used per liter. Cultivation was done at 30°C by shaking with 190 rpm for 15 days on a rotary shaker ROR 4-P (Iwashiya).

3. **Ninhydrin test.** Amounts of ninhydrin-positive substances were calculated using D-alanine as standard by following the method of Yemm and Cocking. Optical density was measured at 570 nm.

4. **Activity assay.** Antioxidative activity was assayed by using autoxidation of Na-ascorbate as described by Mishra and Kovachich. Unit activity was defined as the reciprocal of the highest dilution that showed 50% inhibition of the autoxidation of Na-ascorbate.

5. **Mass Spectrometry.** A JEOL JMS-DX300/JMA-DA5000 FAB mass spectrometer and FAB ionization system with xenon was used. The scanned mass range was m/z 0 to 1400 at 5 KeV acceleration energy and first ion acceleration voltage was 2 KV.

6. **Measurement of peroxides in fish oil.** Sardine oil without antioxidant was donated by Nishinohon Uoichi Co., Ltd. Five ml of an aqueous solution of the antioxidative substance (5 mg/ml) was added to 5 g of autoclaved fish oil, and was incubated at 30°C for 5 days on a reciprocal shaker at 300 rpm. A control was incubated with distilled water without the antioxidative substance. Peroxide value (POV) and malonaldehyde (MDA) were measured as described by Rashid *et al.*

**Results**

1. **Production of antioxidative substance**

   Cultivation of *A. oryzae* was done in a 5-liter flask containing 1 liter of Czapek medium at 30°C for 20 days. Antioxidative activity and ninhydrin-positive substances were measured every 4 days. Figure 1 shows that the activity increased with time and reached
a maximum (about 1,600 units/ml) after 16 days of cultivation. A positive correlation was observed with the production of antioxidative substance along with ninhydrin-positive substances as D-alanine.

2. Purification of the antioxidative substance

Step 1. Preparation of cell-free extract. Cells of \textit{A. oryzae} were completely removed from the culture fluid by filtration through filter paper (Whatman No. 1) and the filtrate was concentrated by a rotary evaporator at 30°C. The resulting concentrated fluid was filtered once again to remove the traces of cell debris. The filtrate was preserved at -20°C.

Step 2. Concentration of the culture fluid. The culture fluid was filtered twice through Whatman No. 1 filter paper and concentrated to 1/10th of the initial volume by a rotary evaporator at 30°C. The concentrated filtrate was filtered once again through Whatman No. 1 filter paper to remove a trace of cell debris and used as a stock sample for further purification.

Step 3. DEAE cellulofine A-200 column chromatography. DEAE cellulofine A-200 column of 2.2×28 cm was equilibrated with 50 mM sodium acetate buffer (pH 4.3). Four ml of the concentrated sample was put on the column. With the same buffer the column was washed and the elution was done with a linear gradient of 0~1.0 M of NaCl. The flow rate was adjusted to 36 ml/hr and 4 ml of each fraction volume was collected. Then antioxidative activity and ninhydrin-positive substances were measured. It was found that the substance was eluted from the column at a concentration of 0.52 M NaCl. The active fractions were pooled together and concentrated to 2.6 ml by evaporation.

Step 4. Gel filtration with Sephadex G-15 column. A Sephadex G-15 column of 1.6×80 cm was equilibrated with sodium acetate buffer (pH 4.3) and washed with distilled water. A sample volume of 2.6 ml was put on to the column. Antioxidative substance was eluted from the column with distilled water at a flow rate of 12 ml/hr and 3 ml fractions were collected. The antioxidative activity and ninhydrin-positive substances in each fraction were measured. It was observed that the substance was eluted from the column with only one peak that coincided with activity. The active fractions were combined and concen-
trated to 4 ml by evaporation.

**Step 5.** DEAE Sephadex A-25 column chromatography. A column of 1.8×21 cm packed with DEAE Sephadex A-25 gel was equilibrated with 50 mM sodium acetate buffer (pH 4.3) and 4 ml of the concentrated sample obtained from Step 4 was put on it. The column was washed with the same buffer, and elution was done with a linear NaCl concentration gradient of 0~1.0 M. The flow rate was 20 ml/hr. Each fraction was 6 ml. The substance was eluted from the column with a sharp peak containing an antioxidative activity and ninhydrin-positive substance at a concentration of 0.48 M NaCl. The active fractions were pooled and concentrated to 1.7 ml by evaporation.

**Step 6.** Sephadex G-15 column chromatography. The concentrated sample (1.7 ml) obtained from Step 5 was put on a column of 1.6×80 cm of Sephadex G-15 which was equilibrated with distilled water. Elution was done with distilled water at a flow rate of 12 ml/hr. Each fraction was 3.5 ml. The active fractions were pooled and concentrated to 4 ml by evaporation. Traces of NaCl present in the concentrated sample were removed with a Micro Acilyzer (Asahi Kasei) and then the sample solution was lyophilized.

**Step 7.** High-performance Liquid Chromatography. A reverse phase HPLC was done with a JASCO tri rotor-V type. A Wakosil-II 5C18 HG column of 1.0×25 cm was used. UV monitor was set at 220 nm, column temperature maintained at 30°C, and the mobile phase was distilled water (HPLC grade). For preparative purposes, the lyophilized sample was dissolved in distilled water (50 mg/ml) and a 500 µl portion was injected; the flow rate was 2 ml/min. The active substance was eluted with a major peak. The active fraction was concentrated and then lyophilized. Nineteen-fold purification with 26% recovery was achieved from 850 ml of the culture fluid through different chromatographic steps as shown in the Table 1. The specific activity increased from 380 units/mg to 7,189 units/mg. Purified brownish white powder (204 mg) was obtained on lyophilization.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total NPS (mg)</th>
<th>Activity (× 10^6) (units/ml)</th>
<th>Total Activity (× 10^6) (units)</th>
<th>Specific Activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Fluid</td>
<td>850</td>
<td>3,581.5</td>
<td>1.6</td>
<td>1360.0</td>
<td>380</td>
<td>100</td>
</tr>
<tr>
<td>Conc. by Evap.</td>
<td>80</td>
<td>3,214.4</td>
<td>16.0</td>
<td>1280.0</td>
<td>398</td>
<td>94</td>
</tr>
<tr>
<td>DEAE-Cellulofine A-200a</td>
<td>52</td>
<td>265.0</td>
<td>17.2</td>
<td>894.4</td>
<td>3,375</td>
<td>66</td>
</tr>
<tr>
<td>Sephadex G-15 (1st)a</td>
<td>80</td>
<td>210.0</td>
<td>9.2</td>
<td>736.0</td>
<td>3,505</td>
<td>54</td>
</tr>
<tr>
<td>DEAE-Sephadex A-25a</td>
<td>34</td>
<td>136.6</td>
<td>19.1</td>
<td>649.4</td>
<td>4,754</td>
<td>48</td>
</tr>
<tr>
<td>Sephadex G-15 (2nd)a</td>
<td>80</td>
<td>86.0</td>
<td>6.0</td>
<td>480.0</td>
<td>5,581</td>
<td>35</td>
</tr>
<tr>
<td>HPLCa</td>
<td>45</td>
<td>48.2</td>
<td>7.7</td>
<td>346.5</td>
<td>7,189</td>
<td>26</td>
</tr>
</tbody>
</table>

a Active fractions were concentrated by evaporation.

b Ninhydrin positive substance.
3. Homogeneity of the purified antioxidative substance

The purified substance after preparative HPLC was analyzed with an analytical column (Wakosil-II 5C18 HG, 4.6 mm × 25 cm). The substance was eluted with a single peak that had a retention time of 4.47 min, but the activity recovered was 66% (w/w). The substance showed a single spot on thin layer chromatograms with various solvent systems. The substance was also a single peak on ion exchange chromatography using amino acid analyzer.

4. Properties of the antioxidative substance

Qualitative tests of the antioxidative substance. The substance was positive to ninhydrin\(^9\) and tolidine\(^{11}\) tests, while it was negative to anthrone, iodine, \(p\)-dimethylaminobenzaldehyde, and triphenyltetrazolium chloride.\(^{13}\)

Molecular mass. From mass spectral analysis, molecular mass was estimated about 700.

UV absorption. Figure 2 indicated that the antioxidative substance had an absorption maximum at 198 nm (\(\lambda_{\text{max}}\) water).

Solubility. The purified antioxidative substance was freely soluble in water but insoluble in organic solvents.

Melting point. It had a melting point of 270°C (dec).

5. Thermostability and pH Stability

The sample (1 mg/ml) in distilled water was incubated at different temperatures for 60 min and every 15 min the remaining activity was measured. The substance remained stable up to 80°C for 60 min while the activity was completely lost over 80°C.
Fig. 4. Analysis of the Antioxidative Substance with Amino Acid Analyzer.
A hundred µl of the antioxidative substance was put on an ion exchange column of amino acid analyzer (JEOL JLC-300).
A : Chromatogram of the antioxidative substance (0.1 mg/ml).
B : Chromatogram of the hydrolysate of the antioxidative substance (0.25 mg/ml).

The effects of pH (1~14) on the antioxidative substance was measured by measuring the residual activity on incubation at 50°C for 90 min (Fig. 3). The buffer systems used were: HCl-KCl (pH 1~2), citric acid-Na₂HPO₄ (pH 3~8), Na₂CO₃-H₃BO₃-KCl (pH 9~10), NaOH-Na₂HPO₄ (pH 11~12), and KOH-HCl (pH 13~14). The activity was affected in the pH ranges 1 to 3 and it was found that about 60% activity was lost at pH 1 and about 9% at pH 3. On the other hand, the activity remained completely stable from pH 4 to pH 14.

6. Amino acid composition.
Lyophilized powder of the antioxidative substance (1 mg) was hydrolyzed with 6 N HCl (1 ml) for 8 hr, 24 hr, 48 hr, 72 hr, and 96 hr at 110°C. Residual HCl was completely removed by repeated drying with a rotary evaporator. The residue was dissolved and diluted in citrate buffer (pH 3.15). After adjusting the concentration, the sample solution was filtered through millipore filter (Nihon Millipore Kogyo, 0.45 µm, HV).

Both native (0.1 mg/ml) and the above hydrolyzed (0.25 mg/ml) substances were put on a JEOL JLC-300 amino acid analyzer. Figure 4 shows that the native substance passed through the column as one peak, while the hydrolysate gave two peaks corresponding to aspartic acid and glycine. On TLC, the hydrolysate gave two distinct purplish violet spots with Rf values of 0.59 and 0.53 corresponding to the Rf values of the standard aspartic acid (0.60) and glycine (0.53).
7. N-terminal and C-terminal amino acids analysis.

N-terminal and C-terminal amino acids were analyzed by the methods described by Arai\textsuperscript{14} and Akabori et al.\textsuperscript{15}

On TLC no clear spot of 2,4-dinitrophenyl (DNP) derivatives of the N-terminal residue of the antioxidative substance was found. This phenomenon indicated that the N-terminal of the peptide remains blocked. On hydrazinolysis, aspartic acid and glycine were found as C-terminal amino acids through the amino acid analyzer. These results indicated that the substance might be a branched chain peptide with aspartyl-glycylβ-peptide bond.

8. Effects of various substances on the activity

A number of compounds including EDTA, amino acids, and divalent cations were added at different concentrations to the reaction mixture along with antioxidative substance and autoxidation of Na-ascorbate was measured. No such enhancement in activity was found.

9. Suppression of fish oil oxidation by the antioxidative substance

Figure 5 shows that the peroxide formation in fish oil was suppressed by the addition of the antioxidative substance. The increase in peroxide value and MDA content were inhibited at 70% of control by the addition of the antioxidative substance up to 3 days of incubation. On further incubation, the peroxide value and MDA content gradually increased and after 5 days of incubation, the inhibitory rate decreased to 40% in peroxide value and 52% in MDA content. It was found that after 5 days of incubation, the values rapidly increased and no differences were found in comparison with the control after 7 days of incubation.

Discussion

Purification of the antioxidative substance from the culture fluid of \textit{A. oryzae} was done by the same chromatographic technique as for that of \textit{A. sojae} K.\textsuperscript{2} It was found that the antioxidative substance of \textit{A. oryzae} is a peptide, composed of equimolar ratio of aspartic acid and glycine as it was found in the antioxidative substance of \textit{A. sojae} K. C-terminal
analysis indicated that the peptide possesses two C-terminal ends. On this basis obviously it might be assumed that the two microorganisms possess the same peptide backbone. The antioxidative substance of \textit{A. oryzae} was eluted from the ion exchange column relatively at a little higher NaCl concentration than that of \textit{A. sojae} K. Moreover, thin layer chromatographic behavior of the antioxidative substance was quite different from that of \textit{A. sojae} K. The antioxidative substance of \textit{A. oryzae} clearly moved when developed with a solvent system consisting of n-butanol : water (3 : 1 : 1). Molecular mass was also lower than that of \textit{A. sojae} K. It was found that during partial hydrolysis of the native antioxidative substance of \textit{A. oryzae}, optical density with ninhydrin was found to increase. This phenomenon indicates that the N-terminal of the peptide probably remains blocked. This antioxidative substance was also resistant to various proteolytic enzymes. The possible reason could be that the substance is a small peptide or it is not suitable for the enzymes as a substrate. It would be expected that the antioxidative substance of \textit{A. oryzae} might be analogous to that of \textit{A. sojae} K. The antioxidative substance of \textit{A. oryzae} was able to suppress the oxidation of fish oil and inhibited the autoxidation of Na-ascorbate. Moreover, this antioxidative substance is a very simple peptide, has a wide range of pH stability, and is stable below 80°C. These criteria, as a whole reflected on its wide scope of utilization of this antioxidative substance in various foodstuffs.

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**References**

Aspergillus oryzae の生産する抗酸化物質の精製と性質

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摘　　要

Aspergillus oryzae が菌体外に生産する抗酸化物質を種々のクロマトグラフィを用いて精製した。精製した抗酸化物質は比活性が培養液から 19 倍に上昇し、収量は 26% であった。精製度は逆相 HPLC でシングルピークとして得られたこと及び薄層クロマトグラフィで単一スポットであったことから確認した。抗酸化物質は、等モルのアスパラギン酸とグリシンからなるペプチドであった。質量分析の結果、分子量は約 700 と推定されたが、正確なアミノ酸配列はまだ明らかでない。この物質は、80℃で 60 分加熱しても安定であった。さらに、pH4 から pH14 の間でも活性の低下は認められなかった。また、この抗酸化物質は魚油の酸化を抑制した。