Synthesis and elucidation structure of O-para dehydroguaiacol prepared by crude of Brassica oleracea var alboglabra peroxidase-catalyzed oxidation

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ABSTRACT
Peroxidase was extracted from Brassica oleracea var alboglabra. The potential of crude Brassica oleracea var alboglabra peroxidase as a biocatalyst for the dimerization of guaiacol is presented. The products of the reaction were isolated and have been fully characterized by spectroscopic methods. One new coupling dimer of O-para dehydroguaiacol was obtained. Bioactive of this compound exhibited have strong antioxidant activity on DPPH radicals, with IC$_{50}$ value of 4.69 µM.

INTRODUCTION
Phenolate oxidation becoming a phenoxy using chemical catalyst is the most common way. Recently, peroxidases (E.C 1.11.1.7) are enzyme that catalyzed oxidative coupling reaction of phenols and aromatic amines (Shin, et al., 2004). Phenols, such as 2-methoxyphenols or guaiacol, are oxidized by peroxidase in the presence H$_2$O$_2$ resulting oxidative coupling, thus dimeric, oligomeric, or polymeric products are formed (Cristina, et al., 2010, Daniel, et al., 2004, Ruth, et al., 2003).

Guaiacol has been isolated from guaiacol from guaiakum, including in family of Zygopyllaceae. Guaiacum has 2 species, Guaiacum Officinale and Guaiacum Sanctum. Recently, guaiacol showed to possess potent antioxidant, anti-cancer, anti-inflammatory activities and also as material for drug of expectorant, tuberculosis, antiseptic (Ruth, et al., 2003). Guaiacol belongs to a group of phenolic compound. This phenolic has been proposed that phenolic oxidative coupling reaction is a key step for the biosynthesis of natural products (Jerzy, et al., 2003; Robert, et al., 2005; Lucas, et al., 2008). The aim of this paper was to describe a phenolic compound synthesized from guaiacol using crude Brassica oleracea var alboglabra peroxidase as biocatalyst. This paper demonstrated crude peroxidase and addition of H$_2$O$_2$ on the yield of O-para dimer of guaiacol formation.

MATERIAL AND METHODS
Material
The leaves of Brassica oleracea var alboglabra were obtained from local supermarket. The pure of enzyme peroxidase (hydrogen peroxide oxidoreductase) as synthesis biocatalysis was purchased from Sigma, and 4-hydroxy-5-methoxybenzene or orto-methoxy phenol (guaiacol) was purchased from nacalai tesque and used without any further purification. Sodiumhydrogen phosphat-monohydrat (Na$_2$HPO$_4$.H$_2$O) and di-Natriumhydrogen phosphat-dihydrat (Na$_2$HPO$_4$.2H$_2$O) were purchased from Merck Damstat, Germany for making phosphate buffer. All other chemical (including hydrogen peroxide, HCl) were purchased from Merck.
Methods

General Method

Silica gel column chromatography was carried out on Merck (70-230 mesh and 230-400 mesh). Thin layer chromatography (TLC) was performed on precoated kiesel gel 60 F254 (silica gel plates, 0.25 mm thick, Merck), spots were visualized under UV light (254 and 365 nm) and irradiation and by spraying with 10% sulphuric acid solution followed by heating at 110°C. IR spectra was measured on a FT-IR Shimadzu prestige 21. 1H-NMR (500 MHz) and 13C-NMR (125 MHz) spectra were recorded on a Jeol spectrophotometer using CDCl3 as solvent and TMS as internal standard. The 2D-NMR experiments were conducted using the standard Jeol software for COSY and DEPT. High resolution mass spectra were determined on a Jeol ECA 500.

Preparation of phosphate buffer

13.9 g sodium dithionite was dissolved in 1 litre of aquades (as solvent A) and dilute 35.85 g sodium hydrogen phosphate monohydrate dilute in 1 litre of aquades (as solvent B). The combination solvent A and B to give a gradient of EtOAc to 100 %, followed by EtOAc/MeOH 1:1. The purifie of dimerization products was identified by spectroscopic methods (NMR, FTIR) (Lucas, et al., 2008).

RESULTS AND DISCUSSION

We have isolated peroxidase from Brassica oleracea var albolabala (Indonesian plant). The specific activity of this crude enzyme is 14.577 U/mg determined with Bergmeyer and Lowry methods. The peroxidase was used as biocatalyzed to dimerization of guaiacol, leading to compound 1. Dimer of guaiacol was obtained as yellow liquid and reacted positively to the FeCl3 reagent indicating the presence of a phenolic group. The mass spectrum showed a (M+H)+ at m/z 247 corresponding to a molecular formula of C11H8O9. The broad band decoupled 13C-NMR spectrum of compound 1 (Table 1) showed 14 carbon signals which were attributed by DEPT and and HMOC techniques as two methoxy, has not methylene, seven methines (7-CH) and five quaternary carbons including a hydroxyl (δ = 141.6 ppm). The IR spectrum displayed free hydroxyl (ν = 3455.9 cm⁻¹), aromatic ring/C=C aromatic (1595-1446) absorption, C-O-C (ν = 1257 cm⁻¹), C-H aromatic (ν = 2880 - 3200 cm⁻¹).

Preparation of enzyme from Brassica oleracea var albolabala

Leaves of Brassica oleracea var albolabala were washed and cutted into small size, then mixed with buffer pH 7 using a blender, were then filterd. Store the crude enzyme on ice until used (Marrion, et al., 1997).

Synthesis of dehydrguiaiacol

A total of 50 ml of peroxidase enzyme was reacted with 6 ml of guaiac (4-hydroxy-5-methoxybenzene), and 3 ml of 5% H2O2 was added and stirred for 3 minutes at room temperature (27 °C), after 3 minutes then were added 3 ml of 5% HCI for stopping the reaction, then extracted with mixtures of EtOAc/n-BuOH 9:1. The combined extract was concentrated at 45°C under vacuum to yield brown residues containing of a mixture dimerization products (Mario, et al., 1997; Matthieu, et al., 2001).

Purification of dimerization products

Dimerization products was purified by column chromatography (sila gel Merck 64271) eluting with n-hexane,
Table 1: $^1$H and $^{13}$C NMR (500 MHz in CDCl$_3$) data for compound 1, O-para coupled product guaiacol, dehydrodiguaiacol.

<table>
<thead>
<tr>
<th>S. No</th>
<th>$^1$H-NMR $\delta_H$ (mult, $J$ in Hz)</th>
<th>HMQC</th>
<th>HMBC</th>
<th>COSY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td></td>
<td>C-6, C-5, C-4</td>
<td>H-3, H-6</td>
</tr>
<tr>
<td>2</td>
<td>6.46(dd 2.6; 8.4)</td>
<td>110.6</td>
<td>C-6, C-5, C-4, C-1</td>
<td>H-2</td>
</tr>
<tr>
<td>3</td>
<td>6.83(d 8.4)</td>
<td>114.5</td>
<td>C-3, C-1, C-4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.38 (OH) (broad)</td>
<td>141.6</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>150.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6.65(dd 2.6)</td>
<td>103.0</td>
<td>C-2, C-3, C-4, C-4', C-5</td>
<td>H-2</td>
</tr>
<tr>
<td>7</td>
<td>3.83 (s)</td>
<td>56.1</td>
<td>C-5, C-1</td>
<td></td>
</tr>
<tr>
<td>1'</td>
<td>6.99 (dd 1.3; 7.8)</td>
<td>112.6</td>
<td>C-5', C-6', C-4'</td>
<td>H-2', H-6'</td>
</tr>
<tr>
<td>2'</td>
<td>6.87(dd 1.3; 7.8)</td>
<td>119.1</td>
<td>C-5', C-6', C-4', C-3'</td>
<td>H-3'</td>
</tr>
<tr>
<td>3'</td>
<td>7.06 (m 1.3; 7.8)</td>
<td>123.9</td>
<td>C-5', C-6', C-2'</td>
<td>H-2'</td>
</tr>
<tr>
<td>4'</td>
<td>-</td>
<td>146.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5'</td>
<td>-</td>
<td>150.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6'</td>
<td>6.89(dd 1.3; 7.8)</td>
<td>212.1</td>
<td>C-5', C-6', C-4', C-3'</td>
<td>H-1'</td>
</tr>
<tr>
<td>7'</td>
<td>3.88 (s)</td>
<td>56.1</td>
<td>C-5'</td>
<td></td>
</tr>
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</table>

In the HMBC spectrum (Fig 2b) also showed methoxy group ($\delta_H = 3.88$) was correlated to the quaternary carbons at $\delta_C = 150.5$ (C-5'). Proton 7.06 was correlated to the carbon 150.5 (C-5'), 121.1 (C-6'), 119.1 (C-2'). Proton 6.89. correlated to the carbon 150.5 (C-5'), 112.6 (C-1'), 123.9 (C-3') and 146.8 (C-4').

Proton 6.99 and 6.87 were correlated to the carbon 150.5 (C-5'), 121.1 (C-6'), 123.9(C-3') and 146.8 (C-4'). The HMBC correlation between H-6 ($\delta_H = 6.65$) and C -4' ($\delta_C = 146.8$) demonstrated that the 2-methoxy phenol moiety was connected to the C-4' as oxidented- para couplet correlation (Fig 2), for all HMBC correlation were showed in Figure 2.

Thus, on the basis of the above spectroscopic analysis, the compound 1 was characterized as dimer of 4-hydroxy-5-methoxybenzene with other name dehydrodiguaiacol (Fig 5).

Since guaiacol has been identified have activities as antioxidant. By using 1,1-diphenyl-2-picrylhydrazyl identified have antioxidant activities. The antioxidant activity of dehydrodiguaiacol (IC$_{50}$ 4.69 µg/mL) was higher than the activity of ascorbic acid and guaiacol (as standard, IC$_{50}$ 10.97 and 70.35 µg/mL, respectively).

The COSY spectrum of 1 showed the connection of a doublet doublet signal at $\delta$ 6.46 and doublet signal at $\delta$ 6.83 ($d$, $J$ 8.4 Hz), other connection could be showed between a doublet signal $\delta$ 6.65 and the doublet doublet signal $\delta$ 6.46 ($J$ 2.6 and 8.4). The COSY spectrum of 1 also showed the connection of a doublet doublet signal at $\delta$ 6.87 and multiplet signal at $\delta$ 7.06 ($J$ 2.6 and 7.3 Hz), other connection could be showed between a doublet signal $\delta$ 6.99 and the doublet doublet signal $\delta$ 6.87 and 6.89 ($J$ 1.3 and 7.8). The mechanism of dimerization of guaiacol catalyzed by Crude of Brassica oleracea var alboglabra was showed in fig 4.

![Figure 2: HMBC spectrum of O-para coupled product guaiacol (dehydrodiguaiacol).](image1)

![Figure 3: COSY spectrum of O-para coupled product guaiacol (dehydrodiguaiacol).](image2)

![Figure 5: Structure of O-para coupled product guaiacol, dehydrodiguaiacol.](image3)
CONCLUSION

Crude of *Brassica oleracea var alboglabra* Peroxidase has potency as biocatalyst for dimerization-oxidative coupling. Our group have practiced the potential of using this crude enzyme to synthesis dimer of guaiacol. The product showed O-para coupled guaiacol, leading to O-para dehydroguaiacol. It exhibited strong antioxidant activity on DPPH radicals compared to guaiacol as parent compound.

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REFERENCES


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