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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_2O_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 biosyntthesis 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 demostrated crude peroxidase and addition of H_2O_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-methoxhybenzene or orto-methoxy phenol (guaiacol) was purchased from nacalai tesque and used without any further purification. Sodiumhydrogen phosphat-monohydrat ($Na_2H_2PO_4.H_2O$) and di-Natriumhydrogen phosphat-dihydrat ($Na_2HPO_4.2H_2O$) were purchased from Merck Damstat, Germany for making phosphate buffer. All other chemical (including hydrogen peroxide, HCl) were purchased from Merck.

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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 F_{254} (silica gel plates, 0.25 mm thick, Merck), spots were visualized under UV light (254 and 365 nm) irradiation and by spraying with 10% sulphuric acid solution followed by heating at 110 ^{o}C. IR spectra was measured on a FT-IR Shimadzu prestige 21. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded on a Jeol spectrophotometer using $CDCl_3$ 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 otherwise state δ in ppm, *J* in Hz (Renzo, *et al*., 2010).

Preparation of phosphate buffer

13.9 g sodiumhydrogenphosphat-monohydrate dilute in 1 litre of aquades (as solvent A) and dilute 35.85g di-Natriumhydrogenphosphat dehydrate dilute in 1 litre of aquades (as solvent B). The combination solvent A and B to give a of phosphate buffer pH 7.

Preparation of enzyme from Brassica *oleracea* var alboglabra

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

Sinthesis of dehyrodiguaiacol

A total of 50 ml of peroxidase enzyme was reacted with 6 ml of guaiac (4-hydroxy-5-methoxybenzene), and 3 ml of 5% H_2O_2 was added and stirred for 3 minutes at room temperature (27 ^{o}C), after 3 minutes then were added 3 ml of 5% HCl for stopping the reaction, then extracted with mixtures of EtOAc/n-BuOH 9:1. The combined extract was concentrated at 45^{o}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 (silica gel Merck 64271) eluting with *n*-hexane, 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 *alboglabra* (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 $FeCl_3$ reagent indicating the presence of a phenolic group. The mass spectrum showed a $(M+H)^{+1}$ at m/z 247 corresponding to a molecular formula of $C_{14}H_{14}O_4$. The broad band decoupled ^{13}C-NMR spectrum of coumpound 1 (Table 1) showed 14 carbon signals which were attributed by DEPT and and HMQC 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_{max} = 3455.9 cm^{-1}), aromatic ring/C=C aromatic (1595-1446) absortion, C-O-C (ν_{max} = 1257 cm^{-1}), C-H aromatic (ν_{max} 2880 - 3200 cm^{-1}), Furhermore, ^{1}H and ^{13}C-NMR spectra also displayed the presence of three sets of signals. The first set, a four-proton duoblet doublets at δ_H 6.46/δ_C = 110.6 (1H *dd J* = 2.6 and 8.4 Hz), δ_H 6.87/δ_C = 119.1 (1H *dd J* 1.3 and 7.8 Hz), δ_H 6.89/δ_C = 121.1 (1H *dd J* 1.3 and 7.8) and δ_H 6.69/δ_C = 112.6 (1H *dd J* 1.3 and 7.8 Hz). The second set of signals, consisting of two proton doublet at δ_H 6.65 /δ_C = 103,0 (1H *d J* 2.6) and δ_H 6.83/δ_C =114.5 (1H *d J* 8.4 H), and also the three set of signals, consisting of one proton multiplet at δ_H 7.06 /δ_C = 123.9 (1H,*m J* 2.6 and 7.3 Hz), and also there are six proton singlet at δ_H 3.88/ δ_C = 56.1 (3H,*s*) and δ_H 3.83/δ_C = 56.1 (3H, *s*), established the presence of a two methoxy substituents. A combination of the COSY and HMQC experiments permitted the assignments of all of the protonated carbons (Table 1). It remained to establish the position of the substituents on the dimers of guaiacol skeleton. In the HMBC spectrum (Fig 1a), the hydroxyl group (δ_H = 5.38) was correlated to the quarternary carbons at δ_C = 141.6 (C-4), 114.5 (C-3) and 147.2 (C-1). This finding clearly indicated that the hydroxyl group was located at C-4 position. The other resonance at δ_C = 141.6 also give cross peaks with three of olefinic protons of the guaiacol (at δ = 6.83; 6.46 and 6.65). Methoxy group (δ_H = 3.83) was correlated to the

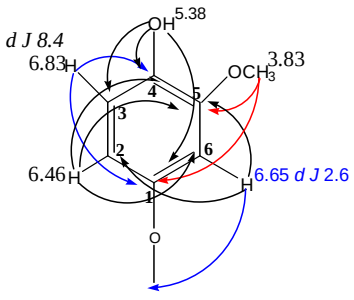


Figure 1a

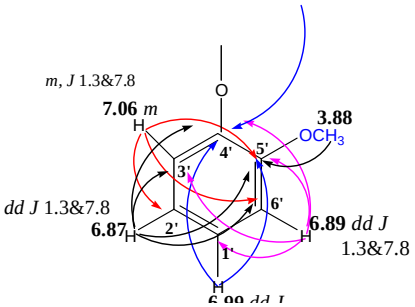


Figure 1b

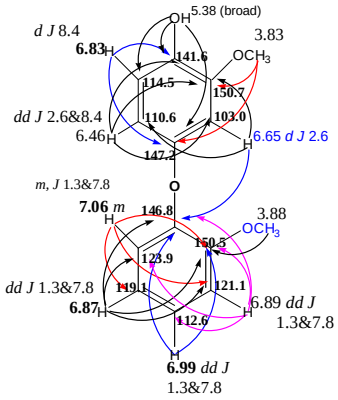
Fig. 1a and 1b: HMBC of the para-*O*- coupled product guaiacol *(dehydrodiguaiecol).*

Table. 1: ^{1}H and ^{13}C NMR (500 MHz in $CDCl_3$) data for compound 1, *O-para* coupled product guaiacol, dehydrodiguaiacol.

S. No	^{1}H-NMR δ_H (mult, *J* in Hz)	HMQC	HMBC	COSY
1	-	147.2		
2	6.46(*dd* 2.6; 8.4)	110.6	C-6, C-5, C-4	H-3, H-6
3	6.83(*d* 8.4)	114.5	C-6, C-5, C-4, C-1	H-2
4	5.38 (OH) (broad)	141.6	C-3, C-1, C-4	
5	-	150.7		
6	6.65(*d* 2.6)	103.0	C-2, C-3, C-4, C-4', C-5	H-2
7	3.83 (*s*)	56.1	C-5, C-1	
1'	6.99 (*dd* 1.3; 7.8)	112.6	C-5', C-6', C-4'	H-2', H-6'
2'	6.87(*dd* 1.3; 7.8)	119.1	C-5', C-6', C-4'; C-3'	H-3'
3'	7.06 (*m* 1.3; 7.8)	123.9	C-5', C-6', C-2'	H-2'
4'	-	146.8		
5'	-	150.5		
6'	6.89(*dd* 1.3; 7.8)	212.1	C-5', C-6', C-4', C-3'	H-1'
7'	3.88 (*s*)	56.1	C-5'	

In the HMBC spectrum (Fig 2b) also showed methoxy group (δ_H = 3.88) was correlated to the quarternary carbons at δ_C = 150.5 (C-5'). Proton 7.06 was corelated to the carbon 150.5 (C-5'), 121.1 (C-6'), 119.1 (C-2'). Proton 6.89. corelated 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 corelated 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 (δ_H 6.65) and C -4' (δ_C 146.8) demonstrated that the 2-methoxy phenol moiety was connected to the C-4' as oxigented- *para* couplet corelation (Fig 2), for all HMBC correlation were showed in Figure 2.



Fig, 2: HMBC spectrum of O-para coupled product guaiacol (dehydrodiguaiacol).

The COSY spectrum of **1** showed the connection of a doublet doublet signal at δ 6.46 and doublet signal at δ 6.83 (*d*, *J* 8.4 Hz), other connection could be showed beetwen a doublet signal δ 6.65 and the doublet doublet signal δ 6.46 (*J* 2.6 and 8.4). The COSY spectrum of **1** also showed the connection of a doublet doublet signal at δ 6.87 and multiplet signal at δ 7.06 (*J* 2.6 and 7.3 Hz), other connection could be showed beetwen a doublet signal δ 6.99 and the doublet doublet signal δ 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.

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).

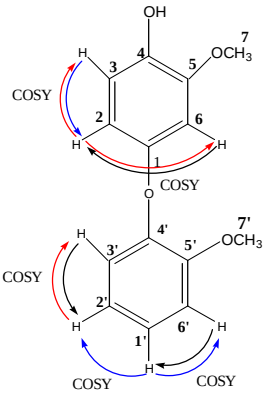


Figure 3. COSY spectrum of *O-para* coupled product guaiacol *(dehydrodiguaiacol)*

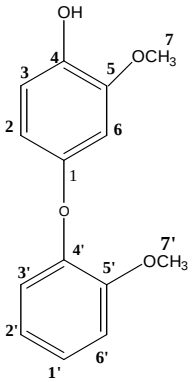


Fig. 5: Structure of *O-para* coupled product guaiacol, dehydrodiguaiacol.

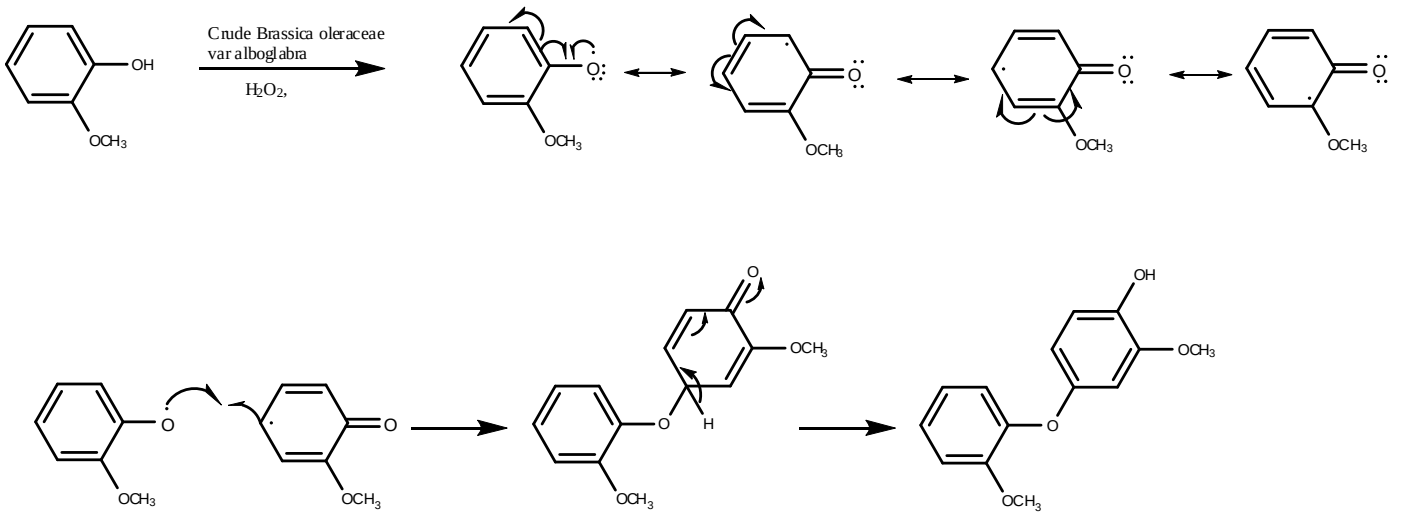


Fig. 4: Proposed mechanism for the oxidative radical *dimerization of guaiacol catalyzed by* Crude of *Brassica oleracea var alboglabra*.

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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