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Enantioselective α-Hydroperoxylation of Long-Chain Fatty Acids with Crude Enzyme of Marine Green Alga *Ulva pertusa*

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Abstract: When palmitic acid was incubated with crude enzyme of marine green alga *Ulva pertusa*, (*R*)-2-hydroperoxyhexadecanoic acid was formed in high enantiomeric purity (>99%ee).

Long-chain fatty acids can be metabolised to the corresponding aldehydes by α-oxidation in a wide range of organisms [1-6]. Although α-oxidation of fatty acids appears to be important for the physiological functions, the reaction pathway has not been fully characterised [7,8].

In an essential oil, which prepared from marine green alga *Ulva pertusa* by simultaneous distillation extraction (SDE) manipulation, long-chain fatty aldehydes such as pentadecanal, (8Z)-heptadecenal, (8Z, 11Z)-heptadecadienal and (8Z, 11Z, 14Z)-heptadecatrienal, were identified by comparison of GC-MS data with those of authentic samples [9]. As expected, long-chain fatty acids; palmitic acid, oleic acid, linoleic acid, and linolenic acid, were converted to the corresponding aldehydes with a loss of one carbon atom [10,11]. Although a partly purification of long-chain aldehyde forming enzyme was examined, the enzyme has not been sufficiently characterised [12,13]. Thus, to elucidate the mechanism and the physiological functions of α-oxidation in *U. pertusa*, the biogenetic intermediate has to be determined. In this paper, we describe highly enantioselective α-hydroperoxylation of long-chain fatty acids with crude enzyme of *U. pertusa*.

The alga *U. pertusa* was collected in the intertidal zone of Aio, Yamaguchi, Japan on 31 July 1997 and immediately frozen at -20°C. The frozen tissue (100g fresh weight) was cut into small pieces and homogenized with 0.1M phosphate buffer (250ml, pH 6.0), which contained 0.1% Triton X-100, in a blender for 10 min. After filtering, the resulting homogenate was centrifuged at 150000 x *g* for 60 min. The pellet was resuspended in the same buffer (50ml) containing 0.1% Triton X-100. The crude enzyme solution was stirred at 5°C for 10 min, and then

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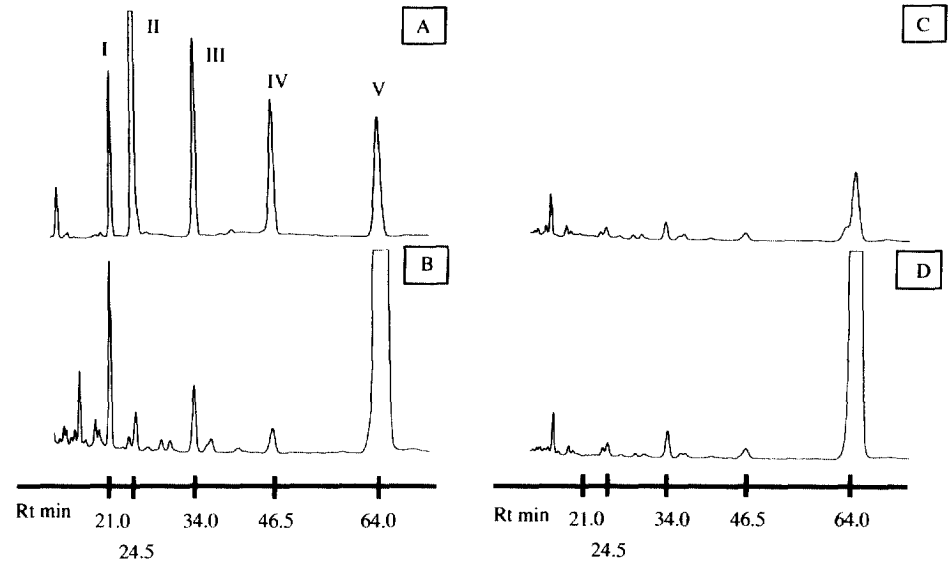


Figure 1 Comparison of 2-oxygenated compounds from extracts of *U. pertusa* with synthetic samples. Condition : column Mightysil RP-18 GP ; eluent CH_3CN / 0.1M $CH_3CO_2NH_4$ (9 / 1) ; flow rate 1.0ml / min ; detector fluorescence Ex 365nm, Em 412nm. A : synthetic samples, I 2-hydroperoxyhexadecanoic acid ; II 2-hydroxyhexadecanoic acid ; III 2-oxohexadecanoic acid ; IV pentadecanoic acid ; V palmitic acid. B : The crude enzyme of *U. pertusa* was stirred with palmitic acid. C : The crude enzyme was incubated in the absence of substrate. D: Heat treatment of the crude enzyme was carried out at 90°C prior to addition of substrate.

palmitic acid (5mg, 0.02mmol) in DMSO (0.5ml) was administered. After stirring at 5°C for 30 min, $(NH_4)_2SO_4$, NaCl, and THF were added to the reaction mixture. The whole mixture was centrifuged at 2000 x *g* for 10 min, the organic layer was separated. The layer was washed with sat. NaCl solution and dried over $MgSO_4$. A portion of the extract was treated with 9-anthryldiazomethane (ADAM) at 0°C for 10 min [14]. Then the reaction mixture was concentrated *in vacuo* and the residue was subjected to HPLC analysis of oxygenated products [15]. With palmitic acid, two major peaks of ADAM esters appeared as shown in Figure 1-B, i.e. the ADAM esters of 2-hyderoperoxyhexadecanoic acid (I ; Rt 21.0 min) and palmitic acid (V; Rt 64.0 min) [16]. These compounds were identified by comparison with the ADAM ester of synthetic samples [17]. Indeed, when a portion of the extract was treated with PPh_3 prior to HPLC analysis, peak I of 2-hydroperoxy form disappeared wheares peak II of 2-hydroxy form increased (Figure 2). In the separate experiment, the extract was esterified with diazomethane at 0°C for 10 min, and the resulting ester was purified by preparative TLC to give methyl ester of 2-hydroperoxyhexadecanoic acid, which exhibited identical properties (TLC[18] and LC-MS[19]) with those of a synthetic sample. Thus, the HPLC and LC-MS data revealed that the addition of palmitic acid to the crude enzyme solution led to an

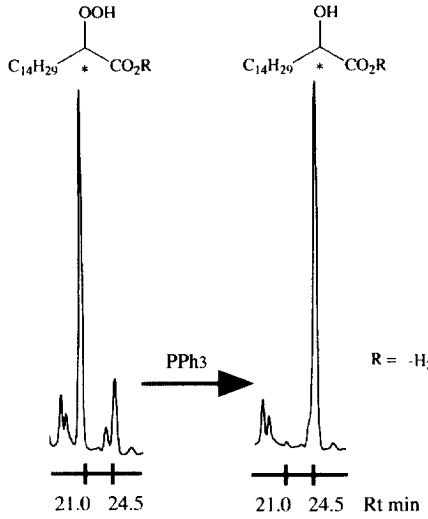


Figure 2 Confirmaion of compounds involving 2-hydroperoxy group with PPh_3. For conditions, see Figure 1 legend.

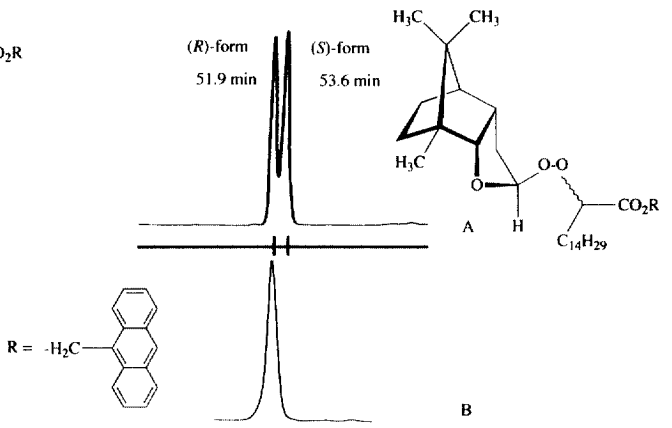


Figure 3 HPLC enantioseparation of 2-hydroperoxyhexadecanoic acid.
condition : column Develosil ODS-5 ; eluent CH_3CN ; flow rate 1.0ml/min ; detector fluorescence Ex 365nm, Em412nm.
A : separation of (±)-2-hydroperoxyhexadecanoic acid.
B : enantiopurity analysis of 2-hydroperoxyhexadecanoic acid obtained from homogenate of *U. pertusa*.

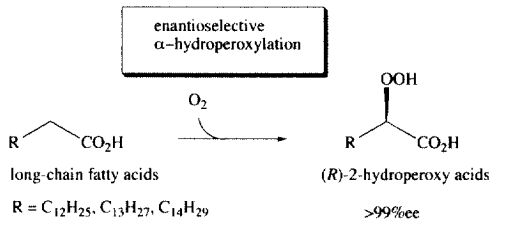
increased yield of the 2-hydroperoxy acid. In turn, incubation of heat-treated crude enzyme prior to addition of palmitic acid did not produce the 2-hydroperoxy acid (Figure 1-D), thus indicating that the 2-oxygenated compound I is formed enzymatically .

On the other hand, a solution of the ADAM ester I in THF was treated with Noe's reagent [20] and TsOH (catalytic amount) at room temperature for 5 min. The reaction mixture was concentrated *in vacuo*, and the concentrate, without further purification, was circulated to determine the absolute configuration and enantiomeric purity by HPLC. On the basis of the HPLC elution pattern

Table 1 Determination of optical purity of 2-hydroperoxy acids using Noe's reagent

fatty acids	2-hydroperoxy acids	
	%ee	confign
myristic acid (C14)	>99	*R*
pentadecanoic acid (C15)	>99	*R*
palmitic acid (C16)	>99	*R*
heptadecanoic acid (C17)	n.d.*	
stearic acid (C18)	n.d.	

* not detected



Scheme 1 Highly enantioselective α-hydroperoxylation of long-chain fatty acids with crude enzyme of *U. pertusa*.

in Figure 3, the peracetal of I from the crude enzyme was attributed to (*R*)-enantiomer of synthetic 2-hydroperoxyhexadecanoic acid and the enantiomeric excess of the product was shown to be >99% [21].

The enantiospecificity of the hydroperoxylation using several other long-chain fatty acids was investigated for the crude enzyme (Table 1). With pentadecanoic and myristic acid, (*R*)-2-hydroperoxypentadecanoic and (*R*)-2-hydroperoxytetradecanoic acid were obtained with an enantiomeric excess of >99%, respectively. However, heptadecanoic and stearic acid were poor substrates.

In coclusion, our results show that long-chain fatty acids (C14-C16) are α–hydroperoxylated with the crude enzyme of *U. pertusa* to afford (*R*)-2-hydroperoxy acids with excellent enantiomeric excess (Scheme 1).

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15. A Mightysil RP-18 GP column (3.0 x 250mm) was used for analysis of oxygenated compounds formed by the α-oxidation, while a Develosil ODS-5 column (4.6 x 250mm) was used to evaluate the absolute configuration of 2-hydroperoxy and 2-hydroxyhexadecanoic acid produced from palmitic acid.
16. In addition, minor peaks were observed and retention times were follows : 24.5 min, 2-hydroxyhexadecanoic acid II ; 34.0 min, 2-oxohexadecanoic acid III ; 46.5 min, pentadecanoic acid IV.
17. Incidentally, racemic 2-hydroperoxyhexadecanoic acid can be conveniently prepared from palmitic acid through sequential formation of dianion with lithium diisopropylamide (LDA) and O_2 bubbling process at -78°C : Konen, D. A., Silbert, L. S. and Pfeffer, P.E. *J. Org. Chem.* **1975**, 40. 3253-3258. Of course, the 2-hydroxy acid can be also obtained by reduction of the 2-hydroperoxy acid with PPh_3.
18. Confirmation of compounds involving hydroperoxy acids was monitored by TLC, using an *N, N'*-dimethyl-*p*-phenylenediamine indicator.
19. LC-MS of the purified compound gave a pseudomolecular ion at m/z 320 $[(M+NH_4)^+]$ in accordance with the synthetic sample.
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21. Futhermore, reduction of the 2-hydroperoxy acid of I from the crude enzyme with PPh_3, followed by treatment with Noe's reagent, gave the corresponding acetal, which coincided with the (*R*)-form of synthetic 2-hydroxy acid : Mori, K. and Funaki, Y., *Tetrahedron* **1985**, 41, 2379-2386. ; Sugai, T. and Ohta, H., *Agric. Biol. Chem.* **1990**, 54, 3337-3338.