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SPECIFICITY OF THE ENZYME SYSTEM PRODUCING LONG CHAIN ALDEHYDES IN THE GREEN ALGA, *ULVA PERTUSA*

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Key Word Index—*Ulva pertusa*; green alga; odour; aldehydes; fatty acids; substrate specificity.

Abstract—The greater part of the enzyme activity producing long chain aldehydes (LCA) such as (8Z)-heptadecenal, (8Z,11Z)-heptadecadienal, (8Z,11Z,14Z)-heptadecatrienal was found in the 4000 *g* precipitate of homogenates of the green alga, *Ulva pertusa*. The LCA-forming activity was readily solubilized from the precipitate by addition of 0.2% Triton X-100. The solubilized LCA-forming activity showed an optimum pH in the range 8.5–9.5. α-Linolenic acid was a very good substrate, but γ-linolenic acid was a poor substrate for the activity. Linoleic acid was the best substrate of an entire series of (ω6Z, ω9Z)-dienoic acids, in which the chain length varied from C_{13} to C_{20}.

INTRODUCTION

Long chain aldehydes (LCA), C_{15}~C_{17}-aldehydes, have been identified in cucumber plants [1–3], germinated peanuts cotyledons [4], pea leaf [5], potato tuber [6], tobacco leaf [7] and rice seedlings [8] as volatile components. Recently, we have identified unsaturated LCA such as (8Z)-heptadecenal (HD), (8Z,11Z)-heptadecadienal (HDD) and (8Z,11Z,14Z)-heptadecatrienal (HDT) as major compounds having the characteristic odour of the essential oils from marine green algae belonging to the Ulvaceae [9]. Our preliminary report [10] has shown that HD, HDD and HDT were enzymatically formed from oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3), respectively, in acetone powders prepared from *Ulva pertusa* fronds. This paper describes substrate specificity for the solubilized LCA-forming activity from the green algal fronds.

RESULTS AND DISCUSSION

LCA-forming activity in U. pertusa *fronds*

Fronds were homogenized with 50 mM sodium-pyrophosphate buffer (pH 9) in a mixer. The homogenate was filtered through four layers of gauze and the filtrate centrifuged at 4000 *g*. Nearly 60% of the LCA-forming activity (LCAA) in the homogenate was found in the 4000 *g* precipitate. The activity (probably membrane-bound) in the precipitate was readily solubilized by an addition of a relatively low concentration (0.2%) of Triton X-100 (Table 1). The solubilized LCAA showed maximum activity in the pH range 8.5–9.5. This is different from the optimum pH of 7 found for LCAA in the homogenate and acetone powder from the fronds [10].

Effects of inhibitors

The effects of inhibitors were examined with 18:2 using the solubilized LCAA. A marked inhibition was observed with 1 mM imidazole (93% inhibition). Cysteine (1 mM) and glutathione (1 mM) showed 50 and 20% inhibition, respectively. Significant inhibition was also observed with certain heavy metal ligands such as 1 mM KCN (90%), 1 mM NaN_3 (38%), and 0.2 mM diphenylthiocarbazone (57%), whereas another chelating agent, 1 mM EDTA (21%) was a poor inhibitor. These results are in agreement with studies on the *Ulva* acetone powder [10]. These observations suggest that the LCAA in the marine alga is a metalloprotein similar to the α-oxidation activity in higher plants, e.g. cucumber fruits [3, 11].

Substrate specificity

The substrate specificity for the solubilized LCAA was examined using natural C_{18} and C_{20} fatty acids, and some derivatives (alcohol, aldehyde, ester) (Table 2) and synthetic (ω6, ω9)-dienoic acids (Fig. 1). The products

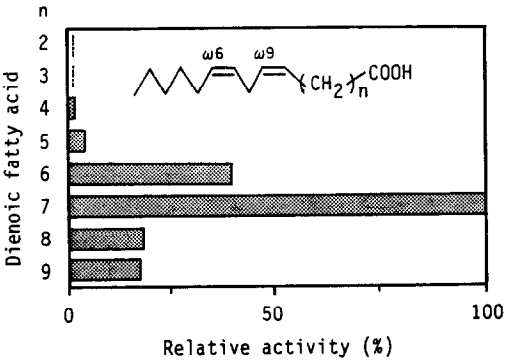

Fig. 1. Substrate specificity of a series of (ω6Z, ω9Z)-dienoic acids for producing long chain aldehydes. Activities are expressed in moles relative to HDD formed during incubation of the solubilized LCA-forming activity and 18:2 ($n=7$) at 35° for 15 min. Enzyme activities were assayed by the same method as Table 1.

Table 1. Long chain aldehyde-forming activity in *Ulva pertusa* fronds

Fraction	HDD formed (n mol/g fr fronds)	Relative activity* (%)
Precipitate (4000 *g*)	7.6	100
Added 0.2% Triton X-100 to 4000 *g* ppt		
20000 *g* supernatant	6.7	88
20000 *g* precipitate	0.3	4

*Activities are expressed in moles relative to HDD formed during incubations of the enzyme solutions and 18:2 at 35° for 15 min. Enzyme activities were assayed by HPLC after products were converted to the corresponding 2,4-DNPHs (see Experimental).

Table 2. Substrate specificity of natural fatty acid and their derivatives

Substrates	Products	Relative activity* (%)
Stearic acid (18:0)	—	0
Oleic acid (18:1)	(8*Z*)-Heptadecenal	8
Linoleic acid (18:2)	(8*Z*, 11*Z*)-Heptadecadienal	100
Methyl linoleate	—	0
Linoleyl alcohol	—	0
Linoleyl aldehyde	(8*Z*, 11*Z*)-Heptadecadienal	21
Linolenic acid (18:3)	(8*Z*, 11*Z*, 14*Z*)-Heptadecatrienal	85
γ-Linolenic acid (γ-18:3)	(5*Z*, 8*Z*, 11*Z*)-Heptadecatrienal†	14
Arachidonic acid (20:4)	—	0
Eicosapentaenoic acid (20:5)	—	0

*Activities are expressed in moles relative to HDD obtained by incubation of the solubilized LCA-forming activity and 18:2 at 35° for 15 min. Enzyme activities were assayed by the same method as in Table 1.

†Tentative identification.

were identified by comparing R_f data of their 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives by HPLC (Zorbax ODS; MeCN–H_2O–THF) with authentic materials, prepared using unequivocal routes [10].

Tests of substrate specificity for the unsaturated C_{18} fatty acids, showed that 18:2 and 18:3 were the best substrates for LCAA (Table 2), γ-18:3, however, was a poor substrate. This reactivity was in agreement with that previously found in *Ulva* acetone powder [10]. With 18:1 and 18:0, the reactivities were low and nonexistent, respectively. This behaviour was different from the α-oxidation enzyme system in higher plants such as cucumber fruits which show a high reactivity for the two C_{18} acids [3, 11]. On the other hand, 18:2 and the aldehyde isomer could act as a substrate, whereas the methyl ester and alcohol isomers show no activity for the LCAA. With polyenoic acids, such as 20:4 and 20:5, the reactivities were slight or nonexistent.

For elucidation of the structural requirement of substrate for the LCAA, an entire series of (ω6*Z*, ω9*Z*)-dienoic acids in which the chain length varied from C_{13} to C_{20} (except for 18:2), were synthesized by a (*Z*)-selective Wittig reaction between (3*Z*)-nonenyltriphenylphosphonium iodide and the appropriate 2-tetrahydropyranyloxyalkanal followed by removal of the protective group [10] and Jones oxidation [12]. The synthetic dienoic acids (over 95% purity) were used as substrates for the enzyme system after purification by silica gel CC.

The $C_{15} \sim C_{20}$-dienoic acids ($n=4 \sim 9$): (6*Z*,9*Z*)-pentadecadienoic acid, (7*Z*, 10*Z*)-hexadecadienoic acid, (8*Z*,11*Z*)-heptadecadienoic acid, (9*Z*,12*Z*)-octadecadienoic acid, (10*Z*,13*Z*)-nonadecadienoic acid, and (11*Z*, 14*Z*)-eicosadecadienoic acid, were found to be converted to aldehydes containing one carbon atom less, i.e. (5*Z*, 8*Z*)-tetradecadienal, (6*Z*,9*Z*)-pentadecadienal, (7*Z*,10*Z*)-hexadecadienal, (8*Z*,11*Z*)-heptadecadienal, (9*Z*,12*Z*)-octadecadienal, and (10*Z*, 13*Z*)-nonadecadienal, respectively (Fig. 1). 18:2 was the best substrate of all the dienoic acids tested. The reactivity of the acids decreased with both an increase and a decrease in chain length with respect to 18:2. C_{13} and C_{14} dienoic acids ($n=2 \sim 3$) did not act as substrates for LCAA production.

These results suggest that the distance between a (*Z*,*Z*)-pentadiene and a carboxy group or a formyl group in the substrate, is an important structural requirement of substrates for LCAA in marine algae. This might partly explain the striking difference in the reactivity between 18:3 and γ-18:3. However this experiment alone, does

not clarify whether or not the LCAA is same as the α-oxidation reported in higher plants. To solve this problem, further purification of the enzyme system is required.

EXPERIMENTAL

Materials. Fresh fronds of *U. pertusa* were collected at Aio Bay in Yamaguchi City, Japan in July 1987. Natural fatty acids (over 98% purity) used as substrates were purchased from Wako Pure Chemicals, Ltd. Imidazole, cysteine, glutathione (reduced form), KCN, NaN_3, diphenylthiocarbazone (DPTC) and ethylenediamine tetraacetic acid (EDTA), all which were used in the inhibition experiment, were also purchased from Wako Pure Chemicals, Ltd.

Preparation of substrates and authentic aldehydes. (ω6*Z*, ω9*Z*)-dienoic acids (C_{13}~C_{20}, except for C_{18}) used as substrates for the LCAA and (6*Z*,9*Z*)-dienal (C_{12}~C_{19}, except for C_{18}) for identification of the enzymatic reaction products, were synthesized according to the same procedure as described for C_{17} dienoic acid and dienals.

(8*Z*, 11*Z*)-*Heptadecadienal.* The (*Z*)-selective Wittig reaction between (3*Z*)-nonyltriphenylphosphonium iodide (2.1 g, 4 mmol) and 8-(2-tetrahydropyranyloxy)-octane-1-al (911 mg, 4 mmol) in THF (70 ml) and HMPA (5.2 ml) at −78° using *n*-BuLi (3.5 ml, 5.3 mmol) under N_2, gave 1-(2-tetrahydropyranyloxy)-(8*Z*,11*Z*)-heptadecadiene (1.05 g, 75%). Stirring of the pyranyl ether (1.05 g, 3 mmol) in 30% H_3PO_4–MeOH (20 ml) for 5 hr at room temp. gave (8*Z*,11*Z*)-heptadecadienol (725 mg, 290 mmol, 96%). Collins oxidation [13] of the dienol (710 mg, 2.82 mmol) using CrO_3 (1.7 g) and pyridine (2.7 g) in CH_2Cl_2 (70 ml) for 20 min at room temp gave (8*Z*,11*Z*)-heptadecadienal (635 mg, 2.54 mmol, 80%). ^{13}C NMR ($CDCl_3$) 202.8 (CHO), 130.2 (CH), 129.8 (CH), 128.2 (CH), 127.8 (CH), 43.9 (CH_2), 31.5 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 27.2 (CH_2), 27.1 (CH_2), 25.6 (CH_2), 22.6 (CH_2), 22.0 (CH_2), 14.0 (Me). The (8*Z*,11*Z*)-geometrical purity was found to be over 95% by HPLC of the corresponding 2,4-DNPH: column ODS 4.6 × 150 mm; solvent MeCN–H_2O–THF; 90:9:1, flow rate 1 ml/min, pres. 100 kg/cm^2, UV detector 350 nm.

(8*Z*,11*Z*)-*Heptadecadienoic acid.* Jones oxidation [12] of (8*Z*,11*Z*)-heptadecadienol (209 mg, 0.83 mmol) using CrO_3 (123 mg, 1.23 mmol) and conc H_2SO_4 (105 μl)-H_2O (0.44 ml) in Me_2CO for 30 min at 0°, gave a crude dienoic acid, which was purified by silica gel CC (Et_2O–hexane) to afford (8*Z*,11*Z*)-heptadecadienoic acid (162 mg, 74%): ^{13}C NMR ($CDCl_3$) 180.3 (COOH), 130.3 (CH), 130.0 (CH), 128.3 (CH), 128.0 (CH), 34.1 (CH_2), 31.6 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 27.3 (CH_2), 27.2 (CH_2), 25.7 (CH_2), 24.7 (CH_2), 22.7 (CH_2), 14.1 (Me).

Preparation of LCAA. Fresh fronds of *U. pertusa* (1.3 kg) were homogenized with 21.50 mM Na-pyrophosphate buffer (pH 9) in a mixer at 7° and filtered through four layers of gauze. The filtrate was centrifuged at 100 *g* for 5 min to remove cell debris. The resulting supernatant was centrifuged at 4000 *g* for 20 min. The ppt. (26.1 *g*) was resuspended in the same buffer containing 2.1 M sucrose. The resulting suspension (144.5 *g*) could be stored at −20° for at least 2 months without significant inactivation. The suspension (10.9 g) was diluted with Na-pyrophosphate buffer (20 ml), pH 9 containing 0.2% Triton X-100 and then sonicated for 3 min. After stirring for a while, the suspension was centrifuged at 20000 *g* for 20 min and the resulting supernatant was used as solubilized LCAA. To the resulting supernatant was added a substrate (18:2, 1 mg/ml) and the mixt. incubated at 35° for 15 min. HDD formed during incubation was measured quantitatively by HPLC analysis of the 2,4-DNPH derivative. Each reaction mixt was added to an EtOH soln of 2,4-DNPH–HOAc (10 ml, excess). The DNPH derivative was extracted with hexane (30 ml × 3). The combined hexane exts were washed with satd $NaHCO_3$ and satd NaCl solns and then dried (Na_2SO_4). The extract was evapd *in vacuo* to leave a solid 2,4-DNPH derivative mixt. The derivatives were dissolved in $CHCl_3$ (0.5 ml) and an aliquot quantitatively analysed by HPLC with a UV detecter at 350 nm: Zorbax ODS column 4.6 × 150 mm; solvent MeCN–H_2O–THF; 90:9:1, flow rate 1 ml/min, pres. 100 kg/cm^2.

pH optimum. LCAA soln (1 ml) was dil. with buffers (3 ml) and individually adjusted to obtain given pH values (5, 6, 7, 7.5, 8, 8.5, 9, 9.5, and 10) by adding dil HCl or NaOH. Buffers used were McIlvaine buffer (5–6), 50 mM Na-Pi buffer (pH 6–8) and 50 mM Na-pyrophosphate buffer (pH 8–10). The activities were measured by HPLC analysis of HDD formed during incubation with 18:2 (1 mg) at 35° for 15 min as described above.

Inhibition of enzyme reaction. To a soln of LCAA (1 ml) in Na-pyrophosphate buffer (3 ml) was added each agent at a given concn. After incubation of the mixt. with 18:2 (1 mg) at 35° for 15 min, HDD was quantitatively analysed by HPLC (under the conditions described above) as a DNPH derivative.

Substrate specificity. To a soln of LCAA (1 ml) dil with Na-pyrophosphate buffer (pH 9, 3 ml) was added each substrate (1 mg). After incubation of the mixt. at 35° for 15 min, a 2,4-DNPH soln (2 ml, excess) was added to the reaction mixt. The LCAA for each substrate was measured by HPLC analysis of the 2,4-DNPH derivative as described for prepn of LCAA.

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