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Is strain DS5 hydratase a C-10 positional specific enzyme? Identification of bioconversion products from α- and γ-linolenic acids by *Flavobacterium* sp. DS5

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SUMMARY

Previously, we reported the isolation of a new microbial strain, *Flavobacterium* sp. DS5 (NRRL B-14859) which converted oleic and linoleic acids to their corresponding 10-keto- and 10-hydroxy-fatty acids. The hydration enzyme seemed to be specific to the C-10 position. Now we have identified, by GC/MS, NMR, and FTIR, the bioconversion products from α-linolenic acid as 10-hydroxy-12(*Z*),15(Z)-octadecadienoic acid and from γ-linolenic acid as 10-hydroxy-6(*Z*),12(*Z*)-octadecadienoic acid. Products from 9(*E*)-unsaturated fatty acids were also identified as their corresponding 10-hydroxy or 10-keto fatty acids. From these results, it is concluded that strain DS5 hydratase is indeed a C-10 positional-specific enzyme and prefers an 18-carbon mono-unsaturated fatty acid. Among the C18 unsaturated fatty acids, an additional double bond on either side of the C-9 position lowers the enzyme hydration activity.

INTRODUCTION

Unsaturated fatty acids are the major components of corn and soybean oils, representing more than 85% of fatty acids present in triglycerides. We are investigating microbial conversion of vegetable oils and their component fatty acids to value-added products.

Microbial conversion of unsaturated fatty acids has been widely investigated. Oleic acid is converted to 10-hydroxy-stearic (10-HSA) acid in 14% yield using *Pseudomonas* sp. [15]. Oleic acid is also converted to both 10-HSA and 10-ketostearic acid (10-KSA) by *Rhodococcus rhodochrous* [12], *Nocardia cholesterolicum* [10], *Micrococcus luteus* and *Sarcina lutea* [1], *Corynebacterium* sp. [14], three microbial strains (*Mycobacterium* and two *Nocardia*) [2], and a *Staphylococcus* sp. [11]. There are reports on oleic acid conversion to 7,10-dihydroxy-8(*E*)-octadecenoic acid [5,7,8] via the 10-hydroxy-8(*Z*)-octadecenoic acid intermediate [6]. Recently, we isolated a new microbial strain *Flavobacterium* sp. DS5 from soil samples taken from Peoria, IL, USA, which converted oleic acid to 10-ketostearic acid in 85% yield [4] and linoleic acid to 10-hydroxy-12(*Z*)-octadecenoic acid in 55% yield [3].

In order to find out the positional specificity of and the effect of substrate carbon chain length on the strain DS5 hydratase activity, we studied the hydration of mono-, di-, and tri-unsaturated C18 fatty acids as well as other carbon chain length mono-unsaturated fatty acids. In this paper, we identified the DS5 bioconversion products from α-linolenic acid as 10-hydroxy-12(Z),15(Z)-octadecadienoic acid and from γ-linolenic acid as 10-hydroxy-6(Z),12(Z)-octadecadienoic acid. Products from other unsaturated fatty acids were also identified. We found that strain DS5 hydratase is indeed a C-10 positional-specific enzyme and prefers an 18-carbon mono-unsaturated fatty acid.

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MATERIALS AND METHODS

Microorganisms

Flavobacterium sp. strain DS5 was grown at 30 °C aerobically in a 5-L Erlenmeyer flask (shaken at 200 r.p.m.) containing 1 L of medium with the following composition (per liter): dextrose, 10 g; K_2HPO_4, 5 g; yeast extract, 5 g; soybean meal, 5 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4$, 0.014 g; $MnSO_4 \cdot H_2O$, 0.008 g; and nicotinic acid, 0.01 g. The medium was adjusted to pH 7.0 with dilute phosphoric acid.

Chemicals

Unsaturated fatty acids (purity >99% by gas chromatography (GC)) were purchased from Nuchek Prep Inc., Elysian, MN, USA. Other chemicals were purchased from Sigma (St Louis, MO, USA). All solvents used were ACS grade and were obtained from commercial sources. Kieselgel 60 and thin-layer precoated Kieselgel $60F_{254}$ plates were obtained from EM Science (Cherry Hill, NJ, USA).

Bioconversion

Flavobacterium sp. strain DS5 was grown for 1 day and then centrifuged at 8000 r.p.m. for 20 min to separate cells and supernatant medium. The cell pellet was washed twice with a 0.05 M potassium phosphate buffer pH 7.5 and resus-

pended in a small amount of the same buffer to $OD_{650\,mm} = 4$. Substrate unsaturated fatty acid was added to the cell suspension, and the mixtures were incubated at 30 °C for 18 h to study the bioconversion. At the end of this time, the culture broth was acidified to pH 2 with 6 N hydrochloric acid. The culture broth was then extracted twice with an equal volume of diethyl ether. The solvent was removed from the combined extracts with a rotary evaporator.

Isolation of products

Crude extracts containing reaction products were subjected to high performance liquid chromatography (HPLC) to isolate individual components for further identification. A Dynamax-60A silica column (25 cm × 21.4 mm i.d., Rainin Instruments, Woburn, MA, USA) and methylene chloride/methanol (97/3, v/v) as solvent were used with refractive index and variable wavelength detectors. Purity of fractions was analyzed with thin-layer chromatography (TLC) and GC.

Analyses of products

The reaction products were analyzed by TLC and GC as described previously [5,7]. Toluene/dioxane/acetic acid (79/14/7, v/v/v) was the TLC solvent system. For GC, the samples were methylated with diazomethane and separated isothermally at 200 °C. For quantitative analysis, palmitic acid was added as internal standard prior to the solvent extraction. A linear relationship was established on the peak area ratios of product vs methyl palmitate.

The product was characterized through GC-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectra. Electron-impact mass spectra were obtained with a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970 Series Mass Selective Detector (Hewlett Packard, Bloomington, IL, USA). The column outlet was connected directly to the ion source. Separations were effected in a methylsilicone column (15 m × 0.25 mm) with a temperature gradient of 8 °C per min from 160 °C to 250 °C after initially holding the column at 160 °C for 3 min. Proton and ^{13}C NMR spectra were determined in deuterated chloroform with a Bruker ARX 400 spectrometer (Billerica, MA, USA) operated at a frequency of 400 and 100.6 MHz, respectively. FTIR analyses of the free acid products were run as films on KBr on a Perkin Elmer infrared Fourier transform Model 1750 spectrometer (Perkin Elmer, Inc., Oakbrook, IL, USA).

RESULTS AND DISCUSSION

Structure determination

Product from alpha-linolenic acid. The purified major product has GC retention time (RT) of 12.7 min and purity of 98.5% by GC. It is a liquid at 4 °C and a solid at freezer temperature (–20 °C). The electron-impact spectrum of the free acid product gave a heaviest ion of m/z 278 (M-18). Ions formed from alpha-cleavage with respect to the hydroxy-group give characteristic fragmentation patterns that provide sufficient information to determine the position of the hydroxy group [13]. Large fragments corresponding to alpha-cleavage with ions m/z 187 and 169 (relative intensity 22% and 100%, respectively) place the hydroxy group at C-10 position (Fig. 1). This was further confirmed by GC/MS of the methyl ester prepared with diazomethane which gave the largest fragments at m/z 201 and 169. These data are consistent with 10-hydroxy-12,15-octadecadienoic acid [9].

FTIR of the free acid showed absorption of the acid dimerization around 2800–3200 cm^{-1} and the alkyl hydroxy group at 3392 cm^{-1}. The carbonyl group was seen at 1710 cm^{-1}. No keto carbonyl was detected. In the absence of a significant absorbance at 970 cm^{-1} which would be evidence of trans double bonds, the unsaturation seen at 3010 cm^{-1} is *cis*.

The reaction product was analyzed by proton and ^{13}C NMR spectroscopy. Resonance signals (p.p.m.) and corresponding

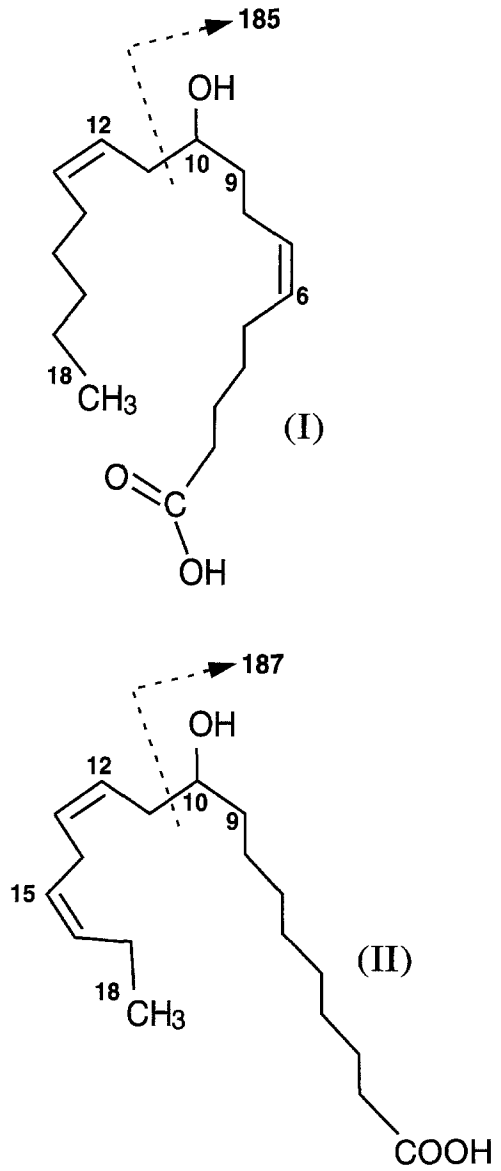

Fig. 1. Microbial conversion products from α- and γ-linolenic acids by strain DS5. (I) 10-hydroxy-6(*Z*),12(*Z*)-octadecadienoic acid. (II) 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic acid.

molecular assignments given in Table 1(A) further confirmed the identity of the bioconversion product as 10-hydroxy-12(Z),15(Z)-octadecadienoic acid [9]. In comparison with the NMR spectrum in the previous paper [9], there are slight revisions and fewer ambiguities in current assignments. The olefinic coupling constant J12,13 = 10.7 Hz and J15,16 = 10.6 Hz confirmed our infrared data that the unsaturations are in a *cis* configuration. A minor product, with a retention time on the GC of 11.97 min, was identified by GC/MS as 10-keto-12,15-octadecadienoic acid [3].

TABLE 1

Proton and ^{13}C nuclear magnetic resonance signals and molecular assignments for products

Carbon number	Resonance signals (p.p.m.)	
	Proton/J(Hz)	^{13}C
(A) From alpha-linolenic acid		
1		179.5
2	2.30 t (2,3 = 7.5)	34.0
3	1.59 m	24.6
4	1.27 m	
5	1.27 m	
6	1.27 m	28.9–29.5
7	1.27 m	
8	1.27 m	25.6
9	1.44 m (9,10 = 7.5)	36.6
10	3.61 m (10,11 = 7.3)	71.5
11	2.22 dd (11,12 = 7.3)	35.1
12	5.38 m (12,13 = 10.7)	125.3
13	5.49 m (13,14 = 7.3)	131.3
14	2.77 m (14,15 = 7.1)	25.6
15	5.28 m (15,16 = 10.6)	126.8
16	5.35 m (16,17 = 7.0)	132.0
17	2.04 m (17,18 = 7.5)	20.5
18	0.94 t	14.2
(B) From gamma-linolenic acid		
1		179.4
2	2.33 t (2,3 = 7.5)	33.9
3	1.63 m (3,4 = 7.5)	24.2
4	1.39 m	29.0
5	2.04 m	26.7*
6	5.36 m (6,7 = 10.9)	129.7
7	5.36 m	129.7
8	2.14 m	23.6
9	1.52 m	36.4
10	3.63 m (10,11 = 7.0)	71.1
11	2.21 m	35.3
12	5.36 m (12,13 = 10.9)	124.8
13	5.54 m (13,14 = 6.9)	133.5
14	2.04 m	27.3*
15	1.33 m	29.3
16	1.27 m	31.5
17	1.27 m (17,18 = 6.6)	22.5
18	0.87 t	14.0

*May be interchanged.

Product from γ-linolenic acid. The purified product has a GC RT of 11.4 min and purity of 98.5% by GC. It is a liquid at 4 °C and is a solid at freezer temperature (–20 °C). The electron-impact spectrum of the free acid of the product gave a heaviest ion at 278 (M-18). Large fragments corresponding to α-cleavage with ions m/z 185 and 167 (relative intensity 55% and 78%, respectively) place the hydroxy group at the C-10 position (Fig. 1). This was further confirmed by GC/MS of the methyl ester which gave the largest fragment at m/z 199 and 167. Therefore, the product is likely 10-hydroxy-6,12-octadecadienoic acid.

FTIR of the free acid showed absorption of the acid dimer group around 2800–3200 cm^{-1} and the alkyl hydroxy group at 3393 cm^{-1}. The carbonyl group was seen at 1710 cm^{-1}. No keto carbonyl was detected. In the absence of a significant absorbance at 970 cm^{-1} which would be evidence of trans double bonds, the unsaturation seen at 3007 cm^{-1} is *cis*.

The reaction product was also analyzed by proton and ^{13}C NMR spectroscopy. Resonance signals (p.p.m.) and corresponding assignments given in Table 1(B) further support the identity of the bioconversion product as 10-hydroxy-6(Z),12(Z)-octadecadienoic acid. The olefinic coupling constant J6,7 = 10.9 Hz and J12,13 = 10.9 Hz confirmed our infrared data that the unsaturations are in a *cis* configuration. Thus, the enzyme hydrated the C9 double bond and presumably did not alter the original olefinic configurations at C6 and C12.

Products from other unsaturated fatty acids. Strain DS5 converted myristoleic acid to two products (GC RT 3.8 min and 4.08 min). The electron-impact spectrum of the methyl ester of RT 3.8 min gave a molecular ion of m/z 256. The presence of a large fragment corresponding to α-cleavage, with m/z 199, places the keto group at the C-10 position. The methyl ester of product RT 4.08 min gave a molecular ion of m/z 258. Large fragments corresponding to α-cleavage with m/z 201 and 169 (relative intensity 15% and 70%, respectively) also place the hydroxy group at the C-10 position. Therefore,

TABLE 2

Bioconversion products from unsaturated fatty acids by strain DS5

Substrate	GC retention time (min)		Relative activity (%)
	Substrate	Product	
Myristoleic acid	2.70	3.8	5.5
		4.08	12.1
Palmitoleic acid	4.21	6.9	31.8
		7.27	2
Oleic acid	7.25	12.6	100
		13.4	9
Linoleic	6.92	12.2	2
		12.9	38.4
α-Linolenic acid	6.99	11.97	4.7
		12.7	39.9
γ-Linolenic acid	6.58	11.4	36.4

products RT 3.8 and 4.08 min, are likely 10-ketomyristic and 10-hydroxymyristic acid, respectively.

Palmitoleic acid also gave two bioconversion products on GC (RT 6.9 min and RT 7.27 min). The electron-impact spectrum of the methyl ester of RT 6.9 min gave a molecular ion of m/z 284. A large fragment corresponding to α-cleavage with m/z 199, places the keto group at the C-10 position. A methyl ester with product RT 7.27 min gave a molecular ion of 286. Large fragments corresponding to α-cleavage with m/z 201 and 169 (relative intensity 20% and 60%, respectively) place the hydroxy group at the C-10 position. Therefore, the products RT 6.9 and 7.27 min, are likely 10-ketopalmitic and 10-hydroxypalmitic acid, respectively.

Previously, the *Flavobacterium* sp. strain DS5 bioconversion products from oleic acid and linoleic acid were identified as 10-ketostearic [4] and 10-hydroxy-12(*Z*)-octadecenoic acid [3], respectively. It is interesting to find that all unsaturated fatty acids tested are hydrated at the C-10 position despite their varying degrees of unsaturation. *Flavobacterium* sp. strain DS5 hydratase was not active on saturated fatty acids and other non-9(*Z*)-unsaturated fatty acids such as: elaidic [9(*E*)-octadecenoic], arachidonic [5(*E*), 8(*E*), 11(*E*), 14(*E*)-eicosatetraenoic], and erucic [13(*E*)-docosenoic] acids [4]. From all of the data gathered, it is concluded that the hydratase is indeed a C-10 positional-specific enzyme. The fact that elaidic acid was not hydrated indicated that the unsaturation must be in the *cis* configuration for DS5 hydratase activity.

The *Flavobacterium* sp. strain DS5 system produced more keto product from palmitoleic and oleic acids and more hydroxy product from myristoleic, linoleic, and α- and γ-linolenic acids (Table 2). The reason for the product preference is not clear and is currently under investigation.

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