Novel pathways of oleic and *cis*-vaccenic acid biosynthesis by an enzymatic double-bond shifting reaction in higher plants

Akira Shibahara[1], Kohei Yamamoto[2], Mariko Takeoka[3], Akemi Kinoshita[1], Goro Kajimoto[1], Takao Nakayama[2] and Manjiro Noda[3]

[1]*Department of Nutrition, Kobe-Gakuin University, Arise, Ikawadani-cho, Nishi-ku, Kobe 673,* [2]*General Testing Research Institute of Japan Oil Stuff Inspectors' Corporation, Mikagetsuka-machi, Higashinada-ku, Kobe 658* and [3]*Department of Agricultural Chemistry, Kinki University, Naka-machi, Nara 631, Japan*

Received 15 January 1990; revised version received 20 March 1990

The novel pathways of oleic acid formation from *cis*-vaccenic (*cis*-11-octadecenoic) acid and of *cis*-vaccenic acid formation from oleic acid by enzymatic positional isomerization have been proposed in higher plants, based on stable-isotope experiments using [2,2-$^2H_2$]*cis*-vaccenate or [2,2-$^2H_2$]oleate as an immediate precursor. A pulp homogenate and also pulp slices prepared from developing kaki (*Diospyros kaki*) fruit could catalyze these hitherto unknown isomerizations. This suggests the presence of a new type of isomerase responsible for the double-bond shifting reaction without *cis-trans* isomerization in the middle of fatty acid carbon chains.

Oleic acid; *cis*-Vaccenic acid; Biosynthetic pathway; Stable isotope; Positional isomerization; Developing kaki pulp

1. INTRODUCTION

Our previous study [1] first proved the pathways of *cis*-vaccenic [2,3] (*cis*-11-octadecenoic) acid biosynthesis by chain elongation of palmitoleic (*cis*-9-hexadecenoic) acid in higher plants. In that study [1], unexpected data were obtained: oleic acid labeled with two deuterium atoms attached at a carbon atom between the double bond and the carboxyl group was synthesized along with the labeled *cis*-vaccenate by incubation with [2,2-$^2H_2$]palmitoleate and kaki (*Diospyros kaki*) pulp homogenate. In view of the present knowledge, the mechanism of incorporating two deuterium atoms from the substrate ([2,2-$^2H_2$]palmitoleate) into oleate cannot be explained.

The present study aims to elucidate this mechanism, and results in the proposal of novel pathways for oleic and *cis*-vaccenic acid biosynthesis by enzymatic double-bond shifting in kaki pulp.

2. MATERIALS AND METHODS

Oleic and *cis*-vaccenic acids, ATP, NADPH and CoA were obtained from commercial sources. The [2,2-$^2H_2$]fatty acids were prepared according to the methods given in [4,5].

Kaki fruit samples grown in the experimental farm of Kyoto Prefectural University were harvested in October 1988 (just before maturation). A kaki fruit (ca 150 g) was peeled and divided into pulp and seed parts.

Correspondence address: A. Shibahara, Department of Nutrition, Kobe-Gakuin University, Arise, Ikawadani-cho, Nishi-ku, Kobe 673, Japan

A piece of kaki pulp (10 g) was cut off and homogenized with 10 ml of 0.4 M phosphate buffer (pH 6.5) in a Waring blender for 3 min at 0°C. The homogenate was filtered through cotton cloth and used immediately for stable-isotope experiments. Protein was determined by the method of Lowry et al. [6]. A typical assay medium contained 0.4 M phosphate buffer (pH 6.5), 0.2 mM ammonium [2,2-$^2H_2$]*cis*-vaccenate or [2,2-$^2H_2$]oleate, 1.2 mM ATP, 0.4 mM NADPH, 0.4 mM CoA and the filtered homogenate (1 ml, corresponding to 3–4 mg protein) in a total volume of 2.5 ml. After incubation at 30°C for 20 min, the reaction was stopped by the addition of 2 M HCl, and a known amount of pentadecanoic acid was added as an internal standard.

For another assay system, slices of kaki pulp (ca 7 mm cubes, 2 g in total) were added to a solution (5 ml) containing 0.2 M phosphate buffer (pH 6.5) and 0.4 mM ammonium [2,2-$^2H_2$]*cis*-vaccenate or [2,2-$^2H_2$]oleate. After incubation at 30°C for 180 min, the reaction was stopped in the same manner as described above.

The extraction of lipids, the preparation of fatty acid methyl esters and the quantitative analysis of each fatty acid were carried out by the same methods as those in [1]. The carbon atom carrying the two deuterium atoms in the newly synthesized oleic or *cis*-vaccenic acid was checked by capillary gas chromatography-mass spectrometry according to the method given in [5]. After methylthiolation of the fatty acid methyl esters [7], the resulting dimethyl disulfide adducts were analyzed by gas chromatography-mass spectrometry with a packed column to determine their structures and to estimate their isotope contents under the same conditions as those in our previous work [1].

3. RESULTS AND DISCUSSION

Fig. 1 shows the pathways of monounsaturated fatty acid synthesis in higher plants. A combination of fatty acid β-oxidation and de novo synthesis to explain the unexpected data in our previous study [1] (cf. section 1) can be ruled out as follows. If the labeled substrate ([2,2-$^2H_2$]palmitoleate) was β-oxidized, only one molecule among the resulting acetyl-CoA molecules

Published by Elsevier Science Publishers B.V. (Biomedical Division)

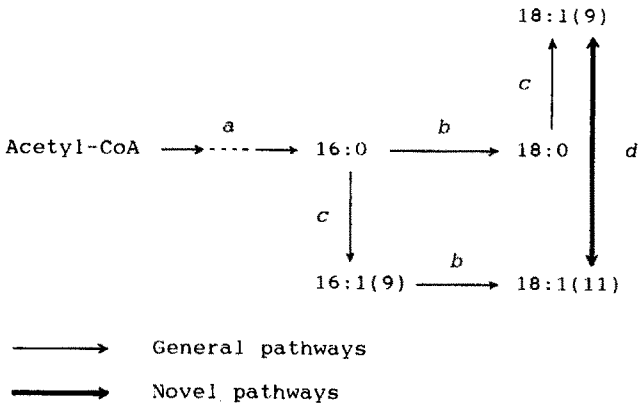

Fig. 1. Biosynthesis of monounsaturated fatty acids in higher plants. *a*, de novo synthesis; *b*, chain elongation; *c*, desaturation; *d*, isomerization. The novel pathways (*d*) are proposed based on the data in Table I and the considerations given in the text.

from one molecule of the substrate must contain one deuterium atom. Therefore, it was practically impossible that the dideuterated oleate with the specific labeling pattern (cf. section 1) was formed by de novo synthesis from the deuterated acetyl-CoAs, each containing one deuterium atom.

Consequently, we assumed that the labeled *cis*-vaccenate originating from [2,2-$^2H_2$]palmitoleate changed directly into oleate through unknown enzymatic reactions.

To test this hypothesis, the kaki pulp homogenate was incubated with ammonium [2,2-$^2H_2$]*cis*-vaccenate or [2,2-$^2H_2$]oleate under conditions similar to those in our previous work [1]. As shown in Table I, dideuterated oleate was biosynthesized from [2,2-$^2H_2$]*cis*-vaccenate, and similarly labeled *cis*-vaccenate was also formed from [2,2-$^2H_2$]oleate. Only the native homogenate from fresh pulp had the catalytic activity for this isomerization, and the presence of exogenous cofactors in the reaction mixture slightly stimulated this isomerization between the labeled octadecenoates. No exchange between the labeled octadecenoates was observed in the absence of the raw pulp homogenate or the labeled substrate. These results suggested that *cis*-vaccenate was converted into oleate by the action of an unknown enzyme system existing in the pulp homogenate, and that the same system or another different system was responsible for the reverse reaction, converting oleate into *cis*-vaccenate.

For another assay system, the kaki pulp slices were incubated with [2,2-$^2H_2$]*cis*-vaccenate or [2,2-$^2H_2$]oleate (Table I). The experiments showed that *cis*-vaccenate markedly changed into oleate even in the absence of cofactors. This in vivo evidence further supports the above findings that the isomerization between *cis*-vaccenate and oleate occurs actually in kaki pulp. The hitherto unknown isomerization cannot be ascribed to microbial contamination (data not shown). This will be discussed in detail in our next paper.

Table I

Enzymatic isomerization between [2,2-$^2H_2$]*cis*-vaccenate and [2,2-$^2H_2$]oleate with kaki pulp homogenate and slices

Substrate and assay system	[2,2-$^2H_2$]18:1(9)/[2,2-$^2H_2$]18:1(11) after incubation	[2,2-$^2H_2$]Fatty acid formed			
		Labeled 18:1(9)		Labeled 18:1(11)	
		% in total 18:1(9)	nmol[a]	% in total 18:1(11)	nmol[a]
Experiment with homogenate:					
[2,2-$^2H_2$]18:1(11)					
Complete					
(+ homogenate, ATP, NADPH, CoA)	12.2/87.8	11.6	1.0		
ATP, NADPH, CoA	10.4/89.6	10.3	0.6		
Homogenate	0/100	0			
Homogenate + boiled homogenate	0/100	0			
Homogenate + BSA[b]	0/100	0			
Substrate	-	0			
[2,2-$^2H_2$]18:1(9)					
Complete					
(+ homogenate, ATP, NADPH, CoA)	94.8/5.2			4.2	2.3
ATP, NADPH, CoA	98.4/1.6			1.2	0.4
Homogenate	100/0			0	
Homogenate + boiled homogenate	100/0			0	
Homogenate + BSA[b]	100/0			0	
Substrate	-			0	
Experiment with slices:					
[2,2-$^2H_2$]18:1(11)	73.9/26.1	56.2	nd[c]		
[2,2-$^2H_2$]18:1(9)	94.0/6.0			2.7	nd[c]

Each value is an average of duplicate assays. Incubation conditions are given in section 2.
[a] nmol/mg protein after 20-min incubation. [b] Bovine serum albumin. [c] Not determined.

The structural elucidations of the biosynthesized fatty acids labeled with deuterium atoms (Table I) were accomplished by means of several chromatographic, spectrometric and chemical methods which had been used in the confirmation of the presence of *cis*-vaccenic acid in the pulp lipids of mango (*Mangifera indica*) [7] and other varieties of commonly available fruits (including kaki) [8]. In addition, the position of the carbon to which two deuterium atoms were attached in the octadecenoate molecule could be specified by capillary gas chromatography-mass spectrometry according to the shift of the McLafferty rearrangement ion (*m/z* 74 to *m/z* 76) [5].

As a possible explanation for the data in Table I, someone may suppose that a biohydrogenation, as reported in microorganisms [9,10], occurs even in higher plants to yield stearate from an octadecenoate, which is then desaturated to give other octadecenoate isomer by the action of a Δ9- or Δ11-desaturase (cf Fig. 1). However, the occurrence of biohydrogenation and the existence of a Δ11-desaturase in higher plants have not yet been pointed out. Furthermore, the presence of [2,2-$^2H_2$]stearate in this study or [2,2-$^2H_2$]palmitate and [4,4-$^2H_2$]stearate in the previous study [1] could not be observed in the reaction mixtures. Additional experiments with ammonium [2,2-$^2H_2$]*cis*-6-, *trans*-9- and *trans*-11-octadecenoates as substrates were carried out under the same conditions as those in Table I. The results showed no formation of labeled oleate or *cis*-vaccenate from these deuterated octadecenoate isomers (data not shown). These findings suggest that the double-bond shifting of the octadecenoates by biohydrogenation and subsequent desaturation is improbable in kaki pulp.

From these considerations and the experimental evidence in Table I, we conclude that the novel pathways for the direct isomerizations of *cis*-vaccenate into oleate and of oleate into *cis*-vaccenate exist in kaki pulp, as illustrated in Fig. 1. It is unclear whether the same enzyme system participates in both reactions (reversible reaction) or whether two enzyme systems catalyze each reaction independently. These enzymatic positional isomerizations between *cis*-vaccenate and oleate up to now have not been reported in the literature. This suggests the presence of a new type of isomerase responsible for the double-bond shifting reaction, keeping the *cis* configuration of the double bond in the middle of fatty acid carbon chains. Detailed studies on characterization and purification of the enzyme system are in progress in our laboratories.

In 1928, Bertram [11] isolated *trans*-11-octadecenoic acid from beef tallow, etc., and named it as vaccenic acid. Since then, the occurrence of its geometrical isomer, *cis*-vaccenic acid, has been recognized in several living materials (cf [7,8,12–14] and references therein). The pathway of *cis*-vaccenic acid biosynthesis in microorganisms was proved to be an anaerobic pathway by Bloch and his coworkers [12]. On the other hand, *cis*-vaccenic acid was aerobically synthesized by chain elongation of palmitoleate derived from palmitate in animals [13], and by a similar route in higher plants [1]. None of the literature [12–19] referred to the conversion of the newly synthesized *cis*-vaccenate or oleate into another octadecenoate, keeping the *cis* configuration of the double bond, as shown in Fig. 1.

It may be necessary to reexamine the well-investigated metabolism of *cis*-vaccenic and oleic acids in microorganisms and also in animals, in view of the double-bond shifting reaction reported in this study. In higher plants, we have obtained preliminary data on the same pathways (Fig. 1) being present in various developing fruit pulps and seed tissues. The generally accepted theory on the formation of common oleic acid will be modified with annotation as follows: oleic acid is synthesized from stearate, and in some higher plants, also from *cis*-vaccenate.

REFERENCES

[1] Shibahara, A., Yamamoto, K., Takeoka, M., Kinoshita, A., Kajimoto, G., Nakayama, T. and Noda, M. (1989) Lipids 24, 488–493.
[2] IUPAC-IUB Commission on Biochemical Nomenclature (1977) Eur. J. Biochem. 79, 11–21.
[3] Slover, H.T. (1983) Lipids 18, 913.
[4] Noda, M. and Miyake, K. (1982) J. Jpn. Oil Chem. Soc. 31, 154–158.
[5] Noda, M. and Inazu, Y. (1985) J. Jpn. Oil Chem. Soc. 34, 42–47.
[6] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[7] Shibahara, A., Yamamoto, K., Nakayama, T. and Kajimoto, G. (1986) Lipids 21, 388–394.
[8] Shibahara, A., Yamamoto, K., Nakayama, T. and Kajimoto, G. (1987) J. Am. Oil Chem. Soc. 64, 397–401.
[9] Hughes, P.E., Hunter, W.J. and Tove, S.B. (1982) J. Biol. Chem. 257, 3643–3649.
[10] Janssen, G., Parmentier, G., Verhulst, A. and Eyssen, H. (1985) Biomed. Mass Spectrom. 12, 134–138.
[11] Bertram, S.H. (1928) Biochem. Z. 197, 433–441.
[12] Bloch, K. (1969) Acc. Chem. Res. 2, 193–202.
[13] Holloway, P.W. and Wakil, S.J. (1964) J. Biol. Chem. 239, 2489–2495.
[14] Fulco, A.J. (1983) Prog. Lipid Res. 22, 133–160.
[15] Wakil, S.J. (1970) in: Lipid Metabolism (Wakil, S.J. ed.) pp. 1–48, Academic Press, New York.
[16] Stumpf, P.K. (1980) in: The Biochemistry of Plants, vol. 4. (Stumpf, P.K. ed.) pp. 177–204, Academic Press, New York.
[17] Stumpf, P.K. (1984) in: Fatty Acid Metabolism and Its Regulation (Numa, S. ed.) pp. 155–179, Elsevier, Amsterdam.
[18] Harwood, J.L. (1986) in: The Lipid Handbook (Gunstone, F.D., Harwood, J.L. and Padley, F.B. eds) pp. 485–525, Chapman and Hall, Cambridge.
[19] Harwood, J.L. (1988) Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 101–133.