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Metabolism of Limonoids: Conversion of Nomilin to Obacunone in *Corynebacterium fascians*

Shin Hasegawa,* Alexa M. Dillberger, and Grace Y. Choi

Corynebacterium fascians cells immobilized in acrylamide gel converted nomilin to obacunone in orange juice serum. Cell-free extracts of the organism also catalyzed this conversion. The conversion of nomilin to obacunone is the fourth metabolic pathway of nomilin established in bacteria.

Bitterness due to limonoids in certain citrus juices is one of the major problems of the citrus industry worldwide and has significant economic impact. During the course of our study on limonoid metabolism, we have isolated from soil a strain of Bacterium, *Corynebacterium fascians* (Hasegawa and King, 1983). Unlike other limonoid-metabolizing bacteria (Hasegawa et al., 1972a,b, 1974, 1983; Hasegawa and Kim, 1975; Vaks and Lifshitz, 1981), this bacterium produces constitutive enzymes for limonoid metabolism that is advantageous over the others from a practical viewpoint. Cells, capable of metabolizing limonoids, can be produced conveniently and economically with simple carbon sources and could be used for a biological process that uses immobilized bacterial cells for reduction of limonoid bitterness of citrus juices (Hasegawa et al., 1982a). The enzymes in the other bacteria had to be induced.

At least 29 limonoids have been isolated from *Citrus* and *Citrus* hybrids; four of them, limonin (1) (Figure 1), nomilin (5), (Dreyer, 1963) (Figure 2), ichangin (Dreyer, 1966), and nomilinic acid (Hasegawa and Bennett, 1975), are bitter. Limonin is the major limonoid present in citrus juices and the primary cause of limonoid bitterness. Recently, it was shown that nomilin also appears to play a role in limonoid bitterness (Rouseff et al., 1981; Hashinaga and Itoo, 1981). Therefore, nomilin has been included in our citrus juice debittering study.

Recently, we have found that treatment of orange juice serums with *C. fascians* immobilized in acrylamide gel converted nomilin to three unidentified metabolites. The major metabolites, which consisted of approximately 90% of the total metabolites, was, therefore, isolated and its structure determined.

U.S. Department of Agriculture, Agricultural Research Service, Fruit and Vegetable Chemistry Laboratory, Pasadena, California 91106.

MATERIALS AND METHODS

Valencia oranges were purchased from a local market. The juice was extracted with a Sunkist juicer. Nomilin was dissolved in a minimal portion of CH_3CN and added to the juice to bring its concentration to 20–40 ppm. The serum was obtained from the juice by centrifugation at 5000*g* for 30 min and kept in a freezer until used.

C. fascians was grown and harvested by the procedures described previously (Hasegawa and King, 1983). Cells were immobilized in acrylamide gel by the procedure of Tosa et al. (1974), blended with a Polytron, and packed in a 2 cm diameter column. The serum was passed through the column at a rate of 100 mL/h at room temperature.

The metabolite was extracted from the treated serum with CH_2Cl_2 by the procedure described previously (Hasegawa et al., 1972a) and isolated by column chromatography on a silica gel. The column was eluted, stepwise, by increasing the concentration of EtOAc in hexane. The major metabolite, thin-layer chromatographically pure, was crystallized from MeOH and analyzed by TLC and NMR spectrum, which was run on a JEOL SP-100 spectrometer. Limonoids were quantitatively analyzed by TLC by the procedure of Maier and Grant (1970).

Nomilin acetyl-lyase activity was also demonstrated with cell-free extracts of *C. fascians*. Two grams of cells was suspended in 50 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing 10^{-3} M dithiothreitol and ruptured with a Branson sonifier, J-22. The suspension was cen-

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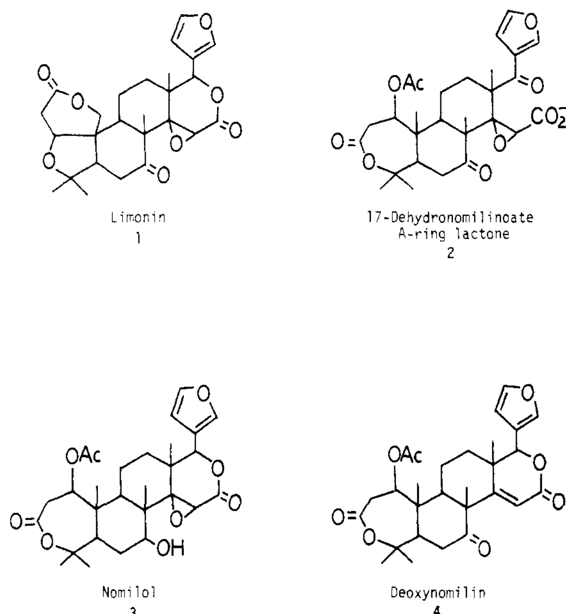

Figure 1. Structures of limonoids.

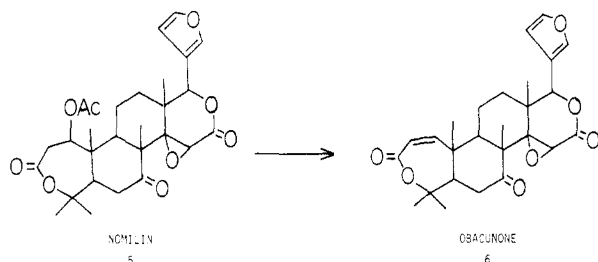

Figure 2. Conversion of nomilin to obacunone in *C. fascians*.

trifuged at 20000*g* for 10 min and the supernatant was brought to 0.9 saturation with solid $(NH_4)_2SO_4$. The mixture was centrifuged at 20000*g* for 15 min: the recovered precipitate was dissolved in a minimum of 0.01 M potassium phosphate buffer (pH 7.0) and dialyzed against the same buffer for 2 h and used for enzyme assay. The lyase activity was assayed in 50 mL of a reaction mixture consisting of 0.1 M potassium phosphate buffer at pH 7.0, 1 mg of nomilin, 1 mg of enzyme preparation protein, and 2% of sucrose. The mixture was incubated at 23 °C. Sucrose was added to increase the solubility of nomilin.

RESULTS AND DISCUSSION

For the isolation of the major metabolite produced from nomilin (**5**) (Figure 2) by immobilized cells of *C. fascians*, an 850-mL portion of the orange juice serum containing 40 ppm of nomilin was passed through a column packed with 5 g of cells (wet weight) immobilized in acrylamide gel. Analysis of the treated serum with TLC showed that approximately 75% of nomilin was metabolized. The major metabolite, which consisted of over 90% of the total metabolites, was isolated on a silica gel column and crystallized from MeOH to give 16 mg. About 95% of the original nomilin was recovered from the serum treated with the control column, which contained acrylamide gel without cells.

The metabolite was neutral and Ehrlich positive. TLC analysis showed that it had the same mobility as that of an authentic sample of obacunone (**6**) with three solvent systems (Table I). Furthermore, its ^1H NMR spectrum was identical with that of authentic obacunone (Dreyer, 1965b). These data showed that *C. fascians* cells immobilized in acrylamide gel convert nomilin to obacunone in orange juice serum (Figure 2).

Table I. R_f Values of Nomilin and Its Metabolite and Limonin[a]

	A	B	C
nomilin	0.30	0.36	0.15
metabolite	0.45	0.54	0.37
obacunone	0.45	0.54	0.37
limonin	0.36	0.30	0.21

[a] Silica gel G plates were used. Composition of solvent systems: A, toluene-EtOH-H_2O-HOAc (200:47:15:1, upper layer); B, EtOAc-cyclohexane (3:2); C, CH_2Cl_2-MeOH (97:3).

The enzyme system in the immobilized cells was very active. Nomilin was completely converted to obacunone when juice serums containing 20–25 ppm of nomilin were treated with the column only once. The same column was used 15 times without losing its effectiveness. One milligram of cell-free extract proteins converted 1 mg of nomilin to obacunone completely at pH 7.0 during 18 h of incubation at 23 °C. The control, boiled extracts, did not catalyze this conversion. The conversion of nomilin to obacunone is most likely catalyzed by the action of nomilin acetyl-lyase. The enzyme has not been isolated yet, but this type of enzyme action is seen in the breakdown of pectic substance by pectin lyase (Hasegawa and Nagel, 1966).

Arthrobacter globiformis converted nomilin to 17-dehydronomilinoate A-ring lactone (**2**) (Hasegawa et al., 1972b) (Figure 1), whereas *Pseudomonas* 321-18 converted to deoxynomilin (**4**) (Hasegawa et al., 1972a, 1974). Immobilized cells of *A. globiformis* II converted nomilin to nomilol (**3**) in citrus juices (Hasegawa et al., 1982b). The conversion of nomilin to obacunone in the immobilized cells of *C. fascians* is the fourth metabolic pathway of nomilin found in bacteria.

Nomilin and obacunone are two of the major limonoids present in seeds of *Citrus* and *Citrus* hybrids (Hasegawa et al., 1980). Particularly, these limonoids are present in high concentration in seeds of lemon and tangelo. Dreyer (1965a) suggested that obacunone is synthesized via nomilin in citrus. The present study suggested that this conversion is most likely catalyzed by the action of nomilin acetyl-lyase.

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Effect of Oxidizing and Reducing Agents on Trimethylamine *N*-Oxide Demethylase Activity in Red Hake Muscle

Linda D. Racicot, Ronald C. Lundstrom,* Kurt A. Wilhelm, Elinor M. Ravesi, and Joseph J. Licciardello

The addition of oxidizing or reducing agents to minced red hake showed that oxidizing agents reduced the rate of dimethylamine (DMA) and formaldehyde (FA) formation, while reducing agents accelerated their formation. To determine the effectiveness of different oxidizing agents, H_2O_2, NaOCl, and $KBrO_3$ were added at four levels to minced red hake. DMA, FA, and trimethylamine oxide values showed that 0.05, 0.10, and 0.25% levels of H_2O_2 were most effective in slowing the reaction rate. Although Instron measurements did not show the oxidizing agents to improve the texture greatly, the sensory panel analysis found the 0.05, 0.10, and 0.25% levels of H_2O_2 to have a better texture than the control.

Investigations into the utilization of red hake (*Urophycis chuss*) as a human food have centered around trimethylamine *N*-oxide demethylase (TMAO-ase), which is thought to contribute to the textural problems associated with this species during frozen storage. This enzyme system catalyzes the breakdown of trimethylamine oxide (TMAO) to form dimethylamine (DMA) and formaldehyde (FA) (Yamada and Amano, 1965; Tomioka et al., 1974). The FA presumably cross-links with the myofibrillar proteins, causing a toughening of the texture and loss of water holding capacity (Childs, 1973).

Various compounds have been reported to be activators or inhibitors of the TMAO–ase system. The system has been found to be catalyzed by flavin mononucleotide, methylene blue, Fe^{2+} or Fe^{3+}, ascorbic acid, and cysteine (Yamada and Amano, 1965; Tomioka et al., 1974, Harada, 1975; Parkin and Hultin, 1981; Spinelli and Koury, 1981; Lundstrom et al., 1982b). Inhibitors of the enzyme system include Cu^{2+}, EDTA, and trimethylamine (TMA) (Tomioka et al., 1974; Parkin and Hultin, 1982a). Of 32 compounds added to minced red hake, the most potent accelerators of DMA formation were phenazine methosulfate, menadione, and methylene blue, while dimethylaniline and TMA were the most potent inhibitors (Parkin and Hultin, 1982b).

The absence of oxygen has also been shown to accelerate the rate of DMA production (Lundstrom et al., 1982a). Red hake packaged in oxygen-permeable film showed decreased rates of DMA and FA formation during iced storage compared to uncooked red hake packaged in cans purged with nitrogen and stored in ice. These investigators also found that while an atmosphere of 100% oxygen gas produced low levels of DMA in minced red hake, an atmosphere of 20% oxygen/80% nitrogen also reduced the rate of DMA formation. Sectioning of blocks of fillets stored at −20 °C in an atmosphere of 100% oxygen or 20% oxygen/80% nitrogen revealed that the oxygen was able to diffuse into the blocks to reduce the DMA formation. Thin-layer sections taken from blocks stored in 100% nitrogen showed no differences in the levels of DMA production in inner and outer layers (Lundstrom et al., 1982a; Racicot and Lundstrom, 1982).

Due to the implications of redox potentiators regulating the enzyme system, one study was initiated to analyze the effects of reducing and oxidizing agents as a means to inhibit DMA and FA production and a second study was designed to determine the effectiveness of varying concentrations of oxidizing agents.

MATERIALS AND METHODS

The studies utilized fresh red hake procured from Gloucester, MA, and Point Judith, RI, day boats. After filleting and skinning by hand, the fillets were minced in a Yanagiya meat bone separator, Model Y-100, to obtain homgeneity. To analyze the effects of oxidizing or reducing agents, hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOCl), sodium erythorbate, or ascorbic acid was added to the mince at a final concentration of 0.1% (w/w). In the second study, which analyzed the level of oxidizing agents required to be effective, H_2O_2, NaOCl, or potassium bromate ($KBrO_3$) was added at final concentrations of 0.01, 0.05, 0.10, or 0.25% (w/w). The additives were dissolved in distilled water and were added to the mince at a level of 20 mL/0.45 kg. A control sample containing an equivalent volume of water was also prepared. Each batch was blended with a Universal Industries Univex mixer, Model 1222, for 2 min at medium speed. Samples were

National Marine Fisheries Service, Northeast Fisheries Center, Gloucester Laboratory, Gloucester, Massachusetts 01930.

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