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# 10-Hydroxyoctadecanoic Acid Produced by *Lactococcus lactis* subsp. *lactis* as a Part of Flocculent Aggregate

**PARK, HEE JUN, YOONG-HO LIM[1], YOUN SOON KIM[2], AND KYU HANG KYUNG***

*Department of Food Science, Sejong University, Seoul 143-747, Korea*
*[1]Department of Agricultural Chemistry, Kon-Kuk University, Seoul 133-745, Korea*
*[2]Department of Home Economics, Chosun University, Kwangju 501-759, Korea*

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**Abstract** A flocculent aggregate produced by *Lactococcus lactis* subsp. *lactis* in broths containing Tween 80, including MRS broth, had a microscopic structure of intertwined thread-like filaments. The filamentous structure was not elongated bacterial cells, but consisted of an organic solvent-soluble portion and an insoluble solid. *L. lactis* subsp. *lactis* grown at 25°C for 15 days in tryptic soy broth with 0.1% Tween 80 and 1.0% malt extract produced 13 mg/l of flocculent aggregate, which contained 0.84 g/g of organic solvent-soluble component. The organic solvent-soluble part was identified as 10-hydroxyoctadecanoic acid.

**Key words:** *Lactococcus lactis* subsp. *lactis*, 10-hydroxyoctadecanoic acid, aggregate

We routinely observed, during the cultivation of a coccus *Lactococcus lactis* subsp. *lactis* in MRS broth, an off-white flocculent aggregate, which upon microscopic observation revealed a thread-like filamentous structure. The structure was not gram stainable, i.e., after gram staining the structure was not seen microscopically, probably due to dissolution of the structure by organic solvents in the staining solution.

There have been several reports concerning the filamentous form of growth of lactobacilli [5, 8, 9], but not of a coccus. The filamentous mass produced by lactobacilli was described as long intertwined chains and filaments microscopically resembling a mass of hair [5, 8, 9] and exhibited considerable tensile strength, and the resultant clot did not break up even when vigorously shaken [5, 8] as also occurred in this study. A single filament contained both gram-positive and -negative segments [8], while the thread-like structure of *L. lactis* subsp. *lactis* in this study was not gram-stainable.

*Corresponding author
Phone: 82-2-3408-3225; Fax: 82-2-3408-3569;
E-mail: kyungkh@cs.sejong.ac.kr

The thread-like filaments formed by *L. lactis* subsp. *lactis* seemed to be similar in physical characteristics to that produced by lactobacilli [5, 8, 9], but different in that it was not gram-stainable. Since *L. lactis* subsp. *lactis* is a coccus and the filamentous structure disappeared upon gram staining, we refrained from interpreting this thread-like structure as elongated cells of the bacterium. The objectives of this study were to isolate the flocculent aggregate from the culture broth and to identify the organic solvent-soluble portion of the aggregate produced by *L. lactis* subsp. *lactis*.

## MATERIALS AND METHODS

### Bacterial Strain and Culture Conditions

*Lactococcus lactis* subsp. *lactis* was subcultured at 25°C in MRS broth (Difco Laboratories, Detroit, U.S.A.) in a 16 mm × 150 mm glass culture tube with a cap. Then, 0.5 ml of overnight grown culture was inoculated into each 500-ml Erlenmeyer flask containing 300 ml tryptic soy broth (TSB; Difco Laboratories) plus 1.0% malt extract and 0.1% Tween 80. The flasks were statically incubated at 25°C for 15 days before the flocculent aggregate was harvested.

### Chemicals

Chloroform and boron trifluoride (20%) in methanol were obtained from Merck Co. (Hobenbrunn, Germany). Hexane, Tween 80 and sodium hydroxide were purchased from Sigma Chem. Co. (St. Louis, U.S.A.). Tryptic soy broth, malt extract and lactobacilli MRS broth were purchased from Difco Laboratories (Detroit, U.S.A.).

### Separation of Flocculent Aggregate and Isolation of the Organic Solvent-Soluble Part from the Dried Aggregate

The aggregate was separated from the culture broth by filtering the growth medium through Doutor coffee filter

paper (Korea Doutor Coffee Co., Seoul, Korea) which allowed the passage of bacterial cells but not of the aggregate. The aggregate was washed by vortexing with distilled water to remove the remaining bacterial cells until the wash water became clear. The washed aggregate was dried in a desiccator at room temperature.

The organic solvent-soluble portion was extracted from the dried aggregate with chloroform and recrystallized three times.

### Identification of the Organic Solvent-Soluble Portion of the Aggregate

NMR data were recorded on a spectrometer (DPX400, Bruker Analytische Messtechnick GMBH, Karlsruhe, Germany) in $CHCl_3$-d using 5 mm dual probehead at 298 K. Elemental analysis data were obtained on Foss Heraeus CHN-O Rapid. The crystalline product was methylated by the boron trifluoride method [1] before GC/MS analysis. Total ion chromatogram (TIC) and mass spectra of methylated sample were obtained using a Hewlett-Packard Model 5890 Series II plus capillary gas chromatograph equipped with a model 5972 mass selective detector (Hewlett-Packard Co., Wilmington, U.S.A.). The GC column (30 m capillary, J & W Scientific Inc., Folsom, U.S.A.) coated with DB-5 (0.25 μm thickness) was coupled directly to the MSD capillary interface. The oven temperature was programmed from 100 to 250°C at 5°C/min with the final temperature maintained for 20 min. The injector port and detector temperatures were 280 and 250°C, respectively. Electron impact ionization (potential 70 ev) was used and the mass range scanned was 40~500 daltons.

### Microscopy

Phase-contrast microscopy (1000×) was performed using the Nikon Labophot microscope equipped with 1.25-numerical aperture phase-contrast objective lens.

## RESULTS

### Flocculent Aggregate Formation

*L. lactis* subsp. *lactis* formed flocculent aggregates when it was statically grown in MRS broth (Fig. 1) during the prolonged period of incubation. The clot began to appear in 7~10 days and was fully formed in about 15 days. The bacterium also formed the aggregate in TSB with 1.0% malt extract and 0.1% Tween 80, but not in the same medium without Tween 80 which was the choice of the medium for the economic production of the aggregate. The aggregate showed a thread-like filamentous structure microscopically (Fig. 1).

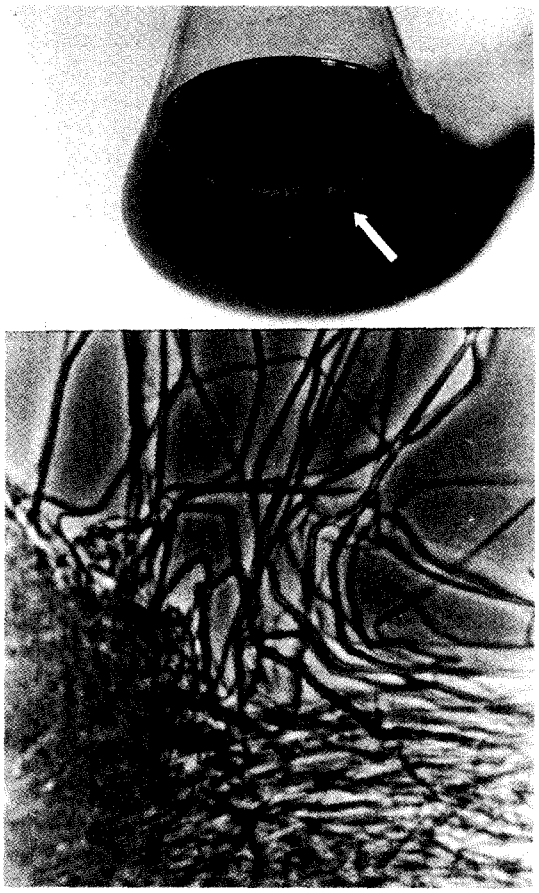

**Fig. 1.** (Top) Flocculent aggregate (arrow) formed by *Lactococcus lactis* subsp. *lactis* in TSB supplemented with 0.1% Tween 80 and 1.0% malt extract at 25°C for 15 days. (Bottom) Photomicrograph (1000×) of thread-like filaments of flocculent aggregate produced by *L. lactis* subsp. *lactis*.

### Isolation and Identification of Organic Solvent-Soluble Portion of the Thread-Like Structure

The dried aggregate was extracted with chloroform. The portion extracted with chloroform formed needle-shaped crystals upon evaporation of the solvent. The residue remaining after extraction was a brown powdery solid which was not soluble either in water nor in organic solvents like chloroform, ether, or ethanol.

The structure of the chloroform-soluble fraction of the thread-like filaments was identified by GC/MS, elemental analysis, and several NMR experiments such as $^{1}$H-NMR, $^{13}$C-NMR, DEPT [7], COSY [2], HMQC [3], and HMBC [4]. Elemental analysis showed that the fraction contained a C:H:O ratio of 6.0:11.9:0.9 and did not contain nitrogen and sulfur. The number of carbon was determined to be 18 by $^{13}$C-NMR. DEPT45, DEPT90, and DEPT135 experiments revealed the type of the carbons: 1 methyl, 15 methylenes, 1 methine, and 1 quaternary carbon. The chemical shifts are listed in Table 1. The peak of 179.5 ppm was caused by a

**Table 1.** The $^{13}C$-NMR and $^{1}H$-NMR chemical shifts of the choloroform-soluble portion.

| $^{13}C$ | Multiplicity | $^{1}H$ | Assignments |
|---|---|---|---|
| 14.3 | q | 0.88 | 18-Methyl |
| 22.9 | t | 1.25 | 16-$CH_2$ |
| 24.9 | t | 1.61 | 3-$CH_2$ |
| 25.8 | t | 1.44 | $CH_2$ |
| 25.84 | t | 1.27 | $CH_2$ |
| 29.2 | t | 1.28 | $CH_2$ |
| 29.3 | t | 1.28 | $CH_2$ |
| 29.5 | t | 1.28 | $CH_2$ |
| 29.55 | t | 1.28 | $CH_2$ |
| 29.7 | t | 1.28 | $CH_2$ |
| 29.8 | t | 1.28 | $CH_2$ |
| 29.9 | t | 1.28 | $CH_2$ |
| 32.1 | t | 1.29 | 17-$CH_2$ |
| 34.2 | t | 2.34 | 2-$CH_2$ |
| 37.5 | t | 1.43 | $CH_2$ |
| 37.6 | t | 1.43 | $CH_2$ |
| 72.3 | d | 3.59 | C-OH |
| 179.5 | s | - | COOH |

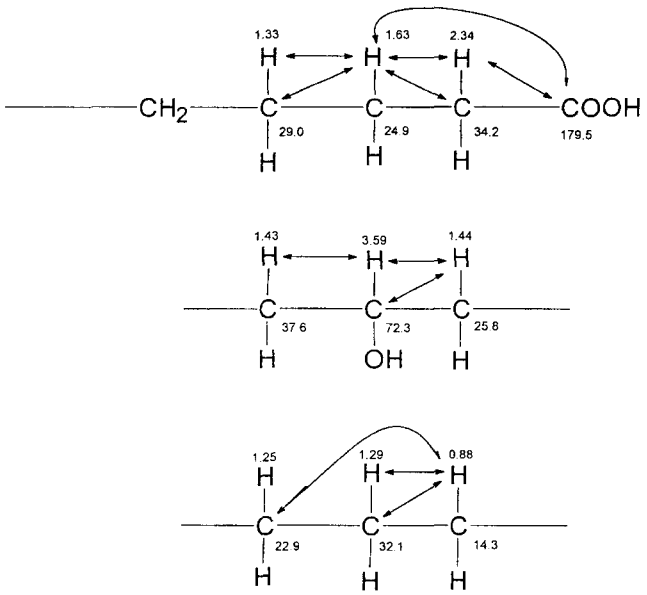


**Fig. 2.** The partial structures obtained from the interpretation of COSY, HMQC, and HMBC.

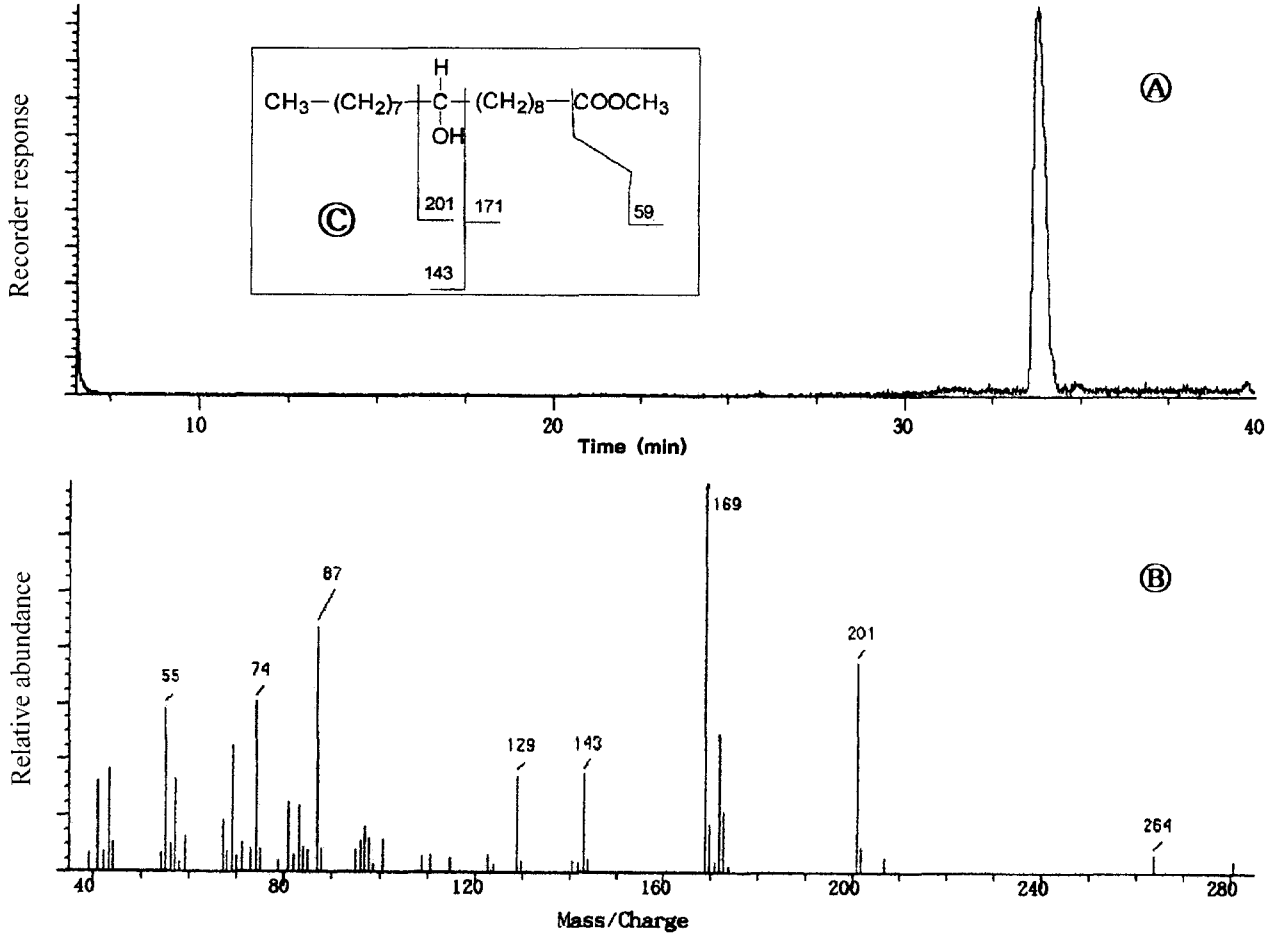


**Fig. 3.** Total ion chromatogram (A) of methylated chloroform-soluble portion of the aggregate, mass spectrum (B) of the methylated product, and molecular structure (C) of methyl 10-hydroxyoctadecanoate.

carboxylic group; and the peak of 72.3 ppm, a hydroxyl group. The chemical shifts of 15 methylene peaks are in the range between 20 and 40 ppm, so that it can be expected that the fraction has a long chain of methylene carbons. The partial structures obtained from the interpretation of COSY, HMQC, and HMBC spectra are shown in Fig. 2. NMR data suggests that the structure of chloroform-soluble fraction of the filament is a hydroxyoctadecanoic acid.

Total ion chromatogram of the methylated product showed a single peak (Fig. 3A), suggesting that the chloroform-soluble fraction of the thread-like filaments is a single compound. The mass spectrum (Fig. 3B) of the methylated product matched well the published spectra of methyl 10-hydroxyoctadecanoate [13]. The proportions (%) of individual ion fragments for chloroform-soluble portion of the aggregate were: 55 (44), 74 (46), 87 (63), 129 (24), 143 (24), 169 (100), 201 (52), 264 (5). The mass spectrum of the methyl ester of the chloroform-soluble fraction of the filaments does not show the expected molecular weight peak (M.W.=314). From the interpretation of MS data, the hydroxyl group seems to be located at the 10th carbon (Fig. 3C).

## DISCUSSION

Flocculent aggregate produced by *L. lactis* subsp. *lactis* is found to be of intertwined thread-like filaments under the microscope and not elongated bacterial cells, consisting of an organic soluble part, 10-hydroxyoctadecanoic acid (10-HODA), and an insoluble solid of unknown identity. There have been several studies [5, 8, 9] reporting the observation of hair-like filamentous growth of lactobacilli. However, to our knowledge, there has not been any report describing the aggregate formation by a coccus. It seems to be that hydrophobic and water-insoluble fatty acids aggregated and that the low density aggregates float on top of broth, instead of settling down.

There have been reports of microbial and enzymatic conversion of oleic acid to hydroxyoctadecanoic acid (HODA) including 10-HODA. Wallen *et al.* [17] and Davis *et al.* [6] reported the isolation of microorganisms that converted oleic acid to 10-HODA. Niehaus *et al.* [13] reported that an enzyme preparation from a *Pseudomonas* catalyzed the conversion of oleic acid and palmitoleic acid to their corresponding 10-hydroxy saturated acid. Thomas [16] reported that many enteric bacteria, including *Streptococcus faecalis*, vigorously converted oleic acid to HODA, which is important in the pathogenesis of diarrhea associated with steatorrhea (malabsorption or maldigestion of fat) [16, 10, 11]. It was suggested that the mechanism for 10-HODA formation is the direct hydration of oleic acid [13, 14, 17]. This may explain why 10-HODA is produced only in a broth with Tween 80, in which oleic acid is included as a part of its structure [18].

The presence of other hydroxy fatty acids in cellular lipids in microorganisms has also been reported. 3-Hydroxyoctadecanoic acid was reported as a component of cellular lipids of *Campylobacter pylori* [12] and *Mycelia sterilia* [15]. However, none of the previous authors reporting the microbial conversion of oleic acid to HODA mentioned filamentous thread-like structure formation as the flocculent aggregate by the microorganisms.

The production of flocculent aggregate by other microorganisms in media containing Tween 80 and the bioconversion of oleic acid to 10-HODA are currently under investigation.

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