Identification of Ground Beef–Derived Fatty Acid Inhibitors of Autoinducer-2–Based Cell Signaling

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MS 07-349: Received 5 July 2007/ Accepted 9 September 2007

ABSTRACT

Autoinducer-2 (AI-2) molecules are used by several microorganisms to modulate various processes, including bioluminescence, biofilm formation, and virulence expression. Certain food matrices, including ground beef extracts, possess compounds capable of inhibiting AI-2 activity. In the present study, we identified and characterized these AI-2 inhibitors from ground beef extract using hexane solvent extraction and gas chromatography. Gas chromatographic analysis revealed the presence of several fatty acids such as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1ω9), and linoleic acid (C18:2ω6) that were capable of inhibiting AI-2 activity. These fatty acids were tested (using Vibrio harveyi BB170 and MM32 reporter strains) at different concentrations (1, 5, and 10 mM) to identify differences in the level of AI-2 activity inhibition. AI-2 inhibition ranged from 25 to 90%. A mixture of these fatty acids (prepared at concentrations equivalent to those present in the ground beef extract) produced 52 to 65% inhibition of AI-2 activity. The fatty acid mixture also negatively influenced Escherichia coli K-12 biofilm formation. These results demonstrate that both medium- and long-chain fatty acids in ground beef have the ability to interfere with AI-2-based cell signaling.

A variety of bacterial cell processes such as growth, sporulation, toxin production, virulence, antibiotic synthesis, and motility are coordinately regulated at the gene expression level by a variety of intra- and intercellular autoinducer (AI) molecules in a process termed quorum sensing (QS) (24–26). Different AI molecules such as AI-1, AI-2, AI-3, oligopeptides, indole, cyclic dipeptides, and the Pseudomonas quinolone signal have been described (2, 14, 17, 24–26). Among these AI molecules, AI-1 is thought to be highly species specific (5), whereas AI-2 is thought to serve as a universal bacterial signal for interspecies communication (3, 22). AI-3 is thought to be involved in communication between bacterial cells and the host’s signaling system (24). Among the AI molecules, AI-2 has been considered the universal signaling molecule, and the luxS gene, which is involved in the production of AI-2, is widely conserved among bacterial species (29).

New information is being gathered about the bacterial species that possess QS systems, the levels of AI molecules within the bacterial cells, the genes that are controlled by QS, and the identity of the signaling molecules (1, 4, 15, 24, 29). However, very little is known about how cell signaling influences spoilage and pathogenic bacteria in foods (17, 23). Previously, we determined that certain foods such as fresh ground beef and fresh poultry meat contain compounds capable of inhibiting AI-2 activity (10). The focus of the present study was to identify and characterize the compounds present in ground beef extracts that interfere with AI-2–based cell signaling.

MATERIALS AND METHODS

Preparation of aqueous ground beef extracts. AI bioassay (AB) medium was prepared as described previously (10). Commercially purchased ground beef (~15% fat content) was homogenized with an equal volume (1:1, wt/vol) of AB medium in a stomacher (Seward Stomacher 400 LAB System, Norfolk, UK) for 2 min. The homogenate was centrifuged (10,000 × g for 10 min), and the supernatant was collected and filter sterilized with a 0.2-μm-pore-size filter.

In vitro synthesis of AI-2. The AI-2 molecule was synthesized in our laboratory by using a previously published protocol (22). His-tagged LuxS and Pfs (proteins required for the formation of AI-2) were isolated and purified using a nickel resin column (Qiagen, Inc., Valencia, Calif.). The purified enzymes were incubated with 1 mM S-adenosyl homocysteine (Sigma-Aldrich, St. Louis, Mo.) for 1 h at 37°C, and AI-2 was further separated from the enzymes in a centrifuge column (Biomax-5, Millipore, Billerica, Mass.). AI-2 activity was confirmed with the Vibrio harveyi BB170 reporter strain (10, 11).

AI-2 activity inhibition assays. V. harveyi reporter strains BB170 (luxN::Tn5; provided by Dr. Bonnie Bassler, Princeton University, Princeton, N.J.) and MM32 (luxN::cm luxS::Tn5; ATCC BAA-1121, American Type Culture Collection, Manassas, Va.), which exhibits bioluminescence in the presence of AI-2 molecules, were used to screen for AI-2 activity. The experimental method to determine inhibition of AI-2 activity was based on that used in our previous study (10). Freshly diluted (1:5,000) culture (90 μl) of the of the reporter strain in AB medium was mixed with 5 μl of in vitro–synthesized AI-2 and 5 μl of the test samples.
in a 96-well plate. A negative control (10 \(\mu\)l of AB medium) and a positive control (5 \(\mu\)l of in vitro AI-2 plus 5 \(\mu\)l of AB medium) were included in each experiment. Six replicates were used for each measurement. The microtiter plates were incubated at 30°C with moderate shaking (100 rpm), and the luminescence response of the reporter strains was monitored with a Wallac 1420 plate reader (PerkinElmer, Shelton, Conn.). Inhibition of AI-2 activity was expressed as a percentage relative to the corresponding positive control and was calculated as 100 \(-\) [(light unit measurement of sample/light unit measurement of positive control) \(\times\) 100] \(\times\) 100).

**Identification and characterization of inhibitory compounds.** Identification and characterization of inhibitory compounds present in the beef extracts were based on the results of a solvent extraction procedure. Equal volumes (1 liter) of ground beef extracts (prepared as described) were mixed with solvents of various polarities such as hexane (minimal polarity), ethyl acetate (medium polarity), and methyl ether ketone (highest polarity), followed by rigorous mixing (200 rpm) for 1 h and a solvent phase separation for 30 min. The solvents were evaporated in a chemical hood. The extracts were dissolved (1 mg/ml) in sterile water (80°C) and were analyzed for AI-2 inhibition. When assayed, the hexane-extracted material had the highest level of AI-2 inhibition, suggesting that the inhibitory compounds may be hydrophobic. The remaining aqueous phase of the hexane-ground beef extract also was checked for AI-2 inhibition. The gas chromatography (GC) analysis of the hexane extracts was performed as described previously (8). The GC system (model 3400, Varian, Palo Alto, Calif.) was equipped with a split injector, a flame ionization detector, and a fused silica capillary column (100 m, 0.25 mm inside diameter, 0.20-\(\mu\)m film; SP 2560, Supelco, Bellefonte, Pa.). Methyl esters of the fatty acids were generated using hydrogen as a carrier gas, and the extracted fatty acid esters were injected into a GC system with a flame ionization detector to pass through a fused silica capillary column. Fatty acid concentrations in the materials extracted with the hexane solvent were determined using standard American Oil Chemists' Society official method Ce 1h-05 under conditions described by Hossen and Hernandez (8). Reference fatty acid methyl esters were used to identify and quantify the fatty acids present in the sample.

**Inhibition of AI-2 activity by selected fatty acids.** The fatty acids, palmitic (C16:0), stearic (C18:0), oleic (C18:1\(\Delta\)9), and linoleic (C18:2\(\Delta\)9\(\Delta\)12), that accounted for the major proportions in the GC analysis were commercially purchased (Sigma) and tested at different concentrations (1, 5, and 10 mM) using *V. harveyi* reporter strains (BB170 and MM32) to screen for inhibition of AI-2 activity. These fatty acids were mixed together (7.16 + 3.71 + 9.97 + 0.58 \(\mu\)g/ml, respectively) in water to obtain a 1X concentration, which corresponded to 0.027, 0.013, 0.035, and 0.002 mM concentrations of the four fatty acids, respectively, and was based on the stoichiometric relationships of these fatty acids obtained during the GC analysis. Both 10X and 100X mixtures also were prepared, and their inhibitory effect on AI-2 activity was measured. To determine whether the observed inhibition of AI-2 was due to acid functionality of selected fatty acids, a portion of the 100X fatty acid mixture (pH 5.9) was neutralized with 0.1 N NaOH and tested for AI-2 inhibition. To rule out the possibility that the observed inhibition of AI-2 activity was due to a bactericidal effect on the reporter strains, portions of the bioassays were plated on Luria-Marine medium when an inhibition of AI-2 activity was observed. The plates were incubated at 30°C, and *V. harveyi* colonies were enumerated after 24 h of incubation.

**Effect of fatty acids on E. coli K-12 biofilm formation.** The biofilm formation assay was based on that previously reported (20). *E. coli* K-12 cells were grown overnight in Luria-Bertani (LB) medium, and 95 \(\mu\)l of the freshly diluted (1:100) culture was mixed with 5 \(\mu\)l of test sample in a sterile round-bottom 96-well polystyrene plate (Nunclogon surface, Nalge Nunc International, Roskilde, Denmark). A positive control (95 \(\mu\)l culture plus 5 \(\mu\)l of deionized water) was included for each experiment. The plates were incubated at 37°C for 48 h without shaking. At the end of the incubation period, the unattached cells were removed, and the plates were washed with distilled water. The remaining biofilm was stained for 15 min with 100 \(\mu\)l of a 1% (wt/vol) crystal violet solution. Stained cells were solubilized with 200 \(\mu\)l of an ethanol-acetone (80:20) solution for quantifying the biofilms. The samples from each well (125 \(\mu\)l) were pipetted to a new flat-bottom 96-well plate (Corning, Inc., Corning, N.Y.), and the optical density at 570 nm was recorded. To rule out the possibility of a bactericidal effect of the fatty acids on the *E. coli* K-12 cells, *E. coli* cells were grown for 24 h in the presence of the ground beef extract and the mixture (100X) of fatty acids. After incubation, the cultures were serially diluted and plated for enumeration.

**Statistical analysis.** Six replicates were included for the AI-2 inhibition and biofilm assays. The experiments were repeated at least twice. Statistical analyses (paired \(t\) test) were performed using SPSS version 12.0 (SPSS, Chicago, Ill.).

**RESULTS**

**Solvent extraction of the ground beef extract.** The inhibitory compounds present in the beef extract were extracted using a solvent extraction procedure and employing solvents of various polarities: hexane, ethyl acetate, and methyl ether ketone. When the different ground beef-solvent extracts were tested for inhibition of AI-2 activity, the hexane extracts had the highest level of AI-2 inhibition (95%; Fig. 1). There was no difference in the plate counts of the *V. harveyi* BB170 reporter strain, confirming that the observed inhibition of AI-2 activity was not an experimental artifact due to reduced viability of the reporter strain (Table 1). When tested, the aqueous content of hexane-ground beef extract did not result in appreciable AI-2 inhibition (18%).

**Identification of fatty acids by GC analysis.** GC analysis of the hexane extracts revealed that C18:1\(\Delta\)9
TABLE 1. Populations of V. harveyi and E. coli after different treatments

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Treatment</th>
<th>Population (log CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. harveyi BB170</td>
<td>Positive control</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Negative control</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Ground beef-hexane extract</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>10 mM linoleic acid (C18:2w6)</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>10 mM palmitic acid (C16:0)</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10 mM oleic acid (C18:1w9)</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>10 mM stearic acid (C18:0)</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Mixture of fatty acids (100x)</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>V. harveyi MM32</td>
<td>Positive control</td>
<td>7.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Negative control</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>10 mM linoleic acid (C18:2w6)</td>
<td>7.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>10 mM palmitic acid (C16:0)</td>
<td>7.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10 mM oleic acid (C18:1w9)</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>10 mM stearic acid (C18:0)</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Mixture of fatty acids (100x)</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>Control</td>
<td>8.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Ground beef extracts</td>
<td>8.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Mixture of fatty acids (100x)</td>
<td>8.7 ± 0.1</td>
</tr>
</tbody>
</table>

a Mean (± standard error, n = 3) population of the V. harveyi (BB170 and MM32) reporter strains at the end of the AI-2 inhibition bio-assay and of the E. coli cells in LB plates at the end of 24 h.

(41.6%), C16:0 (29.9%), and C18:0 (15.5%) acids were the primary fatty acids, and their concentrations were 9.97, 7.16, and 3.71 μg/g, respectively (Table 2). Linoleic, myristic, palmitoleic, linolenic, and pentadecanoic acids also were present at various concentrations in the hexane extracts from the ground beef (Table 2).

Inhibition of AI-2 activity by fatty acids. Linoleic, oleic, palmitic, and stearic acids were tested for their ability to inhibit AI-2 activity in the V. harveyi (BB170 and MM32) reporter strains. The fatty acids at various concentrations (1, 5, and 10 mM) exhibited some level of AI-2 inhibition, and the level of inhibition increased as the concentration of the fatty acids increased (Fig. 2A and 2B). Oleic acid (C18:1w9) in general caused greater AI-2 inhibition than did linoleic (C18:2w6), palmitic (C16:0), and stearic (C18:0) acids. Rather than testing these fatty acids singly, mixtures of these fatty acids were prepared (1x, 10x, and 100x) and tested for their ability to inhibit AI-2 activity. Using V. harveyi BB170 as the reporter strain, inhibition of AI-2 activity averaged 64.5% (±5.2%), 81.5% (±4.7%), and 86.6% (±4.9%) for the 1x, 10x, and 100x solutions, respectively (Fig. 3). When V. harveyi MM32 was used as the reporter strain, AI-2 inhibition averaged 52.3% (±3.9%), 74.4% (±4.1%), and 91.3% (±8.2%), respectively (Fig. 3). Statistical analysis (paired t test) revealed that the reporter strain had no significant effect on the level of AI-2 inhibition for a particular fatty acid mixture concentration. Inhibition of AI-2 activity was higher at

FIGURE 2. Inhibition of AI-2 activity by linoleic, palmitic, oleic, and stearic acids. The y axis represents the percent inhibition of AI-2 activity (± standard error, n = 6). (A) Results with the Vibrio harveyi BB170 reporter strain. (B) Results with the Vibrio harveyi MM32 reporter strain.

FIGURE 3. Mean levels of inhibition of AI-2 activity by fatty acid mixtures based on results with Vibrio harveyi BB170 and MM32 as reporter strains. The y axis represents the percent inhibition of AI-2 activity (± standard error, n = 6). The 1x mixture of fatty acids consists of 0.58, 9.97, 7.16, and 3.71 μg/ml C18:2w6, C18:1w9, C16:0, and C18:0 acids; respectively. Bars with a superscript indicate no significant differences based on a paired t test (P = 0.05).
the 10× concentration than at the 1× concentration, but no differences were observed between the 10× and 100× concentrations. AI-2 inhibition did not differ significantly between 100× fatty acid solutions (86.6% ± 4.9% AI-2 inhibition) and neutralized 100× fatty acid solution (98.6% ± 8.7% AI-2 inhibition), suggesting that observed AI-2 inhibition was not due to the acid functionality of fatty acids. Bacterial counts of the BB170 and MM32 reporter strains (in the positive control, negative control, and fatty acid treatments) averaged between 7.1 and 7.6 log CFU/ml (Table 1), suggesting that the growth of the reporter strains was not influenced by the fatty acid concentrations used in this study.

Influence of fatty acids on E. coli K-12 biofilm formation. Figure 4 shows the influence of fatty acids on biofilm formation by E. coli K-12 cells. The addition of inhibitory compounds from the aqueous extracts or fatty acids mixture resulted in a two- to fourfold reduction in biofilm formation compared with the positive control. There was no difference in the E. coli K-12 plate counts in the presence of ground beef extracts or fatty acids compared with the positive control, which indicates that cell growth was not affected by the experimental treatments (Table 1).

DISCUSSION

Screening and identification of QS inhibitory compounds from natural and man-made sources is of continuing interest (10, 18–20). Lu et al. (10) reported that some food matrices, including ground beef, are capable of inhibiting AI-2–like activity. Halogenated furanones produced by the alga Delisea pulchra and synthetic furanones have the ability to interfere with both AI-1– and AI-2–mediated QS systems (12, 20). Microarray analysis revealed that approximately 79% of the QS-regulated genes in E. coli were repressed by the presence of synthetic furanones (20). Lu et al. (10) studied the effect of different food preservatives such as sodium acetate, benzoic acid, propionic acid, and sodium nitrate on AI-2–like activity. These preservatives inhibited AI-2 activity by 75 to 99%.

In the present study, we identified and characterized some of the specific fatty acids in ground beef that are responsible for the inhibition of AI-2 activity. These fatty acids inhibited AI-2 activity when tested either individually (1, 5, and 10 mM) or in a mixture at various strengths (1X, 10X, and 100X) (Figs. 2A, 2B, and 3). Tenfold increases (1X to 10X, and 10X to 100X) in fatty acid concentrations did not result in a corresponding 10-fold decrease in AI-2 activity, suggesting that there might be point of saturation in the inhibitory activity. The GC analysis of the hexane extracts yielded eight fatty acids. The inhibition by the aqueous extract was 88%, that of the hexane extract was 95%, and that of the 1× mixture of the four fatty acids was 65% (Figs. 1 and 3). This variation in inhibition is not surprising because the hexane extract contains a high concentration of inhibitors. We acknowledge that the hexane extract may contain inhibitors in addition to those identified by the GC analysis. The fatty acids used in this study were among the predominant fatty acids that were extracted. Saturated and unsaturated fatty acids such as caprylic and linoleic acids have been reported to possess antimicrobial activity (16). However, the concentrations that have been used in previous studies were much higher than those tested in the present study (13, 16). The inhibitory effect of the fatty acids on AI-2 activity in the present study is significant because our results indicate that these fatty acids are able to modulate AI-2–based cell signaling without affecting bacterial cell viability.

Microbial biofilm formation has been linked to AI-2–based cell signaling and has been implicated in pathogenicity (6, 7, 9, 18, 21). The E. coli K-12 strain used in this study produces AI-2 molecules and has been used by other investigators to study QS processes (6, 20, 27). Biofilm formation in E. coli K-12 cells has been reported to be under the direct control of AI-2–based cell signaling (6, 7). Our results suggest that a mixture of C16:0, C18:0, C18:1ω9, and C18:2ω6, which was capable of interfering with biofilm formation, may be useful for controlling biofilm formation. Concentrations of these inhibitory fatty acids above those concentrations found in ground beef did not result in greater inhibition of biofilm formation, suggesting that there might be saturation point for the amount of inhibitory activity. Like fatty acids, furanones (which can inhibit biofilm formation) are now considered to be of clinical significance. Synthetic furanones are now undergoing extensive clinical trials as therapeutic agents against biofilms associated with cystic fibrosis (28). We are currently attempting to delineate the specific mechanism(s) by which fatty acids inhibit AI-2 activity and inhibit biofilm formation.

These results demonstrate that both medium-chain and long-chain fatty acids isolated from ground beef can inhibit AI-2–based cell signaling and biofilm formation. Further research, however, will be needed to fully understand the mode of action and potential suite of applications for these fatty acids as AI-2 inhibitors in maintaining food quality and food safety. Such studies can lead to the development of high-value-added products from ground beef, which can be utilized to enhance the safety and quality of foods.

ACKNOWLEDGMENTS

We thank Hema Marwaha and Nathalie Quezada (Food Protein Research & Development Center, Texas A&M University) for assisting with
REFERENCES


