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Anti-Listerial Effect of 4-Hydroxyphenylpropanoic Acid Esters Synthesized by Lipase-Catalyzed Esterification †

Bartłomiej Zieniuk *, Ewa Białecka-Florjańczyk and Agata Fabiszewska

Department of Chemistry, Institute of Food Sciences, Warsaw University of Life Sciences, 159c Nowoursynowska St., 02-776 Warsaw, Poland; ewa_bialecka_florjanczyk@sggw.edu.pl (E.B.-F.); agata_fabiszewska@sggw.edu.pl (A.F.)

* Correspondence: bartlomiej_zieniuk@sggw.edu.pl; Tel.: +48-22-59-37-621

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Abstract: *Listeria monocytogenes*—a Gram-positive intracellular bacteria is one of the most virulent foodborne pathogens. A common strategy used to control the growth of bacteria in food is using food additives. However, many bacterial species are resistant to food preservatives, and bacterial resistance can be natural or acquired. Therefore, there is a constant need for searching for new compounds with antimicrobial properties. The purpose of the present study was to evaluate the efficiency of five (ethyl, butyl, hexyl, octyl, and decyl) esters of 4-hydroxyphenylpropanoic acid against *L. monocytogenes* PCM 2191. Esters were obtained in reactions catalyzed by *Candida antarctica* lipase B. Purified esters with structures confirmed by ^{1}H NMR were applied to determine minimal inhibitory concentrations (MIC) using microdilution broth method. MIC values ranged 0.0625–16 mM. The results of this study have demonstrated the potential application of 4-hydroxyphenylpropanoic acid alkyl esters in inhibiting the growth of *L. monocytogenes*. Octyl 4-hydroxyphenylpropanoate proved to be the best antimicrobial agent and was used in time-kill assay with different concentrations of 1 x MIC, 4 x MIC, and 16 x MIC. Three to nine log reduction of cell number were observed compared to control medium without any antimicrobial compound after 24 h, and the possibility of using octyl 4-hydroxyphenylpropanoate in food applications is worth further investigation.

Keywords: biocatalysis; *Candida antarctica* Lipase B; food additives; 4-Hydroxyphenylpropanoic acid esters; *Listeria monocytogenes*

1. Introduction

In 2017 thirty European countries reported 2502 cases of listeriosis, a dangerous infection with a different clinical presentation from febrile gastroenteritis to severe invasive infections including sepsis, meningitis, perinatal infections, and abortions. Mentioned infection is caused by *Listeria monocytogenes*—a Gram-positive intracellular foodborne bacteria. Especially vulnerable groups of risk of bacterial infections are defined by a common abbreviation YOPI, which stands for Young, Old, Pregnant, and Immunosuppressed person. Despite the incidence of listeriosis is low, this disease poses a serious threat to public health due to very high mortality [1–3].

A common strategy used to control the growth of bacteria in food is using food additives. However, many bacterial species are resistant to food preservatives, and moreover, some of the additives arouse controversy among consumers. Given this, there is a constant need for searching for new food preservatives with antimicrobial properties. The most needed are compounds synthesized

in processes, that cause less environmental pollution, less energy consumption, and in which fewer by-products are generated, and milder reaction conditions are used [4]. A great example of such a process is the use of green chemistry methods, e.g., enzymatically catalyzed reactions, which quite match the previously mentioned features [5].

The purpose of the present study was to evaluate the efficiency of five (ethyl, butyl, hexyl, octyl, and decyl) enzymatically obtained esters of 4-hydroxyphenylpropanoic acid against *L. monocytogenes* PCM 2191. Moreover, the antimicrobial potential of one of them—octyl 4-hydroxyphenylpropanoate was also investigated in the time-kill assay.

2. Materials and Methods

2.1. Microorganism

Listeria monocytogenes PCM 2191 was purchased from the Polish Collection of Microorganisms (PCM) of Institute of Immunology and Experimental Therapy Polish Academy of Sciences (Wrocław, Poland).

2.2. Materials

Candida antarctica lipase B (CALB) was purchased from Sigma-Aldrich (Poznań, Poland). Culture media components were bought from BTL Sp. z o. o. (Łódź, Poland). Chemicals were acquired from Avantor Performance Materials Poland S.A. (Gliwice, Poland) and Sigma-Aldrich.

2.3. Esters Synthesis, Purification, and Identification

Syntheses of five esters were carried out with CALB (addition of 5% by weight of substrates) as biocatalyst. 4-Hydroxyphenylpropanoic acid alkyl esters were synthesized by reacting mentioned phenolic acid with alcohol (ethanol, 1-butanol, 1-hexanol, 1-octanol, and 1-decanol) in a molar ratio 1:1.5 (acid:alcohol). Reactions were carried out in flasks in methyl-*tert*-butyl ether at 37 °C (Figure 1).

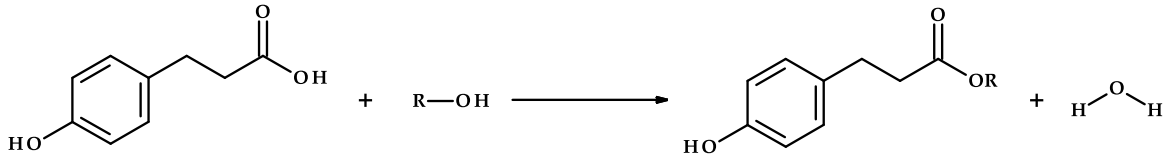

Figure 1. Lipase-catalyzed synthesis of 4-hydroxyphenylpropanoic acid alkyl esters. R = CH_3CH_2-, $CH_3(CH_2)_3$-, $CH_3(CH_2)_5$-, $CH_3(CH_2)_7$-, or $CH_3(CH_2)_9$-.

After the reaction, the enzyme was separated from the reactants by filtration, and the solvent was evaporated. Esters were purified using column chromatography, and silica gel 60 (0.040–0.063 mm; 230–400 mesh) was used as a stationary phase and mixture of chloroform and methanol (9:1) was applied as a mobile phase. Subsequently, ester-containing fractions were dried with $MgSO_4$, filtered and the mixture of solvents was evaporated.

The ^{1}H NMR spectra were measured using Bruker AVANCE 300 MHz (USA) and $CDCl_3$ was used as a solvent. Proton chemical shifts are reported below in ppm (δ) relative to tetramethylsilane (TMS) as an internal standard.

Ethyl 4-hydroxyphenylpropanoate ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.23 (3H, t, *J* = 7.3 Hz), 2.59 (2H, t, *J* = 7.8 Hz), 2.88 (2H, t, *J* = 7.8 Hz), 4.12 (2H, q, *J* = 6.6 Hz), 5.18 (1H, s), 6.70–6.80 (2H, m), 7.00–7.10 (2H, m)

Butyl 4-hydroxyphenylpropanoate ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (3H, t, *J* = 7.3 Hz), 1.34 (2H, m), 1.58 (2H, m), 2.59 (2H, t, *J* = 7.8 Hz), 2.88 (2H, t, *J* = 7.8 Hz), 4.07 (2H, t, *J* = 6.6 Hz), 4.99 (1H, s), 6.70–6.79 (2H, m), 7.02–7.10 (2H, m)

Hexyl 4-hydroxyphenylpropanoate ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (3H, t, *J* = 7.3 Hz), 1.29 (6H, m), 1.60 (2H, m), 2.59 (2H, t, *J* = 7.8 Hz), 2.88 (2H, t, *J* = 7.8 Hz), 4.06 (2H, t, *J* = 6.6 Hz), 4.98 (1H, s), 6.70–6.80 (2H, m), 7.00–7.12 (2H, m)

Octyl 4-hydroxyphenylpropanoate ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (3H, t, *J* = 7.3 Hz), 1.28 (10H, m), 1.60 (2H, m), 2.59 (2H, t, *J* = 7.8 Hz), 2.88 (2H, t, *J* = 7.8 Hz), 4.05 (2H, t, *J* = 6.6 Hz), 4.97 (1H, s), 6.70–6.78 (2H, m), 7.02–7.10 (2H, m)

Decyl 4-hydroxyphenylpropanoate ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (3H, t, *J* = 7.3 Hz), 1.26 (14H, m), 1.61 (2H, m), 2.58 (2H, t, *J* = 7.8 Hz), 2.88 (2H, t, *J* = 7.8 Hz), 4.05 (2H, t, *J* = 6.6 Hz), 4.86 (1H, s), 6.70–6.78 (2H, m), 7.01–7.11 (2H, m)

2.4. Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) of esters and their precursor—4-hydroxyphenylpropanoic acid was determined by the microdilution broth method according to ISO [6]. Furthermore, minimum bactericidal concentrations (MBC) were determined [5].

2.5. Time-Kill Assay

The survival of *L. monocytogenes* PCM 2191 by the time-kill assay was investigated in Mueller-Hinton medium by treating the bacterial strain to the following concentrations of octyl 4-hydroxyphenylpropanoate: 1 x MIC (0.0625 mM), 4 x MIC (0.25 mM), and 16 x MIC (1 mM). In each flask, there was a total of 100 mL of mentioned medium, ester as an antimicrobial agent, and bacterial suspension with an initial density approximately of 10^6–10^7 CFU/mL. During 24 h incubation at 37 °C samples were withdrawn from the cultures at 0, 2, 4, 8, and 24 h, and after diluting in 0.85% saline were cultured in TSA medium at 37 °C for 24 h. Subsequently, the colonies were counted and the results were presented in logarithmic scale as log CFU/mL.

Moreover, optical density measurements at 600 nm were made for comparison using UV/Vis spectrophotometer.

3. Results and Discussion

Due to the growing problem of foodborne microorganisms and their resistance to different antimicrobials, searching for new compounds focus scientific attention. In order to be suitable for use as food additives, the tested compounds should exhibit high biological activity. Therefore, after carrying out enzyme-catalyzed reactions, purifying the compounds and confirming their structure, the MIC and MBC values of the obtained esters were determined and compared with the values obtained for 4-hydroxyphenylpropanoic acid. The results are presented in Table 1.

Table 1. Antimicrobial activities of 4-hydroxyphenylpropanoates and their precursor expressed as minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC).

Compound	MIC [mM]	MBC [mM]
4-Hydroxyphenylpropanoic acid	16	32
Ethyl 4-hydroxyphenylpropanoate	8	16
Butyl 4-hydroxyphenylpropanoate	2	4
Hexyl 4-hydroxyphenylpropanoate	0.5	1
Octyl 4-hydroxyphenylpropanoate	0.0625	0.25
Decyl 4-hydroxyphenylpropanoate	0.25	1

MIC values ranged 0.0625–16 mM, and MBC values were 0.25–32 mM. It has been shown that anti-listerial activity increased with increasing the chain length of the alkyl part of the ester, except decyl 4-hydroxyphenylpropanoate, where MIC and MBC values began to rise again. Octyl 4-hydroxyphenylpropanoate turned out to be the most active compound against *L. monocytogenes* PCM 2191 with MIC = 0.0625 mM and MBC = 0.25 mM. The esterification of phenolic acid allowed

obtaining more active compounds compared to their precursor, which is related to increased lipophilicity, a term that was also discussed by Shi et al. [7].

Shi et al. [7] examined six different ferulic acid alkyl esters against *L. monocytogenes* ATCC 19115 and noted that MIC and MBC values first decreased and then increased with increasing the chain length, where the highest activity was observed for butyl and hexyl ferulates [7].

In the next stage of the research, octyl 4-hydroxyphenylpropanoate was used in the time-kill assay. Growth curves of treatment of *L. monocytogenes* with ester in different concentrations are presented in Figure 2. In addition, the growth of bacteria under the described experimental conditions was also determined by measuring the optical density (Figure 3).

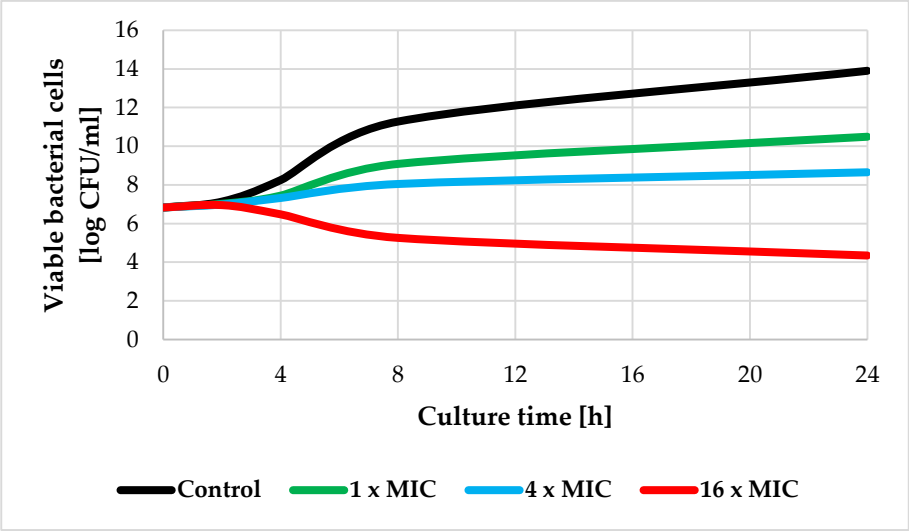

Figure 2. Time-kill curves of *Listeria monocytogenes* PCM 2191 treated with different concentrations of octyl 4-hydroxyphenylpropanoate, i.e., 1 x MIC (0.0625 mM), 4 x MIC (0.25 mM), and 16 x MIC (1 mM).

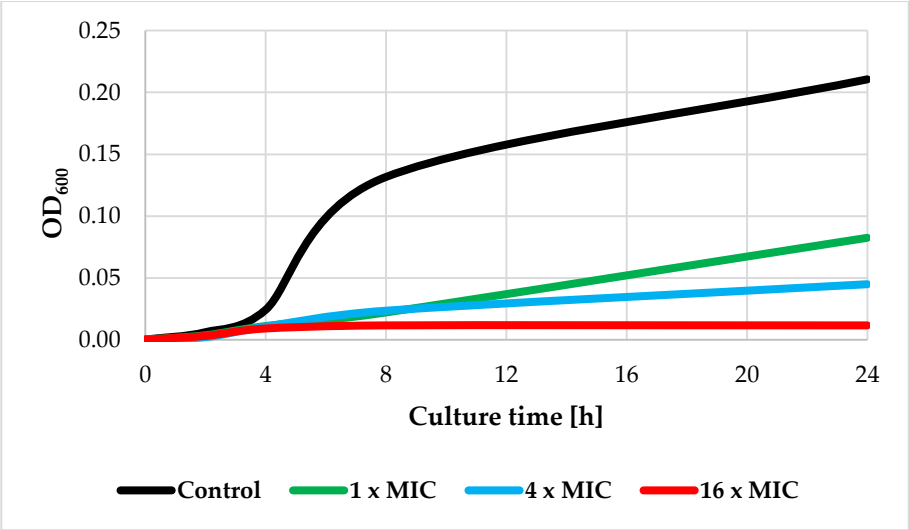

Figure 3. Optical density measurement at 600 nm of *Listeria monocytogenes* PCM 2191 without or with octyl 4-hydroxyphenylpropanoate at 1 x MIC (0.0625 mM), 4 x MIC (0.25 mM), and 16 x MIC (1 mM).

The concentration-dependent anti-listerial activity of octyl 4-hydroxyphenylpropanoate can be observed. Bacteria rapidly grew in the medium without any antimicrobials, and after 24 h number of viable cells increased by 7 log cycles. A significant difference between the media was also noticeable by observing the optical density measurement (Figure 3). Tested ester at 1 x MIC and 4 x MIC led to limiting the growth of bacteria. This indicates that synthesized molecule demonstrated bacteriostatic activity, which is also confirmed by the MBC/MIC ratio, which amounted 4. According to Konate et

al. [8] when the MBC/MIC ratio was 1–2 the effect of the antimicrobial agent was defined as bactericidal, and when this ratio ranged 4–16 the effect was considered as bacteriostatic. The use of octyl 4-hydroxyphenylpropanoate at 1 x MIC and 4 x MIC reduced the growth of *L. monocytogenes* PCM 2191 by 3 and 5 log CFU/mL compared to the control medium, and the bacterial cell number was 10.49 and 8.66 log CFU/mL, respectively.

Only the use of octyl 4-hydroxyphenylpropanoate at the highest concentration of 16 x MIC (1 mM) resulted in the reduction of the number of bacteria. The killing effect has been already observed after 4 h, when the number of cells was reduced compared to their initial value. The decline in cell numbers progressed over time. After 24 h, cell viability in this medium was determined to be 4.33 log CFU/mL, which is about 2.5 log cycles less compared to the number of the cell at 0 h. When comparing the number of bacteria after 24 h in the control medium and the 16 x MIC medium, a radical difference of approx. 9 log cycles is noticeable.

4. Conclusions

To summarize, it was proven that 4-hydroxyphenylpropanoic acid esters synthesized by lipase-catalyzed esterification exhibited antibacterial activity towards *L. monocytogenes* PCM 2191. Enzymatic reactions allowed obtaining more active compounds compared to their carboxyl precursor, and anti-listerial activity increased with increasing the alkyl chain length. Time-kill assay revealed that octyl 4-hydroxyphenylpropanoate was able to limit the number of bacteria cells, and concentration-dependent activity was observed. Moreover, the possibility of using octyl 4-hydroxyphenylpropanoate in food applications is worth further investigation. Economical and environmentally friendly methods of enzymatic synthesis of new food additives should be also further developed.

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