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

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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 to search 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 ¹H NMR were applied to determine minimal inhibitory concentrations (MIC) using the 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 a time-kill assay with different concentrations of 1 × MIC, 4 × MIC, and 16 × 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*

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1. Introduction

In 2017, thirty European countries reported 2502 cases of listeriosis, which is a dangerous infection with a different clinical presentation from febrile gastroenteritis to severe invasive infections including sepsis, meningitis, perinatal infections, and abortions. The 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 persons. Although 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 to search 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 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 = CH₃CH₂-, CH₃(CH₂)₃-, CH₃(CH₂)₅-, CH₃(CH₂)₇-, or CH₃(CH₂)₉-.

After the reaction, the enzyme was separated from the reactants by filtration, and the solvent was evaporated. Esters were purified using column chromatography, silica gel 60 (0.040–0.063 mm; 230–400 mesh) was used as a stationary phase, and a mixture of chloroform and methanol (9:1) was applied as a mobile phase. Subsequently, ester-containing fractions were dried with MgSO₄, filtered, and the mixture of solvents was evaporated.

The ¹H NMR spectra were measured using Bruker AVANCE 300 MHz (USA) and CDCl₃ 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 ¹H NMR (300 MHz, CDCl₃): 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 ¹H NMR (300 MHz, CDCl₃): 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 ¹H NMR (300 MHz, CDCl₃): 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 ^1H 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 ^1H 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: $1 \times \text{MIC}$ (0.0625 mM), $4 \times \text{MIC}$ (0.25 mM), and $16 \times \text{MIC}$ (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, they were cultured in TSA (Tryptic Soy Agar) 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 is a focus of 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 for 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 × MIC (0.0625 mM), 4 × MIC (0.25 mM), and 16 × MIC (1 mM).

Figure 3. Optical density measurement at 600 nm of *Listeria monocytogenes* PCM 2191 without or with octyl 4-hydroxyphenylpropanoate at 1 × MIC (0.0625 mM), 4 × MIC (0.25 mM), and 16 × 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, the 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 × MIC and 4 × 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 to 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 $1 \times \text{MIC}$ and $4 \times \text{MIC}$ 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 $16 \times \text{MIC}$ (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 $16 \times \text{MIC}$ medium, a radical difference of approximately 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 toward *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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References

1. Kuch, A.; Goc, A.; Belkiewicz, K.; Filipello, V.; Ronkiewicz, P.; Gołębiewska, A.; Wróbel, I.; Kiedrowska, M.; Waśko, I.; Hryniewicz, W.; et al. Molecular diversity and antimicrobial susceptibility of *Listeria monocytogenes* isolates from invasive infections in Poland (1997–2013). *Sci. Rep.* **2018**, *8*, 14562.
2. Allerberger, F.; Wagner, M. Listeriosis: A resurgent foodborne infection. *Clin. Microbiol. Infect.* **2010**, *16*, 16–23.
3. European Centre for Disease Prevention and Control. Listeriosis—Annual Epidemiological Report for 2017. Available online: <https://www.ecdc.europa.eu/sites/default/files/documents/listeriosis-annual-epidemiological-report-2017.pdf> (accessed on 9 October 2020).
4. Anastas, P.T.; Zimmerman, J.B. The United Nations sustainability goals: How can sustainable chemistry contribute? *Curr. Opin. Green Sustain. Chem.* **2018**, *13*, 150–153.
5. Zieniuk, B.; Wołoszynowska, M.; Bialecka-Florjańczyk, E.; Fabiszewska, A. Synthesis of Industrially Useful Phenolic Compounds Esters by Means of Biocatalysts Obtained Along with Waste Fish Oil Utilization. *Sustainability* **2020**, *12*, 5804.
6. International Organization for Standards. ISO 20776–1. *Clinical laboratory Testing and In Vitro Diagnostic Test Systems—Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices—Part 1: Reference Method for Testing the In Vitro Activity of Antimicrobial Agents against Rapidly Growing Aerobic Bacteria Involved in Infectious Diseases*; International Organization for Standards: Geneva, Switzerland, 2006.
7. Shi, Y.G.; Bian, L.Q.; Zhu, Y.J.; Zhang, R.R.; Shao, S.Y.; Wu, Y.; Chen, Y.W.; Dang, Y.L.; Ding, Y.; Sun, H. Multifunctional alkyl ferulate esters as potential food additives: Antibacterial activity and mode of action against *Listeria monocytogenes* and its application on American sturgeon caviar preservation. *Food Control* **2019**, *96*, 390–402.
8. Konate, K.; Hilou, A.; Mavoungou, J.F.; Lepengue, A.N.; Souza, A.; Barro, N.; Datte, J.Y.; M'Bachti, B.; Nacoulma, O.G. Antimicrobial activity of polyphenol-rich fractions from *Sida alba* L. (Malvaceae) against co-trimoxazol-resistant bacteria strains. *Ann. Clin. Microbiol. Antimicrob.* **2012**, *11*, 5.