Novel Approach to Control Microbial Contamination of Germinated Wheat Sprouts: Photoactivatedchlorophillin-Chitosan Complex

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Abstract: High resistance of bacteria and fungi to antimicrobial treatments has led to the development of innovative and alternative antimicrobial technologies. It is well-known that chitosan itself is an antimicrobial agent, and chlorophyllin is water-soluble food additive (E140) and food component which in the presence of light exerts antimicrobial properties. Complex of these two antimicrobials can be background for the development of edible active biodegradable coating.

The aim of this study was to evaluate antimicrobial efficiency of photoactivated chlorophyllin-chitosan complex against food pathogens and fungi on the surface of germinated wheat seeds. Obtained data indicate that chlorophyllin-chitosan complex in the presence of visible light (400nm) exhibits strong antimicrobial activity which can be used for decontamination of sprouts.

Keywords: Antimicrobial, Photoactivated chlorophyllin-chitosan complex.

1. INTRODUCTION

The sprouts are outstanding sources of protein, vitamins and minerals. Therefore, germinated seeds are offered as a natural nutritive product which is beneficial to health [1]. Seed contamination with harmful and pathogenic microorganisms is important as it often reduces product quality and shelf-life. Moreover it can be source of food-borne diseases and presents a potentially serious health risk to humans. However, methods recently applied for inactivation of harmful and pathogenic microorganisms are not always efficient and ecologically friendly [1, 2]. In this context, photosensitization treatment seems promising. Antimicrobial photosensitization approach is based on the interaction of three agents: photosensitizer (photoactive compound), light and oxygen. There are two main routes for photosensitizer-cell interaction. In the first case, it could form a tight complex with the surface of the cell wall. In another way, the photosensitizer is transported inside the cell, where it associates with the key structures and irreversibly damages them after photosensitization [3]. Two oxidative mechanisms of photoinactivation are considered to be implicated the inactivation of microbe. The type I pathway involves electron/hydrogen atom-transfer reactions from the photosensitizer triplet state and produce radical ions of surrounding biomolecules. Type II pathway involves energy transfer from triplet state to molecular oxygen and produce singlet oxygen. As a result, a series of cytotoxic reactions occurs in the cell [3].

Na-chlorophyllin (Chl) is effective photosensitizer, water-soluble food additive (E140) and food component used as food colorant, in dietary supplements and in cosmetics [4]. According to our previous results, photoactivated Chl exhibited high antimicrobial activity against Gram (+) food pathogens *Listeria monocytogenes Bacillus cereus, in vitro* and *in vivo* [5-7]. Inactivation of Gram (-) bacteria with more complex cell wall structure needs longer treatment time [8].

Chitosan (CHS), a natural cationic linear polysaccharide, is produced commercially by deacetylation of chitin and is insoluble in water at pH above 6 [9]. It is characterized as nontoxic antimicrobial tool, which do not change pH, color, improves quality of fruits and vegetables and can be applied in food technologies, agriculture, medicine and environment protection [10, 11]. In addition, CHS is able to control decay of different fruits and vegetables extending their storage time [12]. Moreover, CHS can form antimicrobial films and serve as carrier of wide range of food additives, including various antimicrobials [13, 14]. In this context, chitosan film with inserted Chl may act as more efficient antimicrobial tool to improve food safety and prolong shelf-life of products. The aim of this study was to evaluate antimicrobial efficiency of photoactivetedchlorophyllin-chitosan complex (Chl-KCHS) against food pathogen *Listeria monocytogenes* and microfungus, *Botrytis cinerea*, contaminating wheat sprouts.

2. MATERIALS AND METHODS

2.1. Chemicals

Chlorophyll sodium salt (Chl) was purchased from *ROTH*, Karlsruhe, Germany. Low molecular weight chitosan (CHS, degree of deacetylation 72%,

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Brookfield viscosity of 1% solution in 1% acetic acid at 20°C (140 cP) was purchased from *Aldrich*. 2, 3-epoxypropyl trimethyl ammonium chloride (EPTMAC) was purchased from *Aldrich*. Deionized water used in all experiments had specific conductivity less than 1×10^{-6}S/cm.

2.2. Synthesis of Chl-KCHS Complex

N-[(2-hydroxy-3-trimethylammonium)propyl] chitosan chloride (KCHS) with 35 mol% degree of cationization was prepared from equimolar mixture of CHS and EPTMAC according to [15].

Aqueous stock solution of KCHS containing 1% of KCHS and 0.1% of HCl was prepared dissolving in water appropriate amounts of HCl and then KCHS. Aqueous stock solution of 0.01% Chl was prepared by dissolution of Chl in water. Aqueous stock solution of Chl-KCHS complex (pH 2.90 at 20°C) containing 1% of KCHS, 0.01% of Chl and 0.1% of HCl was prepared by dropwise addition of aqueous 0.05% Chl solution into rapidly spinning aqueous solution containing 1.25% of KCHS and 0.125% of HCl.

2.3. Spectrometric Analysis

Absorption spectrum of Chl-KCHS solution was recorded by spectrophotometer He*λ*ios Gamma & Delta spectrophotometers, *Thermo Spectronic* (Great Britain), fluorescence spectrum was recorded by *Perkin Elmer* fluorescence spectrophotometer LS-55 (Germany). Scan range parameters are as follows: excitation wavelength – 400 nm; emission – 550–750 nm; ex Slit – 10 nm; em Slit – 4 nm; scan speed (nm/min) – 200.

2.4. Cultivation of Microorganism

Listeria monocytogenes $ATC_{L3}C$ 7644 were kindly provided by the National Veterinary Laboratory (3rd passage of ATCC7644-test organism, Vilnius, Lithuania). The bacterial culture was grown at 37°C and maintained on aLuria Bertani Agar (LBA; Liofilchem, Rosetodegli Abruzzi, Italy).

L. monocytogenes cultures were grown overnight (~16 h) at 37°C in 20 mLof Luria-Bertani medium (LB; Liofilchem, Rosetodegli Abruzzi, Italy) with agitation of 120 rev/min (Environmental Shaker-Incubator ES–20; Biosan, Latvia). The overnight bacterial cultures were diluted 20 times by the fresh medium containing 0.001% Chl–0.1% KCHS stock solution and used for the experiments (optical density at 540 nm ($OD_{540)}$ was 0.164 for*L. monocytogenes* ~1.25×10^{8} CFU/mL)

2.5. Evaluation of Chl-KCHS Antibacterial Activity *in vitro*

20 mL of bacterial cells suspension with 0.001% Chl–0.1% KCHS were incubated in the dark at 37°C, with aeration of 120 rev/min (cultivation). Primarily, Chl-KCHS solution was tested against bacteria in the dark. For the photosensitization treatment, 150 µL of the samples were removed at intervals and placed into sterile flat bottom wells and then exposed to the light (λ = 405 nm) for 5 min (*L. monocytogenes*; light dose 2. 9 J/cm^2).

LED based light source for the photosensitization was constructed at the Institute of Applied Sciences of Vilnius University. The emission maximum of the light source was 405 nm and the light intensity reached 9.6 mW/cm^2 (6 cm from the light source) and 11.52 mW/cm^2 (3.5 cm from the light source) at the surface of samples from top and bottom LED, respectively. Light dose was calculated as light intensity multiplied on irradiation time. Light intensity was measured by *3 Sigma* power and energy meter "Coherent" equipped with a piro-electrical detector J25LP04.

The antibacterial effect of Chl-KCH3 on bacteria was evaluated by the spread plate method. Portions (100 µL) were spread on LBA plates, which were incubated at 37°C for 48 h, and the numbers of colonies were counted. The surviving cell populations were enumerated and expressed as $\log_{10}$ CFU/µL.

2.6. Evaluation of Chl-KCHS Antibacterial Activity on Wheat Sprouts

The wheat seeds samples (each sample 100 seeds, ~4.8 g) were soaked in 0.001% Chl-0.1% KCHS solution. The control samples were soaked in 0.9% NaCl. The inoculums were incubated in the shaker (130 rev/min) in the dark at 37°C for 60 min. Then the seeds samples were decanted in the treatment chamber in a sterile Petri dish without cover where they were exposed to 405 nm light for 30 min (light dose 38 J/cm^2). The control sample was not irradiated.

After treatment each sample (also control samples) was mixed with 0. 9% NaCl in a sterile 100 BagPage and homogenized 60 s with a BagMixer. Then, 100 µL of appropriate dilutions (0. 9 % NaCl) of homogenized seeds suspension placed onto dichloran glycerol (DG18) agar (Liofilchem, Italy). All plates were placed

in the thermostat for 144 h at 30°C. The surviving cell populations were enumerated and expressed $\log_{10}$ CFU/g.

2.7. Statistics

All experiments were repeated 3-4 times. A standard error was estimated for every experimental point and marked in a figure as an error bar. Sometimes the bars were too small to be visible. The data were analyzed using Origin 7.5 software (*Origin Lab Corporation*, Northampton, MA 01060, USA).

3. RESULTS

3.1. Absorption and Fluorescence Spectra of Chlorophyllin-Chitosan Complex

The representative absorption spectrum of Chl-KCHS suspension in 0.01 M PBS (1×10^{-5}) is presented in the table at Figure **1**. It is obvious, that absorption maximum at 405 nm is characteristic for Chl-KCHS complex at these experimental conditions. Fluorescence spectrum of Chl-KCHS supports the idea that complex molecules tend to form aggregates. Fluorescence of Chl-KCHS complex at 660 nm is negligible but remarkably increases after addition of Triton-X100 which disassembles CHS-Chl complex.

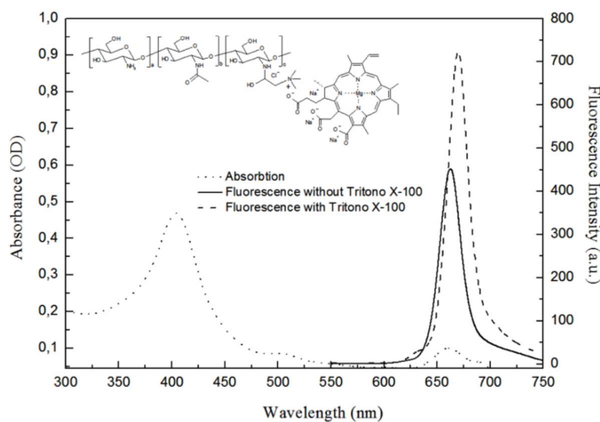

Figure 1: Chemical Formula, Absorption and Fluorescence Spectra of 0.001% Chl–0.1% KCHS Complex in 0.01 M PBS.

3.2. Antibacterial Effect of Photoactivated Chl-KCHS Complex on *L. Monocytogenes*

The antibacterial activity of Chl-KCHS complex against *L. monocytogenes* is illustratedat the Figure **2**. Inactivation of Gram (+) *L. monocytogenes* by this complex in the dark (without light activation)was rather significant and after 4 hours incubation reached 5. 4 log CFU/g. Photoactivated Chl-KCHS complex has remarkably, at much shorter incubation time, inactivated the bacteria. Just 1 h incubation and 2.9 J/cm^2 illumination dose lead to 8 log reduction of viable cell population.

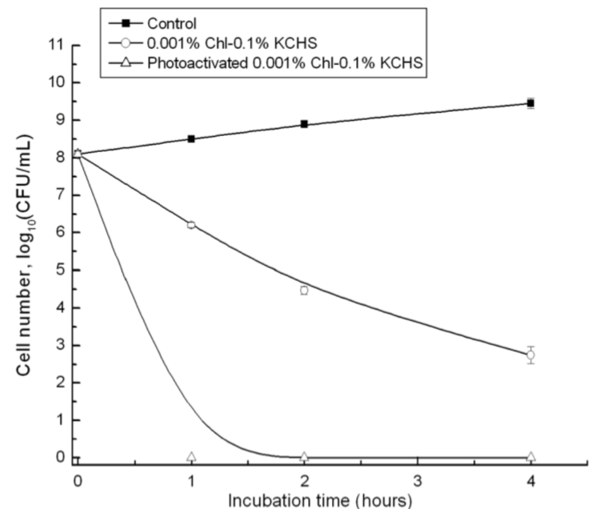

Figure 2: Inactivation of *L. monocytogenes* by photoactivated 0.001% Chl–0.1% KCHS complex *in vitro*.

3.3. Antimicrobial Effect of Photoactivated chl-KCHS Complex

Decontamination of Wheat Sprouts from Total Aerobic Mesophils, Yeasts and Fungi.

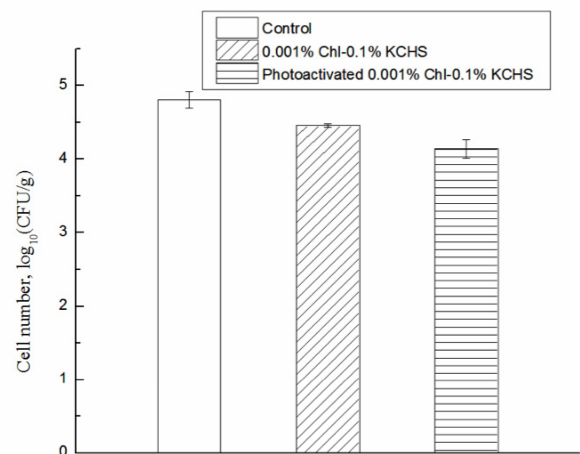

Figure 3: Decontamination of Wheat Seeds from Yeasts/Fungi by Photoactivated of 0.001% Chl–0.1% KCHS Complex.

It was determined whether naturally surface-distributed mesophiles, yeasts and fungi were susceptible to photoactivated Chl-KCHS complex treatment. Data presented in Figure **3** indicated that the growth of total aerobic mesophiles, yeasts and

Figure 4: Decontamination of Wheat Seeds by Photoactivated 0.001% Chl–0.1% KCHS Complex: **a**)- Untreated Seeds and **b**)- Treated Seeds. Incubation Time 60 min, Light Dose 38 J/cm^2.

microfungi in control sprout group increases to 4.81 log. In treated sprout samples (incubation with complex– 60 min, light dose 38 J/cm^2), the amount of yeasts/fungi reduced by 0.68 log_{10} GFU/g sample. Figure **4** illustrates the visual growth of total aerobic mesophils, yeasts and fungi after photoactivated Chl-KCHS complex treatment.

4. DISCUSSION

Despite breath taking progress in biomedical science and technology, the infectious diseases continue to be one of the important problems in the world. Obviously, existing antimicrobial technologies are not enough potent, and development of environmentally friendly and cost-effective antimicrobial technologies is in progress. In this context, photosensitization might serve as an effective and promising antimicrobial tool [16-17].

Na-chlorophyllin (Chl) is water-soluble food additive (E140) and food component used as food colorant, in dietary supplements and in cosmetics [4]. The results obtained in our previous work revealed that Chl-based photosensitization can inactivate Gram (+) pathogens *Listeria monocytogenes*ATC$_{L3}$C 7644 and *Bacillus cereus* ATCC 12826 by 7 log *in vitro* and can clean the surface of food-packaging materials made from polyolefines [5-6]. The data obtained in the present study (Figure **2**) revealed that the population of *L. monocytogenes* decreased after treatment with chlorophyllin-chitosan complex at shorter incubation time in comparison with inactivation found after photosensitization treatment.Moreover, data presented in Figure **3** indicated that it is possible to decontaminate sprouts from total aerobic mesophiles, yeasts and microfungi, since in treated sprout samples (incubation with complex– 60 min, light dose 38 J/cm^2), the amount of yeasts/fungi reduced by 0.68 log_{10} GFU/g sample.

It is well-known that chitosan itself is an antimicrobial agent [18-19]. Antibacterial activity of CHS was assessed for a wide range of Gram (-) and Gram (+) bacteria [20]. The actual mechanism of its antimicrobial activity is not yet fully understood. There are various data concerning the ability of chitosan to disrupt outer membrane of Gram (-) bacteria. Je and Kim [21] found that chitosan and its derivatives disrupt inner and outer bacterial cell membrane.

CONCLUSIONS

Combination of antimicrobial properties of chitosan and chlorophyllin-based photosensitization can be valuable tool to combat food pathogens, yeasts and fungi. This phenomenon can be explained by the enhanced interaction of the positively charged polymer-dye complex with negatively charged bacterial cell wall which probably brings photosensitizer closer to the target cell facilitating photodestruction. Such Chlorophyllin-chitosan complexes have potential to be used for the decontamination and disinfection of different surfaces in industrial environment. Moreover such conjugates can be used for the development of biodestructive antimicrobial "smart" packaging in the future.

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