

## Antimicrobial efficiency of photoactivated chlorophyllin-chitosan complex

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**crossref** <http://dx.doi.org/10.5755/j01.ct.61.3.2710>

Received 17 September 2012; Accepted 24 October 2012

The aim of this study was to assess the antimicrobial efficiency of a photoactivated chlorophyllin–chitosan complex against the food pathogen *Salmonella enterica*.

*Salmonella enterica* was used for experiments. Aliquots of bacterial suspensions ( $\sim 1 \times 10^7$  CFU/ml in 0.9 % NaCl) with 0.001 % chlorophyllin–0.1 % chitosan complex (Chl–CHS) were incubated in the dark at 37 °C for 0–120 min. For the photoactivation, the samples were exposed to light ( $\lambda = 405$  nm) for 30 min (light dose 17.3 J/cm<sup>2</sup>). Microbial viability was evaluated by the spread plate method.

The Chl-based photosensitization treatment reduced the bacterial population by 1.39 log. An extremely high antibacterial efficiency was demonstrated after photoactivation of the Chl–CHS complex (7.3 log reduction of microbial population).

Experimental data support the idea that a photoactivated CHS–Chl complex in a slightly acidic environment can be a useful tool against the gram-negative bacteria *S. enterica*.

**Key words:** photoactivation, chitosan–chlorophyllin complex, *Salmonella*, food safety

### Introduction

Microbiological food safety is an increasing problem worldwide. *Salmonella enterica* is one of the most important foodborne pathogens in many countries. Each year in the United States 1.0 million people are infected with non-typhoidal *Salmonella*, resulting in 19 336 hospitalizations and 378 deaths [1]. In 2010, a total of 1 962 confirmed cases of salmonellosis were reported in Lithuania [2]. The high resistance of *Salmonella* to disinfecting agents has led to the development of alternative antimicrobial technologies. In this context, the photosensitization treatment seems promising. Several studies have demonstrated that bacteria, as well as micromycetes and viruses, could be inactivated by photosensitization *in vitro* [3–5].

Na-chlorophyllin (Chl) is a water-soluble food additive (E140) and food component used as a food colourant, in dietary supplements and in cosmetics [6]. According to our previous results, photosensitized Chl exhibited a very high antimicrobial activity against gram-positive food pathogens *in vitro* and *in vivo* [5, 7, 8]. Meanwhile, photoactivated Chl might be less effective against gram-negative bacteria, which are characterized by a more complex cell wall [9].

Chitosan (CHS) is a natural cationic linear polysaccharide [10]. It is characterized as a nontoxic antimicrobial tool which can be applied in food technologies, agriculture, medicine, and environment protection [11, 12].

The aim of this study was to assess the antimicrobial efficiency of photoactivated chlorophyllin–chitosan complex against the *S. enterica* food pathogen.

### Materials and methods

#### Chemicals

Not copolymerized chlorophyll sodium salt (Chl) was obtained from ROTH, Karlsruhe, Germany. Low molecular weight chitosan (CHS, Brookfield, viscosity of 1 % solution in 1 % acetic acid at 20 °C 140 cP) was obtained from Aldrich. Aqueous stock solution of CHS (pH 2.4 at 20 °C) containing 1 % of CHS and 0.18 % of HCl was prepared dissolving in water appropriate amounts of HCl and then CHS. The aqueous stock solution of 0.01 % Chl was prepared by Chl dissolution in water. The aqueous stock solution of chlorophyllin–chitosan complex (Chl–CHS) (pH 2.4 at 20 °C), containing 1 % of CHS, 0.01 % of Chl and 0.18 % of HCl, was prepared by a dropwise addition of aqueous 0.05 % Chl solution into a rapidly spinning aqueous solution containing 1.25 % of CHS and 0.23 % of HCl.

#### Cultivation of the microorganism

The *Salmonella enterica* serovar Typhimurium strain DS88 [SL5676 SmR (pLM32)], resistant to tetracycline, was kindly provided by Prof. D. H. Bamford (University of Helsinki, Finland).

*S. enterica* was grown in the Luria–Bertani medium (LB) (Liofilchem, Italy) incubated overnight at 37 °C. The

overnight culture was 20 times diluted with fresh LB medium ( $OD_{540} = 0.164$ ) and grown at 37 °C to the mid-log phase ( $5 \times 10^8$  CFU/ml,  $OD_{540} = 1.3$ ) in a shaker (Environmental Shaker-Incubator ES-20; Biosan, Latvia) (120 rev/min). Cells were then harvested by centrifugation (10 min, 3420 g) (Hettich Zentrifugen, Mikro-200, Germany) and resuspended in 0.9 % NaCl to give  $\sim 2.5 \times 10^9$  CFU/ml. These stock suspensions were diluted to  $\sim 1 \times 10^7$  CFU/ml and immediately used for the experiments.

#### Effect of chitosan on bacterial growth

Aliquots of 20 ml of bacterial suspensions ( $\sim 1 \times 10^7$  CFU/ml in 0.9 % NaCl) with 0.1 % chitosan (CHS) were incubated in 50 ml flasks for cell culture cultivation in a shaker (120 rev/min) at 37 °C. The samples were removed at 15 min, 60 min and 120 min intervals.

#### Photosensitization treatment

Aliquots of bacterial suspensions with 10 times diluted CHS-Chl or Chl stock solutions were incubated for cell culture cultivation in a shaker (120 rev/min) in the dark at 37 °C for different periods (0–120 min). Primarily, Chl and Chl-CHS solutions were tested against *S. enterica* in the dark. For the photosensitization treatment, 150  $\mu$ l of the samples were placed into sterile flat-bottom wells and then exposed to light ( $\lambda = 405$  nm) for 30 min (light dose 17.3 J/cm<sup>2</sup>).

A 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 light intensity at the surface of samples reached 9.6 mW/cm<sup>2</sup>.

#### Evaluation of antibacterial activity

The antibacterial activity of photosensitized Chl and Chl-CHS complex against *S. enterica* was evaluated by the spread plate method. Particularly, 100  $\mu$ l of a diluted bacterial test culture after treatment was surface-inoculated

on a separate LB agar (LBA) plate. Afterwards, the LBA plates were kept in a thermostat for 24 h at 37 °C. Bacterial populations were recalculated from CFU/ml into log<sub>10</sub>/ml.

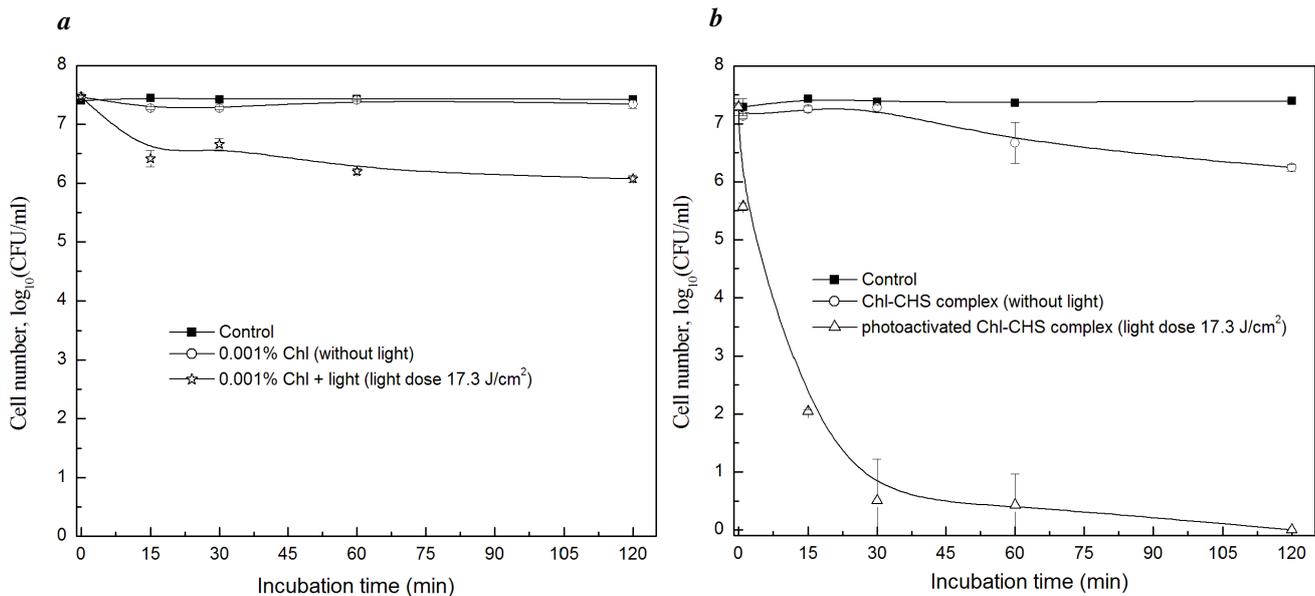
#### Statistical analysis

The experiments were triplicated for each set of exposure. A standard error was estimated for each experimental point and marked in a figure as an error bar. The data were analyzed using Origin 7.5 software (OriginLab Corporation, Northampton, MA 01060, USA).

## Results and discussion

The results obtained in our previous work have shown that Chl-based photoactivation can inactivate the gram-positive pathogens *Listeria monocytogenes* ATCC<sub>L3</sub>C 7644 and *Bacillus cereus* ATCC 12826 by 7 log *in vitro* and can clean the surface of food-packaging materials made of polyolefines [5, 7]. The antibacterial activity of Chl against *S. enterica* is illustrated in Fig. 1a.

It was determined that the dark toxicity of Chl was negligible since the cell number after 120 min of incubation in the dark was reduced only by 0.05 log. The photoactivation treatment (15 min of incubation with Chl and the following illumination) led to a 1.05 log reduction of *S. enterica*. An extension of the incubation time to 120 min favoured the inactivation of *Salmonella* to 1.39 log (Fig. 1a). This means that *S. enterica* is rather resistant to Chl-based photosensitization. These data are in line with those reported by Lopez-Carballo et al. [6] who showed that a photoactivated gelatin film with immobilized Chl exhibited a pronounced bactericidal effect against gram-positive *Staphylococcus aureus* (4 log reduction), but the inactivation of gram-negative *Escherichia coli* and *Salmonella* was just marginal.

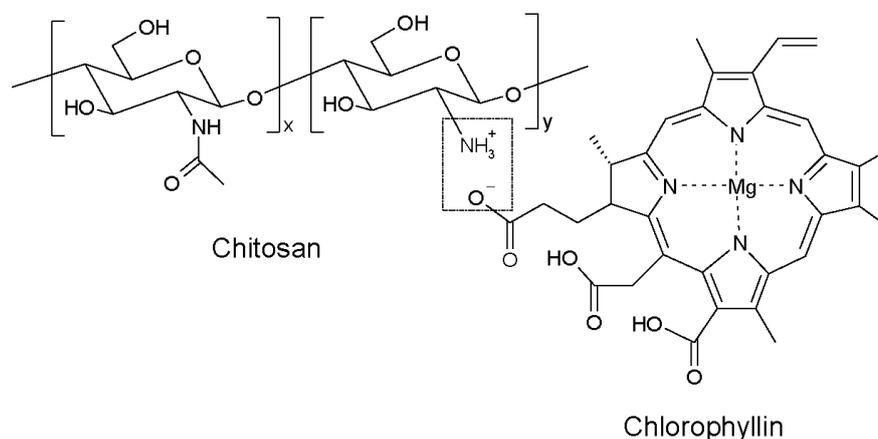


**Fig. 1.** Inactivation of *Salmonella enterica* Serovar Typhimurium strain DS88 (SL 5676 Smr pLM2) by 0.001 % Na-chlorophyllin-based photosensitization treatment (a) and by photoactivation of 0.001 % chlorophyllin-0.1 % chitosan complex (b)

The antimicrobial efficiency of CHS (0.1%) was assessed in a 0.9 % NaCl suspension. Generally, inactivation of the gram-negative bacteria *S. enterica* by CHS alone after 120 min of incubation reached 3.19 log (data not shown).

We suppose that the synergetic antibacterial effect of CHS and Chl relies on the complexation of these two compounds (Fig. 2). Due to the presence of amino groups in the molecule, CHS is able to form ionic (salt-like)

complexes with polyvalent anionic species such as chlorophyllin [13]. The formation of soluble complexes is governed by thermodynamic equilibrium and results in a uniform distribution of the short-chain component among the chains of the oppositely charged long-chain component. Such a case occurs in suspensions containing 0.1 % of CHS and 0.001 % of Chl where the long-chain component chlorophyllin is in high excess.



**Fig. 2.** Hypothetical formula of Chl-CHS complex in 0.9 % NaCl solution at pH 3.5

It is well known that the antibacterial activity of chitosan has been assessed for a wide range of gram-negative and gram-positive bacteria [14]. Liu et al. [15] have shown that protonized chitosan may disrupt the outer but not inner membrane of *E. coli*. This seems to be the first and most important step in chitosan's antimicrobial action. CHS ability to bind to the outer membrane of gram-negative bacteria and to increase membrane permeability should generally sensitize bacteria to other antimicrobial agents.

According to the obtained results, the dark toxicity of the Chl-CHS complex was 1.05 log. On the contrary, the incubation (1 min) of *Salmonella* with a Chl-CHS complex and the following illumination (light dose 17.3 J/cm<sup>2</sup>) resulted in a 1.7 log reduction (Fig.1b). The use of 60 min incubation and 30 min illumination time resulted in a 6.86 log inactivation of *S. enterica*. An increase of the incubation time to 120 min diminished the bacterial population by 7.3 log.

## Conclusions

The gram-negative food pathogen *S. enterica*, being resistant to many antimicrobials, can be effectively inactivated (7.3 log) by a photoactivated 0.001 % Chl-0.1 % CHS complex. Such combination of antimicrobial properties of chitosan and chlorophyllin-based photosensitization seems to be a promising tool to combat gram-negative bacteria which are highly resistant to photosensitization treatment.

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## ANTIMIKROBINIS FOTOAKTYVAUS CHLOROFILINO-CHITUZANO KOMPLEKSO POVEIKIS

### S a n t r a u k a

Šio darbo tikslas buvo įvertinti fotoaktyvaus chlorofilino-chituzano (Chl-CHS) komplekso antimikrobinį poveikį *Salmonella enterica* maisto patogeniui. Bakterijų suspensijos (~1×10<sup>7</sup> kfv/ml 0,9 % NaCl) buvo 0–120 min inkubuojamos 0,001 % chlorofilino–0,1 % chituzano kompleksu tamsoje. Paskui mėginiai buvo 30 min (šviesos dozė 17,3 J/cm<sup>2</sup>) švitinami didelės galios reguliuojamo optinio srauto UV šviestukais (λ = 405 nm). Antimikrobinis poveikis buvo įvertintas taikant paskleidimo lėkštelėje metodą.

Įvertinus tyrimų duomenis, buvo nustatyta, kad chlorofilinu indukuota fotosensibilizacija inaktyvuoja bakterijų populiaciją 1,39 log. Antibakterinis poveikis gerokai padidėja fotoaktyvavus Chl-CHS kompleksą (bakterijų populiacija sumažėja 7,3 log). Šio darbo rezultatai parodė, kad fotoaktyvuotas Chl-CHS kompleksas silpnai rūgštinėje aplinkoje efektyviai inaktyvuoja gramneigiamas *S. enterica* bakterijas.