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Modification of lysozyme with cinnamaldehyde: a strategy for constructing novel preservatives for dermatics

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Abstract

Cinnamaldehyde was covalently bound to the ϵ-amino group of lysyl residues of lysozyme by reduction with sodium borohydride. This paper describes the enhanced antimicrobial activity against both the Gram negative *Escherichia coli* and the Gram positive *Staphylococcus aureus* compared with the activity of lysozyme, cinnamaldehyde or mixtures of both. The minimal inhibition concentration (MIC) of the new product against *E. coli* and *S. aureus* was determined for its possible use as a preservative in cosmetics as well as pharmaceuticals. Moreover, microbial efficacy was studied in cream/gel model preparations and a preservative effectiveness test with the complete microbial spectrum from demineralized water for three different dermatics (cream, gel) was used. Samples examined 1, 7 and 28 days after manufacturing demonstrated a ten-fold reduction in bacterial growth. The results suggest a possible utilization of lysozyme-cinnamaldehyde conjugates as preserving agents. © 1997 Elsevier Science B.V.

Keywords: Lysozyme; Cinnamaldehyde; Antimicrobial activity; Protein modification

1. Introduction

The aim of the present study was to generate novel preservatives for dermatics with increased antimicrobial activity. The problems in developing effective preservatives for food, pharmaceuticals and cosmetics are their limited antimicrobial action against Gram negative bacteria as well as their poor water solubility. Lysozyme, a muco-polysaccharidase that acts against Gram positive bacteria by transforming the insoluble polysaccharides of cell wall to soluble mucopeptides, is also active against some viruses and Gram negative bacteria (Reynolds, 1982). It has been used in the treatment of bacterial and viral infections in widely varying oral doses of up to 100–600 mg daily and 125–250 mg have been given parenterally. Topically applied, it is used for treatment of skin and mucous membrane inflammations in the case of *Herpes zoster* and *Herpes simplex* (Morant

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and Ruppanner, 1995). A new histological study showed that treatment of cutaneous thermal wounds with lysozyme ointment led to new collagen fibres (Takahashi et al., 1995). *E. coli* growth is unaffected by lysozyme, but is reduced by cinnamaldehyde (Yousef et al., 1980; Kabara, 1984; Didry et al., 1993).

Combination of the antimicrobial spectrum of the water insoluble cinnamaldehyde with the antimicrobial activity of the water soluble lysozyme required a new preservative agent. It was the aim of this study to develop a potent bactericidal lysozyme-derivative against both Gram negative and Gram positive bacteria based on a reductive coupling of cinnamaldehyde to lysozyme. This paper reports that a lysozyme derivative incorporating an average of 2.46 cinnamaldehyde residues is very active in killing both Gram positive and Gram negative bacteria.

2. Material and methods

2.1. Substances and bacteria

Lysozyme (egg, molecular mass: 14400), cinnamaldehyde (molecular mass 134.2) and *Micrococcus lysodeicticus* were purchased from Sigma (St. Louis, MO). Hydroxypropylmetylcellulose (HPMC) corresponded to Metolose 60 SH 4000 and was delivered from Shin-Etsu (Japan). Goodrich (UK) made Carbopol 934 available and Diprosicc unpreserved was a gift from Aesca (Austria). Sephadex G-10 was purchased from Pharmacia (Uppsala, Sweden). *S. aureus*: DSM 1104 (Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany); *E. coli*: F82 (O 101:K99)pR 9066. All materials used in this study were of reagent grade.

2.2. Preparations

The following preparations were tested: (1) Diprosicc®/demineralized water (1:3); (2) HPMC-gel (metolose 3.0; aqua purificata 97.0); (3) Carbopol-gel (carbopol 934 0.57; glycerol 85% 15.0; ammonia 1.1; aqua purificata 83.33). Aqua purificata (Pharmacopoea Europaea, 1991) prepared by ion-exchange (Seradest Vario) was used. The water samples were stained with 4′,6-diamino-2-phenylindole (DAPI) and the bacterial cells examined and counted using the microscopic technique of Porter and Feig (1980).

2.3. Modification of lysozyme

Cinnamaldehyde was covalently bound to the ϵ-amino group of lysyl residues of lysozyme by forming imine structures followed by reduction with sodium borohydride ($NaBH_4$) in a modified method as previously described (Lane, 1974; Yoon et al., 1993; Means and Feeny, 1995). To obtain lysozyme with various degrees of modification lysozyme/cinnamaldehyde molar ratios of 1:1, 1:3, 1:7, and 1:30 were used. A volume of 0.2 ml of THF containing 1.04 μmol (1:1; **LC-1**), 3.12 μmol (1:3; **LC-3**), 7.28 μmol (1:7; **LC-7**) or 31.2 μmol (1:30; **LC-30**) cinnamaldehyde was added to 1 ml of 50 mM potassium phosphate buffer, pH 7.4 containing 15 mg of lysozyme. The reaction mixture was stirred at room temperature for 30 min. At 30-min intervals three equal volumes of $NaBH_4$ 1 mg/ml in iced 0.3 M potassium phosphate buffer, pH 7.0 containing 2.5 M NaCl were added to the reaction mixture and stirred continuously at 0°C. Lysozyme derivatives were precipitated by adding ammoniumsulphate solution (60% saturated) and incubating at 4°C for 12 h. The resulting precipitates were separated by centrifugation (26000 × *g*, 20°C, 30 min) and resuspended in 3.0 ml distilled water. After purifying by gel permeation chromatography with Sephadex G-10 the lysozyme derivatives were lyophilized.

2.4. Degree of modification

The degree of modification was determined by measuring the free amino groups of pure lysozyme and modified lysozyme, using trinitrobenzenesulfonic acid (TNBS). A 100-μl portion of the lysozyme derivative solution (0.1% in 50 mM potassium phosphate buffer 7.4) was incubated with 1 ml of $NaHCO_3$ and 1 ml of 0.1% TNBS at 37°C for 2 h. Following 1 ml of 10% SDS and 1 ml 1 N HCl were added and the absorbance at 340 nm was measured. Modified

lysine residues were calculated from the difference between the lysine content in unmodified and modified lysozymes.

2.5. Lysozyme activity

The lytic activity of the lysozyme derivatives on *Micrococcus lysodeicticus* cells was determined according to turbidimetric methods based on the decrease in turbidity of the cell suspension following the addition of lysozyme derivatives. The lysis of cells in 50 mM potassium phosphate buffer pH 6.2 was monitored by the addition of 100 μl from each lysozyme conjugate (0.1% in 50 mM potassium phosphate buffer pH 7.4) to 1.9 ml of *Micrococcus lysodeicticus* cell suspension (190 μg/ml). After shaking the decrease in absorbance at 600 nm was monitored at 3-min intervals (Perkin Elmer Lambda 16 spectrophotometer).

2.6. Antimicrobial activity

The antimicrobial activity was tested against *S. aureus* and *E. coli*, respectively, as Gram positive and Gram negative representatives. The organisms were grown in liquid media B (Pharmacopoea Europaea, 1991) to a density of 10^5 colony forming units (CFU)/ml (OD_{600}: 0.5). Cinnamaldehyde was dissolved in dimethylformamide (DMF) which did not affect the growth of the tested microorganisms. A volume of 0.2 ml of 0.1% solution of lysozyme, lysozyme mixture with cinnamaldehyde (mixture 1:7; **LC-Mix-7**) and of the lysozyme derivatives **LC-30**, **LC-7**, **LC-3** in 50 mM potassium phosphate buffer 7.4 was mixed with 0.8 ml bacterial suspension and incubated for 1 h at 37°C. Each sample was serially diluted to $1:10^6$ with 0.9% NaCl. The mixtures were vortexed (reax 2000) and 200 μl aliquots of each bacterial suspension were plated onto Petri dishes containing bacterial media B. Following incubation at 37°C for 12 h the antimicrobial activity was estimated by counting the CFU. Sterile 50 mM potassium phosphate buffer 7.4 was used for control. All experiments were performed four times.

2.7. Minimum inhibitory concentration (MIC)

The MIC for the lysozyme derivative **LC-3** against *S. aureus* and *E. coli* was determined as follows: a volume of 0.2 ml **LC-3** solution (100 μg/ml; 50 μg/ml; 25 μg/ml; 12.5 μg/ml; 0 μg/ml final concentration) in 50 mM potassium phosphate buffer pH 7.4 was added to 0.8 ml *S. aureus* or *E. coli* (OD_{600}: 0.5) and incubated for 1 h at 37°C. The dilution procedure and the further treatment prior to enumeration was described above. The MIC value was determined as the lowest concentration in which growth of bacterial strains was less than 10% of the control value. All experiments were performed in triplicate.

2.8. Preservative effectiveness test in three topical preparations

A preservative effectiveness test was employed for three selected unpreserved dermatical formulations known to be microbially unstable (Bernkop-Schnürch et al., 1995). The inoculum contained the complete bacteria occuring in demineralized water. **LC-3** was added to each ointment (final concentration of the protein 0.0125%, 0.025%, 0.05%) and 1 g of the gel/cream was vigorously mixed with 9.0 ml sterile NaCl/peptone buffer (Pharmacopoea Europaea, 1991). A volume of 1 ml of the resulting solution was plated onto Petri dishes containing bacteria media B and CFU were counted following 3 days incubation at 37°C. All tests were repeated four times. The 0-time level was performed with the unpreserved dermatics immediately after preparing. To ensure that the 0-time level was less than 5×10^5 CFU/g according to Pharmacopoea Europaea (1991), the complete bacteria in demineralized water were determined in advance by the method of Porter and Feig (1980) by staining with DAPI, before using. All samples were examined following 1, 7 and 28 days after preparation.

3. Results

The degree of modification was determined by colour-reaction with TNBS (trinitrobenzene sul-

fonic acid) of the protein solution. Despite the six lysine residues and one N-terminus of lysozyme on average 2.93 NH_2 groups were covalently bound. Incubation of 1, 3 and 7 mol excess of cinnamaldehyde yielded derivatives with an average of 0.74, 2.46 and 2.93 mol modified lysine residues.

Except for **LC-1**, no reduction of enzymatic activity of the derivatives tested by *Micrococcus lysodeicticus* was detected, compared with pure lysozyme. Therefore the antimicrobial effect of **LC-1** was not further investigated.

Lysozyme is a well known bactericidal protein effective against Gram positive, whereas cinnamaldehyde is active against both Gram positive and Gram negative bacterial strains. Fig. 1 illustrates the antimicrobial activity against *S. aureus* and *E. coli* of lysozyme (lys), cinnamaldehyde (ca), physical mixture of lysozyme with cinnamaldehyde in a molar ratio of 1:7 (**LC mix-7**) and the derivatives in which lysozyme was covalently bound to cinnamaldehyde in a molar ratio of 1:30 (**LC-30**), 1:7 (**LC-7**) and 1:3 (**LC-3**). The antimicrobial activity of the derivatives was significantly enhanced compared to pure lysozyme, cinnamaldehyde and a physical mixture of the two compounds. **LC-3** exhibited the strongest antimicrobial activity against the test organisms. The effectiveness was on average ($n = 4$) greater to *S. aureus* than against *E. coli*, with a mean survival rate of 71.63% and 53.74%, respectively, and therefore **LC-3** was used for all further investigations. The antimicrobial profile was in good accordance with the published data by Ibrahim et al. (1994).

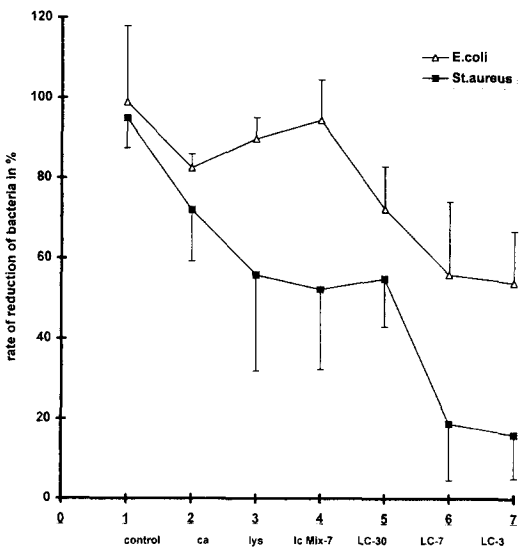

Fig. 1. Antimicrobial activity of lysozyme derivatives against *E. coli* and *S. aureus*. The organisms were grown in liquid media B to an OD_{600}: 0.5, incubated for 1 h with the samples at 37°C, diluted $1{:}10^6$ with 0.9% sodium chloride and 200 μl aliquots were plated onto Petri dishes (media B). Following incubation at 37°C for 12 h, CFU were counted. The rate of reduction of bacteria in % related to a sterile 50 mM phosphate buffer as control is shown. 1, control; 2, cinnamaldehyde (ca); 3, lysozyme (lys); 4, physical mixture of lysozyme and cinnamaldehyde mol ratio 1:7 (**LC Mix -7**); 5, lysozyme covalently bounded to cinnamaldehyde molar ratio 1:30 (**LC-30**); 6, lysozyme covalently bounded to cinnamaldehyde molar ratio 1:7 (**LC-7**); 7, lysozyme covalently bounded to cinnamaldehyde mol ratio 1:3 (**LC-3**). Data incorporates the standard deviation of four experiments.

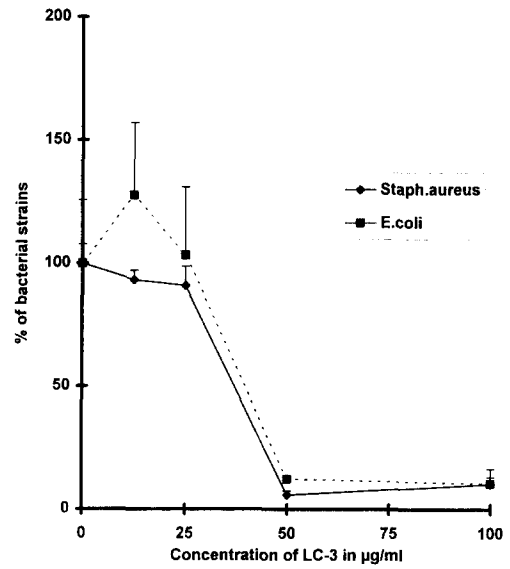

Fig. 2. Minimal inhibition concentration (MIC) of lysozyme derivative **LC-3**. The MICs of **LC-3** were determined against *E. coli* and *S. aureus* which were grown up to an OD_{600} of 0.5, incubated for 1 h at 37°C with the **LC-3** solution (final protein concentration from 0–100 μg/ml), diluted to $1{:}10^6$ and after incubation on Petri dishes at 37° for 12 h, the CFU were counted. Data incorporates the standard deviation of three experiments.

The MICs of the conjugate **LC-3** demonstrated that their mean of 50 μg/ml was equal for both *S.*

aureus and *E. coli* (Fig. 2), and were less than that reported by Didry et al. (1993). The investigations indicated that lysozyme covalently bound to two or more cinnamaldehyde molecules was more effective against Gram negative bacteria than lysozyme alone or a mixture of the two components.

A preservative effectiveness test was determined for three microbially instable dermatica using the complete bacteria in demineralized water. The 0-time level of bacterial growth ranged from 17.5 CFU/g in Carbopol-gel to 1.0×10^2 CFU/g in HPMC-gel and 1.45×10^2 CFU/g in Diprosicc/water.

Addition of **LC-3** resulted in a 5-fold reduction in all three preparations following 1 day and 7 days. The growth of bacteria in Diprosicc/water (Fig. 3) reached 7.4×10^8 CFU/g after 28 days. At a concentration of 250 μg/g and 500 μg/g **LC-3**, a one log and a 11-fold reduction of the bacteria was seen after 28 days, respectively. At a concentration of 125μg/g **LC-3**, a slower reduction was evident. These observations were reproducible using HPMC-gel (Fig. 4), although only a 7.5-fold reduction in bacterial growth was affected in Carbopol-gel (Fig. 5).

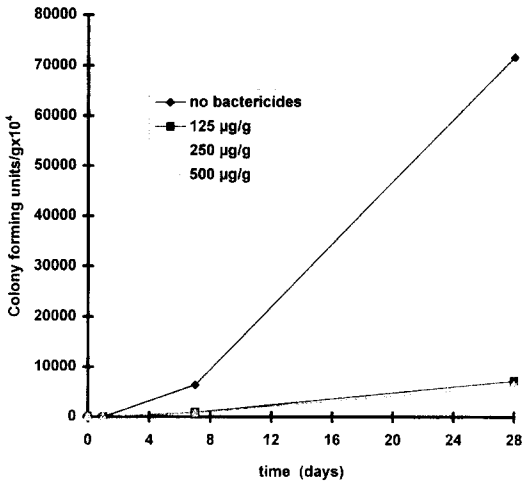

Fig. 4. Microbial growth in HPMC-gel with different concentrations of **LC-3** (μg/g) compared with the unpreserved product. The gel was analyzed 1, 7 and 28 days after preparation. All tests were carried out four times.

4. Discussion

The results of examining three model formulations failed the Pharmacopoea Europaea (1991) efficacy criteria of at least 3 log reduction of bacteria within 7 days (Murray and Cooper, 1994). A disturbing observation was the detection

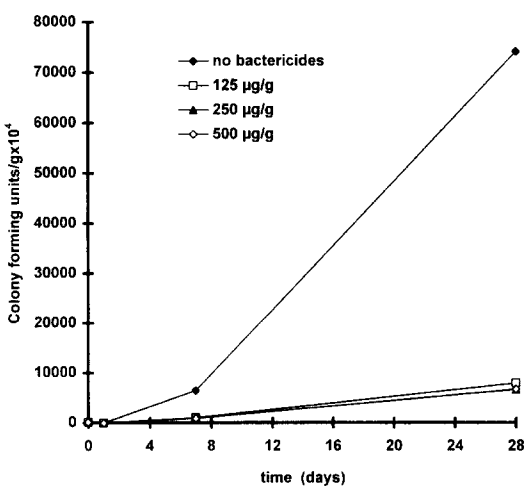

Fig. 3. Microbial growth in Diprosicc/water with different concentrations of **LC-3** (μg/g) compared with the unpreserved product. The cream was analyzed 1, 7 and 28 days after preparation. All tests were carried out four times.

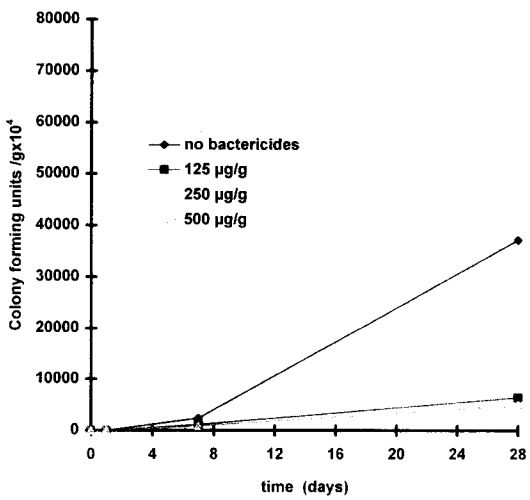

Fig. 5. Microbial growth in Carbopol-gel with different concentrations of **LC-3** (μg/g) compared with the unpreserved product. The gel was analyzed 1, 7 and 28 days after preparation. All tests were carried out four times.

of regrowth between the 7th and 28th day of observation. One reason for this result could be the employment of the complete bacterial spectrum occurring in demineralized water, mainly Gram negative, instead of challenging the products with defined Gram positive organisms (*S. aureus*, *P. aeruginaosa*) as suggested in Pharmacopoea Europaea (1991). A test working with the complete microbial spectrum occurring naturally in water has the advantage of simulating more realistic spoiling-conditions.

Preservatives are important in topical products. These pharmacologically active agents extend product stability permitting their continued use under nonsterile conditions. The presence however in a topical product may pose a risk should they become absorbed through the skin. The antimicrobial activity of many preservatives used in pharmaceuticals and cosmetics depends on their ability to move freely in the aqueous phase, and yet be lipophile enough to partition through the microbial outer cell envelope and the protoplasmic membrane in order to reach their site of action.

Cinnamaldehyde is a water insoluble compound with a high partition coefficient (heptane/water), is 100% soluble in the organic phase of heptane, and has a strong and unpleasant flavour. In contrast the lysozyme-cinnamaldehyde conjugate (**LC-3**) sufficiently water soluble, enzymatic active, and flavourless. It is well known that the percentage dose absorbed through human skin tends to decrease with decreasing partition coefficient. Due to the lipophilic character of the stratum corneum the percutaneous absorption rates of hydrophillic molecules is limited so that possible systemic side-effects are reduced. In addition the new compound has a higher molecular weight (> 14 400) than cinnamaldehyde (134) and therefore passage through the skin could be predicted to decrease. Other antimicrobial active peptides/proteins may also be used as preservatives in dermatics such as the lantibiotics nisin and subtilin (Valenta et al., 1995; 1996). The progress made over the years in biotechnology has permitted many useful peptides/proteins to be produced at an economical scale. It would be interesting to see whether modifying other antimicrobial peptides would similarly result in an increased antimicrobial spectrum as is reported here for **LC-3**.

Creating new compounds by structurally modifying existing drugs is a useful alternative to searching for novel biologically active agents.

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