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Lysozyme-caffeic acid conjugates: possible novel preservatives for dermal formulations

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Abstract

An equivalent mixture of a recently developed lysozyme-caffeic acid conjugate and lysozyme was tested as possible preservative-system for dermal products. Whereas bacterial growth of *Escherichia coli* (ATCC 8793) was strongly inhibited by the lysozyme-caffeic acid conjugate, the efficacy towards *Staphylococcus aureus* (ATCC 6538) was slightly reduced. In order to compensate for this effectivity loss, a combination of lysozyme-caffeic acid conjugate and lysozyme was required. Microbial efficacy was studied in various hydrogels based on the following polymers: hydroxypropylmethylcellulose (HPMC), sodium chitosan-EDTA (NaChito-EDTA) and sodium carbopol 980 (NaC980), and the effectiveness as a preservative tested with *E. coli* as Gram-negative and *S. aureus* as Gram-positive representatives. Colony forming units (cfu) of samples were counted immediately and 1, 7, 14, 21 and 28 days after inoculation. The effectiveness of the preservative-system depended on the polymer type, the bacterial strains (Gram-negative or Gram-positive) and on the applied concentration. Almost no antibacterial effect could be detected in NaC980 gels, which may be caused by the attachment of lysozyme to the polymer. Our results show that a concentration of 0.2% (w/w) of the lysozyme-caffeic acid conjugate/lysozyme mixture (1 + 1) was required for HPMC- and NaChito-EDTA gels to exert a bactericidal effect for Gram-negative as well as for Gram-positive bacteria with the exception of HPMC gels towards *E. coli*, where only a bacteriostatic effect could be detected at this concentration.

Keywords: Lysozyme; Caffeic acid; Antimicrobial activity; Protein modification; Preservative efficacy

1. Introduction

One problem in developing effective preservatives for food, pharmaceuticals and cosmetics is the limited antimicrobial activity of suitable compounds, their allergenic potential and poor water

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solubility (Kabara, 1984; Kabara and Orth, 1997). Progress in recombinant DNA technology and biotechnology has resulted in industrial capability to produce large amounts of antimicrobial active peptides and proteins in commercial quantities. One of these potential candidates is lysozyme. A number of recent studies demonstrates the effectiveness of lysozyme in preparations for treating mouth diseases, ophthalmic infections and bacterial infections of the skin (Sava, 1996). The specifity of lysozyme for bacterial cell walls and the absence of any toxic effect to humans make this molecule a particularly suitable candidate for inclusion in food, dermal formulations and cosmetics that require control for bacterial growth. Further support for lysozyme as a good preservative target comes from studies on wound healing, where the topical application of lysozyme has been practised successfully (Palmieri and Boraldi, 1977). Modified lysozymes in which cinnamaldehyde or different acids were covalently bound to lysozyme have also received attention as antimicrobial agents (Valenta et al., 1997; Bernkop-Schnürch et al., 1998). Limitation for the general use of lysozyme is that although it is very active against Gram-positive, it is much less active against Gram-negative bacteria (Fleming and Allison, 1922; Ibrahim et al., 1994; Ito et al., 1997). In order to increase the activity of lysozyme against Gram-negative bacteria, caffeic- and cinnamic acid have been covalently coupled to lysozyme by forming stable amide-bonds with the available amino-groups of the protein such as ϵ-amino group from lysine (Bernkop-Schnürch et al., 1998). Lysozyme-cinnamic acid conjugates exhibited weaker antimicrobial effects than lysozyme-caffeic acid conjugates. Thus only the latter conjugates are a practical consideration for preservatives. Growth of *E. coli* is strongly inhibited by lysozyme-caffeic acid conjugates. However, their efficacy against *S. aureus* is slightly reduced compared to lysozyme itself (Bernkop-Schnürch et al., 1998). Therefore, the aim of the present study was to create a preserving mixture with a broad antimicrobial spectrum by combining both a lysozyme-caffeic acid conjugate, exhibiting a good activity against *E. coli*, with unmodified lysozyme, which is effective against *S. aureus*.

2. Materials and methods

2.1. Substances and bacteria

Lysozyme (from hen egg, molecular mass: 14400) and caffeic acid (molecular mass: 180.2) were purchased from Sigma (St. Louis, USA). Hydroxypropylmethylcellulose (HPMC) corresponded to Metolose 60 SH 4000 and was from Shin-Etsu (Japan). Carbopol 980 (Goodrich, UK) was neutralised with NaOH as previously reported (Bernkop-Schnürch and Göckel, 1997) and abbreviated as NaC980. Chitosan-EDTA (Na-Chito-EDTA) was synthesised by the covalent attachment of EDTA to chitosan (poly-D-glucosamine) according to Bernkop-Schnürch et al. (1997). Bacterial strains used were *S. aureus* ATCC 6538 and *E. coli* ATCC 8793. All materials used in this study were of reagent grade.

2.2. Formulations

Aqua ad injectabilia (Pharmacopoea Europaea, 1997) was used to prepare 2.5% (w/w) HPMC hydrogels and 0.5% (w/w) hydrogels from NaC980 and NaChito-EDTA. All preparations were sterilised for 15 min at 121°C by autoclaving and stored at room temperature (RT) until evaluation.

2.3. Synthesis of the lysozyme-caffeic acid conjugate

Caffeic acid conjugates were synthesised with a mass ratio lysozyme to caffeic acid $1 + 4$ according to the method described by Bernkop-Schnürch et al. (1998).

2.4. Adsorption of lysozyme or lysozyme-caffeic acid conjugates to the hydrogel polymers

In order to monitor the amount of polypeptide or polypeptide conjugate that might be bound or adsorbed by the hydrogel polymers themselves, a simple method was designed using the protein absorption of UV light at 280 nm (Lamda 16, UV/VIS; Perkin-Elmer). Measurements of 1 ml aqueous stock solutions lysozyme (0.1% w/w),

lysozyme-caffeic acid conjugate (0.1% w/w) and the lysozyme/lysozyme-caffeic acid conjugate mixture (0.05% w/w) were recorded. Then, 1.0 ml of the same stock solutions were added to separate aliquots of each hydrogel (250 mg) and vigorously mixed. After incubation of the protein/hydrogel mixture for 2 h at 20°C on a shaker, samples were centrifuged (30 min; 13500 × *g*; 20°C), the supernatants removed and the UV absorption at 280 nm measured. The amount of protein remaining bound to the polymer (%; w/w) was determined by the difference of the two values at 280 nm between the supernatant exposed to hydrogel and pure polypeptide solution.

2.5. Antimicrobial activity

The antimicrobial activity of the conjugate and the conjugate/lysozyme mixtures was tested against *E. coli* and *S. aureus*, respectively. The organisms were grown in liquid medium B (Pharmacopoea Europaea, 1997) to a density of 10^8 colony forming units (cfu) per ml with an OD600 of 0.4. A volume of 0.8 ml lysozyme, lysozyme-caffeic acid conjugate or a 1 + 1 mixture in 50 mM potassium phosphate buffer pH 7.4 was mixed with 0.2 ml bacterial suspension and incubated for 2 h at 37°C. Each sample was serially diluted to $1{:}10^2$ or $1{:}10^3$ with medium B. The mixtures were vortexed (reax 2000) for 1 min and 100-μl aliquots of each bacterial suspension were plated onto Petri dishes containing medium B. Following incubation at 37°C for 24 h, the antimicrobial activity was estimated by counting the cfu. Sterile medium B was used as control. All experiments were performed five times.

2.6. General procedure of preservation efficacy testing in hydrogels

Sterile HPMC, NaChito-EDTA and NaC980 hydrogels were challenged with bacterial suspensions of *E. coli* and *S. aureus* grown in liquid medium B to an average colony count between 3 and 5.10^8 cfu/ml. A total of 10 g of sterile hydrogel were inoculated with 1 ml of this bacterial suspension. To separate aliquots of each mixture, differing amounts of lysozyme/lysozyme-caffeic acid conjugate (1 + 1) mixture were added aseptically under laminar air flow to give final concentrations of the preservative-system of 0.05, 0.1 and 0.2% (w/w). Then 1 g of the hydrogel was vigorously mixed with 9.0 ml NaCl/peptone buffer (Pharmacopoea Europaea, 1997). Each sample was serially diluted in NaCl/peptone buffer and 500 μl of dilutions plated out to finally give colony counts in the range 20–100 per plate of medium B. Viable counts were determined after 24 h incubation at 37°C. All tests were repeated four times. Samples were examined immediately and 1, 7, 14, 21 and 28 days after inoculation. Inoculated hydrogels without any added preservative served as controls. Only pure cultures were used as inoculum.

2.7. Statistical data analysis

Data are presented as the means ± S.D of results from three experiments for adsorption studies and five experiments for all other tests. Statistical data analysis was performed using Student's *t*-test, with $p < 0.05$ as minimum level of significance.

3. Results

3.1. Lysozyme-binding to hydrogels

Binding studies showed no significant differences between the binding of lysozyme, lysozyme-caffeic acid conjugate or the lysozyme/lysozyme-conjugate mixture to the polymers tested. NaC980 bound protein more strongly at 46–59%, with the protein adsorption to NaChito-EDTA being 25–35%. The lowest binding at 4.9–10.3% was to HPMC (Fig. 1).

3.2. Antimicrobial activity

The antimicrobial activity of the lysozyme-caffeic acid conjugates is markedly enhanced against *E. coli* but reduced against *S. aureus*, compared to pure lysozyme (Bernkop-Schnürch et al., 1998). Fig. 2 illustrates the effect of combining pure lysozyme with the lysozyme-caffeic acid conjugate

on the survival of exposed bacteria as measured by cfu counts. The effectiveness of the mixture was significant greater against *E. coli* with a mean survival of 54% than against *S. aureus* with a mean survival of 69% (Fig. 2).

3.3. Growth of E. coli and S. aureus in unpreserved gels

The growth kinetics of *E. coli* in unpreserved gels is shown in Fig. 3; it can be seen that the cfu in NaChito-EDTA gels are 2-log reduced compared to NaC980 and HPMC.

Although the cell counts of the inoculums were the same for both *E. coli* and *S. aureus*, the growth kinetics of *S. aureus* in NaChito-EDTA and HPMC gels were on average 2-log reduced compared to *E. coli*.

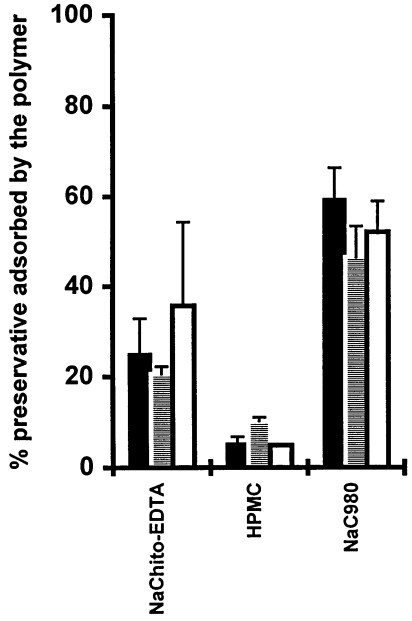

Fig. 1. Adsorption of lysozyme (hatched bars), lysozyme-caffeic acid conjugate (white bars) and lysozyme/lysozyme-caffeic acid conjugate mixture (1 + 1) (black bars) caused by different gelatinising agents (NaChito-EDTA, HPMC, NaC980). Data are the difference between $UV_{280\ nm}$ total protein and UV_{280} nm supernatant given as means ± S.D. of results from three experiments. (UV total − UV supernatant) × 100/UV total = % protein binding.

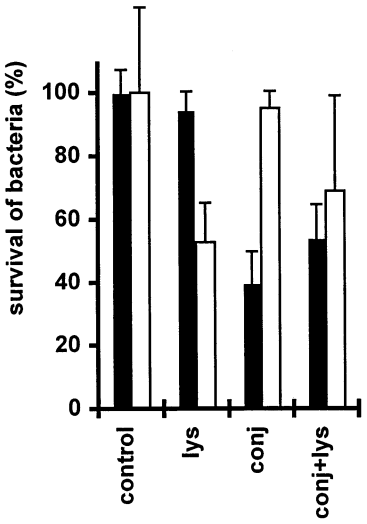

Fig. 2. Antimicrobial activity of lysozyme (lys), lysozyme-caffeic acid conjugate (conj) and lysozyme/lysozyme-caffeic acid conjugate mixture (1 + 1) (conj + lys) against *E. coli* (black bars) and *S. aureus* (white bars). Data are the % of bacterial counts against the control set to 100% and incorporate the standard deviation from five experiments.

During 21 days of observation the growth kinetics of *S. aureus* in the two gel types were similar but reduced in NaChito-EDTA gels. Be-

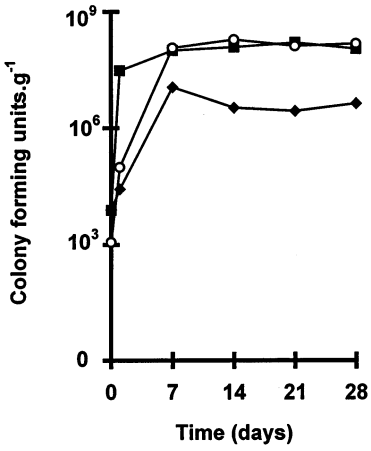

Fig. 3. Viable counts of *E. coli* (ATCC 8739) in hydrogels based on different gelatinising agents: ◆, NaChito-EDTA; ■, HPMC; ○, NaC980. A total of 10 g of sterile hydrogel were inoculated with 1 ml of bacterial suspension (10^8 cfu/ml). The gels were analysed immediately and 1, 7, 14, 21 and 28 days after inoculation. All points are an average of five tests.

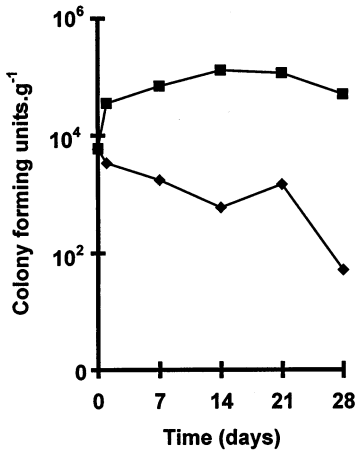

Fig. 4. Viable counts of *S. aureus* (ATCC 6538) in two different hydrogels: ◆, NaChito-EDTA; ■, HPMC. A total of 10 g of sterile hydrogel were contaminated with 1 ml of bacterial suspension (10^8 cfu/ml). The gels were analysed immediately and 1, 7, 14, 21 and 28 days after inoculation. All points are an average of five tests.

tween the 21st and 28th day of observation *S. aureus* was decimated dramatically in NaChito-EDTA gels, whereas the growth rates in HPMC gels stayed approximately equally high (Fig. 4).

3.4. Preservative efficacy

A preservative efficacy test was performed for three hydrogels using the recommended (Pharmacopoea Europaea, 1997) strains of *E. coli* as Gram-negative and *S. aureus* as Gram-positive representatives. Results presented are the mean of five experiments. For the experiments, 0.2, 0.1 and 0.05% (each w/w) of the preservative mixture was tested in NaC980, NaChito-EDTA and HPMC gels. The inoculum contained between 3 and 5.10^8 cfu/ml. The log-reduction of Tables 1 and 2 was calculated in relation to the unpreserved inoculated gels.

3.5. Antimicrobial activity towards E. coli

In HPMC gels the 0.2% preservative mixture could not pass the efficacy criterium B of the European Pharmacopoea (Pharmacopoea Europaea, 1997) entirely, which demands a 3-log reduction after 14 days and no further regrowth after 28 days (Table 1). Although the demand of no regrowth after 28 days was established, only a 1.7-log reduction after 14 days of bacterial counts could be detected.

On the other hand NaChito-EDTA containing 0.05% preservative mixture resulted in growth inhibition of bacteria that could fulfil criterium B. Whereas 0.1 and 0.2% were also sufficient to pass criterium A, which demands a 2-log reduction after 2 days, a 3-log reduction after 7 days and no further regrowth after 28 days of observation. Even the 0.025% preservative met criterium B (data not shown) having killed *E. coli* after 7 days in NaChito-EDTA gels.

Therefore NaChito-EDTA gels with 0.025% preservative mixture are necessary for achieving a bactericidal effect and at least $>0.2\%$ is required for HPMC gels to obtain a bacteriostatic effect.

In NaC980 the preserving mixture did not inhibit the bacterial growth of *E. coli* (Table 1) significantly and therefore, the challenge tests with *S. aureus* were not attempted with NaC980 gels.

3.6. Antimicrobial activity towards S. aureus

Tests conducted using HPMC gels at 0.05 and 0.1% lysozyme/lysozyme-caffeic acid conjugate mixture made the requirements for criterium B and exhibited a strong bactericidal effect against *S. aureus*. Therefore tests with higher concentrations were not required (Table 2). Within 21 days all bacteria were killed effectively by 0.1%.

NaChito-EDTA gels required a minimum concentration of 0.2% preserving mixture to kill all bacteria within 21 days, so that criterium B of the Pharmacopoea Europaea was fulfilled. As illustrated in Fig. 5, NaChito-EDTA itself exhibits a strong antibacterial effect after 28 days in the absence of preservative. Comparison of NaChito-EDTA gels containing 0.2% preservative shows an enhanced effect of 1.7-log reduction to the unpreserved gel at 28 days (Table 2). Lower concentrations of lysozyme/lysozyme-caffeic acid conjugate mixture in NaChito-EDTA gels could not pass any preservative pharmacopoeial efficacy criterium. Therefore from the evidence discussed above, we conclude that for NaChito-EDTA gels

Table 1
Effect of different concentrations of lysozyme/lysozyme-caffeic acid conjugate mixture (1+1) (lys+conj) on the growth of *E. coli* (ATCC 8739) in different gel formulations

	lys+conj (% w/w)	Reduction of viable bacterial (log cfu/g^{-1})				
		1 day	7 days	14 days	21 days	28 days
HPMC	0.2	3.2	1.5	1.7	1.3	1.4
	0.1	2.1	1.1	1.27	0.99	1.39
	0.05	0.8	0.4	0.89	1.12	1.03
NaChito-EDTA	0.2	4.43	7.0	6.5	6.4	6.6
	0.1	4.43	7.0	6.5	6.4	6.6
	0.05	1.43	7.0	6.5	6.4	6.6
NaC980	0.2	0.14	0.05	0.1	0.21	0.5
	0.1	0.0	0.0	0.16	0.24	0.5
	0.05	0.02	0.0	0.11	0.19	0.3

Data are expressed as $\log \frac{\text{cfu/g unpreserved hydrogel}}{\text{cfu/g preserved hydrogel}}$

HPMC, hydroxypropylmethylcellulose; NaChito-EDTA, sodium chitosan-EDTA; NaC980, sodium carbopol 980.

a 0.2% preservative mixture, and at least 0.05% for HPMC, are required to achieve a germicidal effect.

4. Discussion

Lysozyme catalyses the hydrolysis of the β-1 $\rightarrow$ 4-glycosidic linkage of the peptidoglycan in bacterial cell walls. Although this bactericidal activity is limited to Gram-positive bacteria, the bactericidal spectrum can be widened by the covalent linkage of lysozyme to a hydrophobic compound (Ibrahim et al., 1991, 1994; Ito et al., 1997). The activity against Gram-negative bacteria of the resulting lysozyme-derivatives becomes more pronounced with increasing hydrophobicity of the ligand. Lysozyme-caffeic acid conjugates exhibit a broadened antimicrobial spectrum against Gram-negative bacteria which is accompanied by a decrease in activity against Gram-positive bacteria (Bernkop-Schnürch et al., 1998). To overcome this problem a combination of unmodified lysozyme with a lysozyme-caffeic acid conjugate in a mass ratio 1 + 1 was tested.

The results of the preservative efficacy tests demonstrated the strong bactericidal effects of the lysozyme/lysozyme-caffeic acid conjugate mixture when combined with NaChito-EDTA and HPMC hydrogels with the exception of *E. coli* in HPMC, where the effect was only bacteriostatic. Because of the lipopolysaccharide layer of Gram-negative bacteria, they are more resistant to the effects of preservatives than Gram-positive. This may help to explain why Gram-negative bacteria have been so troublesome for the manufacture of pharmaceutics and cosmetics. Besides the bacterial strain, the effectiveness of the mixture also depends on the polymer system. No significant antimicrobial effect of the preservative mixture could be detected in NaC980 gels (Table 1). The reason may be due to the high lysozyme adsorption of NaC980 gels. However, a negative influence to the antimicrobial effect of lysozyme derivatives may be excluded in HPMC gels because of a negligibly low lysozyme binding in HPMC gels. Although 30% lysozyme was bound to NaChito-EDTA in the gel, the inhibitory activity of the preservative mixture was still sufficient to guarantee the microbial stability of the formulation. The very strong bactericidal effect exhibited by the lysozyme mixture towards *E. coli* in NaChito-EDTA gels can be explained by an intensification of the activity from the covalently bound chelator EDTA, which exhibits a high binding affinity to the divalent cations Mg^{2+} and Ca^{2+} (Hart, 1983, 1984; Valenta et al., 1998). Chelators play an important role in product preservatives because they are

Table 2
Effect of different concentrations of lysozyme/lysozyme-caffeic acid conjugate mixture (1+1) (lys+conj) on the growth of *S. aureus* (ATCC 6538) in different hydrogel formulations

	lys+conj (% w/w)	Reduction of viable bacterial (log cfu/g^{-1})				
		1 day	7 days	14 days	21 days	28 days
HPMC	0.1	1.0	1.73	3.0	5.0	4.7
	0.05	1.1	1.23	2.7	3.47	4.7
NaChito-EDTA	0.2	0.0	0.36	0.4	3.17	1.7
	0.1	0	0.36	0	0.38	0
	0.05	0	0	0	1.3	0

Data are expressed as $\log \frac{\text{cfu/g unpreserved hydrogel}}{\text{cfu/g preserved hydrogel}}$

HPMC, hydroxypropylmethylcellulose; NaChito-EDTA, sodium chitosan-EDTA; NaC980, sodium carbopol 980.

believed to increase action by limiting the availability of essential minerals and permeabilising the membrane of Gram-negative bacteria.

Besides the polymer system, the concentration of the preservative also plays an important role and is also dependent upon the bacterial species. Our results show a concentration of 0.2% of the lysozyme-caffeic acid/lysozyme mixture (1 + 1) is the minimum required to exert a bacteriostatic effect for Gram-negative as well as for Gram-positive bacteria. Although this represents a relatively high concentration, the high molecular mass of the components of the preservative system (> 14400) almost excludes dermal absorption, as demonstrated with a comparably smaller peptide (Bernkop-Schnürch et al., 1996), and thus possible side-effects may be minimised. The advantages of negligible absorption and good water solubility fit also for other antimicrobial peptides and proteins such as nisin, which has already been tested by our research group as a preservative for dermal products (Valenta et al., 1996a,b). As well, magainin and defensins, that are known to have broad antimicrobial activity due to their membrane disrupting ability, have been used as preservatives in cosmetics and ophthalmics (Schwab et al., 1992; Haynie et al., 1995). In a decade in which great emphasis is put on naturally derived peptides, it must be stressed that lysozyme (Proctor and Cunningham, 1988) and lysozyme conjugates show important characteristics as compounds which may be used in prevention of bacterial growth in food and pharmaceuticals.

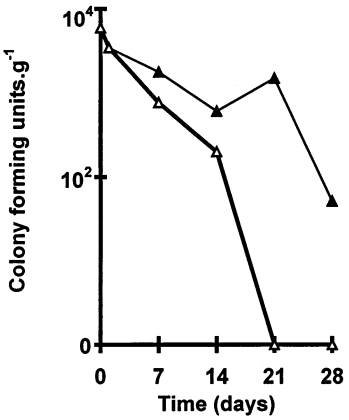

Fig. 5. Antimicrobial effect against *S. aureus* of the NaChito-EDTA hydrogel alone and in the presence of 0.2% lysozyme/lysozyme-caffeic acid mixture (1 + 1). ▼, Unpreserved gel; △, preserved gel.

Thus our work demonstrates that a mixture of lysozyme-caffeic acid conjugate with pure lysozyme (1 + 1) can be recommended as alternative preservative for HPMC and NaChito-EDTA gels. The mixture exhibits improved antimicrobial efficacy in comparison to the lysozyme-caffeic acid conjugate alone.

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