

Interactions Between Peptide and Preservatives: Effects on Peptide Self-Interactions and Antimicrobial Efficiency In Aqueous Multi-Dose Formulations

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ABSTRACT

Purpose Antimicrobial preservatives are known to interact with proteins and potentially affect their stability in aqueous solutions. In this systematic study, the interactions of a model peptide with three commonly used preservatives, benzyl alcohol, phenol and m-cresol, were evaluated.

Methods The impact on peptide oligomerization was studied using GC-MALS, SEC-MALS and DLS, antimicrobial efficiency of different formulations were studied using the Ph. Eur. antimicrobial efficacy test, and the molecular adsorption of preservative molecules on reversible peptide oligomers was monitored using NMR.

Results The hydrodynamic radius and molar mass of the peptide oligomers was shown to clearly increase in the presence of m-cresol but less significantly with phenol and benzyl alcohol. The increase in size was most likely caused by peptide self-interactions becoming more attractive, leading to reversible oligomerization. On the other hand, increasing the concentration of peptide in multi-dose formulations led to

reduced molecular mobility and decreased antimicrobial efficacy of all preservatives.

Conclusions Peptide-preservative interactions not only affect peptide self-interactions, but also antimicrobial efficiency of the preservatives and are thus of significant relevance. Adsorption of preservatives on oligomeric states of peptides is proposed as a mechanism to explain this reduced antimicrobial efficacy.

KEY WORDS antimicrobial efficacy · interaction · oligomerization · peptide · preservative

ABBREVIATIONS

\bar{M}_w	Weight average molar mass
\bar{r}_H	Hydrodynamic radius
A_2	Second virial coefficient
AET	Antimicrobial efficacy testing
C_0	Preservative concentration required to kill/inactivate all microbes in the absence of peptide
CFU	Colony forming unit
CG-MALS	Composition-gradient multi-angle light scattering
C_i	Preservative concentration required to kill/inactivate all microbes in the presence of peptide
D_{free}	Preservative diffusion coefficient in the absence of peptide
DLS	Dynamic light scattering
D_{obs}	Preservative diffusion coefficient in the presence of peptide
DOSY	Diffusion ordered NMR spectroscopy
D_{pep}	Peptide diffusion coefficient
f	Fraction of live microbes after incubation
f_s	Sequestered preservative fraction (in AET experiments)

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M_w	Apparent molar mass
NMR	Nuclear magnetic resonance
pI	Isoelectric point
$P_{O/W}$	Octanol-water partition coefficient
P_{pep}	Peptide-bound preservative fraction (in NMR experiments)
SEC-MALS	Size-exclusion chromatography, coupled with multi-angle light scattering detector

INTRODUCTION

Antimicrobial preservatives must be added to parenteral multi-dose formulations in order to inhibit the growth or kill or inactivate any microbes which may be inadvertently introduced into the drug product during repeated product use (1–3). Currently, the most commonly used preservatives in parenteral protein and peptide products are m-cresol, phenol and benzyl alcohol (molecular structures shown in Fig. 1), whereas chlorobutanol and thiomersal (thimerosal) are less frequently used for various reasons (4).

One challenge often encountered when using antimicrobial preservatives in protein formulations is their potential impact on protein stability. Several examples can be found in literature where commonly used aromatic preservatives exhibit a negative effect on the physicochemical stability of proteins (e.g., 4–7). These preservative-protein interactions can lead to changes in protein conformation and increased aggregation tendency (5), decreased stability at higher temperatures (6, 7) and/or chemical modifications (8). Several studies comparing the effects of phenol, m-cresol and benzyl alcohol on protein stability have suggested that benzyl alcohol generally appears to cause fewer instability issues than phenol or m-cresol (6, 7, 9, 10).

Different hypotheses have been proposed regarding the molecular mechanisms of interactions between proteins or peptides and preservatives. These include the preservatives forming hydrogen bonds with carbonyl and/or amide moieties of amino acid residues (5), their aromatic rings forming π -interactions and/or hydrophobic attractions with amino acids (11–13) and phenolic compounds oxidizing into quinones and subsequently covalently bonding with peptides as well as deprotonated preservatives forming ionic interactions with charged amino acid residues (14). As a number of groups

conclude (e.g., 9, 11, 12), it is also likely that a combination of these mechanisms contribute to the interaction simultaneously. Overall, the intensity of interaction seems relatively weak (9, 15) and it is not limited to only certain interaction sites even though certain structural “hot-spots” in the peptide or protein may bind preservatives more strongly (11, 13).

It should be noted that preservative-polypeptide interactions do not always lead to unfavorable effects on API stability. Phenolic compounds have been shown to stabilize the R₆-Zn-insulin hexamer (11), which displays the highest physicochemical stability of the commonly manifesting Zn-insulin oligomers (16). Similarly, it has been postulated that benzyl alcohol stabilizes a partially unfolded state of lysozyme due to preferential interactions with that particular conformation, thus inhibiting its further conversion towards more aggregation-prone states (17, 18). Data from our own group also shows that the addition of excipients which can act as preservatives can increase protein stability, for example by scavenging radicals and thus acting as antioxidants (unpublished results). In conclusion neither the interactions between a given preservative and a protein or peptide nor their practical consequences on API stability are easy to predict.

The purpose of this study was to explore the effects of three antimicrobial preservatives commonly used in parenteral products, benzyl alcohol, m-cresol and phenol, on the oligomerization state of a model peptide. Furthermore, the antimicrobial efficacy (i.e., microbial inactivation rates after inoculation with bacteria) of different formulations containing peptides and preservatives were compared in order to assess whether peptide/preservative interactions can have an effect on the antimicrobial efficacy of preservatives and thus on bacterial viability.

MATERIALS AND METHODS

Peptides and Excipients

The model peptide in the studies was provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland). The peptide ($M_w \sim 4.5$ kDa, 40 amino acid residues) was acylated with palmitoyl (C16) at the C-terminus. The peptide backbone contained 4 proline residues and 6 residues with aromatic side groups (including phenylalanine, tyrosine and tryptophan), and with an isoelectric point (pI) of 3.2, its overall electrostatic charge was negative at pH 7.0. Peptide purity was >95% and it was formulated in 20 mM His/His-HCl buffer (pH 7.0) with 150 mM NaCl. Under these formulation conditions the peptide manifests mainly as a pentamer, and the oligomeric state does not change measurably during 12 months of storage at either 2–8°C or 25°C (data not shown). To study whether the buffer composition affects preservative interaction tendency, the peptide was also formulated in 20 mM Na-phosphate

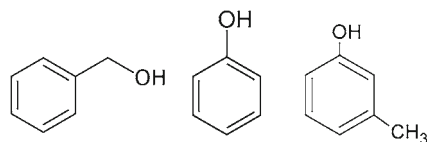


Fig. 1 Molecular structures of benzyl alcohol (left), phenol (center) and m-cresol (right).

buffer (pH 7.0) with 130 mM NaCl in selected experiments. Benzyl alcohol (Ph. Eur. excipient grade, Merck KGaA, Darmstadt, Germany), m-cresol (Ph. Eur. excipient grade, Lanxess Deutschland GmbH, Leverkusen, Germany) and phenol (Ph. Eur. excipient grade, Merck KGaA, Darmstadt, Germany) were combined with the peptide formulations to produce different peptide/preservative mixing ratios. All formulations were manufactured in water for injections (WFI).

Peptide-Preservative Assembly Size and Self-Interaction Tendency Measurement

CG-MALS

The self-interactions of the peptide in the presence and absence of antimicrobial preservative were studied using composition-gradient multi-angle light scattering (CG-MALS). The background of the analysis method has been comprehensively described by Some and Kendrick (19). Briefly, the excess Rayleigh scattering ($R_s - R_0$) of point-like, monodisperse and self-interaction-free polypeptide monomers can be used to calculate their molar mass according to the following equation:

$$R_s - R_0 = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4} \left(\frac{dn}{dc} \right)^2 M c = K^* M c \quad (1)$$

where n_0 is the refractive index of the solution, N_A is Avogadro's number, λ_0 is the wavelength of incident light in vacuum (here 659 nm) and dn/dc is the refractive index increment of the polypeptide (here 0.185), M its molar mass and c its concentration. The constant K can be used to distill all other constants into a single term.

However, in real solutions the polypeptide monomers interact non-specifically with each other, leading to a deviation from the ideal relationship between excess Rayleigh scattering and concentration. This deviation can be taken into account by applying one or more virial expansion coefficients (i.e., A_2 , A_3 etc.) to Eq. 1 as follows (19):

$$\frac{K^* c}{R_s - R_0} = \frac{1}{\overline{M}_w} + 2A_2 c + 3A_3 c^2 + \dots \quad (2)$$

It should be noted that since polypeptide self-interactions lead to an equilibrium state where several different oligomeric states exist simultaneously due to monomer association and dissociation, Eq. 2 now yields the weight average molar mass (\overline{M}_w) of all oligomer units present in the solution. The virial coefficients can be used to draw conclusions regarding the nature of the self-interactions, where positive coefficient values indicate dominantly repulsive interactions and negative values dominantly attractive ones.

Concentration gradients of the model peptide were created using two of the three pumps of Calypso II system (Wyatt Technology Corporation, CA, USA), while using the third pump to vary the concentration of antimicrobial preservatives in the solution. The solutions were analyzed at 25°C with in-line Optilab® rEX differential refractometer and DAWN® Heleos® II multi-angle light scattering (MALS) detector (Wyatt Technology Corporation, CA, USA), which measured simultaneously the light scattering intensity and refractive index of the solutions. The practical details of such measurements are described, for example, in Attri and Minton (20). 12 gradient steps (concatenated from two separate measurements) of the peptide at 0.17–10 mg/ml concentration range were used in the measurements, and these concentration gradients were produced while varying the antimicrobial preservative concentrations (0, 2, 4, 6, 8 or 10 mg/ml) in formulation buffer. To take into account the effect of antimicrobial preservatives on the refractive index of the solution, the reference cell of the refractometer was flushed before each measurement with buffer containing an equal amount of preservative as in the peptide solution. All stock solutions were prefiltered through 0.1 µm filters (Anotop, GE Healthcare AB, Uppsala, Sweden) before measurement.

Data procession was carried out using Calypso 2 software (Wyatt Technology Corporation, CA, USA). A_2 values were calculated using Calypso 2 –software by assuming 5-mer as the main oligomer size (see Fig. 4 for the size-exclusion chromatography results which support this claim) and by plotting the data in reversible dimerization models (i.e., modeling for 5-mer / 10-mer equilibrium). This model was selected because its goodness of fit (χ^2) was low for all preservative concentrations and the model was relatively simple. On the other hand, no molar mass restrictions were used when calculating the weight average molecular weight. The residuals of model fits at different peptide concentrations were observed to be in most cases <0.05 of the total light scattering signal.

SEC-MALS

Characterization of different self-assembly subspecies was attempted by using size-exclusion chromatography (SEC). Waters 2695 HPLC (Waters Corp., MA, USA) was connected to Biosuite 125 column (7.8 × 300 mm, Waters Corp., MA, USA) and the analysis was carried out using Optilab® rEX differential refractometer and DAWN® Heleos® II MALS detector, while the data was processed using Astra 6.1 software (Wyatt Technology Corporation, CA, USA). 20 µl of 50 mg/ml peptide solutions were injected in triplicate and the respective formulation buffers were used as mobile phases at 1.0 ml/min flow rate. To take into account the effect of the antimicrobial preservatives on the refractive index of the solution, the reference cell of the refractometer was flushed with the mobile phase before each measurement.

Hydrodynamic Radius Measurements

Combination of dynamic light scattering (DLS) and rheometry was used to calculate the hydrodynamic radius of peptide self-assemblies in the absence and presence of antimicrobial preservatives. Peptide and preservative solutions in formulation buffers were filtered through 0.1 μm filters (Anotop, GE Healthcare AB, Uppsala, Sweden) and mixed on 96-well plates (Sensoplate™ Black, Greiner Bio-One Inc., NC, USA) in a laminar airflow cabinet to produce different mixtures with 0, 1.7, 3.3, 5, 6.7, 8.3 or 10 mg/ml of peptide and 0, 2, 4, 6, 8 or 10 mg/ml of preservative. Diffusion coefficients (D) of the light scattering units were measured using Dynapro™ II DLS plate reader (Wyatt Technology Corporation, CA, USA) at 25°C. The dynamic viscosities of solutions containing 0, 1.7, 5 or 10 mg/ml of peptide and 0, 5 or 10 mg/ml of different preservatives (in formulation buffer) were measured in triplicate with Anton Paar Physica 301 cone-plate rheometer (Anton Paar GmbH, Graz, Austria) using 50 mm measurement head with 0.5° angle. Measurement temperature was 25°C, rotation speed 1000/min, cone-plate gap 0.025 mm and measurement time 120 s. The apparent hydrodynamic radius (r_H) was calculated according to the Stokes-Einstein equation describing the Brownian motion of spherical particles:

$$r_H = \frac{k_B T}{6\pi\eta D} \quad (3)$$

where k_B is the Boltzmann constant, T is temperature and η is the dynamic viscosity measured with cone-plate rheometer. The apparent hydrodynamic radii calculated using Eq. 3 were then used to estimate the hydrodynamic radius at infinite dilution (\bar{r}_H) by linear extrapolation from 3.3 to 8.3 mg/ml peptide concentration range, where the correlation between the peptide concentration and apparent hydrodynamic radius was linear.

Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) measurements were carried out by using a 600 Mhz Avance II+ spectrometer (Bruker BioSpin AG., Fällanden, Switzerland) equipped with a cryogenically cooled QCI probehead. Experiments were performed at 300 K. A standard stimulated echo diffusion ordered spectroscopy (DOSY) type sequence was used with bipolar gradients for diffusion editing (21). The gradient strength of these pulses was incremented in 32 steps from 5 to 95% of the available gradient strength of the gradient amplifier in use. The diffusion time was set to 300 ms. Solvent suppression was achieved by use of presaturation. Diffusion constants were obtained as the slope of a linear fit of the logarithm of the signal of the NMR amplitude in dependence

on the square of the gradient strength (22). Examples of such plots are shown in Supplement 1 for 5 mg/ml of each antimicrobial preservative in the absence and presence of 10 mg/ml peptide.

To study the effect of peptide concentration on translational diffusion of the preservatives, the mobility of the preservatives at 5 mg/ml were measured in the presence of different peptide concentrations (0, 1.25, 2.5, 5 and 10 mg/ml) using DOSY measurements. If a preservative is in fast exchange between a peptide-bound and a free state, the observed diffusion constant (D_{obs}) is affected by the equilibrium between the population-weighted averages of the peptide-bound (D_{pep}) and free (D_{free}) preservative molecule diffusion constants, as shown by Eq. 4:

$$D_{obs} = P_{pep} * D_{pep} + P_{free} * D_{free} \quad (4)$$

where P_{pep} and P_{free} refer to the fractions of peptide-bound and free preservative molecules, respectively. By substituting:

$$P_{free} = 1 - P_{pep} \quad (5)$$

the following relationship can be established:

$$P_{pep} = \frac{D_{obs} - D_{free}}{D_{pep} - D_{free}} \quad (6)$$

Since peptide-bound preservative molecules can be assumed to diffuse at the same rate as the peptide itself, Eq. 6 can be solved by measuring the diffusion coefficients of the preservative molecules in the presence (D_{obs}) and absence (D_{free}) of peptide and the diffusion coefficient of the peptide (D_{pep}). The diffusion coefficients were viscosity-corrected by measuring the effect of peptide concentration on histidine mobility.

Antimicrobial Efficacy Testing (AET)

The viability of two bacterial strains, *E. coli* (ATCC 8739) and *S. aureus* (ATCC 6538), in different peptide formulations were investigated according to a modified version of Ph. Eur. 8th Ed. 5.1.3. "Efficacy of antimicrobial preservation". Sterile peptide formulations (C=0, 1, 5 or 10 mg/ml) containing benzyl alcohol (C=8–12 mg/ml), phenol (C=4–6 mg/ml) or m-cresol (C=1.5–3 mg/ml) were inoculated with microbes, and samples were drawn after 24 and 72 h of incubation at 20–25°C. Samples were diluted, spread on agar medium B plates (Ph. Eur. 2.6.12.) and incubated until the number of colony forming units (CFU) could be calculated. When comparing the AET results to Ph. Eur. limit values, the reductions in CFU counts 24 h after the inoculation were expressed in logarithmic units, because the pharmacopoeia values are also given in this scale. Additionally, a read out after 72 h was used to verify the trend (i.e., decrease / no decrease in CFU) observed at the 24 h time point. In order to comply with Ph. Eur.

acceptance criteria, the log CFU reduction values had to exceed either limits of level A (suggested antimicrobial efficacy) or level B (acceptable antimicrobial efficacy in case level A limit cannot be reached because of the risk of adverse reactions), which are log 3 and log 1 after 24 h, respectively. 72 h time point is not included in Ph. Eur. 5.1.3 so no acceptance criteria could be attributed to it.

The AET results were also used to calculate how the addition of the peptide affected the antimicrobial efficacy of the formulation. In this case, the results were expressed as fraction of microbes alive 24 or 72 h after inoculation (f):

$$f_{24h/72h} = \frac{n(CFU, 24h/72h)}{n(CFU, 0h)} \quad (7)$$

Within the linear range of the AET setup, f can be plotted as a function of preservative concentration:

$$c_{pres} = xf + C \quad (8)$$

where c_{pres} is the molar concentration of antimicrobial preservative, x is the slope of the linear fit and C is the preservative concentration required to kill or inactivate all inoculated microbes after 24 h (i.e., $f=0$). By plotting f as a function of $c(pres)$, C can be calculated for a given antimicrobial preservative when including either 0 mg/ml, 1 mg/ml, 5 mg/ml or 10 mg/ml peptide in the formulation. C at a given peptide concentration (denoted as C_i) was used to calculate the sequestered (i.e., not being able to diffuse freely and kill/inactivate microbes) preservative fraction (f_S) as follows:

$$f_S = \frac{(C_i - C_0)}{C_i} \quad (9)$$

where C_0 is the preservative concentration required to kill or inactivate all inoculated microbes in the absence of peptide.

RESULTS

Effect of Antimicrobial Preservatives on the Size of Peptide-Preservative Assemblies

Figure 2 shows the hydrodynamic radii (\bar{r}_H) of the peptide oligomers measured with DLS when using different concentrations of preservatives. An increase in preservative concentration led to an increase in \bar{r}_H when m-cresol was used as an excipient, whereas the effects of benzyl alcohol and phenol on \bar{r}_H were negligible. The samples containing >8 mg/ml m-cresol were visibly turbid so they were excluded from the analysis.

Figure 3 shows the weight average molar mass (\bar{M}_w) and the second virial coefficient (A_2) of the peptide oligomers as a function of antimicrobial preservative concentration, measured with CG-MALS. An increase in benzyl alcohol

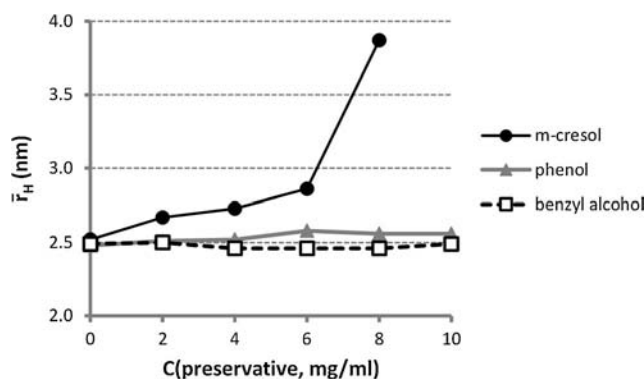


Fig. 2 The hydrodynamic radii (\bar{r}_H) of peptide oligomers in formulations containing different concentrations of benzyl alcohol, phenol or m-cresol, calculated using DLS and cone-plate rheometer and extrapolating to infinite dilution in peptide concentration.

concentration had practically no impact on \bar{M}_w , and the effect of phenol was very small. However, a clear effect on the light scattering signal was observed with m-cresol, meaning that the order in which the preservatives affected the CG-MALS results was similar in which they affected the \bar{r}_H of light scattering entities (Fig. 2). The measurable onset of A_2 decrease as a function of preservative concentrations was lower for m-cresol (>2 mg/ml) than for phenol or benzyl alcohol (>4 mg/ml), and the attractive self-interactions of peptide oligomers were dominant when ~ 6 mg/ml of m-cresol was added (i.e., $A_2 < 0$). As in the DLS measurements, the samples containing 10 mg/ml m-cresol could not be measured, as the formulations became turbid immediately upon preservative addition.

To study the effect of buffer composition on peptide-preservative interaction, phenol ($C=0-10$ mg/ml) was combined with the peptide ($C=1.7-10$ mg/ml) in both 20 mM His/His-HCl (pH 7.0)+150 mM NaCl buffer and 20 mM Na-phosphate (pH 7.0)+130 mM NaCl buffer, and the peptide oligomerization tendency was studied using CG-MALS. These NaCl concentrations were used because peptide A_2 was almost identical in them ($6.0 \cdot 10^{-4}$ mol*ml/g $^{-2}$ in His/His-HCl vs. $6.3 \cdot 10^{-4}$ mol*ml/g $^{-2}$ in Na-phosphate), signifying that the screening of electrostatic repulsions was equally effective in both buffers. Adding up to 10 mg/ml phenol decreased peptide A_2 in both buffers (to $2.0 \cdot 10^{-4}$ mol*ml/g $^{-2}$ in His/His-HCl and to $7.6 \cdot 10^{-5}$ mol*ml/g $^{-2}$ in Na-phosphate) and increased 5-mer to 10-mer conversion similarly (data not shown). Although the strengthening of self-attractive peptide potential may be slightly more pronounced in Na-phosphate buffer as a function of phenol concentration as depicted by the greater decrease in A_2 , the data did not indicate a significant difference in peptide aggregation propensity between the buffers.

Figure 4 shows the SEC elution profiles and the apparent molar mass (M_w) when the mobile phase contained different antimicrobial preservatives (note that no preservatives were included in the injected formulations). The preservative concentration in mobile phase was 10 mg/ml except for m-cresol

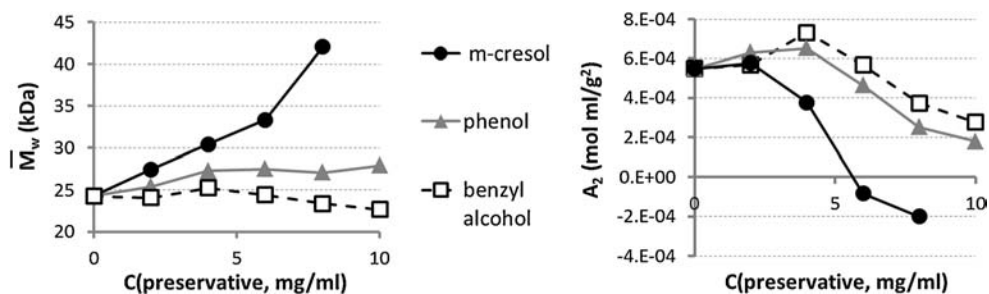


Fig. 3 Weight average molecular weight (\overline{M}_w) and second virial coefficient (A_2) of the peptide oligomers as a function of antimicrobial preservative concentration, measured using CG-MALS. Each \overline{M}_w and A_2 value was calculated by fitting 12 measurements at different peptide concentrations (between 0.17 and 10 mg/ml) into a reversible association model (see Materials and methods for details).

where lower concentration (5 mg/ml) was used due to peptide aggregation at 10 mg/ml. The numerical results of the SEC analysis are shown in Table I. Without preservatives in the mobile phase, the peptide eluted mostly as 5-mers ($M_w \sim 23$ kDa, observed at the main part of the elution peak) and 4-mers ($M_w \sim 18$ kDa, observed at the tail of the peak), as shown in Fig. 4(a). Including preservatives in the mobile phase (Figs. 4(b–d)) did not cause any high molecular weight (aggregate) peaks to appear. Very similar molar masses were observed at the elution peaks of the SEC measurements as were seen in the CG-MALS experiments, when

comparing the peptide M_w (Table I) to the \overline{M}_w results (Fig. 3) at respective preservative concentrations.

Inclusion of 10 mg/ml phenol and 5 mg/ml m-cresol affected the elution profile of the peptide by causing the peptide to elute earlier, increasing the half-width of the peak and decreasing the peak height (Table I). This could have been caused either by an increase in average peptide oligomer size or stronger column interactions when using a mobile phase containing phenol or m-cresol. All preservatives increased peak area slightly, possibly due to a formation of a preservative-rich layer around the peptide oligomers which changed the refractive index of the solution at elution peak.

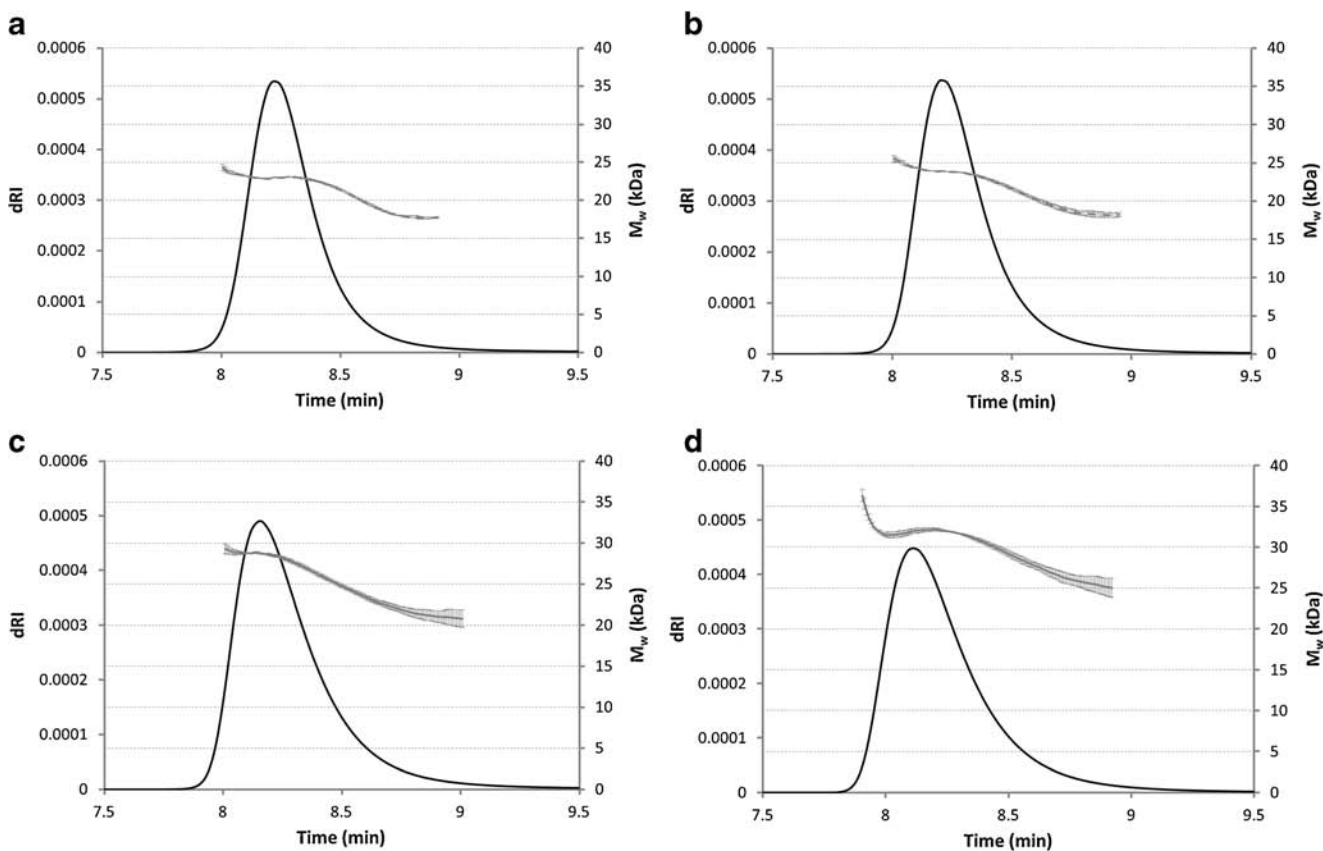


Fig. 4 Average ($n = 3$) SEC elution profiles of the peptide ($M_w \sim 4.5$ kDa) when using no preservatives in the mobile phase (a), or when including 10 mg/ml benzyl alcohol (b), 10 mg/ml phenol (c) or 5 mg/ml m-cresol (d). The solid lines show the signal from dRI detector, whereas the dashed lines represent the molar mass (M_w) calculated from dRI and MALS detectors.

Table I The Numerical Results of SEC Analysis, Including the Onset of Elution [T(onset)], Peak Elution Time [T(max)], Peak Half-Width [W(1/2)], Normalized Peak Height [dRI(max)], Normalized Peak Area and Apparent Molar Mass (M_w) at peak ($n = 3$)

Preservative in mobile phase	T(onset, min)	T(max, min)	W(1/2, min)	dRI (max)	peak area	M_w at peak (kDa)
none	7.88	8.21	0.30	100%	100%	22.9
10 mg/ml benzyl alcohol	7.90	8.20	0.30	100%	102%	23.8
10 mg/ml phenol	7.86	8.15	0.35	91.7%	104%	28.7
5 mg/ml m-cresol	7.83	8.11	0.36	83.7%	101%	31.9

Antimicrobial Efficacy of Peptide/Preservative Formulations

The antimicrobial efficacy testing (AET) was carried out by spiking *E. coli* and *S. aureus* into the sterile formulations and analyzing the remaining fractions of live microbes after 24 h and 72 h incubation period. These time points were used because preliminary tests showed that achieving sufficiently low microbial viabilities was most difficult when the incubation times were relatively short. Low microbe viability after incubation therefore depicts better antimicrobial efficacy for a given formulation. *E. coli* and *S. aureus* were selected because, as confirmed in preliminary testing, these strains were more resistant to the antimicrobial preservatives than the other strains which are accepted in the Ph. Eur. AET scheme (i.e., *P. aeruginosa*, *C. albicans* and *A. brasiliensis*, data not shown). Therefore, using *E. coli* and *S. aureus* in the AET allowed the study to focus on the strains which were most difficult to kill or inactivate. Additionally, *S. aureus* is a gram positive and *E. coli* a gram negative microbial strain, and it was important to check whether their different cell wall structures affected the outcome of the AET results when using different antimicrobial preservatives.

The results of the antimicrobial efficacy test are shown in Table II. As expected, higher preservative concentrations generally resulted in better antimicrobial efficacy (i.e., higher log CFU reduction values). At the same time, the antimicrobial efficacy decreased in most cases when increasing the peptide concentration. This decrease was often significant enough to lower a sample from one Ph. Eur. acceptance criterion class to another (e.g., from level A to level B, or from level B to non-compliance). While at certain preservative concentrations no changes in microbial viability were observed between low and high peptide concentrations (e.g., 8 mg/ml benzyl alcohol), the antimicrobial efficacy of such formulations was so low that it had only a minor effect on microbial viability. Therefore it was difficult to estimate whether the addition of peptide had a negative effect on their (already low) antimicrobial efficacy. The preservatives were not always equally effective against both bacterial strains, and especially benzyl alcohol seemed less effective against *S. aureus* than *E. coli* in this study. The log CFU reductions after 72 h incubation were always higher than or equal to the ones obtained 24 h after inoculation.

To study whether the decrease in antimicrobial efficacy as a function of peptide concentration could be caused by the microbes using the peptide as nutrient for faster replication, selected *E. coli* AET tests were repeated for formulations containing 5 mg/ml phenol and 1–10 mg/ml peptide by also adding 250 mM trehalose in the buffer. Since *E. coli* can utilize trehalose as its sole carbon and energy source (23), such formulations contained excess nutrients and thus minimized the need for the microbe to use the peptide as an energy source. 24 h after inoculating the trehalose-containing formulations with *E. coli*, the log CFU reduction values were 1.2, 0.7 and 0.5 for 1, 6 and 10 mg/ml peptide concentrations, respectively. These values were lower than those of 5 mg/ml phenol formulations without trehalose (Table II), suggesting microbe multiplication was faster in such nutrient-rich (i.e., trehalose-containing) formulations. Still, an increase in peptide concentration was shown to decrease the antimicrobial efficacy of the formulation even in the presence of trehalose.

Comparison Between Peptide-Bound and Sequestered Preservative Fractions

The results of Table II suggest that antimicrobial efficacy of preservatives was lowered by the peptide. This might mean that in such formulations the preservatives exist either as freely diffusing molecules and thus being able to affect microbial viability, or as peptide-bound molecules which are sequestered to the vicinity of peptide oligomers and thus unable to affect microbial viability. To test this hypothesis, the peptide-bound preservative fractions (P_{pep} , calculated from the NMR results using Eqs. 4–6) and sequestered preservative fractions (f_s , calculated from AET results using Eqs. 7–9) were plotted in Fig. 5. This was done to analyze whether the percentage of peptide-bound preservative molecules correlated with the extent of lost antimicrobial efficacy. It should be noted that only the linear regions of f vs. $c(\text{pres})$ curves (Eq. 8) were used in calculating the sequestered preservative fractions (Eq. 9), because at low preservative concentrations there was no observable bacteriocidal effect. On the other hand, when the preservative concentration exceeded C (Eq. 8), all microbes were eliminated and increasing the concentration further had no additional effect. Such preservative concentrations were therefore also excluded.

Table II Antimicrobial Efficacy Testing (AET) Results for *E. coli* and *S. aureus* in the Presence of Different Peptide (C_{pep}) and Preservative (C_{pres}) Concentrations. The Results are Expressed as Logarithmic Colony Forming Unit (log CFU) Reductions and Fractions of Live Microbes (f) After 24 h or 72 h Incubation. The log CFU Columns for 24 h Incubation Time Points Also Include a Marking Whether the Formulations Fulfilled Ph. Eur. level A (Marked with “A”) or level B (Marked with “B”) Acceptance Criteria, or Whether they Failed to Comply with Both (Marked with “F”). The 72 h Incubation Point is not Included in the Ph. Eur. criteria so no Acceptance Criteria Could be Attributed for this Time Point

Benzyl alcohol											
<i>E. coli</i>		log CFU reduction		f		<i>S. aureus</i>		log CFU reduction		f	
C_{pres}	C_{pep}	24 h	72 h	24 h	72 h	C_{pres}	C_{pep}	24 h	72 h	24 h	72 h
8 mg/ml	0 mg/ml	0.3 (F)	1.2	0.50	0.06	8 mg/ml	0 mg/ml	0.0 (F)	1.3	1.00	0.05
	1 mg/ml	0.3 (F)	1.0	0.50	0.10		1 mg/ml	0.0 (F)	0.6	1.00	0.25
	5 mg/ml	0.3 (F)	1.7	0.50	0.02		5 mg/ml	0.0 (F)	1.2	1.00	0.06
	10 mg/ml	0.3 (F)	1.7	0.50	0.02		10 mg/ml	0.1 (F)	1.8	0.79	0.02
10 mg/ml	0 mg/ml	0.8 (F)	3.7	0.16	0.00	10 mg/ml	0 mg/ml	0.1 (F)	3.3	0.79	0.00
	1 mg/ml	0.6 (F)	3.7	0.25	0.00		1 mg/ml	0.0 (F)	2.0	1.00	0.01
	5 mg/ml	0.4 (F)	1.5	0.40	0.03		5 mg/ml	0.3 (F)	2.4	0.50	0.00
	10 mg/ml	0.3 (F)	1.9	0.50	0.01		10 mg/ml	0.2 (F)	1.7	0.63	0.02
11 mg/ml	0 mg/ml	3.8 (A)	3.8	0.00	0.00	11 mg/ml	0 mg/ml	1.7 (B)	3.8	0.02	0.00
	1 mg/ml	1.8 (B)	3.8	0.02	0.00		1 mg/ml	0.8 (F)	3.8	0.16	0.00
	5 mg/ml	0.8 (F)	3.8	0.16	0.00		5 mg/ml	0.6 (F)	2.2	0.25	0.01
	10 mg/ml	0.5 (F)	3.8	0.32	0.00		10 mg/ml	0.6 (F)	1.7	0.25	0.02
12 mg/ml	0 mg/ml	3.7 (A)	3.7	0.00	0.00	12 mg/ml	0 mg/ml	0.8 (F)	3.3	0.16	0.00
	1 mg/ml	3.7 (A)	3.7	0.00	0.00		1 mg/ml	0.1 (F)	2.2	0.79	0.01
	5 mg/ml	3.7 (A)	3.7	0.00	0.00		5 mg/ml	0.9 (F)	3.4	0.13	0.00
	10 mg/ml	2.4 (B)	3.7	0.00	0.00		10 mg/ml	0.7 (F)	3.1	0.20	0.00
Phenol											
<i>E. coli</i>		log CFU reduction		f		<i>S. aureus</i>		log CFU reduction		f	
C_{pres}	C_{pep}	24 h	72 h	24 h	72 h	C_{pres}	C_{pep}	24 h	72 h	24 h	72 h
4 mg/ml	0 mg/ml	1.6 (B)	3.7	0.03	0.00	4 mg/ml	0 mg/ml	0.3 (F)	3.3	0.50	0.00
	1 mg/ml	0.4 (F)	3.4	0.40	0.00		1 mg/ml	0.0 (F)	1.7	1.00	0.02
	5 mg/ml	0.3 (F)	2.3	0.50	0.01		5 mg/ml	0.0 (F)	1.2	1.00	0.06
	10 mg/ml	0.2 (F)	1.5	0.63	0.03		10 mg/ml	0.2 (F)	2.1	0.63	0.01
5 mg/ml	0 mg/ml	3.7 (A)	3.7	0.00	0.00	5 mg/ml	0 mg/ml	2.8 (B)	3.3	0.00	0.00
	1 mg/ml	2.6 (B)	3.7	0.00	0.00		1 mg/ml	1.1 (B)	3.3	0.08	0.00
	5 mg/ml	1.9 (B)	3.7	0.01	0.00		5 mg/ml	0.4 (F)	3.3	0.40	0.00
	10 mg/ml	1.0 (B)	3.7	0.10	0.00		10 mg/ml	0.8 (F)	3.4	0.16	0.00
6 mg/ml	0 mg/ml	3.7 (A)	3.7	0.00	0.00	6 mg/ml	0 mg/ml	3.3 (A)	3.3	0.00	0.00
	1 mg/ml	3.7 (A)	3.7	0.00	0.00		1 mg/ml	2.6 (B)	3.3	0.00	0.00
	5 mg/ml	3.7 (A)	3.7	0.00	0.00		5 mg/ml	3.4 (A)	3.4	0.00	0.00
	10 mg/ml	3.7 (A)	3.7	0.00	0.00		10 mg/ml	2.5 (B)	3.4	0.00	0.00
m-cresol											
<i>E. coli</i>		log CFU reduction		f		<i>S. aureus</i>		log CFU reduction		f	
C_{pres}	C_{pep}	24 h	72 h	24 h	72 h	C_{pres}	C_{pep}	24 h	72 h	24 h	72 h
1.5 mg/ml	0 mg/ml	1.6 (B)	3.8	0.03	0.00	1.5 mg/ml	0 mg/ml	0.5 (F)	1.1	0.32	0.08
	1 mg/ml	0.1 (F)	0.2	0.79	0.63		1 mg/ml	0.2 (F)	0.9	0.63	0.13
	5 mg/ml	0.2 (F)	0.2	0.63	0.63		5 mg/ml	0.3 (F)	0.6	0.50	0.25
	10 mg/ml	0.2 (F)	0.8	0.63	0.16		10 mg/ml	0.2 (F)	0.6	0.63	0.25
2 mg/ml	0 mg/ml	3.8 (A)	3.8	0.00	0.00	2 mg/ml	0 mg/ml	1.6 (B)	3.8	0.03	0.00
	1 mg/ml	0.9 (F)	3.8	0.13	0.00		1 mg/ml	0.2 (F)	2.4	0.63	0.00
	5 mg/ml	0.2 (F)	1.2	0.63	0.06		5 mg/ml	0.2 (F)	0.9	0.63	0.13
	10 mg/ml	0.2 (F)	0.8	0.63	0.16		10 mg/ml	0.4 (F)	0.9	0.40	0.13
2.5 mg/ml	0 mg/ml	3.8 (A)	3.8	0.00	0.00	2.5 mg/ml	0 mg/ml	3.5 (A)	3.8	0.00	0.00
	1 mg/ml	3.8 (A)	3.8	0.00	0.00		1 mg/ml	1.1 (B)	3.8	0.08	0.00

Table II (continued)

3 mg/ml	5 mg/ml	3.8 (A)	3.8	0.00	0.00	5 mg/ml	0.6 (F)	3.8	0.25	0.00	
	10 mg/ml	1.2 (B)	3.8	0.06	0.00	10 mg/ml	0.6 (F)	2.2	0.25	0.01	
	0 mg/ml	3.8 (A)	3.8	0.00	0.00	3 mg/ml	0 mg/ml	3.8 (A)	3.8	0.00	0.00
	1 mg/ml	3.8 (A)	3.8	0.00	0.00	1 mg/ml	3.8 (A)	3.8	0.00	0.00	
	5 mg/ml	3.8 (A)	3.8	0.00	0.00	5 mg/ml	2.2 (B)	3.8	0.01	0.00	
	10 mg/ml	3.8 (A)	3.8	0.00	0.00	10 mg/ml	1.1 (B)	3.8	0.08	0.00	

A reasonably good correlation was observed between the NMR and AET data (Fig. 5), even though the peptide-bound fractions (P_{pep}) measured by NMR were sometimes higher than the sequestered preservative fractions (f_s) measured by AET using *E. coli* and *S. aureus*. More specifically, the largest differences between P_{pep} and f_s values were observed for phenol and m-cresol at 10 mg/ml peptide concentrations. It was unclear whether this was caused by the difficulties in f_s calculation in such samples or because some other phenomenon besides peptide binding affected the antimicrobial efficacy of phenol and m-cresol at high peptide concentrations. Nonetheless, it was evident that lowest bound/sequestered fractions were observed with benzyl alcohol, followed by phenol, while m-cresol displayed highest fractions for a given concentration of peptide.

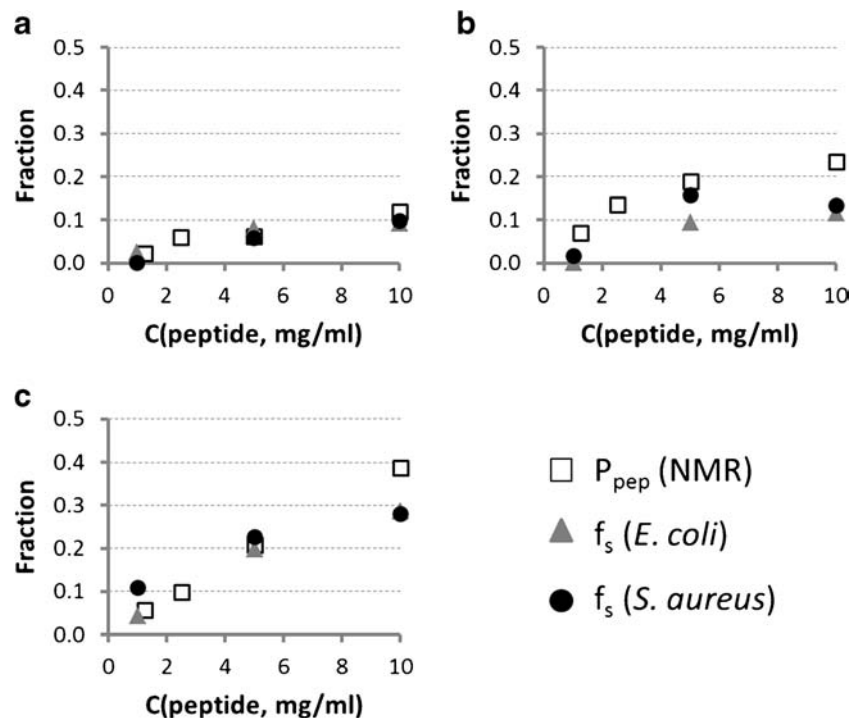
DISCUSSION

The purpose of this study was to explore the effects of three antimicrobial preservatives, benzyl alcohol, m-cresol and phenol, on the oligomerization of a model peptide containing a

covalently-added palmitoyl chain. Furthermore, the antimicrobial efficacy (i.e., the rate of microbial inactivation after inoculation with bacteria) of different preservative- and peptide-containing formulations were compared in order to assess whether peptide-preservative interactions can have an effect on the antimicrobial efficacy of the preservative and thus on bacterial viability.

The three preservatives studied here are commonly used in multidose protein and peptide formulations (4) and their molecular structures resemble each other closely (see Fig. 1), although it should be noted that phenol and m-cresol are classified as phenols and benzyl alcohol as aromatic alcohol. In our study, we observed that the presence of certain preservatives increases the size of the light scattering entities (Figs. 2, 3 and 4). Benzyl alcohol had practically no effect on dynamic (DLS) and static (CG-MALS and SEC-MALS) light scattering results, and only a very small increase in molar mass (both \overline{M}_w and M_w) was observed when including phenol in the peptide solution. On the other hand, a significant size increase in all light scattering studies was observed when m-cresol was used, ultimately leading to visible aggregate formation at C(m-

Fig. 5 Comparison between the fractions of peptide-bound preservative [P_{pep} , C(preservative)=5 mg/ml] calculated from NMR results against the sequestered preservative fractions (f_s) calculated from AET results when incubating either *E. coli* or *S. aureus* with benzyl alcohol (a), phenol (b) and m-cresol (c).



cresol) > 8 mg/ml. No aggregates were observed in SEC analyses with 10 mg/ml benzyl alcohol and phenol, or with 5 mg/ml m-cresol in the mobile phase (Fig. 4). When measured with CG-MALS, the attractive forces between peptide oligomers became stronger at increasing preservative concentrations, as shown in Fig. 3. Still, all the effects observed with light scattering techniques, including changes in \bar{r}_H , M_w , \bar{M}_w and A_2 , were relatively small when benzyl alcohol and phenol were used at 0–10 mg/ml concentrations, and only m-cresol was shown to have an effect which could be linked with clear peptide oligomerization state changes at such concentrations. In order to corroborate the light scattering results, AUC was evaluated as an orthogonal sizing method, but a meaningful interpretation of the peptide signals was not possible due to the strong UV absorption of the preservatives when they were used at similar concentrations as in the DLS, CG-MALS and SEC-MALS experiments (data not shown). Similarly, circular dichroism (CD) was evaluated to study the three-dimensional structure of the peptide in the presence of preservatives, but it could not be effectively employed because the preservatives absorb strongly at far-UV range, and at the near-UV range the peptide CD signal was relatively weak (data not shown).

For the purpose of evaluating the antimicrobial efficacy of peptide/preservative formulations, the Ph. Eur. antimicrobial efficacy test (AET) was used. As expected, higher preservative concentrations generally resulted in better antimicrobial efficacies, but the efficacy was also shown to decrease with increasing peptide concentrations (Table II). Most significant changes in antimicrobial efficacy as a function of peptide concentration were seen with m-cresol, followed by phenol, while least changes were seen when benzyl alcohol was used. This is the same ranking order with which these preservatives have been shown to induce the aggregation of several different proteins (7, 9, 10). However, our study is the first one to propose that the interaction may also lead to the reduction of their antimicrobial efficacy with increasing concentrations of peptide in the same ranking order. This effect was significant, because in many cases it led to non-compliance in the Ph. Eur acceptance criteria (Table II). The decrease in antimicrobial efficacy as a function of peptide concentration was also observed in formulations containing trehalose as additional energy source for *E. coli*. This result indicates that the reduced antibacterial efficacy cannot be explained by the bacteria using the peptide as a carbon and energy source for faster growth. The decrease in antimicrobial efficacy as a function of peptide concentration was generally more clearly seen when using *E. coli* as opposed to *S. aureus*, perhaps because the former has been shown to be more susceptible to the antimicrobial effect of aromatic alcohols (*Sic!*) than the latter (24). Also, pH 7 is not optimal for the antimicrobial efficacy of benzyl alcohol, whereas this is not the case for phenol or m-cresol (4). These may have been possible causes for the relatively low log CFU reduction values for *S. aureus* when using

benzyl alcohol (the only aromatic alcohol in this study) as preservative (Table II). It should also be noted that when the inoculated formulations were incubated for 72 h, the log CFU reduction was always higher than or equal to the ones obtained 24 h after inoculation. This suggests that the number of live microbes continues to decrease after 24 h, making it possible to fulfil the acceptance criteria of the subsequent Ph. Eur. AET time points (7, 14 and 28 days).

Using Eqs. 7–9, the effect of increasing peptide concentration on the sequestered preservative fractions could be compared with the peptide-bound fractions calculated from the NMR data using Eqs. 4–6 (Fig. 5). These datasets show a reasonable correlation, and at 10 mg/ml peptide concentration they also revealed the same ranking order at which the preservatives affected the light scattering signal of peptide assemblies (benzyl alcohol < phenol < m-cresol). This suggests that a given fractions of preservative molecules interact with peptide oligomers, thus becoming sequestered and not being able to diffuse freely in the aqueous solution. This can lead in some cases to an increase in peptide oligomerization state as well as to a decrease in the antimicrobial efficacy of the formulation. The ranking order of preservative molecules' interaction intensities with the peptides (benzyl alcohol < phenol < m-cresol) correlated with their logarithmic octanol/water partition coefficients ($\log P_{O/W}$), which are 1.1, 1.5 and 2.0, respectively (25). Also, it has been previously suggested that aromatic alcohols (*Sic!*) with higher heptane/water partition coefficients tend to display lower minimal concentrations at which they inhibit bacterial proliferation (24). If this trend can also be extended to other classes of molecules, such as phenols, it may mean that more lipophilic preservatives can generally be more effective as antimicrobial agents, at least as long as they remain water-soluble. Indeed, the concentrations (m/V) needed to reach Ph. Eur. antimicrobial efficacy limits for the three preservatives used here decreased in the same order as their $\log P_{O/W}$ values increased (Table II). The flipside of this attribute may be that as their antimicrobial efficacy increases, so does their tendency to interact with peptides and proteins. However, it is unlikely that the intensity of the interaction can be predicted based purely on the hydrophobicity of the preservative molecules, and as mentioned in the Introduction, other intermolecular forces besides hydrophobicity have been suggested to contribute to the interaction (5, 11–13). For example, the propensities of the preservative molecules to form π -interactions and hydrogen bonds would also be different, especially between phenols (i.e., phenol and m-cresol) and aromatic alcohols (i.e., benzyl alcohol).

Furthermore, the peptide structure and assembly might play a role in determining the overall level of interaction. For example, assembling peptide monomers into higher oligomeric structures containing a hydrophobic core might affect their interaction propensity with preservatives. Literature suggests that formulation components containing hydrophobic

cores, such as surfactant micelles, can have a negative effect on the antimicrobial efficacy of preservatives, and the antimicrobial efficacy of methylparahydroxybenzoate has been shown to be weaker in the presence of polysorbate 80 (26). This effect has been attributed to preservative – surfactant interactions, which have been proposed to take place both at the polyoxyethylene - hydrocarbon junction and at the non-polar core of surfactant micelles (27). A comparable interaction mechanism could account for the decrease in antimicrobial efficacy of preservatives in the presence of palmitoylated peptides which, like surfactants, are also relatively amphiphilic molecules. Since acylation has been shown to affect the strength at which peptides bind to hydrophobic surfaces (28), it is likely that it could also affect peptide/preservative interactions. The primary sequence of the peptide is another factor which might play a role in the determination of the overall interaction intensity. It has been suggested that polyphenols interact preferentially with amino acid residues with heterocyclic or aromatic side groups, such as proline and phenylalanine, although the conformation of the peptide dictates whether the reactive moieties of these residues are exposed or buried (29, 30). Since 10 amino acid residues out of 40 in the peptide primary sequence were either heterocyclic or aromatic (in this case proline, phenylalanine, tyrosine and tryptophan), and since its C-terminus was acylated with palmitoyl, the peptide in question may be especially prone to interact with preservatives. Nonetheless, the abundance of polypeptide-structured macromolecules (e.g., 5, 7–9) which have been shown to interact with different antimicrobial preservatives suggests that the binding can be unspecific by nature and should be taken into account regardless of the primary structure of the polypeptide in question.

CONCLUSIONS

Antimicrobial preservatives were shown to interact with an acylated peptide with an intensity which increases in the order benzyl alcohol < phenol < m-cresol. The interaction affected the size of the peptide oligomers as well as the mobility of the preservatives, especially in the case of m-cresol. Furthermore, the antimicrobial efficacy of all preservatives was weakened by the presence of the peptide, and this effect could be correlated with the molecular mobility of the preservatives, suggesting that the reduced mobility of a peptide bound state of the preservatives makes them less effective against microbes. Although the interaction intensity correlates with the overall lipophilicity of antimicrobial preservatives, its molecular mechanism has not been fully elucidated, evoking the need for further studies in this field. Still, taking into account this study and the literature already available on protein-preservative interactions, the intensity with which benzyl alcohol (aromatic alcohol) interacts with polypeptides may often

be weaker than that of phenol or m-cresol (phenols). This is an important point to consider when formulating proteins or peptides which have a strong attractive self-interaction potential, making benzyl alcohol an attractive preservative option in such formulations. On the other hand, since the antimicrobial efficacy of preservatives can decrease at high peptide concentration formulations, their concentration subsequently needs to be higher in order to fulfill the antimicrobial efficacy requirements of pharmacopoeias. Consequently, this may subject patients to larger preservative doses. This can be a problem for example in formulations containing benzyl alcohol, since its use should be limited in pediatric patient groups and a higher dose might lead to a higher prevalence of potentially fatal side-effects in neonates. Understanding and possibly controlling the interaction is therefore of paramount importance.

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