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Physicochemical Properties and Antimicrobial Efficacy of Electrostatic Complexes Based on Cationic ε-Polylysine and Anionic Pectin

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ABSTRACT: ε-Polylysine (ε-PL) is a food-grade cationic antimicrobial that is highly effective against a wide range of food pathogens and spoilage organisms. However, its application within foods and beverages is currently limited because of its tendency to associate with anionic substances, thereby increasing product turbidity or forming sediments. In this study, we examined a potential means of overcoming these problems by forming electrostatic complexes between cationic ε-PL and anionic pectin. The nature of the complexes formed depended on the mass ratio of pectin to ε-PL (R_{P-PL}), since this determined their electrical characteristics, aggregation stability, and antimicrobial efficacy. The electrical charge on the complexes went from positive to negative with increasing R_{P-PL}, with the point of zero charge being around $R_{P-PL} \sim 8$. Soluble complexes or stable colloidal dispersions were formed at low and high R_{P-PL} levels, but insoluble complexes were formed at intermediate levels (i.e., $4 \leq R_{P-PL} \leq 16$). The complexes maintained good antimicrobial activity (minimum inhibitory concentration of ε-PL < 10 μg/mL) at $R_{P-PL} \leq 20$ against two acid resistant spoilage yeasts: *Zygosaccharomyces bailli* and *Saccharomyces cerevisiae*. Finally, we showed that certain ε-PL−pectin complexes (10 μg/mL ε-PL; $R_{P-PL} \geq 2$) could be incorporated into green tea beverages without adversely affecting their appearance or physical stability. This work has shown that the function of a cationic antimicrobial agent (ε-polylysine) can be improved by incorporating it within electrostatic complexes using a food-grade anionic biopolymer (pectin).

KEYWORDS: ε-polylysine, pectin, electrostatic complex, antifungals, antimicrobials, yeast, *Zygosaccharomyces bailli*, *Saccharomyces cerevisiae*, beverages

INTRODUCTION

ε-Polylysine (ε-PL) is a food-grade cationic antimicrobial of great interest to the food and beverage industry because of its strong antimicrobial activity. ε-PL was discovered in culture filtrates of *Streptomyces albulus* ssp. *lysinopolymerus* strain 346.[1] The compound is now produced industrially by aerobic fermentation, using a mutant derived from strain 346 that exhibits increased production of ε-PL.[2−4] Chemically, ε-PL is a homopolymer consisting of L-lysine monomers (typically between 25 and 35) linked together by isopeptide bonds between ε-amino and α-carboxyl groups.[1,3,5] A number of studies have shown that ε-PL is highly effective against a broad spectrum of food pathogens and spoilage organisms, and thus has great potential for utilization in food and beverage products.[3,6−10] Based on absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies, ε-PL has been shown to be safe for human consumption.[11] For this reason, it has been approved as generally recognized as safe (GRAS) within the United States for certain food applications (GRAS No. 000135).[12]

Because of its high antimicrobial activity, nontoxicity, water-solubility, and good thermal stability, ε-polylysine has the potential to be a highly effective ingredient for controlling or preventing microbial growth in food products. ε-PL is cationic at pH values below its isoelectric point (p$I \approx 9.0$) because of the presence of primary amine groups along its backbone.[3] The antimicrobial ability of ε-PL is highly dependent on its cationic nature, because it is thought to absorb onto negatively charged microorganisms through electrostatic interactions where it promotes cell membrane disruption.[9] On the other hand, the cationic nature of ε-PL may cause problems when it is incorporated into some food and beverage systems because it may form undesirable precipitates if it interacts with anionic components within the food matrix, leading to an increase in product cloudiness or to the formation of sediments. In addition, precipitate formation may reduce the antimicrobial activity of ε-PL. Therefore, both the beneficial attributes (antimicrobial) and detrimental attributes (precipitate formation) of ε-PL are likely to depend on its electrical characteristics. There is thus a need to control the electrical attributes of ε-PL to reach a balance between high antimicrobial efficacy and good aggregation stability.

A potential solution to this problem is the creation of antimicrobial delivery systems consisting of electrostatic complexes of cationic ε-PL and anionic biopolymers.[13,14] The electrical characteristics of these complexes can be controlled by varying the nature and ratio of the biopolymers used to form them. If these electrostatic complexes are going to be utilized within the food industry, it is important to establish conditions where physically stable complexes can be formed that still maintain their antimicrobial efficacy. The purpose of our study was to systematically examine the influence of complex composition on the physicochemical properties and antimicrobial activity of ε-PL−pectin complexes. Pectin was selected because it is an anionic polysaccharide that is readily available and widely used within the food and beverage industries. Chemically, pectin is a heteropolysaccharide of partially esterified α-1,4 linked D-galacturonides, containing varying amounts of covalently attached rhamnose

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and branches of L-arabinose, D-galactose, D-xylose, and L-rhamnose.[15] It has carboxylic acid side groups that are negatively charged at pH values around and above their pK_a value (p$K_a \approx 3.5$).[16]

Our previous work showed that cationic ε-PL can interact with anionic pectin and form either soluble or insoluble electrostatic complexes depending on the total concentration of pectin and the mass ratio of pectin to ε-PL (R_{P-PL}), as well as environmental factors such as pH and ionic strength.[14] In this study, we formed ε-PL−pectin electrostatic complexes with different charge characteristics by varying the ratio of high methoxyl pectin (HMP) to ε-polylysine (ε-PL). We then determined the influence of charge characteristics on the aggregation stability and antifungal activity of these electrostatic complexes. Finally, we incorporated these complexes into a commercial green tea beverage to test their aggregation stability in a real food system. The information generated in this study will be useful for the rational design of effective antimicrobial delivery systems based on ε-PL−pectin electrostatic complexes.

MATERIALS AND METHODS

Materials. The cationic antimicrobial ε-polylysine (PuraQ Xtend FX50P; lot #: 1091102) was provided by Purac America (Lincolnshire, IL). High methoxyl pectin (HMP) with a degree of esterification (DE) of approximately 71% (Pectin 1400) was provided by TIC Gums (Belcamp, MD). The manufacturer reported that the pectin in this ingredient had an average molecular weight of 170 kDa and contained no sugar additives. Difco malt extract agar (MEA) and malt extract broth (MEB) from Becton Dickinson (Sparks, MD) were used as media to grow yeast cells. Lipton green tea with citrus (ready to drink) was purchased from local stores to examine the influence of ε-PL−pectin complexes on the appearance of commercial tea beverages.

Solution Preparation. Stock solutions of ε-polylysine (1.0 w/v %, pH 3.5) and pectin (0.5 w/v %, pH 3.5) were prepared by dispersing appropriate amounts of powdered ingredients into double distilled water, followed by pH adjustment by addition of HCl or NaOH solution. The stock solutions were then filter sterilized (0.45 μm pore size; Corning Incorporated, Corning, NY) and kept refrigerated until used.

Turbidity, Electrical Charge and Size Measurements. Appropriate volumes of ε-polylysine, pectin stock solutions, and sterile water (adjusted to pH 3.5) were mixed to create complexes with different pectin-to-PL mass ratios (R_{P-PL}). The concentration of ε-PL was fixed at 100 μg/mL, and the pectin concentrations were varied (R_{P-PL} = 0.25, 0.5, 1, 2, 4, 6, 8, 12, 16, 20, 24, and 32). The resulting ε-PL−pectin complex solutions were then mixed thoroughly and stored for 24 h prior to analysis. Before measurements, the complex solutions were vortexed to ensure the complexes were evenly distributed in the solution.

The optical turbidity (at 600 nm) of the ε-PL and ε-PL−pectin complexes solutions was measured using a UV−visible spectrophotometer (Ultraspec 2000, Pharmacia Biotech) at ambient temperature (≈23 °C). The samples were contained within 1 cm path length optical cells, and distilled water was used as a control. Turbidity measurements were carried out on at least two freshly prepared samples.

The electrical charges (ζ-potentials) and mean diameter (Z-average) of the complexes in the solutions were then measured using a commercial instrument capable of electrophoresis and dynamic light scattering measurements (Zetasizer Nano-ZS, model ZEN3600, Malvern Instruments, Worchester, U.K.). The ζ-potential was calculated from measurements of the electrophoretic mobility of particles in an applied oscillating electric field using laser Doppler velocimetry. The mean diameter of the complexes was calculated from their Brownian motion via the Stokes−Einstein equation. All measurements were conducted on at least two freshly prepared samples and repeated three times per sample.

Yeast Strains. Two strains of acid resistant spoilage yeasts, *Zygosaccharomyces bailli* (ZB) and *Saccharomyces cerevisiae* (SC), were used to examine the antimicrobial effects of ε-PL−pectin complexes. Both strains were obtained from the Pepsico R&D Culture Collection (Valhalla, NY). Yeast cultures were kept frozen at −70 °C in 25% glycerol. The yeast strains were refreshed on MEA plates before the following tests of MICs. A single yeast colony from the plate was then inoculated into 10 mL of MEB broth, which was adjusted to pH 3.5 by citrate buffer (10 mM in final media). The culture was incubated at 25 °C under mild agitation (150 rpm in a rotary shaker) for 2 to 3 days until the optical density (turbidity) at 600 nm (OD_{600}) was around 1.0 cm^{-1}. As a guideline, an OD_{600} of 1.0 cm^{-1} corresponds to approximately 5×10^6 CFU/mL for cultures of yeast strains.

Determination of the Minimum Inhibitory Concentration (MIC) of ε-Polylysine−Pectin Complexes. The ε-PL−pectin complexes were prepared as described previously. The antimicrobial effectiveness of ε-PL−pectin complexes was then determined by calculating the minimum inhibitory concentration (MIC) using a microbroth dilution assay against the two yeast strains. Briefly, sterile microtiter plates were inoculated with ε-PL−pectin complex solutions (100 μL) of varying concentrations, and an equal volume of inoculated 2× MEB broth (preadjusted to pH 3.5 using 20 mM citrate buffer) was added and then mixed thoroughly. The resulting 1× MEB media contained 10 mM citrate buffer. The target yeast cell inoculation level was around 5×10^4 CFU/mL in the final 1× MEB medium. For each microtiter plate, a specific ε-PL−pectin complex was tested, which had a specific pectin-to-ε-PL ratio (either of 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 16, 24, or 32) and its ε-PL concentration ranged from 100 to 1.6 μg/mL (100, 50, 25, 12.5, 6.4, 3.2, 1.6 μg/mL). Plates were incubated at 25 °C for 6 days, and the MICs were assessed visually as the lowest concentration of ε-PL showing complete inhibition of growth. All MIC experiments were repeated twice with freshly prepared materials.

Surface Charge (ζ-Potential) Measurements of Yeast Cells. The surface charge (ζ-potential) of yeast cells (ZB and SC) was measured using the particle electrophoresis instrument (Zetasizer Nano-ZS) mentioned above. Briefly, freshly grown yeast cells in MEB broth (pH 3.5) were collected and washed twice by sterile citrate buffer (10 mM; pH 3.5), and were then suspended in an appropriate volume of sterile citrate buffer to achieve an $OD_{600\ nm}$ of around 0.5 cm^{-1}. The ζ-potential of the resulting yeast cell suspensions was then measured at room temperature. Each measurement was conducted in duplicate by collecting freshly grown yeasts.

Influence of ε-PL−Pectin Complexes on Stability of Tea Beverages. 250 μg/mL of ε-polylysine was combined with different ratios of pectin (R_{P-PL} = 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 16, 20, 24, and 32; pH 3.5), was vortexed to mix evenly, and then stood for at least 1 h to enable complex formation. A non-food grade antimicrobial (0.04% sodium azide) was added to the green tea samples to ensure that the influence of the ε-PL−pectin complexes on the physical appearance of the products was not influenced by any microbial growth. Appropriate amounts of ε-PL−pectin solutions were added to 40 mL of green tea (pH 3.6), to obtain a final ε-PL concentration of 10 μg/mL. After vortexing for about 1 min to mix thoroughly, the resulting solutions were stored at room temperature. The turbidity of the resulting solutions over time was measured at 600 nm using a spectrophotometer in 1 cm path length optical cells using distilled water as a blank. The formation of precipitates or sediments was also recorded by visual observation and digital photography.

RESULTS AND DISCUSSION

The overall purpose of our experiments was to characterize the physicochemical and antifungal properties of ε-PL−pectin complexes with different compositions, so as to establish whether

effective ε-polylysine delivery systems could be developed. The physicochemical properties of the ε-PL−pectin complexes were characterized using electrical charge, turbidity, and visual appearance measurements. The antifungal efficacy of each ε-PL−pectin complex was then determined by measuring their MIC against two representative yeast strains: *Zygosaccharomyces bailli* (ZB) and *Saccharomyces cerevisiae* (SC). Finally, the potential application of ε-PL−pectin complexes was established by incorporating them into a commercial green tea beverage to determine whether they influenced product appearance and stability.

Aggregation Characteristics of ε-Polylysine−Pectin Complexes. The stability of ε-PL−pectin complexes to aggregation was characterized using turbidity measurements and visual observation. Small soluble complexes do not scatter light strongly and so lead to the formation of clear solutions with low turbidity, whereas large insoluble complexes scatter light strongly and so lead to the formation of cloudy colloidal suspensions with high turbidity and possibly to sedimentation. A series of solutions was prepared containing fixed ε-PL contents (100, 50, 25, 12.5, or 6.25 μg/mL) and varying amounts of pectin (R_{P-PL} from 0 to 32), and then the turbidity and visual appearance of the solutions (pH 3.5) were measured. For the turbidity measurements, the samples were mixed thoroughly prior to analysis to ensure that any complexes were evenly distributed throughout the entire sample. For visual observation, the samples were stored at ambient temperature for 24 h to determine the stability of the complexes to gravitational separation.

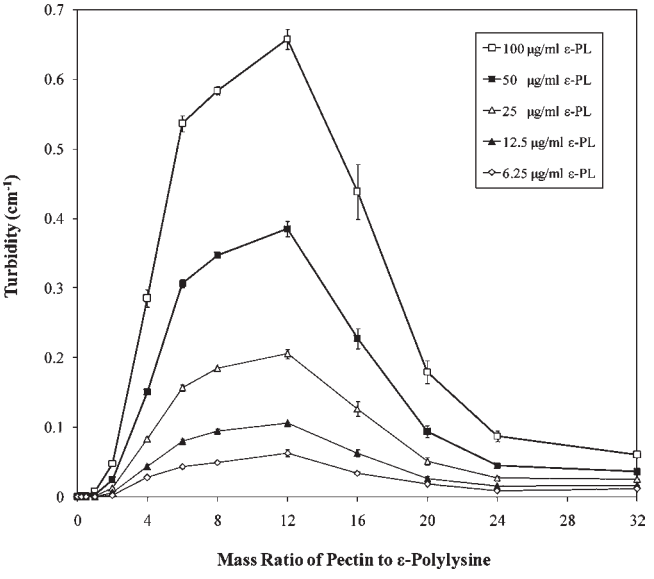

Figure 1. Dependence of the turbidity (at 600 nm) of ε-PL−pectin complexes aqueous solutions on mass ratios of pectin to ε-PL for different concentration of ε-PL. ε-PL molecules (100−6.25 μg/mL) were complexed by pectin molecules with varying ratios of pectin to ε-PL (0.25−32) at pH 3.5.

For the ε-PL−pectin complex solutions containing 100 μg/mL ε-PL, the turbidity (OD_{600}) increased slowly when R_{P-PL} was increased from 0 to 2, but the values were very low (<0.05 cm^{-1}) (Figure 1). This suggested that the size of the ε-PL−pectin complexes formed was so small that they did not scatter light strongly. The turbidity then increased steeply when R_{P-PL} increased from 2 to 12, suggesting that large insoluble complexes were formed that scattered light strongly. Finally, the turbidity progressively decreased as R_{P-PL} was increased further, suggesting that the size of the complexes decreased. Visual observation of the solutions (Figure 2) indicated that they were transparent at low pectin concentrations ($R_{P-PL} < 4$), formed turbid colloidal suspensions or white sediments at intermediate pectin concentrations ($4 \leq R_{P-PL} \leq 16$), and formed relatively stable colloidal dispersions at high pectin concentrations ($R_{P-PL} \geq 20$).

A similar type of general behavior was observed when lower levels of ε-PL were present: with increasing pectin concentration, the turbidity initially increased until it reached a maximum value, and then decreased (Figure 1). Nevertheless, the magnitudes of the turbidity values decreased as the concentration of ε-PL in the systems decreased, which can be mainly attributed to a dilution effect, i.e., fewer particles present to scatter light. At ≤12.5 μg/mL ε-PL, all the ε-PL−pectin complexes solutions remained transparent and stable to aggregation (maximal OD_{600} < 0.1 cm^{-1}) (Figure 1), irrespective of pectin concentration. This result suggests that complexes containing low ε-polylysine concentrations may be suitable for application within clear acidic beverages.

Electrical Characteristics of ε-Polylysine−Pectin Complexes. The electrical characteristics (ζ-potential) of ε-PL−pectin complexes (100 μg/mL ε-PL with varying R_{P-PL}; pH 3.5) were measured by particle electrophoresis to obtain more information about the nature of the interactions involved (Figure 3). When ε-PL was combined with low concentrations of pectin ($R_{P-PL} \leq 1$), the charge of the resulting complexes was highly positive ($\zeta \approx +48$ mV). As the pectin concentration was increased further, the ζ-potential went from positive to negative (Figure 3). These measurements showed that ε-PL−pectin complexes were positively charged at low R_{P-PL}, but negatively charged at high R_{P-PL}, with charge neutralization occurring at

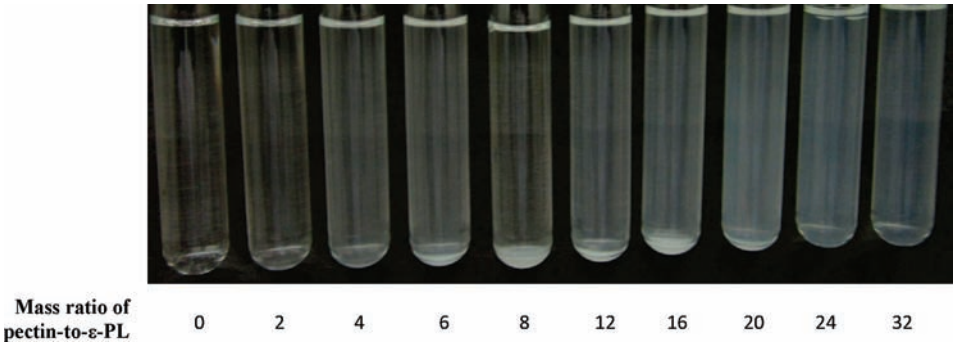

Figure 2. Visual appearance of aqueous solutions containing ε-PL−pectin complexes (pH 3.5) composed with 100 μg/mL of ε-PL and pectin with varying mass ratios of pectin to ε-PL as indicated.

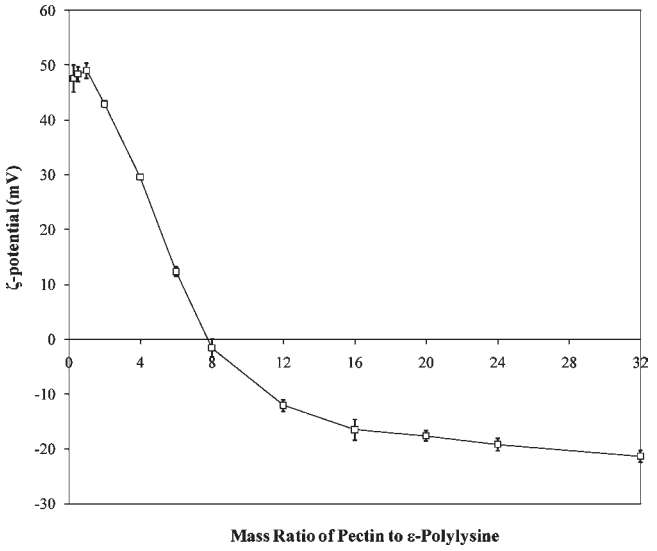

Figure 3. Dependence of the ζ-potential of ε-PL−pectin complex solutions on the mass ratio of pectin to ε-PL for 100 μg/mL of ε-PL (pH 3.5).

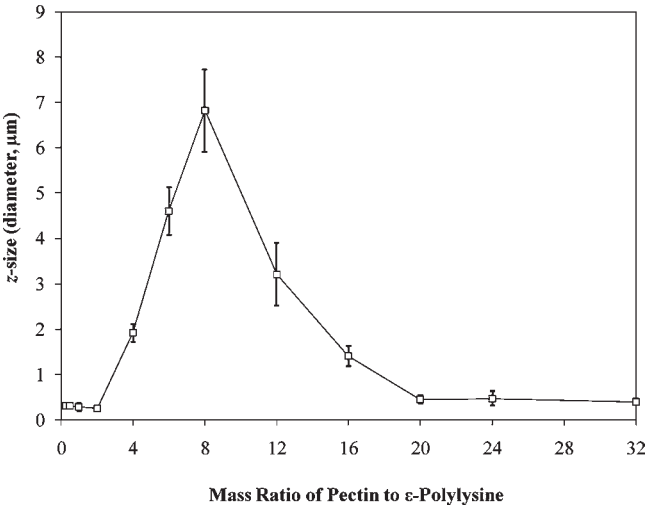

Figure 4. Dependence of the particle size (*Z*-average diameter) of ε-PL−pectin complexes on the mass ratio of pectin to ε-PL for 100 μg/mL of ε-PL (pH 3.5).

$R_{P-PL} \sim 8$. We propose that electrostatic repulsion plays an important role in maintaining the stability of these ε-PL−pectin complexes. The complexes were stable when their net charges were either highly positive or highly negative; otherwise they became turbid and/or precipitated and formed sediments at the bottom of the tubes (Figure 2). Extensive biopolymer aggregation may have occurred due to charge neutralization or ionic bridging effects.

Knowledge of the electrical characteristics of the complexes has important consequences for understanding the physicochemical properties and functional behavior of ε-PL−pectin complexes in food and beverage applications. The formation of anionic complexes may alter the tendency for ε-polylysine to bind to the negative surfaces of bacteria, which could reduce its antimicrobial efficacy. On the other hand, the formation of anionic complexes might reduce the undesirable tendency for ε-polylysine to bind to anionic biopolymers in human saliva (causing astringency) or to bind to anionic ingredients in foods (causing precipitation).

Size Characteristics of ε-Polylysine−Pectin Complexes. Dynamic light scattering (DLS) was used to measure the mean diameter of ε-polylysine−pectin complexes. DLS measurements indicated that the size of the complexes remained relatively small and constant (*Z*-diameter ≈300 nm) when the mass ratio of pectin to ε-polylysine (R_{P-PL}) increased from 0.25 to 2 (Figure 4). This was probably because ε-polylysine−pectin complexes were highly positively charged (ζ > +42 mV) (Figure 3) so they electrostatically repelled each other thereby inhibiting aggregation. When R_{P-PL} was further increased to 4−16, the size of the complexes increased sharply (>1.4 μm), indicating the formation of large aggregates (Figure 4). The formation of large aggregates was probably due to the decreased net electrical charge of the ε-polylysine−pectin complexes (Figure 3), leading to aggregation through charge neutralization or ionic bridging effects. When the R_{P-PL} was further increased to 20 or higher, the solution turned transparent or only slightly turbid (Figure 1 and Figure 2), and the size of complexes decreased to around 400 nm (Figure 4). This was probably the result of increased negative charges on the complexes (Figure 3) increasing the electrostatic repulsion between them.

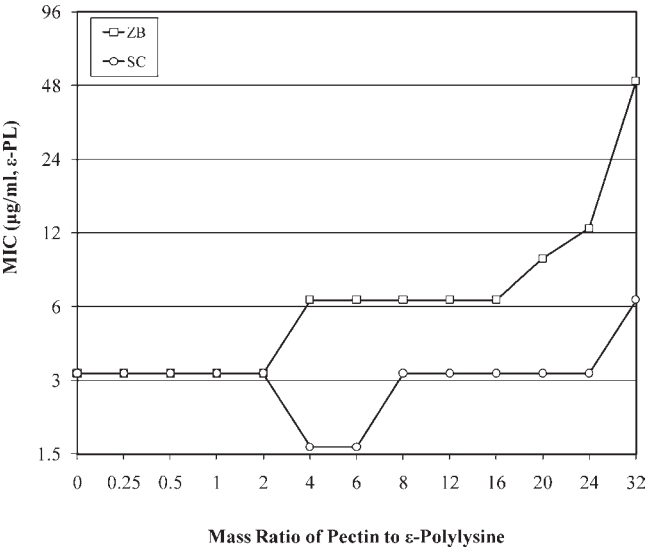

Figure 5. Dependence of the minimal inhibitory concentration (MIC) of ε-PL on ε-PL−pectin complexes with varying mass ratios of pectin to ε-PL. The determination of MICs were performed in a nutrient MEB medium (pH 3.5) against two acidic resistant yeasts, *Zygosaccharomyces bailli* (ZB) and *Saccharomyces cerevisiae* (SC).

Antifungal Efficacy of ε-Polylysine−Pectin Complexes. We determined the antimicrobial effects of different ε-PL−pectin complexes by measuring their minimum inhibitory concentration (MIC) in a nutrient MEB medium (pH 3.5), against two yeast strains: *Zygosaccharomyces bailli* (ZB) and *Saccharomyces cerevisiae* (SC). These two yeasts were selected as target organisms, because they are acid resistant and may cause spoilage in acidic beverages.[17,18]

In the absence of pectin, ε-polylysine was found to be highly effective at inhibiting the growth of both yeast strains, with a MIC of 3.2 μg/mL (Figure 5). The antimicrobial efficacy of cationic polymers has previously been attributed to their ability to interact with and disrupt anionic cell membranes, thereby leading to cell

leakage.[9,19] Generally, the antimicrobial efficacy of ε-polylysine decreased in the presence of increasing levels of pectin, as indicated by increasing MIC with increasing R_{P-PL} (Figure 5). For ZB, the MIC of ε-PL increased from 3.2 to 6.4 μg/mL when ε-PL was complexed with between 4 and 16 times of pectin. The MIC of ε-PL was further increased to 9.6, 12.5, and 50 μg/mL when ε-PL was complexed with 20, 24, and 32 times of pectin, respectively. For SC, the MIC of ε-PL increased from 3.2 to 6.4 μg/mL when ε-PL was complexed with 32 times of pectin. The observed decrease in antimicrobial activity may be attributed to the reduction in the positive change and then increase in the negative charge on the complexes with increasing pectin levels (Figure 3), which would be expected to reduce the electrostatic attraction and then increase the electrostatic repulsion between ε-PL−pectin complexes and negatively charged yeast cell surfaces.

Interestingly, we found that the complexes still had relatively high antifungal efficacy even when their net charge was neutral or negative (Figures 3 and 5). This could be due to either of the following: (i) the cationic ε-PL molecules were at least partially released from the complexes because of a competition between the anionic pectin and anionic yeast cell surfaces; or (ii) even though the complexes as a whole were negative, there were some localized parts that were positively charged, which could be attracted to the negatively charged yeast cell surfaces.

In general, increased levels of pectin tended to reduce the antifungal efficacy of ε-PL for both ZB and SC, however this effect was strain specific. SC was much less sensitive to increased levels of pectin. For SC, the MIC of ε-PL did not increase when ε-PL was complexed with ≤24 times of pectin. Even when ε-PL was complexed with 32 times of pectin, its MIC only increased from 3.2 to 6.4 μg/mL. This phenomenon could result from differences in the surface charges of the two yeasts. We observed the morphology of the two yeasts using differential interference contrast (DIC) optical microscopy, and found that their shape and size were very similar to each other (data not shown). However, by measuring the ζ-potential of the yeast cells, we found that their surface charges were quite different: -15.6 ± 1.8 mV (SC) versus -9.2 ± 1.1 mV (ZB). The cell surface of SC had a higher negative charge, and may therefore have been able to attract ε-PL−pectin complexes more strongly. It is also possible that the surface microstructures differed between the two yeasts, resulting in different steric repulsion.

Another interesting finding was that ε-PL−pectin complexes had better antifungal activity than pure ε-PL against SC at $R_{P-PL} = 4$ or 6 (Figure 5). The physicochemical origin of this effect is currently unknown. At these R_{P-PL} values the net charge on the complexes was slightly positive (Figure 3), which suggests that they could still be electrostatically attracted to the anionic surfaces of the yeast cells.

Although the antimicrobial efficacy of ε-polylysine was decreased with increasing amounts of pectin, the ε-PL−pectin complexes were still highly effective against both yeast strains tested. When complexed with ≤16 times of pectin, relatively low levels of ε-PL (6.4 μg/mL) were still able to inhibit the growth of both yeasts, suggesting that these complexes could be utilized in food and beverage systems. The net charge of ε-PL−pectin complexes was negative for $R_{P-PL} > 8$, while their antimicrobial efficacies were still strong for R_{P-PL} between 8 and 20 (i.e., MIC < 10 μg/mL for both ZB and SC). This result suggests that ε-PL−pectin complexes with $8 \leq R_{P-PL} \leq 20$ may be able to solve the problems of ingredient precipitation and astringency (because they are anionic), while still maintaining favorable antimicrobial activity.

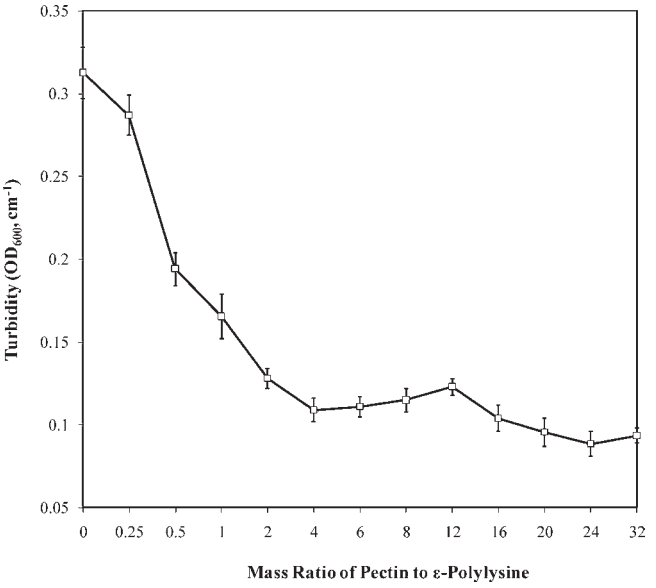

Figure 6. Dependence of the turbidity (at 600 nm) of green tea beverages on presence of ε-PL−pectin complexes with varying mass ratios of pectin to ε-PL (0−32). ε-PL molecules (10 μg/mL) were complexed by different levels of pectin as indicated. The optical densities were measured after 2 weeks storage at room temperature. As a control, the original green tea beverage had a turbidity of around 0.066 cm^{-1} after storage under same conditions.

Influence of ε-PL−Pectin Complexes in a Green Tea Beverage Model. Finally, we tested the potential for incorporating ε-PL−pectin complexes into commercial products. A series of ε-PL−pectin complexes with different R_{P-PL} were incorporated into a commercial green tea beverage to test their impact on product turbidity and sediment formation. We hypothesized that, if the complexes adversely affected the appearance or stability of a commercial product, then they would be unlikely to be utilized in practice.

We observed an increase in turbidity and sediment formation in the tea samples when ε-PL (10 μg/mL) was added, which suggested that there were some components within the tea that could interact with the cationic ε-polylysine and form large aggregates (Figures 6 and 7). Indeed, the use of as little as 1 μg/mL ε-PL caused the formation of visible precipitates in the green tea beverage after storage for only four days (data not shown), indicating that ε-polylysine could not be directly applied into these products. However, the use of ε-PL−pectin complexes led to appreciable improvements in the stability of the products to cloudiness and sedimentation. There was an appreciable decrease in the turbidity of the samples and a reduction in the amount of sediment formed as the amount of pectin in the complexes was increased (Figures 6 and 7). For $R_{P-PL} \geq 2$, the turbidity remained low, the samples appeared clear, and no sediments were visible after 2 weeks storage (Figures 6 and 7). These results indicate that ε-polylysine can be incorporated into tea beverages, provided it is first complexed with a sufficient amount of an anionic biopolymer. The ε-PL−pectin complexes therefore have a weaker tendency to interact with any anionic components within the green tea than pure ε-PL, presumably because there are fewer cationic groups present to react. The nature of the components in the green tea that interact with ε-polylysine and form precipitates is currently unknown. They could

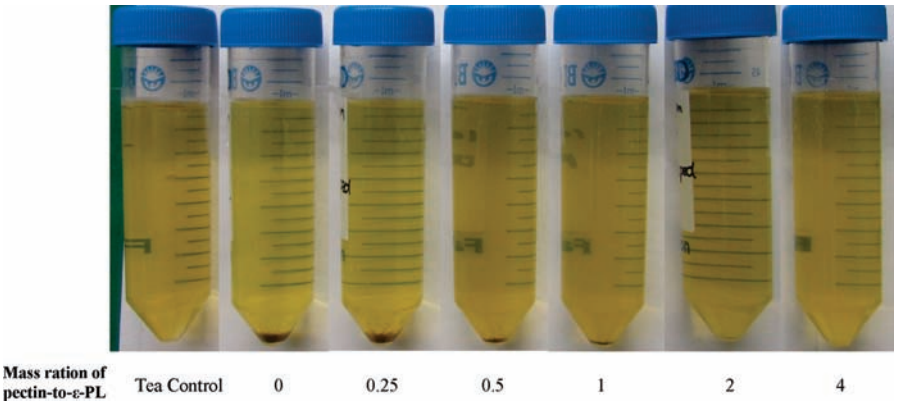

Figure 7. Visual appearance of green tea beverages containing representative ε-PL−pectin complexes (pH 3.5) composed of 10 μg/mL of ε-PL and varying levels of pectin with the mass ratios of pectin to ε-PL indicated. Please note the formation of a dark sediment in the bottom of some samples.

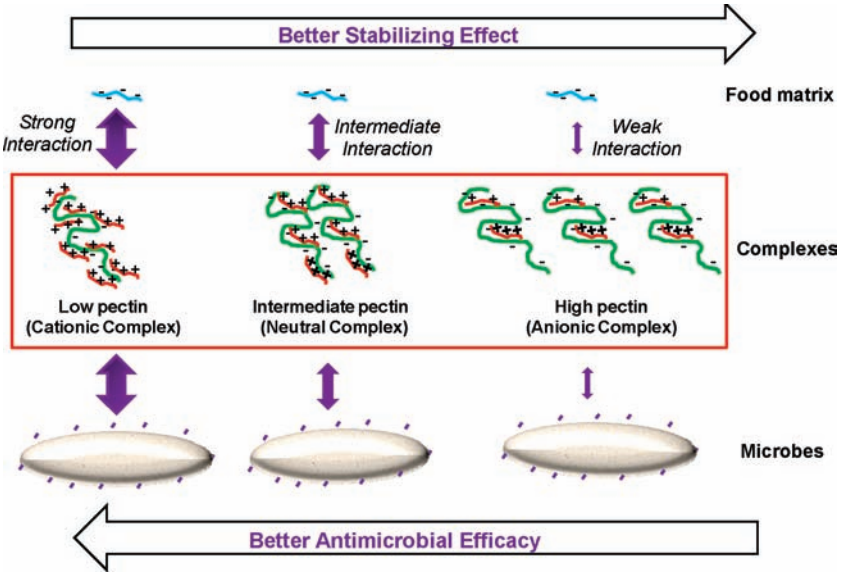

Figure 8. Schematic diagram of interaction of ε-polylysine−pectin complexes with microbes and anionic substances in a food matrix. Complexes of different electrical characteristics were formed by complexing ε-polylysine (red) with varying ratios of pectin (green). As the concentration of pectin increases, there should be less interaction of the complexes with anionic components in the food matrix, but also less interaction with microbe surfaces.

be anionic species (such as polysaccharides or minerals) or other types of molecular species, such as polyphenols. Further work is required to identify the molecular origin and physicochemical basis of these interactions.

In terms of physical stability, the higher the levels of pectin present in the ε-PL−pectin complexes, the better the stability to cloudiness or precipitation. On the other hand, in terms of antimicrobial effects, the lower the levels of pectin present in the complexes, the higher the antimicrobial efficacy (Figure 5). These two effects therefore oppose each other, as indicated schematically in Figure 8. These results suggest that ε-PL−pectin complexes should be specifically designed to obtain an optimum balance between these two opposing effects. Our results suggest that ε-PL−pectin complexes containing 10 μg/mL ε-PL and R_{P-PL} from 2 to 20 have good physical stability in a green tea model, while still demonstrating appreciable antifungal efficacy against two acid resistant yeasts in MEB medium.

We did not test the antifungal efficacy of ε-PL−pectin complexes in the green tea beverage, because this commercially available product already contained antimicrobial preservatives, making the performance of antifungal experiments impractical. Nevertheless, we have good reason to believe that ε-PL−pectin complexes would exhibit good antimicrobial activity in commercial tea beverages. The complexes were highly effective at inhibiting microbial growth in the MEB medium that contained a nutrient composition specifically designed to promote yeast growth. The complexes would therefore be expected to be even more effective in a green tea beverage which contains fewer nutrients especially lacking of proteins. Indeed, it has previously been reported that the antimicrobial activity of ε-PL in food extracts was better than that in nutrient culture media, and the antimicrobial activity of ε-PL was found to be more pronounced in food extracts that contained low protein levels.[7] In practice, it will be important to test the antimicrobial efficacy of ε-PL−pectin complexes under the specific conditions pertaining to the particular commercial product that they are to be incorporated into (e.g., pH, ionic composition, biopolymer composition, and storage temperature).

CONCLUSIONS

This study has shown that antimicrobial delivery systems can be fabricated by electrostatic complexation of cationic ε-polylysine and anionic pectin. The electrical charge, aggregation stability, and antifungal activity of the complexes depended on the ε-PL concentration and ratio of pectin to ε-PL (R_{P-PL}) in the complexes:

(1) At relatively low ε-PL concentrations, transparent solutions could be formed at all pectin-to-ε-PL ratios. On the other hand, at relatively high ε-PL concentrations the stability and appearance of the biopolymer solutions depended on the pectin-to-ε-PL ratio: at low R_{P-PL}, small cationic complexes were formed that gave transparent solutions; at intermediate R_{P-PL}, large complexes were formed with a low net charge that gave turbid or opaque systems prone to sedimentation; at high R_{P-PL}, small anionic complexes were formed that gave transparent solutions or slightly turbid stable colloidal suspensions.

(2) The antimicrobial efficacy of the ε-PL−pectin complexes tended to decrease as the pectin level (R_{P-PL}) increased. Even so, electrostatic complexes were still highly effective at inhibiting yeast growth (i.e., MIC < 10 μg/mL ε-PL) as long as $R_{P-PL} \leq 20$.

(3) The appearance and physical stability of commercial green tea beverages also depended on the composition of ε-PL−pectin complexes added. Electrostatic complexes (10 μg/mL ε-PL) with low levels of pectin ($R_{P-PL} \leq 1$) led to increased turbidity and precipitate formation, whereas higher levels ($R_{P-PL} \geq 2$) did not have an adverse impact on product appearance or stability.

Overall, our results indicate that high levels of pectin in the complexes led to improved physical stability, but reduced antimicrobial activity. Consequently, electrostatic complexes must be carefully formulated to balance these two effects so as to optimize their antimicrobial activity, without adversely affecting product appearance or stability. We found that complexes containing 10 μg/mL of ε-PL with R_{P-PL} between 2 and 20 had good physical stability in a commercial green tea beverage, while maintaining good antifungal efficacy in antimicrobial tests using model systems. Overall, this study demonstrates an effective strategy for overcoming some of the limitations of cationic antimicrobials, while maintaining their antimicrobial efficacy.

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