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Cationic Antimicrobial (ε-Polylysine)−Anionic Polysaccharide (Pectin) Interactions: Influence of Polymer Charge on Physical Stability and Antimicrobial Efficacy

Yuhua Chang,[†,§] Lynne McLandsborough,[†] and David Julian McClements*[,†]

[†]Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003, United States

[§]College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an, Shaanxi 710062, People's Republic of China

ABSTRACT: The cationic biopolymer ε-polylysine (ε-PL) is a potent food-grade antimicrobial that is highly effective against a range of food pathogens and spoilage organisms. In compositionally complex systems such as foods and beverages, cationic ε-PL molecules may associate with anionic substances, leading to increased turbidity, sediment formation, and reduced antimicrobial activity. This study therefore characterized the interactions between cationic ε-PL and anionic pectins with different degrees of esterification (DE) and then investigated the influence of these interactions on the antimicrobial efficacy of ε-PL. The nature of the interactions was characterized using isothermal titration calorimetry (ITC), microelectrophoresis (ME), and turbidity measurements. High (DE 61%), medium (DE 51%), and low (DE 42%) methoxyl pectins interacted with ε-PL molecules through electrostatic forces, forming either soluble or insoluble complexes with various electrical charges, depending on the relative mass ratio of pectin and ε-PL. The interaction of pectin with ε-PL increased as the negative charge density on the pectin molecules increased, that is, with decreasing DE. The antimicrobial efficacy of ε-PL against two acid-resistant spoilage yeasts (*Zygosaccharomyces bailii* and *Saccharomyces cerevisiae*) decreased progressively in the presence of increasing levels of all three pectins. Nevertheless, the low DE pectin decreased the antimicrobial efficacy of ε-PL much more dramatically, likely due to strong electrostatic binding of ε-PL onto low DE pectin molecules reducing its interaction with anionic microbe surfaces. This study provides knowledge that will facilitate the rational application of ε-PL as an antimicrobial in complex food systems.

KEYWORDS: *ε-polylysine, pectin, electrostatic complex, antimicrobial, interactions, yeast*

INTRODUCTION

ε-Polylysine (ε-PL) is a naturally occurring antimicrobial produced by *Streptomyces albulus*.[1] Chemically, ε-PL is a cationic homopolymer consisting of L-lysine monomers (typically 25−35) linked together by isopeptide bonds between ε-amino and α-carboxyl groups.[1−3] 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 products.[2,4−8] On the basis of absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies, ε-PL has been shown to be safe for consumption,[9] and it has been approved as generally recognized as safe (GRAS) within the United States for certain food applications (GRAS No. 000135).[10]

ε-PL is cationic when the environmental pH is lower than its isoelectric point (p$I \approx 9.0$), because of the presence of primary amine groups ($-NH_3^+$) along its backbone.[2] The antimicrobial ability of ε-PL is highly dependent on its cationic nature, because ε-PL is thought to function by adsorbing onto negatively charged cell surfaces of microorganisms through electrostatic interactions, where it promotes cell membrane disruption.[7] On the other hand, the cationic nature of ε-PL may cause problems when it is applied into some food systems: (i) it may interact with anionic components within the food matrix, forming precipitates that cause cloudiness or sediment formation; (ii) it may interact with anionic food components, leading to reduced antimicrobial activity; (iii) it may interact with anionic mucin molecules in human saliva, leading to astringency.[11,12] Therefore, both the beneficial attributes (antimicrobial activity) and detrimental attributes (precipitate formation and astringency) of ε-PL are likely to depend on its electrostatic interactions with other molecules in the food systems in which it will be utilized. It is therefore important to characterize the interactions between ε-PL and anionic food ingredients and to investigate how these interactions influence the antimicrobial efficacy of ε-PL, so as to obtain a better understanding of how to control the functionality of ε-PL in compositionally complex food products.

In recent studies, we characterized the interactions of ε-PL with a high-methoxyl pectin (degree of esterification (DE) 71%) and studied the influence of these interactions on the antimicrobial activity of ε-PL.[11,12] These studies indicated that ε-PL forms molecular complexes with pectin and that the nature of these complexes (i.e., charge, size, and solubility) depends on the ratio of ε-PL to pectin used. We also showed that the formation of electrostatic complexes reduced the antimicrobial efficacy of ε-PL at sufficiently high pectin levels, presumably because it prevented the cationic antimicrobial from interacting with the anionic surfaces of the microbial cells.[11] The origin of the interaction between cationic ε-PL and anionic pectin is electrostatic, and it should therefore depend

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on the electrical characteristics of the polymers involved. For this reason, we examined the influence of the electrical characteristics of pectin on its ability to interact with ε-polylysine, and on the antimicrobial efficacy of ε-PL. The term "pectin" covers a group of compositionally and structurally complex molecules with similar biological origins and molecular characteristics. One of the most important features of pectin molecules is the presence of a partially esterified α-1,4-linked D-galacturonic acid linear backbone, with various amounts of covalently attached neutral sugar side branches.[13] The carboxylic acid side groups on the D-galacturonic acids are negatively charged across a wide range of pH values ($pK_a \approx 3.5$). The charge density of the anionic backbone depends on the fraction of carboxylic acid groups that are esterified (methylated). In this study, three pectin ingredients with various degrees of esterification were used: high-methoxyl pectin (HMP, DE 61%), medium-methoxyl pectin (MMP, DE 51%), and low-methoxyl pectin (LMP, DE 42%). The purpose of this study was to obtain a better understanding of the interactions between cationic ε-polylysine and anionic pectins of various electrical characteristics and to establish the influence of these interactions on the antimicrobial efficacy of ε-PL.

MATERIALS AND METHODS

Materials. The cationic antimicrobial ε-polylysine (50:50 mixture with maltodextrin) was provided by Purac (Puraq Xtend FX50P; lot 1100402). Three different types of pectin were provided by CPKelco (Denmark): USP/100 (DE 61%), 103-01080 (DE 51%), and 72-02090 (DE 42%). According to the manufacturer's report, pectin samples with different DE values were prepared by partial de-esterification of high-methoxyl pectin isolated from lemon peel. De-esterification of the pectin samples was carried out using a pectin-esterase of fungal origin that attacks the methyl groups on the pectin molecules randomly, resulting in a random distribution of non-methylated D-galacturonic acid residues. None of the three pectin samples contained any additives such as sugars or starch, and all were reported to have a moisture content of around 10%. Double-distilled water was used for the preparation of all aqueous solutions.

Solution Preparation. All biopolymer solutions were prepared in citric buffer solution (0.5 mM, pH 3.5). 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 citric buffer solution, followed by pH adjustment by addition of HCl or NaOH solution. The stock solutions were then filter sterilized (0.45 μm pore size; Corning Inc., Corning, NY) and kept refrigerated until use.

Isothermal Titration Calorimetry (ITC). An isothermal titration calorimeter (VP-ITC, Microcal Inc., Northampton, MA) was used to measure enthalpies of mixing at 30 °C. Fifty-eight 5 μL aliquots of ε-PL solution (1.0 w/v %, pH 3.5) were injected sequentially into a 1480 μL titration cell initially containing 0.1 w/v % pectin (pH 3.5). Each injection lasted 12 s, and there was an interval of 360 s between successive injections. The solution in the titration cell was stirred at a constant speed of 315 rpm throughout the experiments. The resulting heat flow−time curves were integrated using the instrument's software to give interaction enthalpy−polylysine concentration curves. The ε-polylysine sample used in this study contained 50% maltodextrin, and therefore a preliminary experiment was carried out to determine the potential influence of this component on the observed enthalpy changes. We found that injection of maltodextrin solution (1.0 w/v %, pH 3.5) into 0.1 w/v % pectin solutions (pH 3.5) gave negligible enthalpy changes (data not shown), indicating that there was no significant contribution of maltodextrin to the measured enthalpy changes.

Microelectrophoresis (ME) and Turbidity Measurements. ME and turbidity measurements were used to provide information about the electrical charge and aggregation state of ε-PL−pectin complexes, respectively. These experiments were designed to mimic the ITC experiments, that is, using a similar total ε-PL concentration range and ε-PL-to-pectin ratio. Aliquots of 1.0 w/v % ε-PL solution (0−1500 μL, pH 3.5) were injected into glass test tubes initially containing 7.5 mL of 0.1 w/v % pectin solution (pH 3.5). The resulting solutions were then mixed thoroughly and stored overnight prior to analysis. Solutions were thoroughly mixed again immediately prior to analysis to ensure that any complexes were evenly distributed throughout the entire sample.

The electrical charge (ζ-potential) of the particles in the solutions was measured using a particle electrophoresis instrument (Zetasizer Nano-ZS, model ZEN3600, Malvern Instruments, Worchester, U.K.). The ζ-potential was calculated from the measurement of the electrophoretic mobility of particles in an applied oscillating electric field using laser Doppler velocimetry. All measurements were conducted at least twice on freshly prepared materials and repeated three times per sample.

The optical turbidity (at 600 nm) of ε-PL−pectin complex solutions was measured using a UV−visible spectrophotometer (Ultraspec 2000, Pharmacia Biotech) at room temperature. 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.

Yeast Strains. Two strains of acid-resistant spoilage yeasts, *Zygosaccharomyces bailii* (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 malt extract agar (MEA) plates before the following tests of minimum inhibitory concentration (MIC). A single yeast colony from the plate was then inoculated into 10 mL of malt extract broth (MEB), which was adjusted to pH 3.5 by citrate buffer (10 mM in final medium). The culture was incubated at 25 °C under mild agitation (150 rpm in a rotary shaker) for 2−3 days until the optical density (turbidity) at 600 nm (OD_{600}) was around 1.0 (1 cm path length). As a guideline, an OD_{600} of 1.0 corresponds to approximately 5×10^6 CFU/mL for cultures of yeast strains. The cultures were then diluted 100-fold using fresh MEB (pH 3.5) to determine the minimum inhibitory concentration (MIC) as stated below.

Determination of the MIC of ε-Polylysine−Pectin Complexes. The ε-PL−pectin complexes were prepared by mixing appropriate amounts of ε-polylysine, pectin stock solution, and sterile citric buffer solution (0.5 mM, pH 3.5), creating final solutions composed of 200 μg/mL ε-polylysine and different pectin-to-PL mass ratios ($R_{P\text{-}PL}$). After storage at room temperature for 24 h, ε-PL−pectin complexes were examined for their individual antimicrobial efficacy by testing the MIC against the two yeast strains (ZB and SC). The MIC tests were carried out using 96-well microtiter plates by a 2-fold serial dilution method. The microtiter plate wells of the 1st, 2nd, 11th, and 12th columns were filled with 180 μL of blank MEB medium to monitor potential contamination. The wells from the 4th to 10th columns were filled with 180 μL of MEB medium (preadjusted to pH 3.5 using 10 mM citrate buffer), which was inoculated with yeast cells (ZB or SC) at levels around 5×10^4 CFU/mL. Each well of the third column was filled with 180 μL of inoculated 2× MEB broth (preadjusted to pH 3.5 using 20 mM citrate buffer), and an equal volume of a certain type of complex solution was then added and mixed thoroughly. The resulting solution in the third column was composed of 1× MEB broth (pH 3.5) and ε-PL−pectin complexes (100 μg/mL ε-polylysine and pectin with various $R_{P\text{-}PL}$). A volume of 180 μL of the mixed medium from the third column was then transferred to the wells of the fourth column, and this procedure was continued so as to make successive 2-fold dilutions up to the 10th column. For each microtiter plate, a specific ε-PL−pectin complex was tested, which had a specific pectin type (DE 61%, 51%, or 42%) and a specific pectin-to-ε-PL ratio (0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, or 32), and its ε-PL concentration ranged from 100 to1.6 μg/mL (100, 50, 25, 12.5, 6.4, 3.2, or 1.6 μg/mL) from the 3rd to the 10th column. Plates were incubated at 25 °C for 6 days, and the MICs were

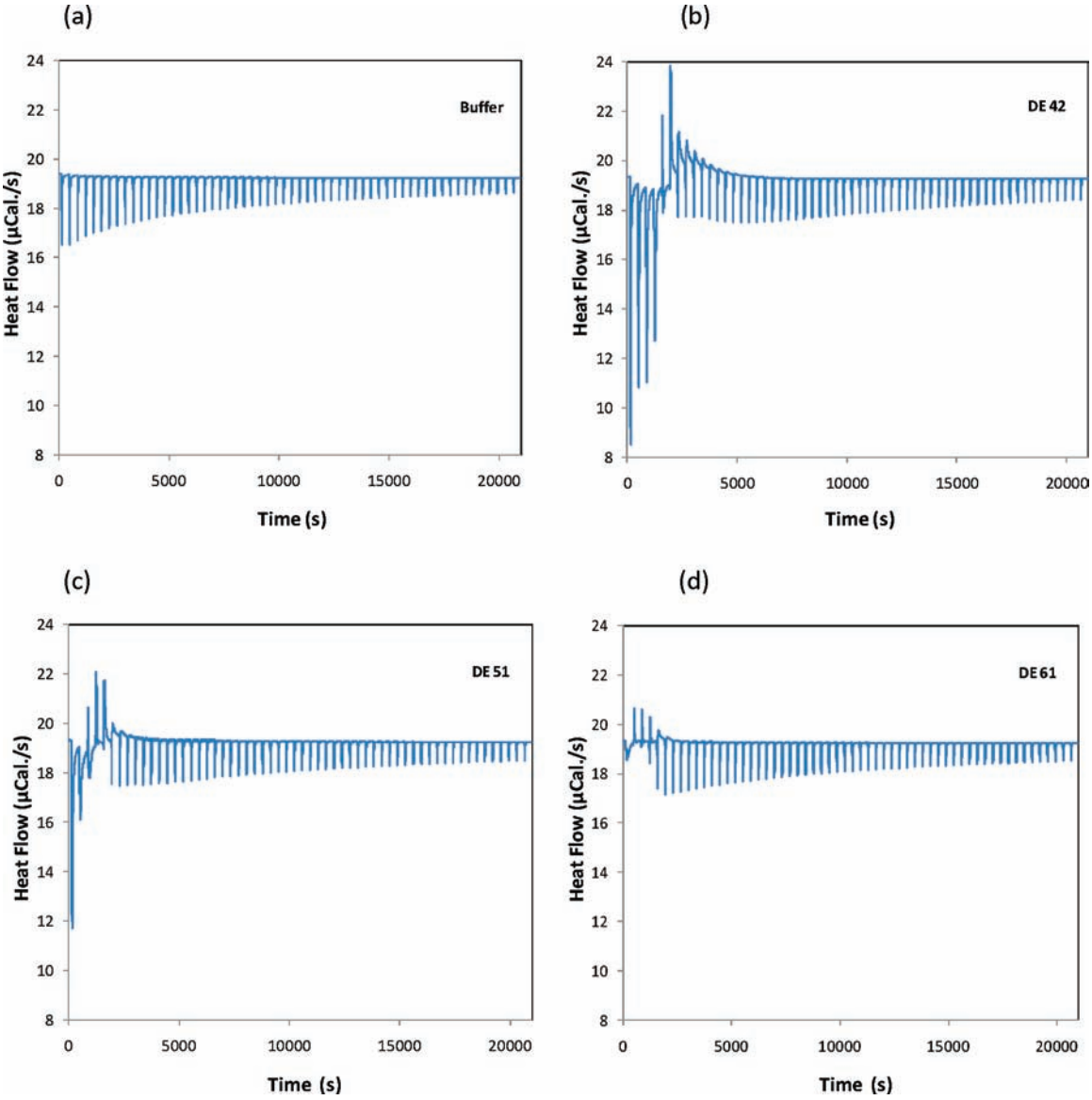

Figure 1. Heat flow versus time profiles resulting from injection of 5 μL aliquots of 1.0 w/v % ε-PL into a 1480 μL titration cell (30.0 °C) containing either (a) citric buffer (0.5 mM) at pH 3.5, (b) 0.1 w/v % pectin of DE 42% at pH 3.5, (c) 0.1 w/v % pectin of DE 51% at pH 3.5, or (d) 0.1 w/v % pectin of DE 61% at pH 3.5.

assessed visually as the lowest concentration of ε-PL showing complete inhibition of growth. All MIC experiments were repeated twice with freshly prepared materials.

RESULTS AND DISCUSSION

In this study, we characterized the interactions between cationic ε-polylysine (ε-PL) and anionic pectins of various degrees of esterification and investigated the influence of these interactions on the antimicrobial efficacy of ε-PL as well.

Enthalpy Changes Associated with ε-Polylysine–Pectin Interactions. The enthalpy changes associated with the interactions between ε-polylysine and pectin molecules with different DE values were quantified by ITC at 30 °C. Heat flow versus time profiles resulting from sequential injections of 5 μL aliquots of ε-PL solution (1.0 w/v %) into a 1480 μL titration cell initially containing either citric buffer (0.5 mM; pH 3.5) or 0.1 w/v % pectin in buffer (pH 3.5; DE 42%, 51%, or 61%) were measured (Figure 1). The dependence of the interaction enthalpy on the ε-PL concentration in the reaction cell was calculated by integration of the heat flow versus time profiles and subtracting the values for the blank (buffer) from the sample (pectin): $\Delta H_{\text{interaction}} = \Delta H_{\text{pectin}} - \Delta H_{\text{buffer}}$ (Figure 2).

In the absence of pectin, enthalpy changes resulting from titration of ε-PL into buffer solutions were initially slightly exothermic and became progressively less exothermic with increasing ε-PL concentrations (Figures 1 and 2), which can be attributed to solute dilution effects.[12] In the presence of pectin, the interaction enthalpy profiles were qualitatively similar for all three pectins (Figure 2). In general, the interaction of ε-polylysine with pectin could be divided into three concentration regimens (Figure 2). At relatively low ε-PL concentrations, the interaction enthalpy was exothermic and gradually decreased with increasing ε-PL concentration. At intermediate ε-PL concentrations, the interaction enthalpy was endothermic, initially increasing to a peak and then gradually decreasing to zero with increasing ε-PL concentration. At relatively high ε-PL concentrations, the interaction enthalpy was close to zero, suggesting that little further interactions occurred. These results

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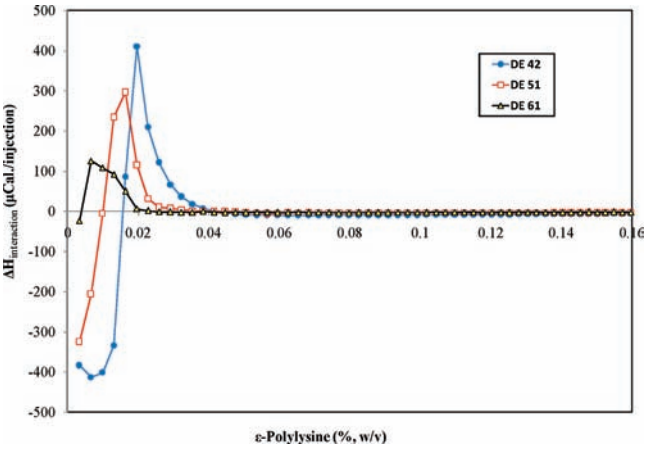

Figure 2. Interaction enthalpy change ($\Delta H_{interaction}$) versus ε-PL concentration (w/v %) when 1.0 w/v % ε-PL solution (pH 3.5) was injected into a reaction cell containing 0.1 w/v % pectin of various DE values (pH 3.5, 30 °C). Profiles of interaction enthalpy change ($\Delta H_{interaction}$) were obtained by subtracting the ΔH profile of ε-PL solution titrated into citric buffer (0.5 mM, pH3.5) from that into each pectin solution (i.e., $\Delta H_{interaction} = \Delta H_{pectin} - \Delta H_{buffer}$), to highlight the ε-PL–pectin interactions.

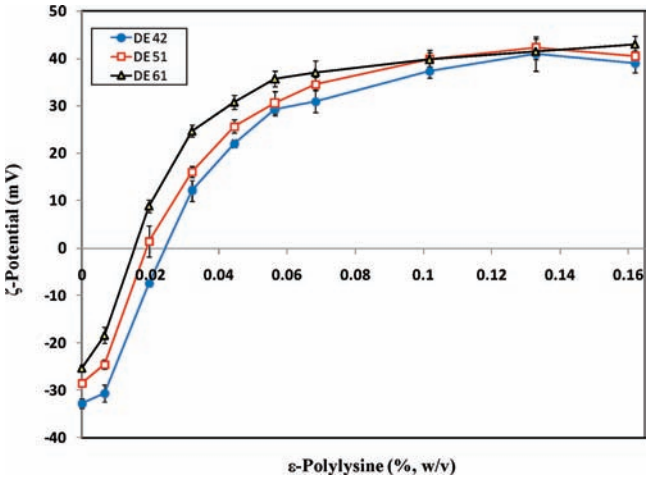

Figure 3. Dependence of the ζ-potential of ε-PL–pectin solutions on the ε-PL concentration in the reaction cell. A ε-PL solution (1.0 w/v %, pH 3.5) was titrated into aqueous pectin solution (0.1 w/v %, pH 3.5). Three pectins of different DE values (i.e., DE 42%, 51%, and 61%) were titrated by ε-PL individually.

are in agreement with previous experiments using a high-methoxyl pectin (DE 71%) and ε-PL,[12] indicating that pectin molecules with different DE values interact with ε-PL in a similar qualitative manner.

However, there were some quantitative differences in the interaction enthalpy profiles of different kinds of pectin (Figures 1 and 2). The main differences existed in the low and intermediate ε-PL concentration regimens: the range of ε-PL concentrations for which an interaction was observed increased with decreasing pectin DE; the magnitude of the exothermic and endothermic peaks increased with decreasing pectin DE (Figure 2).

ITC data provide valuable information about the interactions involved. First, the results suggest that there are at least two different physicochemical phenomena occurring: one at low ε-PL levels, at which exothermic enthalpy changes occurred, and one at intermediate ε-PL levels, at which endothermic enthalpy changes occurred. Second, the results suggest that the pectin molecules became saturated with ε-PL molecules above a certain level of ε-PL, because no further enthalpy changes were observed (Figure 2). The fact that more ε-PL molecules had to be titrated into the reaction cell to reach this saturation level when pectin molecules with lower DE values were used can be attributed to the fact that there was a greater number of negative groups on these pectin molecules available for the cationic groups on the ε-PL molecules to interact with. Similarly, the fact that the magnitudes of the endothermic and exothermic peaks were greater for pectin molecules with lower DE values can be attributed to a stronger interaction between ε-PL and pectin molecules with higher charge densities.

Electrical Characteristics of ε-Polylysine–Pectin Complexes. The electrical characteristics (ζ-potential) of ε-PL–pectin complexes were measured by microelectrophoresis to obtain additional information about the nature of the interactions involved (Figure 3). A titration experiment was carried out that mimicked the ITC experiment: the ζ-potential was measured when increasing amounts of 1.0 w/v % ε-PL solution were titrated into a series of 0.1 w/v % pectin solutions (pH 3.5).

In the absence of ε-PL, the ζ-potential was highly negative, which can be attributed to the presence of partially charged carboxylic acid groups along the pectin chain at pH 3.5.[14] The ζ-potentials of pectin ingredients with low (42%), medium (51%), and high (61%) DE values were −32, −28, and −25 mV, respectively. As expected, the low-methoxyl pectin was most negatively charged and the high-methoxyl pectin was least negatively charged, because the former is less esterified and thus has more carboxylic acid groups per unit chain length.

For all three pectins, the ζ-potential went from negative to positive as the ε-PL concentration was increased, indicating that the cationic ε-PL formed complexes with the anionic pectin. These measurements show that the ε-PL–pectin complexes were negatively charged at low ε-PL-to-pectin mass ratios, but positively charged at high mass ratios. Charge neutralization for high-, medium-, and low-methoxyl pectin occurred when the ε-PL concentration reached 0.015, 0.020, and 0.025 wt %, respectively. These values correspond to mass ratios of ε-PL-to-pectin of ≈0.15-to-1, 0.20-to-1, and 0.25-to-1 for the high-, medium-, and low-methoxyl pectins, respectively. The pectin of lower DE value therefore required addition of more ε-PL to reach charge neutralization, which can be attributed to its higher charge density.

Aggregation Characteristics of ε-Polylysine–Pectin Complexes. The aggregation characteristics of ε-polylysine–pectin complexes were determined by turbidity measurements. A titration experiment was carried out to mimic the ITC and microelectrophoresis experiments: increasing amounts of 1.0 w/v % ε-PL solution were titrated into a series of 0.1 w/v % pectin solutions (pH 3.5), and the turbidity was measured after the samples were stored at room temperature for 24 h. Prior to the turbidity measurements, the samples were vortexed to ensure that any complexes present were evenly distributed throughout the whole sample.

For all three pectins, their turbidity (τ_{600}) changed in a similar qualitative manner upon addition of ε-PL. The turbidity increased steeply when the ε-PL concentration was increased from 0 to 0.02 w/v % to reach a maximum value (Figure 4). This suggested that the size and/or number of the ε-PL–pectin complexes increased. When the ε-PL concentration was

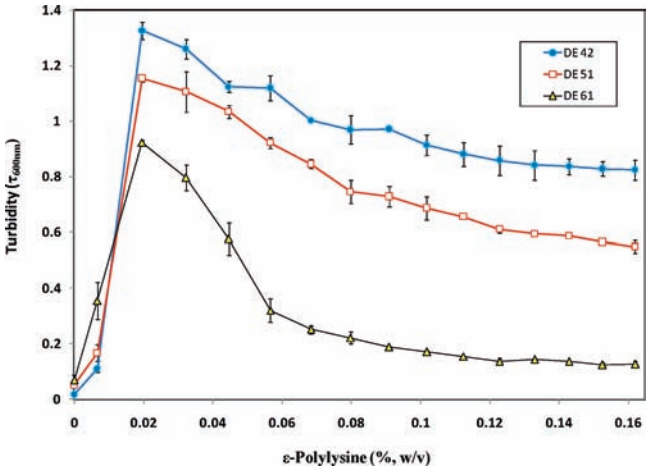

Figure 4. Dependence of the turbidity (at 600 nm) of ε-PL−pectin solutions on the ε-polylysine concentration for pectins of various DE values. A ε-PL solution (1.0 w/v %, pH 3.5) was titrated into the three aqueous pectin solutions (0.1 w/v %, pH 3.5) individually.

increased further (from 0.02 to 0.16 w/v %), the turbidity progressively decreased, suggesting that the size and/or number of the complexes decreased. For the high-methoxyl pectin (DE 61%), the turbidity decreased rapidly, and when the ε-PL levels exceeded 0.07 w/v %, the turbidity remained low ($\tau_{600} \approx 0.2$ cm^{-1}). On the contrary, for the medium -methoxyl pectin (DE 51%) and low-methoxyl pectin (DE 42%), after reaching peak turbidity, their turbidities decreased slowly as the ε-PL concentration was further increased, and it remained high at the end of titration (Figure 4).

Classification of ε-Polylysine−Pectin Interactions. On the basis of previous studies[15−17] and our experimental data, it was possible to divide the interactions between ε-polylysine and pectin into three different regions:

Region I: Exothermic Enthalpy Change Region. The ε-PL concentrations in this range were 0−0.004, 0−0.01, and 0−0.015 w/v % for HMP, MMP, and LMP, respectively. When the ε-PL concentration was increased in this region, (i) the enthalpy change became less exothermic until close to zero (Figure 2), (ii) the electrical charge was highly negative (Figure 3), and (iii) the turbidity increased but remained relatively low (Figure 4). We postulate that the cationic ε-PL molecules bound to the anionic pectin molecules and formed relatively small soluble primary complexes that did not scatter light strongly. The formation of larger secondary complexes was inhibited due to the relatively high negative charge on the primary complexes, which generated a strong electrostatic repulsion between them.

Region II: Endothermic Enthalpy Change Region. The ε-PL concentrations in this range were 0.004−0.020, 0.010−0.030, and 0.015−0.040 w/v % for HMP, MMP, and LMP, respectively. When the ε-PL concentration was increased in this region, (i) the enthalpy change became increasingly more endothermic, reached a maximum value, and then became gradually less endothermic until it reached zero (Figure 2); (ii) the electrical charge on the complexes became gradually less negative until charge neutralization occurred and became slightly positively charged (Figure 3); and (iii) the turbidity was relatively high (Figure 4). We postulate that many ε-PL−pectin primary complexes associated together to form large secondary complexes that scattered light strongly. These large complexes were able to form because of the relatively low net charge on the primary complexes, which meant that the electrostatic repulsion between them was weak. The resulting complexes were large enough to sediment to the bottom of the test tubes and form a coacervate phase because they were denser than the surrounding water.

Region III: Zero Enthalpy Change Region. The ε-PL concentrations in this range were >0.020, >0.030, and >0.040 w/v % for HMP, MMP, and LMP, respectively. When the ε-PL concentration was increased in this region, (i) the enthalpy change remained close to zero (Figure 2), (ii) the electrical charge on the complexes became increasingly positive (Figure 3), and (iii) the turbidity decreased (Figure 4). In this case, the primary complexes contained considerably more positive charges from ε-polylysine than negative charges from pectin, and so their net charge was highly positive. We postulate that large ε-PL−pectin complexes dissociated at higher ε-polylysine concentrations because the positive charge on the primary complexes became increasingly large, which would have generated a strong electrostatic repulsion between them.

In summary, the complexes contain an excess of anionic groups from pectin in region I, a fairly even number of anionic groups from pectin and cationic groups from ε-polylysine in region II, and an excess of cationic groups from ε-polylysine in region III. The analytical methods used in this study were not able to provide detailed information about the conformation or structural organization of the two component biopolymers within the complexes, but this would certainly be a useful area of research in future studies.

Antifungal Efficacy of ε-Polylysine−Pectin Complexes. We determined the antimicrobial effects of different ε-PL−pectin complexes by measuring their MIC in a nutrient MEB medium (pH 3.5) against two yeast strains: *Z. bailii* (ZB) and *S. cerevisiae* (SC). These two yeasts were selected as target organisms, because they are acid resistant and may cause spoilage in acidic beverages.[18,19]

In this series of experiments, a different approach was used to prepare complexes with various pectin-to-PL mass ratios ($R_{P\text{-}PL}$): the ε-PL concentration was fixed and the pectin concentration was varied (rather than the other way around). This approach was not used in the previous set of experiments because it was not possible to put highly viscous pectin solutions in the titration unit of the isothermal titration calorimeter, so we had to titrate ε-PL into pectin solutions. For this reason, we measured the electrical characteristics and turbidities of the complexes again by titrating pectin into ε-polylysine solutions, so that we could directly compare the electrical and turbidities characteristics with the antimicrobial experiments under the same conditions.

The electrical characteristics of ε-polylysine−pectin complexes (100 μg/mL ε-PL with various $R_{P\text{-}PL}$; pH 3.5) were measured by microelectrophoresis. For all three pectins, the dependence of the electrical charge on pectin concentration was qualitatively similar (Figure 5). When low levels of pectin were added, the charge of the resulting complexes was highly positive. As the pectin concentration was increased further, the ζ-potential went from positive to negative (Figure 5). These

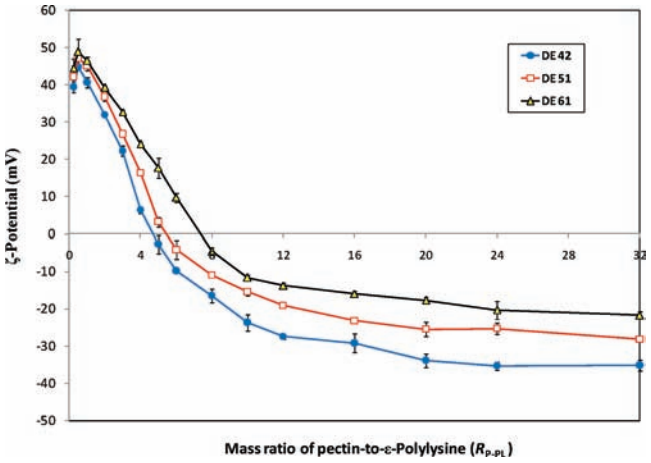

Figure 5. Dependence of the ζ-potential of ε-PL–pectin complex solutions on the mass ratio of pectin-to-ε-PL ($R_{P\text{-}PL}$) for 100 μg/mL of ε-PL (pH 3.5).

measurements showed that ε-PL–pectin complexes were positively charged at low $R_{P\text{-}PL}$, neutral at intermediate $R_{P\text{-}PL}$, and negatively charged at high $R_{P\text{-}PL}$. Charge neutralization occurred at $R_{P\text{-}PL}$ values of 4.5, 5.5, and 7.0 for LMP, MMP, and HMP, respectively (Figure 5), which correspond to mass ratios of ε-PL-to-pectin of 0.22, 0.18, and 0.14. These values are similar to those found when ε-polylysine was titrated into pectin solutions (Figure 3), suggesting that the order of addition did not have a large impact on the electrical characteristics of the complexes. As discussed earlier, the fact that a smaller amount of low DE pectin was needed to neutralize ε-PL can be attributed to its greater anionic charge density. At high pectin-to-ε-PL ratios, the negative charge on the complexes increased with decreasing DE on the pectin molecules (Figure 5).

The stability of ε-PL–pectin complexes (100 μg/mL ε-PL with various $R_{P\text{-}PL}$; pH 3.5) to aggregation was characterized using turbidity measurements. For all three pectins, the aggregation behavior of the complexes was qualitatively similar (Figure 6). The turbidity (τ_{600}) increased slowly when $R_{P\text{-}PL}$ was increased from 0 to 2 (for HMP) or from 0 to 1 (for LMP and MMP), but the values were very low (<0.05 cm^{-1}) (Figure 6). 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 $R_{P\text{-}PL}$ was increased further, suggesting that large insoluble complexes were formed that scattered light strongly. Finally, the turbidity progressively decreased as $R_{P\text{-}PL}$ was increased to higher levels, suggesting that the size of the complexes decreased. 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 (Figure 6). All of the samples had some cloudiness ($\tau \approx 0.18$ cm^{-1}) at high $R_{P\text{-}PL}$, which may have important implications for their utilization in certain types of products, for example, clear beverages.

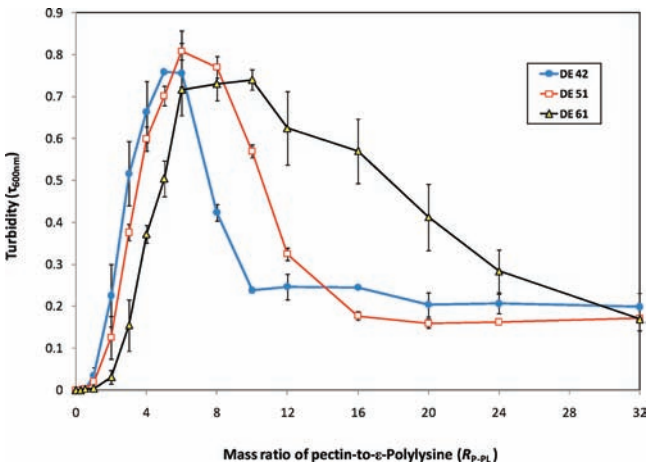

Figure 6. Dependence of the turbidity (at 600 nm) of ε-PL–pectin complex aqueous solutions on mass ratios of pectin-to-ε-PL ($R_{P\text{-}PL}$). ε-PL molecules (100 μg/mL) were complexed by pectin molecules with various mass ratios of pectin-to-ε-PL (0.25–32) at pH 3.5.

We then examined the antimicrobial efficacy of the complexes by MIC tests. In the absence of pectin, ε-polylysine was found to be highly effective at inhibiting the growth of both yeast strains, with MIC values of 3.2 and 1.6 μg/mL against ZB and SC, respectively (Figure 7). The antimicrobial efficacy of cationic ε-PL has previously been attributed to their ability to interact with and disrupt anionic cell membranes, thereby leading to cell leakage.[7,20] Generally, the antimicrobial efficacy of ε-polylysine decreased in the presence of increasing levels of pectin, as indicated by increasing MIC with increasing $R_{P\text{-}PL}$ (Figure 7). The observed decrease in antimicrobial activity may be attributed to the reduction in the positive charge and then increase in the negative charge on the complexes with increasing pectin levels (Figure 5), 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. The MICs of ε-PL present within ε-PL–pectin complexes appeared to depend on the electrical characteristics of the complexes. However, there appeared to be no direct correlation between the antimicrobial efficacy of ε-polylysine and its tendency to aggregate with pectin (Figure 6): the antimicrobial efficacy of ε-PL decreased with increasing pectin concentration, but the turbidity increased and then decreased.

Interestingly, when complexed with pectin of lower DE value, the antimicrobial efficacy of ε-PL was decreased much more rapidly than that of pectin of higher DE value (Figure 7). For example, when ε-PL was complexed with LMP (DE 42%), its antifungal efficacy against ZB decreased steeply when $R_{P\text{-}PL} \geq 2$, with MIC achieving 100 μg/mL at $R_{P\text{-}PL} = 6$, and the MIC against ZB was >100 μg/mL when $R_{P\text{-}PL}$ increased further (Figure 7a). In contrast, when ε-PL was complexed with HMP (DE 61%), its antifungal efficacy against ZB decreased much more slowly: the MIC increased only to 12.5 μg/mL when $R_{P\text{-}PL}$ increased to 20. Even at relatively high HMP levels, the ε-PL–pectin complexes still had relatively strong antifungal efficacy, with MIC against ZB being 25 and 50 μg/mL at $R_{P\text{-}PL}$ = 24 and 32, respectively. For the medium-methoxyl pectin (DE 51%), the antifungal efficacy of ε-PL against ZB also decreased with increasing levels of pectin, with the impact of the MMP being between that of the LMP and HMP (Figure 7a). For the other tested target yeast, SC, a similar phenomenon was observed: pectins with higher charge densities (lower DE) were more effective at inhibiting antimicrobial efficacy (Figure 7b). This phenomenon could be due to (i) the differences in the net electrical charges of

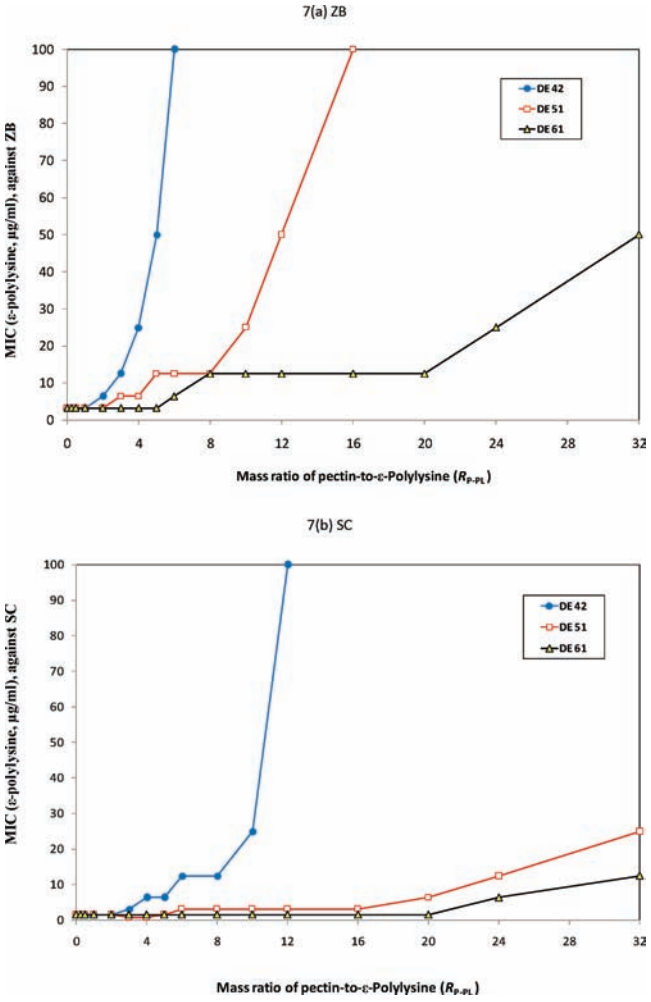

Figure 7. Dependence of the minimal inhibitory concentration (MIC) of ε-PL against the acidic resistant yeasts (a) *Zygosaccharomyces bailii* (ZB) and (b) *Saccharomyces cerevisiae* (SC) on ε-PL−pectin complexes with various mass ratios of pectin-to-ε-PL ($R_{P\text{-}PL}$). The ε-PL−pectin complexes were prepared by mixing 200 μg/mL of ε-PL with pectin of different DE values (DE 42%, 51%, or 61%) in various mass ratios of pectin-to-ε-PL, and the ε-PL−pectin complexes of different concentrations were then added to a nutrient MEB medium (pH 3.5) to determine the MICs.

complexes (when $R_{P\text{-}PL}$ is the same, complexes containing LMP are less positively charged or more negatively charged (Figure 5); therefore, there are weaker electrostatic attractions between the ε-PL (in the complexes) and yeast cells with anionic surfaces); (ii) the differences of the binding strength of ε-PL onto pectin molecules (Figures 1 and 2) (ε-PL binds onto LMP pectin strongly; therefore, ε-PL is more difficult to be released out of the complexes to function against the yeast cells). We propose that the second reason is dominant, because even with the same net electrical charges, the complexes containing ε-PL and pectins of lower DE value were less effective in terms of antifungal efficacy.

In general, for all three pectins, the antifungal efficacy of ε-PL against both ZB and SC decreased with increasing levels of pectin; however, this effect was strain specific. ZB was much more sensitive to increased levels of pectin than SC (Figure 7). For example, when complexed with the medium-methoxyl pectin (DE 51%) at $R_{P\text{-}PL}$ = 16, the MIC of ε-PL was 3.2 μg/mL for SC, but 100 μg/mL for ZB (Figure 7). This phenomenon could result from differences in the surface charges of the two yeasts. As we reported previously, the surface charges of the two yeasts were different: −15.6 ± 1.8 mV (SC) versus −9.2 ± 1.1 mV (ZB), although their shape and size were similar.[11] The cell surface of SC had a higher negative charge and may therefore have been able to attract the ε-PL−pectin complexes more strongly. It is also possible that the surface microstructures differed between the two yeasts, resulting in different interactions. Clearly, further research is needed to establish the biological differences between different strains of microbial cells that lead to different sensitivities to specific cationic antimicrobials.

In summary, this study characterized the interactions between cationic ε-PL and anionic pectin molecules of various degrees of esterification using a variety of analytical techniques and used this information to understand the influence of complexation on the antimicrobial efficacy of ε-PL. The main results of this study are the following:

(1) All pectin samples interacted with ε-PL molecules through electrostatic interactions. The nature of the complexes formed (e.g., solubility, electrical charge) depended on the mass ratio of pectin to ε-PL and the electrical characteristics of the pectin (DE) used.

(2) The antimicrobial efficacy of ε-PL against two acid-resistant spoilage yeasts (*Z. bailii* and *S. cerevisiae*) was reduced in the presence of increasing levels of pectin. The ability of pectins to reduce the antimicrobial efficacy of ε-PL increased as their anionic charge density increased (lower DE). The origin of this effect is most likely to be due to strong binding of cationic ε-PL to anionic pectin molecules, eliminating its interaction with anionic microbes.

The results of this study have provided an improved understanding of the interactions between cationic ε-PL and anionic pectin, as well as the consequences of these interactions on the antimicrobial efficacy of ε-PL, which will prove useful in the rational application of ε-polylysine as a functional ingredient in food systems. The results suggest that ε-PL is not suitable to be used in food systems containing low-methoxyl pectin, because ε-PL molecules tend to bind strongly to low-methoxyl pectin, reducing the antimicrobial efficacy of ε-PL dramatically. This study also indicates that high-methoxyl pectin reacted with ε-PL to form ε-PL−pectin complexes that maintained their favorable antimicrobial activities. This form of pectin may therefore be useful for overcoming some of the problems associated with using cationic ε-PL in foods, for example, ingredient precipitation and potential astringency.

AUTHOR INFORMATION

Corresponding Author

*Phone: 413 545 1019. Fax: 413 545 1262. E-mail: mcclements@foodsci.umass.edu.

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Notes

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