Antibacterial characteristics and mechanisms of ε-poly-lysine against *Escherichia coli* and *Staphylococcus aureus*

Ying-Qiu Li a,*, Qing Han a, Jian-Ling Feng a, Wen-Li Tian b, Hai-Zhen Mo c

a Shandong Provincial Key Laboratory of Microbial Engineering, School of Food & Bioengineering, Qiu University of Technology, No. 3501 University Road of Changqing District, Jinan 250333, Shandong Province, China

b Library, Qiu University of Technology, Shandong Province, Jinan 250333, China

c School of Food Science, Henan Institute of Science and Technology, Xinxiang 453003, China

**Abstract**

The aim of this study was to investigate the antibacterial characteristics and antibacterial mechanisms of ε-poly-lysine against *Escherichia coli* and *Staphylococcus aureus*. The diameters of inhibition zones of *E. coli* (10 ± 0.5 mm) and *S. aureus* (12 ± 0.1 mm) treated by 200 μg/ml ε-poly-lysine were much larger than control (5 ± 0.3 mm) (p < 0.05). Minimum inhibition concentration of ε-poly-lysine against *E. coli* and *S. aureus* was 12.5 μg/ml. Scanning electron microscopy showed that ε-poly-lysine damaged the morphology of tested bacterial cells. The increase in electric conductivity of bacterial cells suspension indicated that the cytoplasmic membranes were broken by ε-poly-lysine, which caused leakage of ions in cells. SDS-PAGE of bacterial proteins demonstrated that ε-poly-lysine could damage bacterial cells through the destruction of cellular proteins. These results indicated that ε-poly-lysine has good potential to be as a natural food preservative.

**Keywords:**
ε-Poly-lysine
Antibacterial characteristics
Antibacterial mechanisms
*Escherichia coli*
*Staphylococcus aureus*

**1. Introduction**

There is a growing recognition that the continuous use of chemical synthetic preservatives in food industry may cause various hazards to human being health (Ho, Ishizaki, & Tanaka, 2000). Thus, safe natural food preservatives had become the priority in food industry to improve the safety of food products for decades (Najjar, Kashtanov, & Chikindas, 2007).

ε-Poly-lysine (ε-PL) is a cationic homopolyamide of 25–30 ε-lysine residues, having amid linkage between ε-amino and ε-carboxyl groups (Nishikawa & Ogawa, 2006; Shih, Shen, & Van, 2006; Shima & Sakai, 1981a, 1981b; Szökán et al., 1997; Takehara et al., 1999). Shima and Sakai (1981a) and Shima (1977) found that ε-PL was produced by filamentous bacterium *Streptomyces albus* strain 346, originally isolated from soil. ε-PL exhibited high solubility in water and thermal stability at higher temperature. Moreover ε-PL solution was still stable even when it was boiled at 100 °C for 30 min or autoclaved at 120 °C for 20 min (Hiraki, 2000; Shih et al., 2006).

As a natural antimicrobial food additive, ε-PL was already approved by Food and Drug Administration (FDA, USA) for safe food in 2003, and been used widely in Japan (Hiraki et al., 2003). ε-PL can be decomposed into lysine without any side effects on the human body, and serve as a kind of lysine sources. Chronic toxicity and carcinogenicity joint test showed that a person taking ε-PL 6500 μg/kg on diet every day was safe. When 20,000 μg/kg of ε-PL was taken, no obvious pathological tissue changed and no possible carcinogenicity was found (Fukutome, Kashima, & Aiuchi, 1995). These results were in agreement with the findings of Hiraki et al. (2003) and Neda, Sakurai, Takahashi, Ashiuchi, and Ohgushi (1999). Hiraki et al. (2003) found there was no significant difference between the mice of long-term taken high dose ε-PL (20,000 ppm) on diet and the normal mice. Neda et al. (1999) reported that ε-PL was almost no toxicity by mutability in rat toxicity test and bacterial reversion assays.

ε-PL has a wide antimicrobial spectrum against microorganisms, including yeast, fungi, Gram-positive and Gram-negative bacteria, even molds (Hiraki, 2000; Shima, Matsuoka, Iwamoto, & Sakai, 1984; Yoshida & Nagasawa, 2003). It has strong inhibition against many disease-causing bacteria such as *Salmonella Typhi-* murium, *Listeria monocytogenes*, *Escherichia coli* (E. coli) O157:H7, *Staphylococcus aureus* (S. aureus), *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Vibrio cholera*, *Bacillus cereus* and fungi *Candida*...


albicans (Chang, Lu, Park, & Kang, 2010; Geornaras & Sofos, 2005; Geornaras et al., 2007; Najjar et al., 2007; Shih et al., 2006; Zhou et al., 2011). The Minimum inhibition concentration (MIC) of 3-PL in against bacteria was less than 100 μg/ml with the higher MIC against fungus than bacteria (Hiraki, 2000). Similarly, Zhou et al. (2011) reported that the MIC of 3-PL solution against various pathogens (including E. coli, P. aeruginosa, Serratia marcescens, S. aureus, C. albicans, Fusarium solani) was less than 100 μg/ml, except fungus F. solani 109 μg/ml, indicating that 3-PL was highly effective at killing the pathogens.

Some literatures on antimicrobial activity and properties of 3-PL were published (Chang et al., 2010; Hiraki, 2000; Shima et al., 1984; Zhou et al., 2011). However, to date, few reports could be found on the possible inhibitory mechanisms of 3-PL against some specific bacterium. The purpose of this work was to investigate antibacterial effects and mechanisms of 3-PL against E. coli and S. aureus. Antibacterial activity of 3-PL was examined by the value of MICs and Oxford cup test. And antimicrobial mechanism was evaluated by changes of electrical conductivity, bacterial proteins and the bacteria cell surface morphology by scanning electron microscopy.

2. Materials and methods

2.1. Bacteria cultures

The bacteria cultures, E. coli (ATCC 8739) and S. aureus (ATCC 6538) were obtained from the Culture Collection, Jilin University of Technology. Freeze-dried bacteria were activated according to the ATCC guidelines. Bacteria were inoculated into 100 ml of beef extract peptone (BEP) medium (0.3% beef extract, 1% peptone, 0.5% NaCl), and cultivated with shaking (170 rpm) in a 300 ml flasks at 37 °C for 6 h at 4 °C. An aliquot of 0.1 ml (107 CFU/ml) of bacterial suspension was 3-PL against E. coli and S. aureus were incubated at 37 °C with gentle shaking for 9 h. Bacterial cells were collected by centrifugation at 6000 × g for 15 min at 4 °C. The control was prepared as described above, but in the absence of 3-PL. Bacterial cells were fixed again with 0.1 M phosphate buffer solution (PBS) more than 2 h for three times. Then bacterial cells were fixed with 10% glutaraldehyde for 12 h. The 3-PL fixed bacterial cells were washed with 0.1 M phosphate buffer solution (PBS) more than 2 h for three times. Bacterial cells were dehydrated by two rounds of serial dehydration, in 50%, 70%, 80%, 90%, 100% alcohol solutions, at 1 min intervals, followed by final isoamyl acetate rinse for 30 min. Cells were dried by CO2 critical point drying (HCP-2, Hitachi, Tokyo, Japan), mounted, and platinized with ion sputter coater (IB-5) and observed by SEM (S-570, Hitachi, Tokyo, Japan).

2.2. Measurement of inhibition zone diameter

3-PL powder (purity, 99%, Zhejiang Silver Elephant Bioengineering Co., Ltd., Zhejiang, China) was dissolved in sterile water to final concentrations (w/v) of 6.25, 12.5, 25, 50, 100, and 200 μg/ml, respectively. Antimicrobial activity of 3-PL against E. coli and S. aureus was tested by the Oxford cup method of Wang, Lu, Wu, and Lv (2009) with some modifications. An aliquot of 0.1 ml (107 CFU/ml) of the diluted inoculum from the bacteria cultures was transferred to the surface of LBA medium plates. Each strain was spread uniformly onto individual plates through sterile cotton swabs. Sterile Oxford cups (5 mm) were placed on the surface of plates poured with LBA medium. Aliquots of 200 μl of varying 3-PL concentrations (6.25, 12.5, 25, 50, 100 and 200 μg/ml) were transferred into each Oxford cup, respectively, and equivalent amounts of sterile water was used in place of 3-PL as control. These plates were placed in the refrigerator for 6 h at 4 °C then incubated at 37 °C for 20 h. The inhibitory activity of 3-PL against E. coli and S. aureus was evaluated by measuring the diameter of the transparent inhibition zone, and the antibacterial effect was evaluated by comparing inhibition zone diameters.

2.3. Determination of minimum inhibition concentration (MIC)

MIC was measured by referring the conventional broth dilution method of Sitohy, Mahgoub, and Osman (2012) and Wang et al. (2009) with some modifications. E. coli and S. aureus were incubated at 37 °C for 8–10 h to approximately 10⁹–10⁶ CFU/ml in LB medium previously. 3-PL of serial dilutions, previously dissolved in sterile distilled water, were prepared to final concentrations of 0, 6.25, 12.5, 25, 50, 100, and 200 μg/ml of LB medium. After incubated for 18–24 h at 37 °C in a well shaken (130 rpm), MIC was the lowest concentration of 3-PL which visibly inhibited microorganism growth and confirmed by measuring the OD₆₀₀ of all treatments with a spectrophotometer (V-1100, Meipuda Instrument Co. Ltd., Shanghai, China). The sample of no adding 3-PL was termed as control.

2.4. Scanning electron microscopy (SEM) analysis of E. coli and S. aureus cells

To further explore the morphology changes of E. coli and S. aureus cells, SEM analysis (Benli, Yigit, Geven, Guney, & Bingöl, 2008; Sitohy et al., 2012) was performed. E. coli and S. aureus were inoculated into LB and cultivated at 37 °C with gentle agitation for 12 h. Samples containing E. coli and S. aureus (approximately 10⁹ CFU/ml) in LB with 200 μg/ml 3-PL were incubated at 37 °C with gentle shaking for 9 h. Bacterial cells were collected by centrifugation at 6000 × g for 15 min at 4 °C. The control was prepared as described above, but in the absence of 3-PL. Bacterial cells were fixed in 2.5% glutaraldehyde for 12 h. The fixed bacterial cells were washed with 0.1 M phosphate buffer solution (PBS) more than 2 h for three times. Then bacterial cells were fixed again with osmic acid for 1.5 h and washed with double-distilled water for 2 h (three times). Bacterial cells were dehydrated by two rounds of serial dehydration, in 50%, 70%, 80%, 90%, 100% alcohol solutions, at 1 min intervals, followed by final isoamyl acetate rinse for 30 min. Cells were dried by CO2 critical point drying (HCP-2, Hitachi, Tokyo, Japan), mounted, and platinized with ion sputter coater (IB-5) and observed by SEM (S-570, Hitachi, Tokyo, Japan).

2.5. Measurement of electrical conductivity

Cellular leakage of the bacteria cells was determined by measuring electrolyte leakage into the incubation medium with a conductivity meter (DDS-307, Precision & Scientific Instrument Co. Ltd., Shanghai, China) following the method of Lee, Choi, and Cho (1998). After incubation in LB medium at 37 °C for 12 h, E. coli and S. aureus were separated by centrifugation at 6000 × g for 20 min, washed with triple 10 mM PBS (pH 7.0) and diluted with the same buffer to approximately 10⁹ CFU/ml. 3-PL was then added to bacterial cells suspension to final concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/ml. The samples were mixed and incubated with shaking (130 rpm) at 37 °C, and the conductivity was measured at 0, 10, 20, 30, 45, 60, 90 and 120 min, respectively.

2.6. SDS-PAGE of bacterial proteins

SDS-PAGE of the bacterial proteins was carried out after incubation with the antibacterial agent according to Sitohy et al. (2012). S. aureus strain was incubated in 10 ml LB medium overnight at 37 °C to approximately 1 × 10⁹ CFU/ml. 3-PL were added to all culture solutions of bacterial cells (200 μg/ml), except the control, and incubated at 37 °C for 3 h. Bacterial cells were separated by centrifugation (6000 × g) for 20 min at 4 °C and were re-suspended in PBS (10 ml, pH 7.0). An aliquot (50 μl) of bacterial suspension was combined with 25 μl of the sample buffer (pH 6.8; 1 M Tris–HCl, 50% glycerol, 10% SDS, 10% β-mercaptoethanol, 0.1% bromophenol blue), heated to 100 °C, cooled to 25 °C, then treated with ultrasonic for 3 min, and loaded onto a 3% stacking and 12% resolving gel. The proteins were run at 10 mA for 30 min on the stacking gel and 20 mA for 2 h on the resolving gel. The gel was dyed with Coomassie Brilliant Blue R250 and destained with destaining agent (glacial acetic acid methanol distilled water). After 2 days, protein bands were visualized on the gels.
2.7. Statistical analysis

Every experiment was performed in triplicates and average value with standard errors was reported. SPSS17.0 software was used for all statistical analysis. The data were analyzed by analysis of variance (ANOVA) and Duncan’s post hoc analysis, besides, graphs were produced using Microsoft Excel 2010, and regression analysis was used to determine the significant difference at 5% confidence intervals ($p < 0.05$).

3. Results and discussion

3.1. Antimicrobial activities of $\varepsilon$-PL against $E. coli$ and $S. aureus$

The antibacterial effects of $\varepsilon$-PL on $E. coli$ and $S. aureus$ were evaluated by the diameter of the inhibition zone as shown in Fig. 1(a)–(d) and Table 1. The diameters of inhibition zones against $E. coli$ (Fig. 1(b)) and $S. aureus$ (Fig. 1(d)) treated with 200 $\mu$g/ml $\varepsilon$-PL were much larger than controls (Fig. 1(a) and (c)). As observed in Table 1, the diameters of inhibition zones against $E. coli$ and $S. aureus$ increased from $5 \pm 0.3$ to $10 \pm 0.5$ mm and from $5 \pm 0.3$ to $12 \pm 0.1$ mm, respectively, with increasing concentrations of $\varepsilon$-PL, from 0 to 200 $\mu$g/ml. The diameters with 200 $\mu$g/ml $\varepsilon$-PL were much twice times bigger than those of controls. These results showed that antimicrobial activities increased with the increase of $\varepsilon$-PL concentration. Moreover, the diameters of the inhibition zones against $S. aureus$ were larger than $E. coli$, indicating that Gram-negative bacteria $S. aureus$ was more sensitive to $\varepsilon$-PL than $E. coli$ O157:H7 (Gram-negative bacterium). The reason of difference in antimicrobial activities of $\varepsilon$-PL against Gram-positive and Gram-negative bacterium might be they had different cell membrane constituents and structure.

3.2. Minimum inhibition concentration (MIC) of $\varepsilon$-PL against $E. coli$ and $S. aureus$

MIC of $\varepsilon$-PL against $E. coli$ and $S. aureus$ was measured by conventional broth dilution assay as depicted in Fig. 2. OD$_{600}$ values decreased with the increase of $\varepsilon$-PL concentration, and both MICs of $\varepsilon$-PL against two bacteria were 12.5 $\mu$g/ml, showing $\varepsilon$-PL had significant antibacterial activity at very low dosage. Moreover, OD$_{600}$ value of $S. aureus$ (0.339) was smaller than that of $E. coli$ (0.441) at MIC 12.5 $\mu$g/ml, indicating that $S. aureus$ was more susceptible to $\varepsilon$-PL than $E. coli$. This result was in agreement with that of inhibition zone. Moreover, the inhibitory effect of $\varepsilon$-PL against $E. coli$ and $S. aureus$ increased with increasing concentration of $\varepsilon$-PL. Zhou et al. (2011) indicated that the MIC value of $\varepsilon$-PL for E. coli was 8 $\mu$g/ml and for S. aureus was 16 $\mu$g/ml. Similarly, The MIC of $\varepsilon$-PL for Streptococcus mutans was 20 $\mu$g/ml (Najjar, Kashtanov, & Chikindas, 2009) and for L. monocytogenes was 15 $\mu$g/ml (Najjar et al., 2007), which were bigger than those of $E. coli$ and $S. aureus$ in this study. However, Shima et al. (1984) and Hiraki (1995) reported that MICs

<table>
<thead>
<tr>
<th>$\varepsilon$-PL($\mu$g/ml)</th>
<th>$E. coli$</th>
<th>$S. aureus$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5 $\pm$ 0.3</td>
<td>5 $\pm$ 0.3</td>
</tr>
<tr>
<td>6.25</td>
<td>6 $\pm$ 0.1</td>
<td>6 $\pm$ 0.2</td>
</tr>
<tr>
<td>12.5</td>
<td>6 $\pm$ 0.5</td>
<td>6 $\pm$ 0.4</td>
</tr>
<tr>
<td>25</td>
<td>7 $\pm$ 0.2</td>
<td>7 $\pm$ 0.1</td>
</tr>
<tr>
<td>50</td>
<td>8 $\pm$ 0.2</td>
<td>8 $\pm$ 0.5</td>
</tr>
<tr>
<td>100</td>
<td>9 $\pm$ 0.4</td>
<td>10 $\pm$ 0.2</td>
</tr>
<tr>
<td>200</td>
<td>10 $\pm$ 0.5</td>
<td>12 $\pm$ 0.1</td>
</tr>
</tbody>
</table>

Table 1: The diameters of the inhibition zones with different concentration $\varepsilon$-PL against $E. coli$ and $S. aureus$.

Fig. 1. Photos of inhibition zone of $E. coli$ and $S. aureus$ treated with and without $\varepsilon$-PL. (a)–(d) were control of $E. coli$, $E. coli$ treated with 200 $\mu$g/ml $\varepsilon$-PL, control of $S. aureus$, and $S. aureus$ treated with 200 $\mu$g/ml $\varepsilon$-PL, respectively.
of e-PL against E. coli K-12, E. coli F-2, E. coli B, B. subtilis, and S. aureus were 1 μg/ml, 2 μg/ml, 1 μg/ml, 1 μg/ml and 4 μg/ml, respectively, which were much lower than those of E. coli and S. aureus in this study. These results demonstrated e-PL had different MIC to different bacteria. The possible reason for the different MIC of e-PL against tested bacteria might be the differences in purity of e-PL, strains, and structure of bacteria cell.

3.3. Effect of e-PL on the morphology of S. aureus and E. coli cells

The morphology changes of S. aureus and E. coli cells were evaluated by SEM analysis. Fig. 3 shows SEM photomicrographs of E. coli and S. aureus cells treated with and without e-PL. As observed in Fig. 3b, S. aureus cells treated by e-PL exhibited irregularly wrinkled outer surface, with fragmentation, adhesion and aggregation of damaged cells or cellular debris. Moreover, these cells were not uniform in size and distribution. However, bacterial cells without e-PL (Fig. 3a) had regular and spherical morphology, with plump and smooth surface, which were uniform in size and distribution. Similarly, as seen in Fig. 3c, the bacterial cells of E. coli without e-PL displayed typical bacilliform morphology with uniform in size and distribution. Furthermore, the surface appeared full and glossy. In contrast to the control, E. coli cells treated with e-PL (Fig. 3d) had irregular, withered, and coarse surfaces, forming aggregations and adhesions. Furthermore, these cells were not uniform in size and distribution. These results indicate that e-PL treatment results in the damage to S. aureus and E. coli cells.

The present findings are in support of the report of Zhou et al. (2011) that the bacteria and fungi of the control appeared smooth and rounded, whereas, those on the e-PL-graft-methacrylamide exhibited wrinkled and withered surfaces by SEM analysis. A SEM study by Duval, Zatlyn, Laurencin, Baudy-Floc’h, and Henry (2009) demonstrated also that the numerous fragments as well as spherical element, probable microsomes, were observed in E. coli cultures treated with K4, whereas, there were no fragments for control. Similarly, Sitohy et al. (2012) found that the B. subtilis cells with basic subunit of soybean protein exhibited irregular wrinkled outer surface, with fragmentation and adhesion.

3.4. Effect of e-PL on the membrane permeability of E. coli and S. aureus

To evaluate the relationship between antibacterial activity of e-PL and membrane permeability of E. coli and S. aureus, electrical conductivities of samples were determined as shown in Fig. 4a and b. The electric conductivities of E. coli and S. aureus samples were basically stable about 2.4 and 3.4 ms/cm, respectively, in the first 20 min. The reason might be that e-PL did not affect two bacteria during period. For control, the electric conductivities of E. coli and S. aureus were essentially constant throughout 120 min. However, the conductivity values of E. coli suspensions treated with 6.25,
12.5, 25, 50, 100, 200 µg/ml ε-PL increased significantly (p < 0.05) with treatment time (20–45 min) from 2.4, 2.4, 2.5, 2.6, 2.5 ms/cm to 3.4, 4.6, 5.6, 7.1, 8.2, 8.9 ms/cm, respectively (Fig. 4a). Similarly, the electric conductivities of S. aureus suspensions treated with 6.25, 12.5, 25, 50, 100, 200 µg/ml ε-PL increased significantly (p < 0.05) with treatment time (20–45 min) from 3.5, 3.6, 3.6, 3.5, 3.4 ms/cm to 5.0, 5.5, 6.4, 7.1, 7.6, 8.5, respectively (Fig. 4b). The electric conductivities of all samples almost were stable after 45 min. The increase in electric conductivity of samples with increasing concentrations of ε-PL suggested that the cytoplasmic membranes were disrupted, which caused cellular leakage (Dayan et al., 1999; Galindo et al., 1999). Moreover inhibitory effects of ε-PL on E. coli and S. aureus were very remarkable at front 45 min. The leakage of bacterial cells was probably caused by the interaction between ε-PL and the cytoplasmic membrane.

This present study was similar to Kong et al. (2008) who reported that oleoyl chitosan microspheres with degree of substitution of 5% caused the increase of E. coli membrane permeability to 96% compared with the control by electric conductivities. Sawai (2003) evaluated the antibacterial activities of metallic oxide (ZnO, MgO and CaO) powders against S. aureus and E. coli quantitatively by measuring the change in electrical conductivity of the growth medium caused by bacterial metabolism. Cloete, Thantsha, Maluleke, and Kirkpatrick (2009) studied the leakage of tested cells of P. aeruginosa and E. coli by determining electrical conductivity, evaluating that the dilution of halide derived anolyte was effective in killing the test bacteria.

3.5. SDS-PAGE of protein patterns of bacteria treated with ε-PL

SDS-PAGE profiles of proteins of S. aureus cells are shown in Fig. 5. Lane 1, 2, 3 were the control, treated sample, and Marker, respectively. As seen in Fig. 5, the protein profiles of bacteria treated with ε-PL differed from those of the control. There were two thick bands (approximately 10.5 kDa, 14.4 kDa) in lane 1 for untreated bacteria. However, these two bands disappeared in lane 2 for treated bacteria with ε-PL. Two new bands (approximately 36 kDa, 110 kDa) appeared in lane 2. Moreover, there were more kinds and amounts of bands above 31 kDa in treated sample than the control. This demonstrated that ε-PL could damage bacterial cells through the destruction of cellular proteins. The reason of protein bands disappearance might be that ε-PL interfered synthesis of bacterial cell proteins, or resulted in the leakage of proteins from bacterial cells. And the reason of new protein bands appearance probably attributed to aggregation of bacterial proteins by ε-PL.

These results were in agreement with the findings of Cloete et al. (2009) who observed the disappearance of protein bands after exposure of P. aeruginosa to halide anolyte and suggested that anolyte caused bacterial death by complete destruction of proteins or partially degrading proteins. Zinkевич, Beech, Tapper, and Bogdarina (2000) also found the disappearance of protein bands after exposing E. coli to an anolyte solution with an ORP of 1000 mV. Sitoloh et al. (2012) reported that protein patterns of L. monocytogenes and S. Enteritidis treated with glycinin and basic subunit faded most bacterial protein bands.

4. Conclusions

These results showed that ε-PL had antibacterial activities against E. coli and S. aureus. The antimicrobial activities increased with the increase of ε-PL concentration with MIC 12.5 µg/ml. On the one hand, the antibacterial action of ε-PL not only involved intercellular reaction, but may also affect the structure of the phospholipid bilayer of the cell membrane, thereby changing the cell membrane permeability to result in the release of some cellular components such as proteins and ions. On the other hand, the mechanism of bacterial cells damage might be that ε-PL interfered synthesis of bacterial cell proteins or induced aggregation of bacterial proteins.

Acknowledgments

This work was supported by Science and Technology Development Planning of Shandong Province (2011GGH22110 and 2012YD07012) and Science and Technology Development Planning of Shandong Province Colleges and Universities (J13LE02), and National Natural Science Foundation of PR China (31371839).

References


