Potential of a novel and thermostable recombinant phage endolysin of Escherichia phage KW1E_UTAR against *Shigella* spp.

Dharshini Selvam[1,*], *Nor Ismaliza* Mohd Ismail[1], and *Michelle* Y. T. Ng[2]

[1]Department of Biological Science, Faculty of Science, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Baru Barat, 31900, Kampar, Perak, Malaysia
[2]Department of Allied Health Sciences, Faculty of Science, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Baru Barat, 31900, Kampar, Perak, Malaysia

Abstract. Shigella causes millions of cases and hundreds of thousands of deaths annually, mainly affecting children in impoverished countries. Shigellosis outbreaks have been linked to waterborne transmission, and chlorination is commonly used to disinfect water. However, the emergence of chlorine-resistant *Shigella* spp. is a concern. Therefore, it is imperative to develop an alternative antibacterial agent such as endolysin against the *Shigella* spp. In this study, the Escherichia phage KW1E_UTAR gene encoding for endolysin was successfully cloned and expressed in *Escherichia coli* pET-28a. The purified endolysin was characterized, and its antibacterial activity was determined. The purified endolysin remained stable between pH 4 and 9, and at a broad range of temperature (4°C–75°C). The endolysin's storage stability was tested for one week, one month, and three months at different temperatures (4°C, −20°C, and −80°C). Despite over 50% drop in the lytic activity, the purified endolysin outperformed the commercial lysozyme. Besides, all four *Shigella* spp. tested were susceptible to the purified endolysin, with *Shigella flexneri* being the most prominent. Subsequently, the purified endolysin's effectiveness in removing *S. flexneri* from spiked water samples was evaluated. After an hour of treatment, the number of viable bacteria in the water samples was reduced by 99.9%. Therefore, endolysin could be effective at eradicating *Shigella* spp. in various water sources.

1 Introduction

Shigella is the commonest waterborne or foodborne bacterial infection, and it is frequently associated with water and food-contaminated consumption [1]. Of the four Shigella spp., S. flexneri is the major cause of shigellosis outbreaks which involve more than 2 million patients per year, with most patients being children aged less than five years, including babies [2]. The Global Enteric Multicentre study determined that S. flexneri accounted for more than 67% of Shigella isolates globally, especially in poor-resourced countries [3]. The number of people infected with pathogenic microorganisms in industrialized countries was

[*]Corresponding author: dharshini_selvam@yahoo.com

© The Authors, published by EDP Sciences. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (https://creativecommons.org/licenses/by/4.0/).

significantly lower compared to developing countries.

This is because most of the people in developing nations unable to access clean water and poor sanitation due to low financial resources. Chlorine treatment, ultraviolet radiation, distillation, and carbon filtration are methods to remove biological pollutants in water. However, their byproducts such as halogenated organic molecules, can be carcinogenic and mutagenic, thus they have been linked to various health illness [4]. Multidrug resistance in Shigella infections is a growing concern, hence, necessitating scientists to explore the development of new antimicrobial agents, such as endolysin to combat this issue.

Endolysins are lytic enzymes produced by bacteriophages near the end of their replication cycle. It can cleave the peptidoglycan layer, breaking the bacterial cell wall, and resulting in cell lysis. Endolysin is regarded as a unique antibacterial agent due to its quick action, minimal resistance, and great selectivity to the target organism [5]. This study aimed to clone and express the novel KW01-E endolysin gene. The efficacy and stability of the recombinant endolysin produced were evaluated to ensure its long-term success as alternative antibacterial agent in water treatment aiming to control waterborne infections, particularly against *S. flexneri.*

2 Methods

2.1 Genomic analysis of Escherichia phage KW1E_UTAR

The Escherichia phage KW1E_UTAR (GenBank accession No. MZ506873.1) was isolated from a hot spring at Kuala Woh Recreational Forest, Perak, Malaysia. Whole phage genome was sequenced and the open reading frames (ORFs) for putative protein were annotated using Prokka version 1.12. The gene encoding for endolysin were identified and the sequence were obtained from the NCBI database.

2.2 Molecular cloning, expression, and purification of protein

The endolysin sequence was cloned into a pET-28a expression vector with a C-terminal His6 tag. *E. coli* BL21(DE3) cells containing the plasmid harboring the gene encoding for endolysin were grown under kanamycin selection (50 µg/mL). The protein was induced with IPTG (0.75 mM) and purified using Nickel resin (NEB Express) column based on the manufacturer's protocol with slight modifications. The protein was dialyzed, concentrated, and quantified. Then the protein fractions were analyzed using 15% SDS-PAGE.

2.3 Endolysin Activity

The assay was conducted following the method described by Zhang et al. [6], with modifications. Briefly, the purified endolysin was used to assess the lytic ability against the *Shigella* spp., and both Gram-negative and Gram-positive bacteria. The overnight bacteria cells were sub-cultured and grown to log phase OD_{600nm} 0.65–0.7 at 37°C with shaking. Bacterial suspension was then placed into a 96-well plate and purified endolysin (100 µg/µL) were mixed with the culture to a final volume of 200 µl per well. The commercial lysozyme was used as the positive control, whereas phosphate buffer was used as the negative control. The changes in turbidity were measured at OD_{600nm} at every 5 min interval for a duration of 1 h with the Microplate Absorbance Reader (Bio-Rad).

2.4 Effects of temperature, pH, and storage stability on the lytic activity

The activity of purified endolysin over different temperatures (4-95°C) upon 30 min treatment was evaluated. Additionally, the activity of purified endolysin over different pHs (4-11) upon 2 h treatment was also tested. The enzyme activity was determined by measuring the time-dependent turbidity changes in a suspension containing a mixture of *S. flexneri* and purified endolysin. Changes in turbidity were measured at OD_{600nm} at every 5 min intervals for an hour and commercial lysozymes were used as comparison. The purified endolysin was stored in 20 mM Tris HCI, 100 mM KCI and 50% glycerol at pH 7.5. The storage stability of the purified endolysin was assessed based on different storage temperatures (4, -20 and -80°C) for one week, one month and three months. Antimicrobial activities were assessed by turbidity reduction assay as described in section 2.3.

2.5 Antibacterial assessment in *S. flexneri*-spiked water samples

Water samples were collected from various sources at Kampar, Perak Malaysia, which included the lake water (W1), wet market drain water (W2), and waterfall (W3). The collected water samples were tested for its pH value. Prior to spiking them with bacteria, the water samples were centrifugated at 10,000×*g* for 30 min at 4°C to remove any sediments and autoclaved to eliminate any existing bacteria. Subsequently, 0.1 mL of overnight *S. flexneri* culture were seeded into the 10 mL sterile water samples and grown to log phase. Then, the purified endolysin was added into the mixture and treated for 4 h. The efficiency of enzyme activity was calculated as: % reduction = (A-B) x 100/A, where A is the number of viable bacteria before treatment, B is the number of viable bacteria after treatment. The log-linear Chick-Watson (CW) model was used to determine the disinfection kinetics as described by Park et al. [7].

3 Results and discussion

3.1 Genomic analysis of Escherichia phage KW1E_UTAR

Escherichia phage KW1E_UTAR (GenBank accession No. MZ506873.1) has a genome size of approximately 45 kb, with 67 predicted ORFs which is shown in Figure 1. Each of the gene products were compared to the protein database using BLAST. Five of the putative proteins have no sequence similarity to any other proteins, thus indicating its novelty. The putative genomic sequence of endolysin derived from this phage was identified to be 495 bp and its product is predicted to be 18 kDa (164 amino acids) in size.

3.2 Molecular cloning, expression, and purification of protein

The identified endolysin sequence was successfully cloned into the pET-28a. This was confirmed by the band located at 18 kDa on SDS-PAGE gel in Figure 2. Based on Table 1, the final concentration and yield of the purified endolysin obtained was 2.178 mg/mL and 67.58%, respectively.

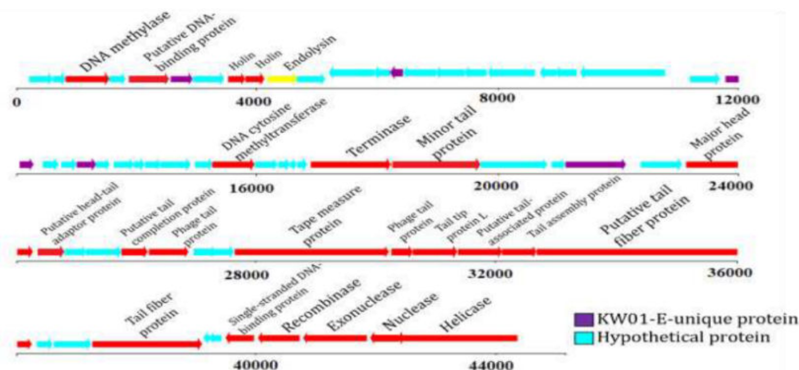

Fig. 1. Genome map of Escherichia phage KW1E_UTAR (GenBank accession No. MZ506873.1). Arrows indicate the direction of each ORF. Predicted proteins were based on BLAST hits from the NCBI non-redundant protein sequences database. The "KW01-E-unique proteins" were proteins with no matches from the database.

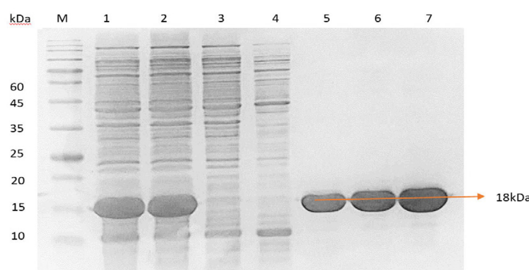

Fig. 2. Expression and purification of recombinant endolysin. Shown is the SDS-PAGE (15%) gel image of endolysin, M: ladder, lane 1: unclarified sample; lane 2: supernatant (clarified sample), lane 3: flowthrough 1, lane 4: flowthrough 2, lane 5: purified protein, lane 6: dialyzed protein using SnakeSkin Dialysis Tubing (Thermo Scientific), lane 7: concentrated protein using Amicon® Ultra Centrifugal Filter (Merk Millipore Ltd).

Table 1. Concentrations and yields of purified recombinant endolysin.

Samples	Protein concentration (mg/mL)	Yield (%)
Unclarified homogenate	3.223	100
Clarified homogenate	3.098	96.12
Concentrated and purified protein	2.178	67.58

3.3 Endolysin Activity

The purified endolysin exhibited lytic activity against several Gram-negative and Gram-positive bacteria, including, *S. dysenteriae, S. flexneri, S. sonnei, S. boydii, Escherichia coli* strains (BL21(DE3), Top 10, TGI, EPEC), *P. aeruginosa, K. pneumoniae, S. aureus and M. luteus*. All four *Shigella* spp. tested were susceptible to purified endolysin, with *S. flexneri* being the most prominent. Based on Figure 3, *S. flexneri* cells treated with purified endolysin (100 µg/ml) showed a 50% decrease in the absorbance at OD_{600} within the first 30 min of incubation. Whereas the absorbance of the cell culture treated with lysozyme (100 µg/mL) only dropped by 50% after 1 h of incubation. This clearly demonstrated that purified endolysin was more effective than lysozyme in eradicating *S. flexneri*.

Furthermore, purified endolysin in the present study exhibited a good lytic activity at a considerably low concentration. This is evident when the effective concentration of another *E. coli* phage endolysin, PlyE146, was four times (400 µg/mL) higher than the purified endolysin. Similarly, a study using endolysin P28 was able to lyse several Gram-positive and a few selective Gram-negative bacteria [8].

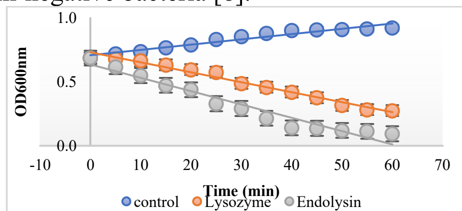

Fig. 3. The lytic activity of purified endolysin and lysozyme against *S. flexneri*. The error bars represent the standard deviation of triplicate readings. Results of the experiment were performed in triplicate are presented as mean ± SD.

3.4 Effect of temperature, pH and storage stability on the lytic activity of endolysin

The purified endolysin showed a wide range of thermal and pH stability compared to the commercial lysozyme as shown in Figure 4 (a) and (b). Eighty percent of the purified endolysin activity was retained even after incubating it at 4°C to 40°C for 30 min. However, the purified endolysin activity decreased with an increase in temperature, whereby no activity was detected after incubation at 95°C. Similar finding was observed in previous studies whereby, the endolysin activity declined at temperatures over 60°C [9]. Additionally, the current purified endolysin had significantly higher thermostability compared to another thermophilic endolysin, such as LysPA26, which only able to maintain 20% of its activity after incubation at 60°C for 30 min [10]. Based on Figure 4 (b), the optimum pH of the purified endolysin activity ranges from pH 6 to 8.5. At pH 10, the activity decreased to 14% and no lytic activity was detected at pH 11. The purified endolysin's thermal and pH stability could be attributed by the origin and characteristics of its bacteriophage. The Escherichia phage KW1E_UTAR remained viable at temperatures below 75 °C and pH 4-9 [11]. Apart from that, endolysins' storage stability is also vital to their application as an antibacterial agent. Based on Figure 4 (c), after incubation for 3 months, the purified endolysin still retained 42%, 54% and 28% of its activity after storage at 4°C, −20°C, and −80°C, respectively. On the contrary, in another study with similar storage conditions, showed that LysAm24's lytic activity was nearly undetectable after three months [12]. Overall, based on the temperature, pH and storage stability assessments, the purified endolysin demonstrated a greater lytic activity against *S. flexneri* compared to lysozyme.

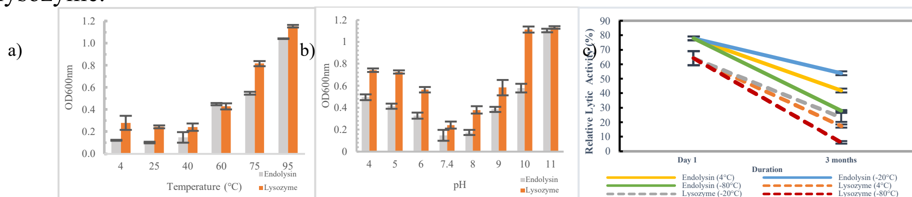

Fig. 4. Enzyme stability assessment of purified endolysin and lysozyme against *S. flexneri.* (a) effects of different temperatures on enzyme lytic activity, (b) effects of different pH on enzyme lytic activity and (c) effects of different storage condition on enzyme lytic activity. Results of the experiment were performed in triplicates and presented as mean ± SD.

3.5 Antibacterial assessment in *S. flexneri*-spiked water samples

The water sample W1, W2, and W3 exhibited pH value of 8.15, 6.8 and 7.95, respectively. The antibacterial efficiency of purified endolysin exhibited a dose and time-dependent manner. As shown in Figure 5(a), the viable colony counts of *S. flexneri* cells reduced significantly, around 99% and 100% when treated with 100 µg/mL purified endolysin for 1 h and 4 h, respectively. At a lower concentration (25 µg/mL), purified endolysin showed higher efficiency in eradicating *S. flexneri* at 4 h than at 1 h. Besides, increasing the dose of purified endolysin concentration and lengthening the treatment duration resulted in nearly 100% eradication of *S. flexneri* in different water samples. The log-linear Chick-Watson (CW) model was used to determine the efficacy of purified endolysin. As shown in Figure 5 (b), (c) and (d), a continuous increase in disinfectant efficacy was observed when purified endolysin concentration and treatment time were increased. After 4 h, the lowest log reduction of 0.595-log, 0.786-log and 2.21-log was observed at 25 µg/mL. Meanwhile, the highest log reduction of 20.28-log, 9.11-log and 18.86-log were observed at 100 µg/mL for water sample W1, W2, and W3 respectively. Similarly, several studies have shown that increase in contact time with the concentration of disinfectant increases the efficiency to eradicate desired bacteria in water samples [13].

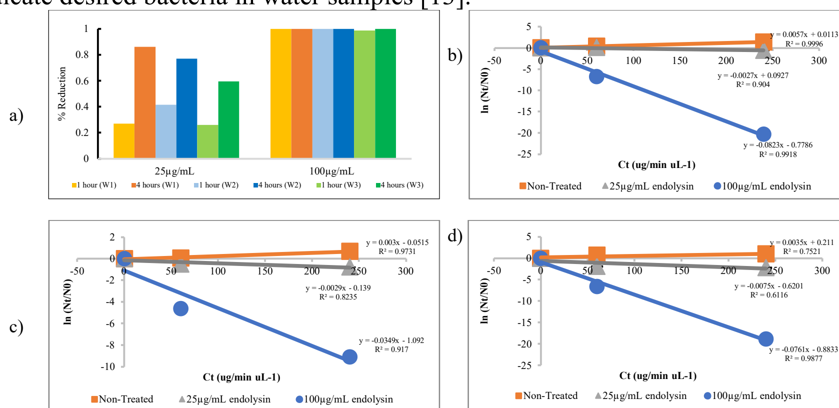

Fig. 5. Antibacterial assessment in *S. flexneri*-spiked water samples. (a) efficiency of purified endolysin in eliminating *S. flexneri* in water samples, W1- UTAR lake water, W2- Kampar wet market drain water, W3- Kampar waterfall, (b) Chick-Watson Coefficient of UTAR lake water (W1), (c) Chick-Watson Coefficient of Kampar wet market drain water (W2), and (d) Chick-Watson Coefficient of Kampar waterfall (W3). Results of the experiment were performed in triplicates and are presented as mean ± SD.

4 Conclusion

In this study, the endolysin gene from a novel Escherichia phage KW1E_UTAR was successfully cloned, expressed, and characterized. The purified endolysin showed a broad lytic spectrum against several Gram-positive and Gram-negative bacteria. It also exhibited a strong antibacterial activity against *S. flexneri*. Additionally, the purified endolysin is stable in a wide range of temperatures (4–75°C) and pHs (4.0–9.0). Despite over 50% drop in the lytic activity, the purified endolysin outperformed the lysozyme after 3 months of storage. Besides, the purified endolysin exhibited good antibacterial efficiency against *S. flexneri* in different water sources. These results suggest that the purified endolysin is a potential antibacterial agent in water treatment to eradicate *S. flexneri*.

This study was supported by UTAR Research Fund (IPSR/RMC/UTARRF/2020-C1/N04) and Fundamental Research Grant Scheme (FRGS/2/2014/SG05/UTAR/02/1).

References

1. Niyogi S. K. Shigellosis. *J. Microbiol.* **43**, 133–143 (2005).
2. Zafar A., Sabir N., and Bhutta Z.A. Frequency of isolation of *Shigella* serogroups/serotypes and their antimicrobial susceptibility pattern in children from slum areas in Karachi. *J. Pak. Med. Assoc*. **55**, 184–188 (2005).
3. Livio S., Strockbine N.A., Panchalingam S., Tennant S.M., Barry E.M., Marohn M.E., Antonio M., Hossain A., Mandomando I., Ochieng J.B., et al. Shigella Isolates From the Global Enteric Multicenter Study Inform Vaccine Development. *Clin. Infect. Dis.* **59**:933–941. doi: 10.1093/cid/ciu468 (2014).
4. Bichi, Mustapha, Agunwamba, Jonah and Muyibi, S.A. Kinetics of water disinfection with Moringa oleifera seed extracts. J Environ Earth Sci. 2. 58-68 (2012).
5. Khan F M, Gondil V S, Li C, Jiang M, Li J, Yu J, Wei H and Yang H. A Novel Acinetobacter baumannii Bacteriophage Endolysin LysAB54 with High Antibacterial Activity Against Multiple Gram-Negative Microbes. Front. Cell. Infect. Microbiol. **11** (2021).
6. Zhang H, Buttaro BA, Fouts DE, Sanjari S, Evans BS, Stevens RH. Bacteriophage ϕEf11 ORF28 endolysin, a multifunctional lytic enzyme with properties distinct from all other identified *Enterococcus faecalis* phage endolysins. Appl. Environ. Microbiol. 85(13), 18–19 (2019).
7. Park S, Ko YS. Jung H, Lee C, Woo K, Ko G. Disinfection of waterborne viruses using silver nanoparticle-decorated silica hybrid composites in water environments. Science of the Total Environment. 625: 477–485 (2018).
8. Dong H, Zhu C, Chen J, Ye X and Huang Y-P. Antibacterial Activity of Stenotrophomonas maltophilia Endolysin P28 against both Gram-Positive and Gram-Negative Bacteria. Front. Microbiol. 6:1299. doi: 10.3389/fmicb.2015.01299. (2015).
9. Lim, JA., Lee, N., Chun, HS. et al. Characterization of a novel endolysin from bacteriophage infecting Vibrio parahaemolyticus, vB_VpaP_KF2. Appl Biol Chem 63, 40. (2020).
10. Guo M, Feng C, Ren J, Zhuang X, Zhang Y, Zhu Y, Dong K, He P, Guo X, Qin J. A Novel Antimicrobial Endolysin, LysPA26, against Pseudomonas aeruginosa. Front Microbiol. doi:10.3389/fmicb.2017.00293. (2017).
11. Ahmad Tarmizi, A.H., MYT Ng, and N.I.Mohd Ismail. Characterization of *E. coli* BL21 (DE3) bacteriophage KW01-E isolated from Kuala Who Hot Spring in Perak, Malaysia. [Unpublished manuscript]. (2022)
12. Antonova NP, Vasina DV, Lendel AM, Usachev EV, Makarov VV, Gintsburg AL, Tkachuk AP, Gushchin VA. Broad Bactericidal Activity of the Myoviridae Bacteriophage Lysins LysAm24, LysECD7, and LysSi3 against Gram-Negative ESKAPE Pathogens. Viruses. doi: 10.3390/v11030284. (2019).
13. S. Kesar, and M.S. Bhatti.Chlorination of secondary treated wastewater with sodium hypochlorite (NaOCl): an effective single alternate to other disinfectants. Heliyon, 8 (11) Article e11162. (2022).