Journal of Experimental Microbiology and Immunology (JEMI) Vol. 9:1-5

The Use of Microwave Radiation in Combination with EDTA as an Outer Membrane Disruption Technique to Preserve Metalloenzyme Activity in *Escherichia coli*

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Ethylenediaminetetraacetic acid (EDTA) is commonly used in the isolation of bacterial proteins to disrupt the outer membrane of gram negative cells by chelating Mg^{2+} ions, essential to outer membrane stability. The chelation properties of EDTA can interfere with metalloenzymatic activity, whose catalysis is dependant upon the cationic cofactors that are chelated by EDTA. An alternative method to disrupt the outer membrane while preserving metalloenzyme activity is athermal microwave radiation. This study demonstrated that athermal microwave radiation succeeds in decreasing membrane integrity in *E.coli C29,* and that maximal enzymatic activity of the metalloenzyme alkaline phosphatase was achieved using a membrane disruption technique that combined EDTA treatment and athermal microwave radiation.

Isolation of periplasmic enzymes from Gram-negative bacteria requires the disruption of the outer membrane. One of the most common methods of isolating periplasmic enzymes is the use of EDTA (7), a chelating agent that sequesters magnesium, zinc, and calcium. This chelator is used to destabilize the outer membrane (OM) because the integrity of the OM relies on Mg^{2+} ions, which electrostatically link LPS molecules together (6). The activity of metalloenzymes also relies on divalent cofactors, and this activity may be compromised when EDTA is used as a means of OM disruption (3).

A plausible alternative to EDTA-induced membrane disruption that would conserve metalloenzymatic activity is to expose cells to microwave radiation. Microwaves are commonly used to completely inactivate bacteria through thermal and theoretical athermal mechanisms including cell membrane rupture, selective heating, electroporation, and magnetic field coupling (5,11). This study was designed to exploit the athermal mechanism of microwave radiation to disrupt the OM of *Escherichia coli* while maintaining the activity of its periplasmic metalloenzymes. The initial focus of this investigation was to determine athermal microwave effects on membrane integrity using differential stain fluorescent microscopy and cell viability assays. Protein release from different microwave radiation exposure times was tracked using SDS-PAGE analysis, and periplasmic metalloenzymatic activity was assessed using an alkaline phosphatase assay.

MATERIALS AND METHODS

Bacterial strain and culture growth. *Escherichia coli* C29 was obtained from the UBC Department of Microbiology and Immunology. Overnight cultures were grown in M9 media (12), 20% w/v glycerol in a 37ºC shaking water bath. Four flasks containing 102 mL of fresh M9 media, 20 %w/v glycerol were inoculated with overnight culture to a starting OD_{460} of 0.2, returned to a 37ºC shaking water bath, and monitored until exponential growth was obtained.

Cell harvest and microwave treatment. The log phase culture was centrifuged for 5 minutes in a Beckman J2-21 ultracentrifuge (Beckman-Coulter) at 13000 x g. The pellets were washed twice in 0.01 M Tris-HCl (pH 7.5), 0.03 M NaCl and resuspended in 31 mL 0.033 M Tris-hydrochloride (pH 7.5). 31 mL of 0.033 M Tris-hydrochloride (pH 7.5), 40 %w/v sucrose was added with rapid mixing and the suspension was incubated for ten minutes at room temperature. Six 10 mL fractions were removed from the suspension, transferred to individual 50 mL beakers weighing between 49.76 g and 50.99 g, and initial temperatures were recorded. Each 50 mL beaker was nested within a 500 mL beaker containing an equivalent amount of ice and placed centrally on the turntable of the *Genius* Panasonic microwave Mn-5853C (120 V, 60 Hz, 1380 watts, 800 watts normal maximum output power). The samples were microwaved at medium-low power for 15, 20, 25, 30, 35, and 40 seconds, removed from the ice, and the final temperature recorded.

Membrane integrity and viability assay. After microwave treatment 8 mL of each fraction was centrifuged for 10 minutes at 13000 x g in a Beckman J2-21 ultracentrifuge. The pellet was resuspended in 1 mL of 0.85% NaCl, and 250 uL of this suspension was added to 10 mL of 0.85% NaCl. The diluted sample was incubated at room temperature for 15 minutes, gently vortexed, incubated for an additional 15 minutes, and centrifuged at 10000 x g for 5 minutes. The pellet was washed twice in 10 mL 0.85% NaCl and resuspended in 5 mL of the same solution. 1.5 uL of 0.01 %w/v acridine orange (Sigma-Aldrich) and 1.5 uL of 20 mM propidium iodide (Sigma-Aldrich) were mixed and added to 1 mL of each resuspended sample, gently vortexed, and incubated in the dark for 15 minutes at room temperature. Wet mounts of each dyed sample solution were prepared and observed under a Zeiss Axiostar Plus fluorescence microscope set with fluoresceine filters. Photographs were taken using a Canon Powershot A95 digital camera aimed through the trinocular tube of the microscope. For the viability assay, each microwave irradiated sample was plated to final plated dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} on Luria-Bertani agar plates (12). Plates were incubated for 18 hours in a 37ºC incubator, after which the plates were observed and colonies were counted.

Isolation of periplasmic proteins. Periplasmic proteins were isolated from *E. coli* C29 growing at 37°C in M9 media, 20 %w/v glycerol by using the osmotic shock procedure. A 360 mL portion of cells was divided into two equal fractions and harvested as previously described for microwave treatment with slight modifications. Both fractions were resuspended in 35 mL 0.033 M Tris-hydrochloride (pH 7.5). 35 mL 0.033 M Tris-hydrochloride (pH 7.5), 40% sucrose was added to one while 35 mL 0.033 M Tris-hydrochloride (pH 7.5), 0.1 M EDTA, 40% sucrose was added to the other fraction. After microwave treatment, each sample was centrifuged at 13000 x g for 10 minutes, rapidly resuspended in 5 mL of 0.5 mM $MgCl_2$, and stirred rapidly while immersed in an ice bath for 10 minutes. The shocked cells were then removed by centrifugation and the supernatant containing periplasmic proteins was decanted and saved. Two mL of each supernatant were dialyzed in Spectra/Por 12000-14000kDa molecular membrane tubing (Cat#132700) for 16 hours against 0.01 M Tris-HC1 (pH 7.5), 1 mM $MgSO_4$ buffer on VWR and VWR 375 (Henry Troemnerll) stir plates with a speed setting of 3. The dialysis buffer was removed and fresh buffer was added after the first 8 hours.

Alkaline phosphatase assay. Two mL of dialyzed cell supernatant from each sample of the microwave-treated cells, microwave and EDTA treated cells, and EDTA-treated cells was added to 1.0 mL of 0.2 mg/mL p-nitrophenyl phosphate in 1 M Tris-HCl (pH 8.0). After color development, 0.2 mL of 13% K_2HPO_4 was added to stop the reaction and OD_{410} readings were taken on a Beckman Coulter DU530 spectrophotometer. One unit is that amount of protein that produces a change in absorbance of 1.0 per minute measured in a cuvette of l-cm path length. The molar extinction coefficient used in the conversion of absorbance and time into enzyme activity was 18,400 $M^{-1}cm^{-1}$.

SDS PAGE analysis. Microwave-treated samples, osmotically-shocked treated samples, and dialysis-treated samples were separated in 11% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (3.7mL mL H_2O, 3.7 mL 30% acrylamide/bis, 2.5 mL pH 8.8 buffer, 0.1mL 10 %w/v SDS, 0.05 mL 10 %w/v ammonium persulfate, 0.005mL TEMED (Bio-Rad Laboratories)) for 1 hour at 200 volts using a Bio-Rad 200/2.0 power supply (Bio-Rad Laboratories). Gels were stained with Bio-Rad Silver Staining reagents following manufacturer's instructions and dried for 2 hours in a Bio-Rad Gel Dryer 583 (Bio-Rad laboratories).

RESULTS

Microwave exposure decreases cell viability and disrupts membrane integrity. Increasing exposure to microwave radiation resulted in progressively decreasing viability (Fig 1). The results of the plating viability assay were consistent with observations of microwave-irradiated cells stained differentially with fluorescent dyes to assess membrane integrity. In Fig 2, a representative field of each sample observed with fluorescence microscopy illustrates the degree of outer membrane damage to the cells in each exposure time. Cells with a damaged outer membrane stained red, whereas cells with an intact outer membrane stained green. The ratio of green to red cells was 9.8:1 in the untreated sample, 4.0:1 in the 15 second sample, 1.9:1 in both the 20 and 25 second samples, 0.3:1 in both the 30 and 35 second samples, and 0.1:1 in the 40 second sample. The decreasing ratio of green to red cells demonstrates outer membrane disruption in each sample increases with longer exposure time to microwave radiation. Consistent bacterial numbers at all time points suggest that little, if any, cellular lysis occurs and it should also be noted that the maintenance of cell shape, observed with microscopy indicates the peptidoglycan layer remained intact with all microwave treatments.

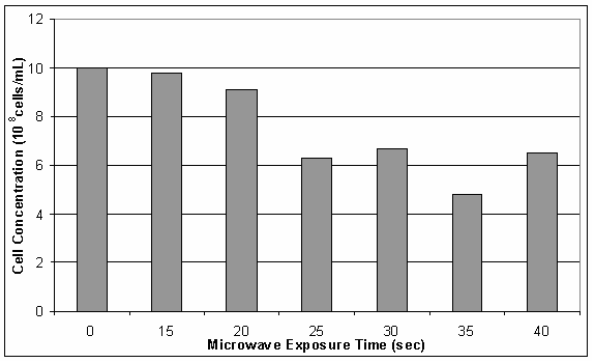

Fig 1. Viability of microwave irradiated *E. coli C29*. Cell concentration represents the average of duplicate plates for each time point, with the exception of the 30 second time point, at which only one plate was available.

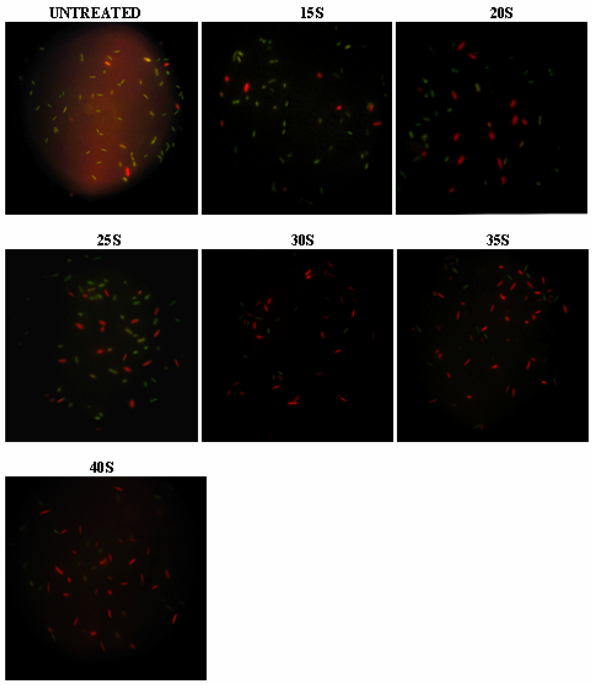

Fig 2. Differential fluorescent staining of *E. coli* C29. Cells were illuminated with a fluoresceine filter and magnified 1000X. Cells with intact outer membranes stained green; cells with disrupted outer membranes stained red. Photos represent an average trend over 15 fields.

Alkaline phosphatase activity and release differs in different cell disruption techniques. In Fig 3, the overall trend in both microwave-only and microwave-

EDTA treatments demonstrate that alkaline phosphatase release increased from 15 to 25 seconds and after maximal release at 25 seconds, enzymatic activity decreased from 30 to 40 seconds. At the 25 second exposure point the microwave treatment alone, or in concert with EDTA, released alkaline phosphatase two-fold more effectively than EDTA and osmotic shock alone. The greatest difference between the microwave-only and microwave-EDTA treatment trends occurring after 25 seconds is the preservation of alkaline phosphatase activity in the microwave-EDTA treated samples. Although there was an initial decrease in both microwave-EDTA and microwave only samples, enzyme activity leveled off in microwave-EDTA samples but continued to decrease in the microwave only samples.

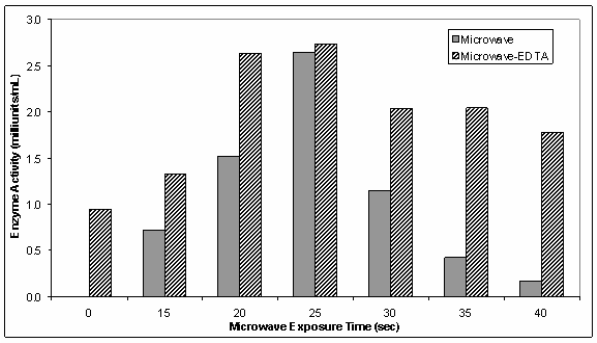

Fig 3. Alkaline phosphatase activity in the supernatants of microwave and microwave-EDTA treated cells. Untreated *E. coli C29* without EDTA demonstrated enzymatic activity which was treated as background and subtracted from all samples.

Microwave exposure time does not affect protein profiles. The SDS-PAGE gels of the protein released to the supernatant at different microwave times and treatments indicate that all three treatments had similar protein profiles, suggesting that the treatments did not differentially cause specific protein release (data not shown). However, the alkaline phosphatase assay suggests that the highest degree of alkaline phosphatase activity was retained in the microwave-EDTA treatment.

DISCUSSION

The trends observed in the cell viability assay suggest that prolonged exposure to microwaves results in decreased viability due to compromised membrane integrity (2). Microwaves cause oscillation of molecules in bacterial outer membranes that can lead to temporary or permanent disruption of the membrane (11). Previous work has shown that decreased viability of cells exposed to microwaves over 25 seconds may be due to protein aggregation as a result of a local increase in temperature (4). In order to negate the effect of temperature on cell viability, we used a temperature-controlled vessel during microwave treatment of the cells. The sample was nested in crushed ice, allowing for the maintenance of a temperature range over all time points between 20°C and 29°C. This suggests that athermal mechanisms are responsible for the decrease in cell viability, most likely due to outer membrane disruption, and not protein aggregation.

The most effective method for protein release was predicted to be the microwave-EDTA treatment because of the plausible synergistic effect of microwave radiation and EDTA on the integrity of OM. The results support this hypothesis, as there is equal or higher enzyme activity in the microwave-EDTA samples at all time points compared to the microwave-only samples (Fig 3). At all time points before maximal release at 25 seconds, similar amounts of protein were released in both samples. The electromagnetic energy of a microwave, when applied to a solution, causes the oscillation of dipolar molecules, such as H_2O, as well as the oscillatory migration of ionic species resulting in heat (1). By removing the effects of temperature, we begin to observe only the energetic effects of molecular oscillation. At 15, 20 and 25 seconds, the microwave energy significantly increases alkaline phosphatase release compared to EDTA osmotic shock, to as great as two-fold at 25 seconds. This suggests additional OM damage is contributed by microwave radiation, which may be due to the oscillation and subsequent disruption of the asymmetric lipid bilayer components. The damage sustained by the microwave could be more severe or irreparable than damage due to the chelation of Mg^{+2} ions by EDTA. Therefore when the cells are osmotically shocked, the OM could be disrupted to a greater extent in the cells exposed to both microwave-only and microwave-EDTA treatments than in the cells exposed to EDTA alone.

Maximum protein release occurred at 25 seconds for both the microwave and microwave-EDTA treatment, but as microwave exposure time increased above 25 seconds, two different trends were observed. The microwave-only treatment displayed a rapid decrease in alkaline phosphatase activity as microwave exposure increased, which translates to a 16-fold decrease between 25 and 40 seconds. The alkaline phosphatase activity of the microwave-EDTA treatment also decreased after 25 seconds but an EDTA buffering effect was observed, as its activity was maintained as microwave exposure increased. The data suggests a threshold of energy that the enzyme can be exposed to and retain its activity for both the microwave-only and microwave-EDTA treatments; this occurs at 25 seconds. If more microwave energy is absorbed by alkaline phosphatase, the enzymatic

activity begins to decrease. Two hypotheses may account for these trends. First, maintenance of alkaline phosphatase activity may be due to the increased ionic strength of the EDTA solution, which could have reflected some of the microwave energy, preventing the disruption of the enzymatic activity (8,10). Alternatively, there may be unidentified effects of divalent cations on microwave activity. Microwave radiation is considered to penetrate into solutions containing ionic compounds more poorly than into pure water because an ionic solution has a higher electroconductivity than pure water and is apt to reflect the energy of microwaves irradiated onto the surface of a solution (10). Secondly, the specific action of EDTA may interfere with alkaline phosphatase cofactors. The cofactors required by alkaline phosphatase for optimal activity are four Zn^{2+} ions and two Mg^{2+} ions, but only two of the Zn^{2+} ions are essential for catalytic activity (9). Since osmotic shock in all treatments was done with magnesium supplemented medium, we are uncertain if the magnesium concentration used affected metalloenzymatic activity.

It is possible that in the microwave-only sample, oscillation of alkaline phosphatase causes the release of two of the Zn^{2+} ions essential for catalytic activity which are replaced by other transition metals, resulting in metalloenzyme with distinct catalytic activities and physiochemical properties (9). In the microwave-EDTA sample the effect of other divalent cations is likely minimized because of the sequestration of divalent cations by EDTA. Although the mechanisms behind the buffering effects of EDTA remain unknown, this result and the others elucidated in our experiment may have positive implications for the future isolation of functional metalloenzymes from *E. coli*.

Microwave radiation in concert with EDTA for 25 seconds is the most effective treatment to maximize the release of proteins and maintain optimal metalloenzymatic activity as determined by alkaline phosphatase.

FUTURE EXPERIMENTS

Future experiments are necessary to elucidate the mechanisms by which athermal microwave radiation affects bacteria and their proteins. While the importance of cations in outer membrane disruption by EDTA has been established, the role of cations in the microwave disruption method and on the activity of the enzymes released using this method remains unclear. Experiments should be performed to investigate the role of cations in microwave-irradiated solution on enzyme activity. Changing the concentrations or identities of the cations in the cellular environment may provide insight into reasons for the changes in enzyme activity observed when EDTA was added to the cells prior to microwave treatment.

It may also be useful to quantify the release of alkaline phosphtase from the cell upon microwave radiation to clarify the reason for the changes in enzyme activity observed in this study. This could be achieved by determining the protein concentration in supernatants of cells that were subjected to different disruption methods in addition to measuring enzyme activity, such the specific activity of the alkaline phosphatase in these samples could be ascertained. Furthermore, an experiment to test the direct effects of athermal microwave radiation on enzyme activity could be performed by exposing alkaline phosphatase purified from *E. coli* C29 to microwaves for variable periods of time and observing the effect of this exposure on enzyme activity.

ACKNOWLEDGEMENTS

We thank the department of Microbiology and Immunology at the University of British Columbia for the funding of this project. We would like to express our gratitude to Jennifer Sibley, Karen Smith and Mike Chow for their guidance, emotional and technical support, early mornings, and encouragement to remain in the Microbiology/Immunology program at UBC. We are grateful to Lando, Nick, Ed, Gilly and Loida from the media room for their contributions and their patience, and to Dr. Gaye Sweet for lending her assistance and wisdom on many occasions. Finally, we owe a great deal of thanks to Dr. William Ramey for his great wealth of knowledge and advice, his role as a mentor and overseer of the Epsilon 1 gaggle of geese.

REFERENCES

1. **Arnold, R. G. et al. 2000.** Kinetics of microbial inactivation for alternative food processing technologies: microwave and radio frequency processing. Report of the Institute of Food Technologists for the Food and Drug Administration of the U.S. Department of Health and Human Services and Center for Food Safety and Applied Nutrition. June 2.
2. **Bogosian, G. and E. V. Bourneuf.** 2001. A matter of bacterial life and death. *EMBO*. **2**:770-774
3. **Derman, A. and J. Beckwith**. 1991. *Escherichia coli* alkaline phosphatase fails to acquire disulphide bonds when it is retained in the cytoplasm. *J. Bacteriol*. **173**: 7719-7722.
4. **Kozempel, M. F., B. A. Annous, R.C. Cook, O.J. Scullen and R.C. Whiting.** 1998. Inactivation of microorganisms with microwaves at reduced temperatures. *J. Food Protect*. **61**: 582-585.
5. **Kozempel, M. F., R.C. Cook, O.J. Scullen and B. A. Annous.** 2000. Development of a process for detecting non-thermal effects of a microwave energy on microorganisms at low temperature. *J. Food Processing*. In Print.
6. **Labischinski, H., G. Barnickel, H. Bradaczek, D. Naumann,E. T. Rietschel and P. Giesbrecht.** 1985. High state of order of isolated bacterial lipopolysaccharide and its possible contribution to the permeation barrier property of the outer membrane. *J. Bacteriol*. **162**:9-20.
7. **Nossal, N. C., and L. A. Heppel**. 1966. The release of enzymes by osmotic shock from *E. coli* in exponential phase. *J. Biol. Chem*. **241**:3055-3062.

8. **Porcelli, M. *et al*.** 1997. Non-thermal effects of microwaves on proteins: thermophilic enzymes as model system. *FEBS Lett.* **402:** 102-106

9. **Simpson, R. T., and B. L. Valle.** 1968. Two differentiable classes of metal ions in alkaline phosphatase of *Escherichia coli*. *Biochem*. **7:** 4343-4350

10. **Watanabe, K., Y. Kakita, N. Kashige, F. Miake and T. Tsukiji.** 2000. Effect of ionic strength on the inactivation of micro-organisms by microwave irradiation. *Lett. App. Microbiol*. **31**: 52-56

11. **Woo, I. S., I. K. Rhee and H. D. Park.** 2000. Differential damage in bacterial cells by microwave radiation on the basis of cell wall structure. *Appl. Environ. Microbiol*. **66**:2243-7.

12. **Zimbro, M. J. and D. A. Power (eds).** 2003. Difco & BBL Manual: Manual of microbiological culture media, 1st ed. Franklin Lakes, New Jersey.