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# The Inhibitory Effect of EDTA and $Mg^{2+}$ on the Activity of NADH Dehydrogenase in Lysozyme Lysis

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**NADH dehydrogenase of *Escherichia coli*, a component of the electron transport chain, oxidizes NADH to $NAD^+$. Previously, it was demonstrated that when *E. coli* K12 cells were lysed by the lysozyme lysis method, NADH dehydrogenase activity was inhibited; however, when cells were lysed by French press, NADH dehydrogenase activity was observed. In this study, components commonly used in lysozyme lysis were examined for their effect on NADH dehydrogenase activity. It was found that the combination of EDTA and $Mg^{2+}$ inhibit the enzyme's activity. Hence, we have developed alternative protocols of lysing cells with lysozyme that do not require EDTA. One method involves the use of polymyxin B, while the other utilizes the mechanical freeze-thaw process. Our alternative methods eliminate the need for EDTA by utilizing different mechanisms of disrupting the outer membrane. Though these alternative methods did not give rise to high NADH dehydrogenase activity, it may be possible to further optimize their conditions to yield lysate containing functional NADH dehydrogenase.**

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The periplasmic enzyme nicotinamide adenine dinucleotide (NADH) dehydrogenase is an important electron transport chain enzyme of *Escherichia coli*. The enzyme, also known as type I dehydrogenase, is an oxidoreductase that oxidizes NADH to $NAD^+$ and its activity is dependent on an iron-sulfur cofactor (3). To study the function of this enzyme, cells need to be lysed without destroying enzyme activity. Mechanical methods, such as French press or sonication, can be used to lyse cells. NADH dehydrogenase activity is retained despite the stresses on the cells induced by these protocols. Alternatively, cells may be lysed using enzymatic methods. However, previous attempts using a lysozyme lysis method (4) yielded no detectable NADH dehydrogenase activity, while alkaline phosphatase activity was retained.

In this paper we confirm the previous results (4), and we explore the possible reasons behind the lack of NADH dehydrogenase activity using the lysozyme lysis protocol. We hypothesized that one or more components in the lysozyme protocol—EDTA, $Mg^{2+}$, DNase and lysozyme—inhibited or destroyed the activity of the enzyme. EDTA disrupts the outer membrane of gram-negative bacteria by removing positive charges, causing the negatively charged heads of the phospholipids to repel one another. This allows lysozyme access to the peptidoglycan to cleave the C1 N-acetyl muramic acid (NAM) to C4-N-acetyl glutamic acid (NAG) glycosidic linkage. The end result is cell wall failure followed by cellular lysis. However, as EDTA is a powerful chelator of divalent cations, it can potentially sequester essential ions away from the NADH dehydrogenase. Therefore, we also hypothesized that EDTA was responsible for inhibiting NADH dehydrogenase activity. Hence, we sought modified lysozyme protocols that did not require EDTA for cell breakage. Two potential candidates were elucidated, based on our hypothesis that EDTA was likely responsible for inhibiting NADH dehydrogenase activity. Both protocols involved a novel way of disrupting the outer membrane of gram-negative bacteria without the use of EDTA. The first candidate was a procedure involving polymyxin B and lysozyme. Polymyxin B is an antibiotic known to disrupt the structure of the outer membrane of gram-negative bacteria. Polymyxins are cyclic, polycationic peptides with fatty acid chains. Their amphipathic structures allow them to interact with lipopolysaccharide and phospholipids. Polymyxins also interact electrostaticallly with the membranes by competitively displacing divalent cations from the negatively charged phosphate groups of the outer membrane lipids, causing disruption of the outer membrane (2). This permits polymyxin B access to the peptidoglycan wall where it can break the glycosidic bonds resulting in cellular lysis. The second protocol involved the use of cellular freeze-thawing combined with lysozyme. Freeze-thawing results in the formation of ice-crystals that disrupt the outer membrane of gram-negative bacteria. This mechanical action allows lysozyme to begin breaking down the cell wall.

In this study we utilized a simple assay to test the hypothesis that one or more of the components from the lysozyme protocol were responsible for loss of NADH dehydrogenase activity. We report that the combination of EDTA and $Mg^{2+}$ together account for the greatest loss of activity. We also present our results regarding two novel methods for isolating NADH dehydrogenase that do not involve the use of EDTA.

## MATERIALS AND METHODS

**Bacterial cell culture and growth.** *Escherichia coli* K12 strain B23 cells were grown in a standard Luria broth (LB) medium (4) to an early to late-log phase. Growth was stopped with a 15-minute incubation on ice. Two hundred and fifty mL of culture was used for French press, and 125 mL of cultures was used for lysozyme, polymyxin B (Sigma), and freeze-thaw lysis procedures. The cells were harvested by centrifugation at 7,000 x g for 10 minutes.

**French press treatment.** The cell pellet was resuspended in 30 ml of 5 mM Tris-HCl buffer, pH 7.4, and the suspension was then passed through a French press (Hancock Lab, University of British Columbia) twice, each time at 15000 psi. The lysate was then centrifuged at 10,000 x g for 10 minutes, and the supernatant was collected for further assay (4).

**Assaying the effects of lysozyme treatment components.** All combinations of EDTA (10 mM), $MgCl_2$ (10 mM), Chicken egg-white lysozyme (53 µg/ml, Sigma), Deoxyribonuclease Type I (0.22 mg/ml, Sigma) were added to French press lysate in a final volume of 100 µl. The suspensions were then incubated (37°C, 10 min), and NADH dehydrogenase activity was assayed. Combinations of only EDTA and $MgCl_2$ were also added to French press lysate with one reagent at fixed concentration (10 mM) and the other at different concentrations (10, 1, 0.5, 0 mM). The suspensions were then incubated (37°C, 10 min), and NADH dehydrogenase activity was again assayed.

**Lysozyme treatment.** The cell pellet was resuspended in 30 ml of 50 mM Tris-HCl buffer, pH 8.2. EDTA (10 mM) and Chicken egg-white lysozyme (53 µg/ml, Sigma) were added and incubated (37°C, 10 min). $MgCl_2$ (10 mM) was added and the suspension was swirled (37°C, 30 s), then Deoxyribonuclease Type I (0.22 mg/ml, Sigma) was added and incubated (37°C, 10 min). The lysate was then centrifuged as in the French press method, and the supernatant was assayed. The protocol was also performed with EDTA (10 mM) and different concentrations of $MgCl_2$ (10, 1, 0.5, 0 mM), and vice versa.

**Freeze-thaw treatment.** The cell pellet was re-suspended in 30 ml of 50 mM Tris-HCl buffer, pH 8.2. Lysozyme (53 µg/ml, Sigma) was added in three different ways: lysozyme was either added before the freeze-thaw, after the freeze-thaw, or half was added before and half was added after. The suspension was frozen in dry ice-acetone bath (2 min), then immediately thawed at 37°C. This process was repeated three times. $MgCl_2$ (10 mM) was added and the suspension was swirled (37°C, 30 s), then DNase (0.22 mg/ml) was added and incubated (37°C, 10 min). The lysate was then centrifuged as in the French press method, and the supernatant was collected for further assay (4).

**Polymyxin B treatment.** The cell pellet was resuspended in 30 ml of 50 mM Tris-HCl buffer, pH 8.2. Polymyxin B (100 µg/ml) was added and incubated (25°C, 25 min). Next, lysozyme (53 µg/ml, Sigma) was also added (37°C, 10 min). $MgCl_2$ (10 mM) was then added and the suspension was swirled (37°C, 30 s), and treated with DNase (0.22 mg/ml, 37°C, 10 min). The lysate was then centrifuged as in the French press method, and the supernatant was collected for further assay (4).

**NADH dehydrogenase activity assay.** We used 1.35 ml of 50 mM Tris-HCl buffer, pH 7.4 and 1.5 ml of $dH_2O$ to blank the spectrophotometer. One hundred µl of 5 mg/ml NADH (Sigma) was added to bring the absorbance ($A_{340}$) to 0.6. One hundred µl of sample was added. The absorbance was continuously monitored for one minute using the SpecX software (Vernier Inc, Seattle) (4).

## RESULTS

**Effects of lysozyme and French press methods of cell breakage on NADH dehydrogenase activity.** Previous studies report that NADH dehydrogenase enzyme activity is undetectable in *E.coli* K12 supernatant lysate derived from lysozyme cell breakage, though the enzyme activity is detectable in lysate obtained using a French press (4). Before experimentally determining potential explanations to these findings, we first repeated the reported experiment (4) to confirm reproducibility of the results. As expected, the lysate obtained from lysozyme treatment did not display NADH dehydrogenase activity, while the French press lysate showed roughly 2 U/ml of enzyme activity (Fig. 1). Spectrophotometric readings ($A_{280}$) indicated that the lysozyme-derived lysate contained 4-fold less protein than the French press-derived lysate. However, the lack of NADH dehydrogenase activity in lysozyme-derived lysate was likely not attributable to the lower protein concentration, as the lysate still contained 0.6 mg/ml of protein. We also found that the cell pellets obtained by both methods did not show NADH dehydrogenase activity, suggesting that the lack of detectable NADH dehydrogenase activity in lysozyme-treated lysate was not due to the enzyme spinning down with the pellet (data not shown). Therefore, NADH dehydrogenase was likely inhibited by components of the lysozyme lysis method.

**Determining components of lysozyme treatment that inhibit NADH dehydrogenase activity.** To determine the cause of NADH dehydrogenase inhibition in lysozyme-lysed cells, we tested individual components used in the lysozyme method in their ability to inhibit NADH dehydrogenase. We also examined the effects of combining two or more reagents used in lysozyme method on NADH dehydrogenase activity. Although we hypothesized that EDTA was responsible for inhibiting NADH dehydrogenase, interestingly, our results show that $Mg^{2+}$, EDTA, lysozyme, and DNase alone all slightly inhibited NADH dehydrogenase activity when added to French press lysate (Fig. 2). However, the combination of EDTA and $Mg^{2+}$ had the most profound effect (Fig. 3 and Fig. 4). EDTA together with $Mg^{2+}$ reduced activity by 88% (Fig. 3). Every sample that contained EDTA and $Mg^{2+}$ together showed decreased activity (Fig. 3 and Fig. 4). Also, the combination of lysozyme with EDTA reduced enzyme activity by 60% (Fig. 3), but the combination of EDTA, lysozyme, and DNase did not appear to inhibit NADH dehydrogenase (Fig. 4). These findings suggest that EDTA may not be the only factor that inhibits NADH dehydrogenase. EDTA in conjunction with $Mg^{2+}$, or perhaps even lysozyme, may result in a more complex mechanism of enzyme inhibition.

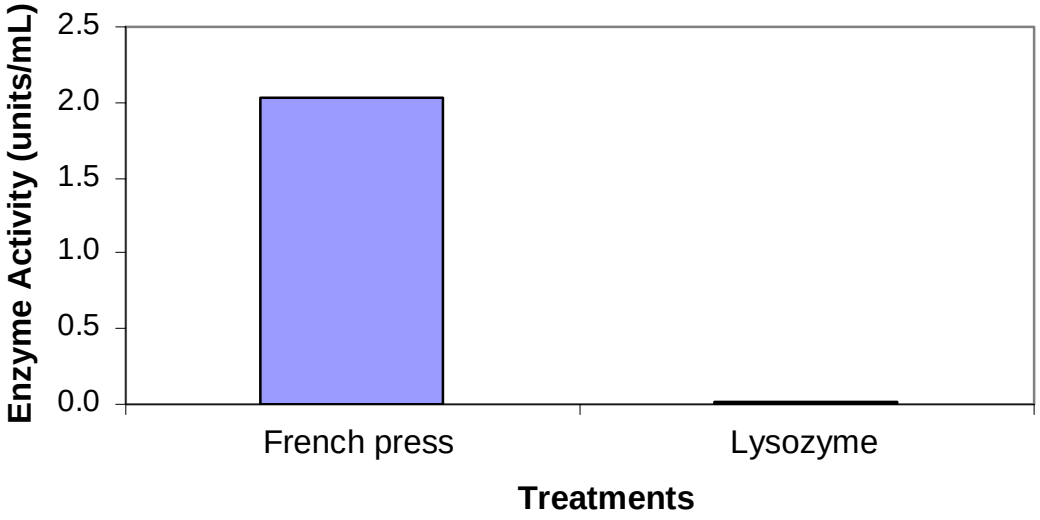


FIG. 1. NADH dehydrogenase activity in supernatants of samples prepared by either French press or lysozyme lysis.

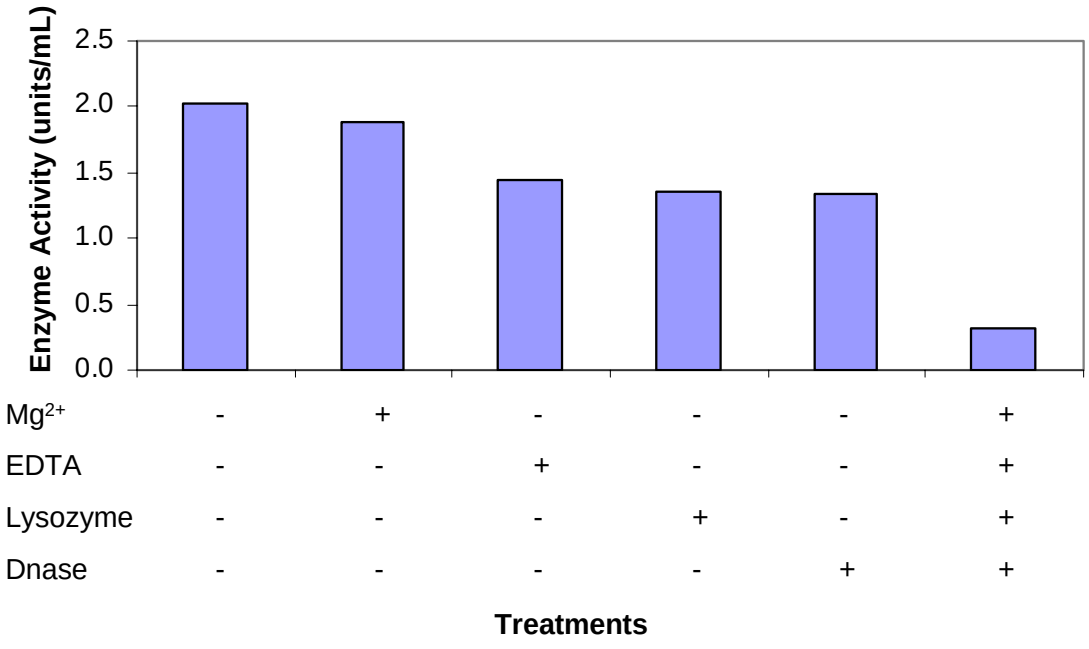


FIG. 2. NADH dehydrogenase activity from French press supernatant with single component addbacks.

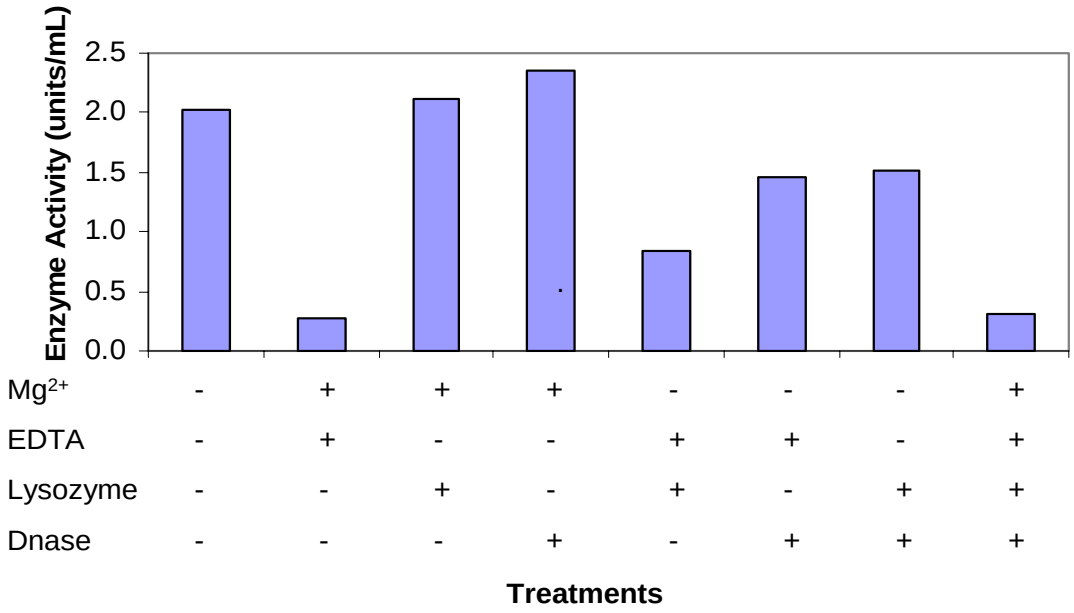


FIG. 3. NADH dehydrogenase activity from French press supernatant with two components addbacks.

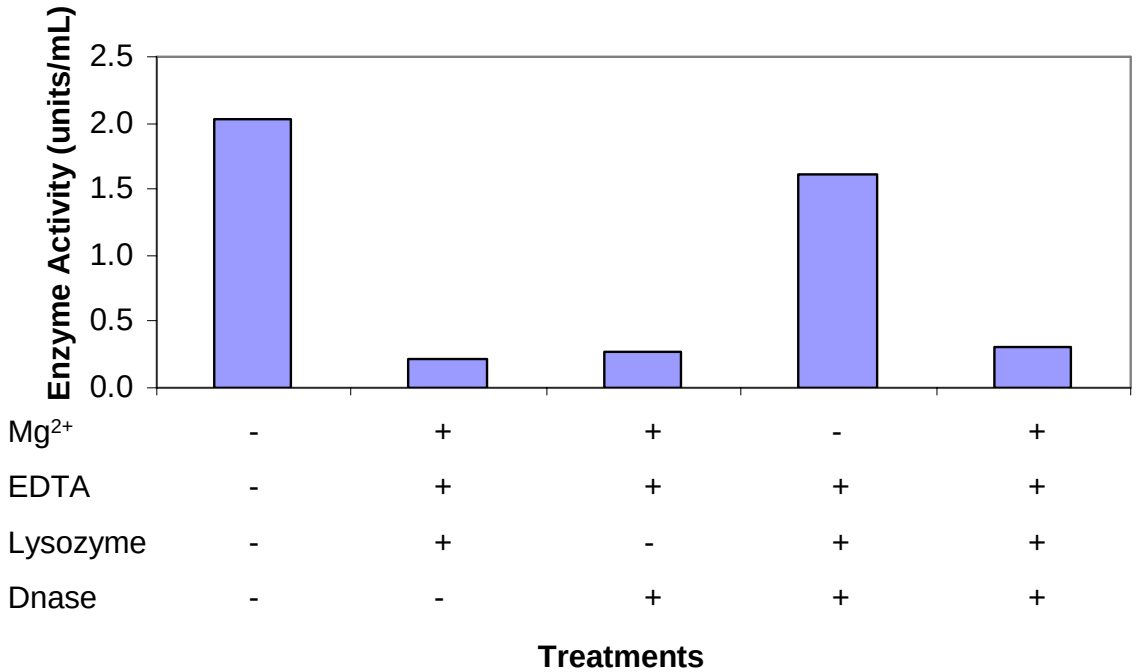


FIG. 4. NADH dehydrogenase activity from French press supernatant with three components addbacks.

**Lowering concentrations of EDTA and $Mg^{2+}$ .** Because the combination of 10 mM EDTA and 10 mM $Mg^{2+}$ added to French press lysate inhibited the activity of NADH dehydrogenase, we considered the possibility that lowering the concentrations of either EDTA or $Mg^{2+}$ may result in the retention of some enzyme activity. Keeping the concentration of $Mg^{2+}$ at 10 mM, we tested EDTA concentrations of 0, 0.5, 1, and 10 mM in French press lysate. As

expected, 0, 0.5, and 1 mM EDTA in conjunction with 10 mM $Mg^{2+}$ did not inhibit NADH dehydrogenase to the extent that was observed with 10 mM of both EDTA and $Mg^{2+}$ (Fig. 5, data not shown). Ten mM of both EDTA and $Mg^{2+}$ resulted in a decrease in activity of 79%, while 1 mM of $Mg^{2+}$ and 10 mM EDTA resulted in no significant decrease in enzyme activity. NADH dehydrogenase activity was not significantly inhibited by 0 or 0.5 mM EDTA with 10 mM $Mg^{2+}$ (data not shown). Similarly, we looked at the effects of 10 mM EDTA in conjunction with lower $Mg^{2+}$ concentrations: 1 and 10 mM $Mg^{2+}$. The addition of 1 mM $Mg^{2+}$ with 10 mM EDTA to French press supernatant lysate did not inhibit enzyme activity (Fig. 5). These results suggest that by lowering the concentration of EDTA or $Mg^{2+}$ from 10 mM to 1 mM or less, NADH dehydrogenase activity may be retained.

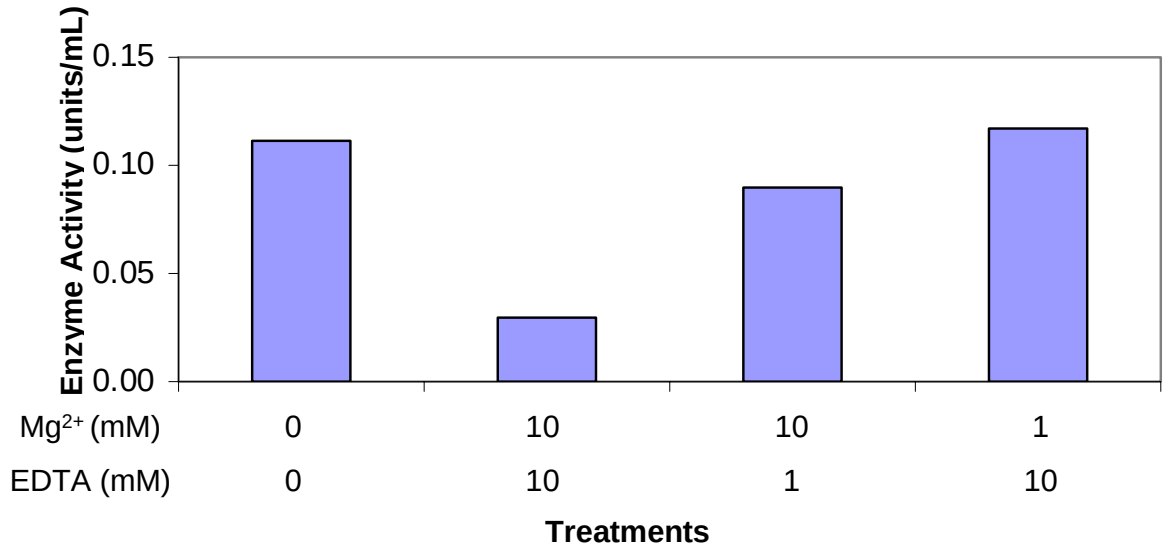


FIG. 5. NADH dehydrogenase activity from French press supernatant with varying concentrations of EDTA and $Mg^{2+}$

To test the relevance of our findings, we attempted to lyse cells using lysozyme with varying concentrations of EDTA and $Mg^{2+}$, as we had done when testing the effects of the components on French press lysate. One concern was the possibility that lowering either EDTA or $Mg^{2+}$ might result in inefficient cell lysis, particularly since EDTA is a requirement for weakening the outer membrane. We did not observe retention of NADH dehydrogenase activity for any of the lysozyme lysate samples (data not shown). Also, no enzyme activity was observed in the pellets (data not shown).

**Using alternatives to EDTA to weaken the outer membrane before lysozyme treatment.** Given that the presence of EDTA and $Mg^{2+}$ inhibited NADH dehydrogenase activity, but that their absence may have resulted in poor cell breakage, we developed alternative methods of weakening the outer membrane to allow lysozyme access to the peptidoglycan layer. Polymyxin B is sometimes used in lieu of EDTA to destabilize the outer membrane (2). Based on Dixon and Chopra's previous results (2), we tested 100 µg/ml polymyxin B in conjunction with lysozyme, in an attempt to generate lysate with NADH dehydrogenase activity (Fig. 6). Our results show that our method of combining 100 µg/ml of polymyxin B with 53 µg/ml of lysozyme results in 5-fold lower NADH dehydrogenase activity compared to the French press lysate. Although activity of polymyxin B lysate appeared to be 5-fold higher than the lysozyme lysate, it is inconclusive as to whether this is significant.

In addition to testing polymyxin B, we also explored a method of freeze-thawing cells to weaken the outer membranes and make cells more susceptible to lysis by lysozyme. In Fig. 6, we show that NADH dehydrogenase activity is 6-fold lower in the freeze-thaw compared to the French press lysate, but 4-fold higher than the lysozyme lysate. Both methods, as carried out, did not give rise to high levels of NADH dehydrogenase when compared to activities yielded by French press.

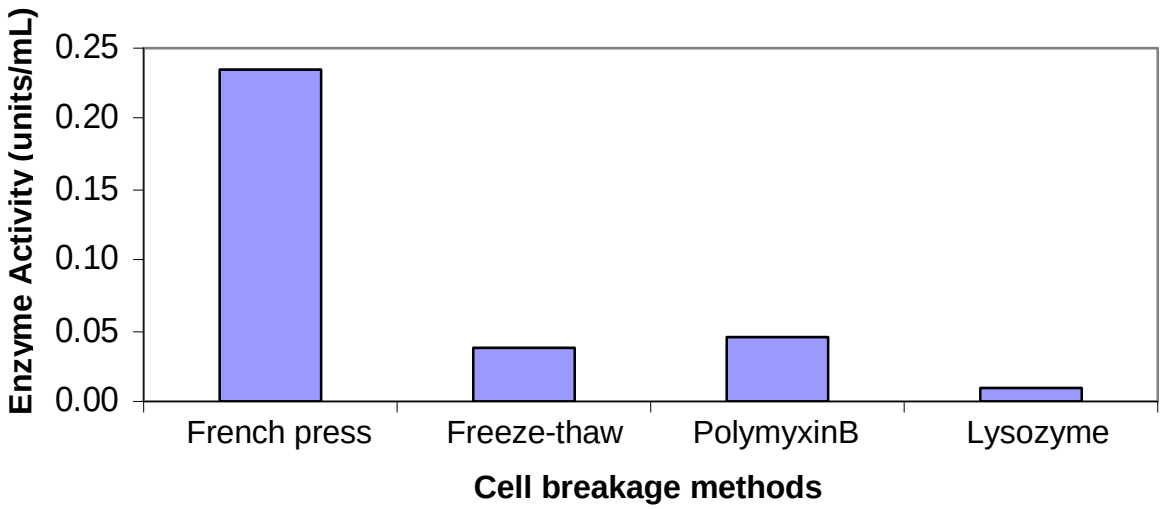


FIG. 6. Effects of different cell breakage methods on NADH dehydrogenase activity.

## DISCUSSION

The goal of this study was to explain the loss of NADH dehydrogenase activity in lysozyme-lysed cells. Our results indicate that the combination of EDTA and $Mg^{2+}$ may drastically reduce NADH dehydrogenase activity in lysozyme lysate, based on our French press addback results (Fig. 2, 3, and 4). Prior to these findings, we first measured lysozyme lysate protein concentrations to rule out the possibility that the undetectable enzyme activity was a result of undetectable levels of protein in the supernatant. There were observable levels of protein in the lysozyme lysate, though the concentration was 4-fold lower than the French press lysate. Still, if active NADH dehydrogenase existed in the supernatant, even in low concentrations, some activity should have been detected. This was not observed. Also, another group of researchers who isolated almost identical yields of protein in their French press supernatants compared to lysozyme supernatants were unable to detect NADH dehydrogenase activity in the lysozyme-lysed cells (4). These results suggest the lack of NADH dehydrogenase activity was not due to the lack of protein in the lysate.

Suspecting that the inhibition of the enzyme was due to components of the lysozyme lysis method, we added all possible combinations of the four reagents in the lysozyme protocol to the French press lysate. Upon treatment with lysozyme and EDTA, the NADH dehydrogenase activity was reduced by 50% compared with the control (Fig. 3). However, when the lysate was treated with lysozyme, EDTA, and DNase together, the enzyme activity was only slightly inhibited (Fig. 4). While it is intriguing that DNase appeared to maintain enzyme activity it is improbable that DNase would restore NADH dehydrogenase activity of the EDTA and $Mg^{2+}$ treated lysate, particularly considering that DNase was not able to restore activity to lysate containing EDTA, $Mg^{2+}$, and DNase (Fig. 4). The decreased enzyme activity observed in the lysozyme and EDTA treatment could be caused by experimental error, as the experiment was not duplicated.

With respect to the EDTA/$Mg^{2+}$ pair reducing NADH dehydrogenase activity, a complex may have formed between the two reagents, as EDTA would tend to chelate the divalent cation $Mg^{2+}$, and the complex could have inactivated NADH dehydrogenase. Additionally, each reagent on its own could have caused the conditions in the solution to be unfavourable and together the two molecules may have acted synergistically, dramatically lowering the activity. In French press samples containing EDTA and $Mg^{2+}$, and inhibited activity, the solution turned cloudy after the 10-minute incubation at 37°C. The identity of the precipitate or the cause of cloudiness remains unknown. It is unclear if the EDTA and $Mg^{2+}$ were binding to one another and forming a precipitate, or if addition of these two components altered the environment of the solution (pH), causing salts or buffer components to come out of solution. The optimal pH range for the enzyme activity is very narrow: between 6.5 and 7 (1). Because cells treated with lysozyme are in a turbid suspension, we cannot confirm if the cloudiness forms when the cells are lysed with lysozyme in the presence of EDTA and $Mg^{2+}$ or if this phenomenon is only observed when the lysozyme lysis conditions are simulated using French press lysate.

Another factor to consider when adding back reagents into the French press lysate to mimic the lysozyme procedure, is the possibility that the reagents react differently in the absence of cells and cell debris. In a normal lysozyme protocol, chemicals such as EDTA, and enzymes such as DNase are added to broken cell suspensions. However, in our protocols, we added back these chemicals or enzymes to cell debris-free supernatant. They may have interacted with the protein lysates differently than they would have in the presence of cell debris.

Despite these challenges, there was still reason to believe that 10 mM EDTA and 10 mM magnesium were responsible for inhibiting NADH dehydrogenase activity; therefore, we tried lowering their concentrations in an attempt to preserve enzyme activity. Decreasing their concentration to 1 mM or lower in French press lysate resulted in detectable enzyme activity. However, when these concentrations were applied to the lysozyme lysis method NADH dehydrogenase activity was not detected. Lowering EDTA concentrations in the lysozyme lysis method may not have been sufficient in disrupting the outer membrane; thus, the cells may not have been effectively lysed. Lowering $Mg^{2+}$ concentrations may have limited DNase activity, causing the sample to remain more viscous, possibly affecting the enzyme. Decreasing EDTA and $Mg^{2+}$ concentrations may have reduced the inhibitory effect on the enzyme; however, since low concentrations may not have been sufficient to lyse cells, minimizing EDTA and $Mg^{2+}$ may not be useful. With this in mind we designed two unique protocols that used alternative methods of disrupting the outer membrane.

Polymyxin B is sometimes used with lysozyme in cell lysis methods instead of EDTA (2, 5, and 7). While we based our experiment on Dixon and Chopra's data (2), there are other variations of the protocol that require different concentrations of polymyxin B and incubation times and temperatures for maximal cell lysis. In our experiment, the concern was that our methods yielded poor cell lysis due to sub-optimal polymyxin B concentration, leading to low NADH dehydrogenase activity (Fig. 6). We used 100 µg/ml of polymyxin B, but Warren *et al.* (7) have reported using 20 µg/ml of polymyxin B in their lysis method. Repeating the experiment using a range of polymyxin B concentrations, incubation times, and incubation temperatures may be worthwhile to determine the optimal conditions to effectively lyse cells.

Freeze-thaw, our other alternative to using EDTA, was a mechanical method of disrupting the outer membrane, which avoids the use of chelating agents so that lysozyme could gain access to its substrate, peptidoglycan. Three separate trials were set up. One had all of the lysozyme added before the freeze-thaw procedure, while another had half the lysozyme added before and half added after. The final version had all the lysozyme added after the freeze-thaw procedure was complete. In all three cases, lysis was observed as the cell suspensions showed significant clearing and had a shimmering quality, suggesting that cells were efficiently lysed in the absence of EDTA and $Mg^{2+}$. There also seemed to be cellular debris in all three of the suspensions after the freeze thawing was complete. Therefore, we expected the freeze-thaw lysate to show more activity than it did. However, there was no activity observed, which would mean that something in the freeze-thaw protocols was inhibitory to NADH dehydrogenase activity. Possibilities include irreversible denaturation of the enzyme due to harsh conditions, and creation of conditions substandard for activity.

During the course of the experiment we observed inconsistent NADH dehydrogenase activity over different experimental days. The activity varied between 0.1 and 2.0 U/ml for French press lysate (Figs. 1-6), however, the trends with respect to what we were observing were always consistent. A possible reason for variation could be differences in the harvest times of our cells, and that our bacterial growth did not always yield maximum NADH dehydrogenase; Wackwitz *et al.* (6) showed that maximal expression of NADH dehydrogenase genes occurred during early log phase (2.5 hours) at $8 \times 10^8$ cells/ml, and that expression of NADH dehydrogenase decreased by approximately one third during mid-log phase, $2.6 \times 10^9$ cells/ml (7). To determine if our low activity levels were caused by harvesting post-log phase cells, we grew cells for 2.5 hours, but still saw little activity in our lysates: we needed to add 2 ml (instead of 100 µl) of lysate to the reaction mix of the NADH dehydrogenase assay to see substantial activity. Perhaps the low activity was due to the fact that we re-suspended the cells in excess volume of buffer in relation to cell density.

In this study we report that NADH dehydrogenase activity is inhibited by the combination of EDTA and $Mg^{2+}$, typically used in methods of cell breakage using lysozyme. Hence, we have explored alternative lysis methods that do not require these reagents. Neither method proved particulary successful; however, these methods might prove more effective if conditions are optimized.

## FUTURE EXPERIMENTS

To further this investigation, our main priority would be to determine conditions that optimize NADH dehydrogenase expression and production. Although we tried growing cells to early log phase, we resuspended cells in too much buffer prior to lysis; hence, we would like to repeat the experiment, but resuspend the cells in less

volume. Once we are able to observe consistent values of enzyme activity in French press lysate, we would then repeat experiments where EDTA and $Mg^{2+}$ are added to French press lysate to confirm their inhibitory effects on the enzyme. However, instead of adding the EDTA and $Mg^{2+}$ to the supernatant of the French press lysate, we would add the EDTA and $Mg^{2+}$ to a cell suspension prior to lysis in the French press. This would allow EDTA and $Mg^{2+}$ to interact with cellular material in a manner more similar to the conditions in the lysozyme lysis method.

Once we are convinced that EDTA and $Mg^{2+}$ are responsible for inhibiting NADH dehydrogenase activity, we would like to re-evaluate the polymyxin B and freeze-thaw lysis methods in order to provide an alternative enzymatic method of lysis. By assaying the activity of other enzymes, we can determine if these current lysis conditions affect all proteins. If activities of other enzymes tested appear low as well, we may simply optimize conditions to improve total protein yield. However, if we find that other enzymes display high activity, this would suggest that these protocols have specific inhibitory effects on NADH dehydrogenase, and new options would have to be explored. Indeed, this was observed for lysozyme lysis (4) where alkaline phosphatase activity was seen in lysozyme lysate while NADH dehydrogenase activity was not.

It may also be worthwhile to consider different mechanisms by which lysozyme method components inhibit NADH dehydrogenase activity. Flavin adenine dinucleotide (FAD), an absolute requirement of the enzyme, may be negatively affected by EDTA or some other component of the lysis method (1). We would also like to further investigate the effects of EDTA with lysozyme. Although it appeared that this combination inhibited NADH dehydrogenase activity, we would like to repeat the experiment, particularly since the combination of EDTA, lysozyme and DNase did not inhibit the activity.

Finally, it would be useful to confirm that the low or undetectable NADH dehydrogenase activity in lysozyme lystate is due to the inhibition of the enzyme and not the absence the protein in the lysate. By western analysis we would be able to examine cell lysates taken from each experiment to compare the various protocols to each other.

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