Assessment of Periplasmic Enzyme Isolation Methods: Isolating L-Asparaginase from *Escherichia coli* using Microwave Irradiation and Potassium Phosphate-Hexane Permeabilization Methods

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The aim of this investigation was to compare the efficacy of the permeabilization methods of microwave irradiation and potassium phosphate (KPi)-hexane solvent system in isolating the periplasmic enzyme, L-asparaginase II. Previous studies have shown that both methods enable the isolation of periplasmic proteins in Gram-negative bacteria. Periplasmic, cytoplasmic, and membrane fractions of *Escherichia coli* K-12 MG1655 cells grown to log phase were assayed for the enzyme activity of inner membrane-bound succinate dehydrogenase, cytoplasmic glucose-6-phosphate dehydrogenase, and L-asparaginase II. Cells treated with KPi-hexane yielded periplasmic fractions with minimal L-asparaginase activity and cytoplasmic fractions with high activity that appear to be consistent with poor periplasmic release. However, as L-asparaginase II is optimally expressed under anaerobic conditions, the aerobic conditions may have facilitated higher expression of the cytoplasmic isozyme, L-asparaginase I, and account for this observation. Contrary to previous studies, our results also indicate that neither method was selective in isolating the periplasm as L-asparaginase activity was detected in both the cytoplasmic fraction and membrane fractions.

Gram-negative bacteria possess a layer of peptidoglycan within the periplasmic space which lies between the inner cytoplasmic and outer cell membranes. The periplasmic space is more closely associated with the inner cytoplasmic membrane side and holds various hydrolytic enzymes and binding proteins essential to nutrient metabolism (8). The surrounding outer membrane is comprised of phospholipids and lipopolysaccharides that provides a critical protective barrier for the hydrolytic enzymes residing in the periplasmic space (8). In addition, it permits the entry of only small molecules like monosaccharides and excludes extracellular toxins like bile salts and antibiotics from permeating the cell (8).

To break down the outer protective barrier, mechanical means, enzymes, chelating agents, or solvents are necessary. Permeabilizing the outer membrane is generally pursued to release significant periplasmic enzymes such as L-asparaginase II (3). Mechanical methods such as sonication and microwave radiation are abrasive methods that introduce contamination from intracellular contents into the periplasmic fraction and as such, require the purification of the desired periplasmic enzyme from the mixture of cellular contents, which can be both demanding and time-consuming. Alternatively, enzymatic permeabilization methods use lytic enzymes that necessitate the removal of the added enzyme during the periplasmic fractionation process. Chelating agents, like EDTA, whose ability to sequester ions that function to anchor membrane lipopolysaccharides together, help destabilize membrane integrity but can often bind to and deprive ions required by certain periplasmic enzymes for their activity. Thus, the use of solvents, such as the potassium phosphate (KPi)-hexane solvent system developed by Geckil *et al*. to permeabilize the outer membrane, offers a cost-effective and less labor-intensive alternative for isolating functionally active periplasmic enzymes (6).

Barnett *et al*. investigated microwave radiation as an alternative to the commonly used EDTA method for bacterial membrane permeabilization and periplasmic protein release (2). The method makes use of microwave exposure over time to disrupt the integrity of the bacterial outer membrane. It has been suggested that the oscillation of the microwaves destabilizes membrane constituents, resulting in the release of periplasmic proteins (10). Their study compared microwave radiation to microwave radiation with EDTA to permeabilize *Escherichia coli* cell membranes and measured the release of the periplasmic enzyme, alkaline phosphatase. However, they did not compare the mechanical microwave radiation method to more cost-effective, gentle, and selective alternative methods such as solvent, ionic, and non-ionic detergent methods. Furthermore, osmotic shock was required as an
The aim of this study was to validate the claim put forth by Barnett et al. that microwave radiation facilitates an effective and selective membrane permeabilization suited to the isolation of periplasmic proteins by testing this method against the straightforward, cost-effective KPi-hexane solvent system (2). Developed by Geckil et al., this solvent system was previously shown to effectively isolating the periplasmic enzyme, L-asparaginase II, while preserving its activity (6). This method uses phase separation extraction, where the desired periplasmic fraction resides in the aqueous salt phase after the volatile hexane component evaporates from the sample (6). The underlying mechanism in disrupting membrane integrity is not fully understood, but is thought to relate to the small size of hexane molecules and their efficiency in permeating the outer lipid bilayer of Gram-negative bacteria (5). The release of active periplasmic enzymes using KPi-hexane also depends on the concentration of salt and hexane, where lower salt and higher hexane concentrations were able to better retain periplasmic enzyme activity (6). Retained specific activity has also been suggested to relate to the dielectric and hydrophobic properties of hexane (5). To verify the purity of the periplasmic fractions isolated via the microwave radiation or the KPi-hexane solvent system, three enzymes from different cellular locations were used as markers. The periplasmic, cytoplasmic and membrane fractions were assayed for the activity of cytoplasmic membrane enzyme succinate dehydrogenase, the cytoplasmic enzyme glucose-6-phosphate (G6P) dehydrogenase and the periplasmic enzyme, L-asparaginase II. Of note, in addition to L-asparaginase II, there is a cytoplasmic isozyme known as L-asparaginase I that may be expressed constitutively under aerobic conditions by E. coli K12 with a characteristically low substrate affinity compared to its periplasmic counterpart (3). Although anaerobic conditions have been shown to be ideal for optimal L-asparaginase II expression, the culture was grown aerobically to allow expression of the control enzymes (3).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Escherichia coli K-12 strain MG1655 was obtained from the MICB 421 Culture Collection at the University of British Columbia Department of Microbiology and Immunology. Cultures were grown in a shaking waterbath at 37°C and 200 rpm in Luria-Bertani (LB) media (10 g/L tryptone, 5 g/L bacto yeast extract, 10 g/L NaCl, pH 7.2) supplemented with 0.4% (w/v) potassium succinate and 0.4% (w/v) maltose.

Whole cell lysis. To confirm the presence of the enzymes, 70 mL of supplemented LB media was inoculated and grown overnight. The cells were harvested by centrifugation in a J2-21 centrifuge (Beckman) at 13000 x g for 5 min at room temperature and washed once with 0.05 M KPi buffer, which was prepared by mixing K2HPO4 and KH2PO4 to adjust the pH to 8.0. The pellet was then resuspended in 0.05 M KPi to an OD600 of 5.0, cooled in an ice bath and sonicated with a Microson Ultrasonic Cell Disruptor (Misonix, Farmingdale, NY) at power level 18 with eight 20 second pulses at 40 second intervals. Whole cell lysates were centrifuged at 13000 x g for 10 min at 4°C and the supernatant was collected (cytoplasm fraction). The pellet was resuspended in the same volume of 0.05 M KPi as the supernatant collected and enzyme assays were performed on the two fractions.

Growth of culture for permeabilization. Culture was grown to OD600 of 0.4 in supplemented LB. The culture was split into two equal fractions for the hexane and microwave treatments and harvested at 13000 x g for 5 min at room temperature.

Hexane/KPi treatment. The hexane/KPi permeabilization was performed as previously described (6). The pellet cultured was washed once with 0.05 M KPi buffer, resuspended in 0.05 M KPi, 1% hexane (v/v) to an OD600 of 5.0 and incubated at room temperature for 1 hour with vortexing every 10 min. The cap was then left off the tube for 5 min to evaporate the volatile upper phase. The sample was centrifuged at 13000 x g for 5 min at room temperature and the supernatant was collected (periplasmic fraction). The pellet was resuspended in 0.05 M KPi to the same volume as the supernatant collected, cooled in an ice bath and sonicated as previously described. The sample was then centrifuged at 13000 x g for 10 min at room temperature and the supernatant was collected (cytoplasm fraction). The pellet (membrane fraction) was resuspended in the same volume of 0.05 M KPi as the two other collected supernatant fractions.

Microwave treatment. The microwave radiation treatment was performed as previously described (2). The pellet cultured was washed once with 0.033 M Tris-hydrochloride buffer (pH 7.5), and resuspended in 50 mL of 0.033 M Tris-hydrochloride buffer (pH 7.5), 20% (w/v) sucrose in a 100 mL beaker. The beaker was placed in another 500 mL beaker filled with ice and heated for 25 sec on power level 3 in a Samsung MW1150WA microwave (power output 1100 Watts). The culture was then split into two equal portions and centrifuged at 13000 x g for 5 min at room temperature. The supernatant for one portion was collected (no-osmotic shock periplasmic fraction) and the pellet was resuspended in 0.05 M KPi in half the volume that was used in the hexane treatment and put aside. The pellet from the second portion was rapidly resuspended with 0.5 mM MgCl2 in half the volume that was used in the hexane treatment and stirred while immersed in an ice bath for 10 min. The osmotic shocked cells were then centrifuged at 12000 x g for 5 min at room temperature, and the supernatant was collected (osmotic shock periplasmic fraction). The pellet was resuspended with 0.05 M KPi in half the volume that was used in the hexane treatment and, along with the non-osmotic shock resuspended pellet, was sonicated as previously described. The sonicated samples were centrifuged at 13000 x g for 10 min at room temperature and the supernatants were collected (cytoplasm fractions). The pellets were resuspended with
0.05 M KPi in half the volume that was used in the hexane treatment (membrane fractions).

Equalization of buffers. All collected fractions were split into two portions for equilibrated and unequilibrated samples to eliminate any chemical differences in the buffers during the enzyme assays. The microwave osmotic shock periplasmic fraction was split into two portions, one of which was equilibrated to the hexane treatment with KPi to a final concentration of 0.05 M KPi. All other collected fractions from both treatments were split into two portions, one of which was equilibrated to the osmotic shock buffer used in the microwave treatment with MgCl₂ to a final concentration of 0.5 mM MgCl₂. The fractions were frozen at -20°C until the enzyme assays were performed.

Total protein assay. A Lowry assay was performed to measure the total protein released in each fraction. Standards were prepared by diluting chicken egg albumin with 0.1 M NaOH. Tubes were prepared with 0.5 mL of undiluted, 1/4 and 1/16 dilutions of the collected fractions, using 0.1 M NaOH for all dilutions. Fresh copper reagent was prepared by combining 1 mL of 2% sodium potassium tartrate, 1 mL of 1% hydrated copper sulfate and 100 mL of 5% sodium carbonate. 2.5 mL of the copper reagent was added to the prepared samples and incubated at room temperature for 10 min. After incubation, 0.2 mL of Folin-Ciocalteu reagent was added to each tube and incubated at room temperature for 30 min before the absorbance was read at 500 nm against the assay blank with 0 µg of protein.

L-asparaginase assay. The activity of L-asparaginase was measured by using the Nesslerization reaction to determine the amount of ammonia liberated by the enzyme (6). 0.1 mL of the isolated fraction was added to 0.9 mL of prewarmed 0.01% L-asparagine in 0.05 M Tris-HCl buffer (pH 8.6), mixed by vortexing and incubated for 30 min at 37°C. The reaction was stopped by adding 0.1 mL of 1.5 M trichloroacetic acid (TCA) and the sample was centrifuged at 13000 x g for 5 min at room temperature to remove the precipitate. 0.2 mL of the supernatant was added to 0.1 mL of Nessler reagent and 0.7 mL dH₂O, vortexed well and incubated at room temperature for 10 min. The absorbance was read at 480 nm against blanks that received TCA before extract addition. The amount of ammonia liberated was calculated from a standard curve generated using ammonium sulfate. One unit of enzyme was defined as the amount of enzyme that liberates 1 µmol of NH₃ in 1 min at 37°C and pH 8.6.

Succinate dehydrogenase assay. Succinate dehydrogenase catalyzes the reaction of succinate to fumarate along with the reduction of 2,6-dichlorophenol-indophenol (DCPIP) (7). A reaction mixture of 0.8 mL of 62.5 mM Tris-HCl (pH 8.0), 5 mM KCN, 37.5 mM potassium succinate was pre-warmed at 25°C for 5 min before the addition of 0.1 mL of the isolated fraction. The reaction was started by adding 0.33 mL of 1.2 mM DCPIP and vortexed briefly before the addition of 0.67 mL of 3 mM phenazine methosulfate (PMS). Enzyme activity was measured by monitoring the decrease in absorbance at 600 nm over 5 min due to the reduction of DCPIP. The absorbance was read against a blank made with 0.1 mL dH₂O instead of the isolated fraction. The extinction coefficient used in the conversion of absorbance and time into enzyme activity was 22 mM⁴ cm⁻¹·min⁻¹. One unit of enzyme was defined as the amount of enzyme that reduces 1 nmol of DCPIP in 1 min at 25°C and pH 8.0.

Glucose-6-phosphate dehydrogenase assay. The enzyme assay to measure glucose-6-phosphate dehydrogenase activity was performed as previously described (11). A reagent mixture of 2.7 mL of 0.055 M Tris-HCl, 0.0033 M MgCl₂, pH 7.8, 0.1 mL of 6 mM NADP, and 0.1 mL of 0.1 M D-glucose-6-phosphate was pre-warmed at 30°C before adding 0.1 mL of the isolated fraction. The increase in absorbance at 340 nm was monitored for 5 min against a blank made using 0.1 mL of the Tris-HCl buffer instead of the fraction, and the change of A₃₄₀/min was calculated from the linear portion of the curve. Enzyme activity is measured by monitoring the appearance of NADPH at 340 nm. The extinction coefficient used in the conversion of absorbance and time into enzyme activity was 6.22 mM⁻¹·cm⁻¹. One unit of enzyme was defined as the amount of enzyme that reduces 1 µmol of NADP in 1 min at 30°C at pH 7.8.

RESULTS

Chemical equilibration has a negligible effect on enzyme activity. To assess whether any differences in enzyme activity observed between permeabilization methods were caused by the different buffers used in each method or by the methods themselves, fractions with and without chemical equilibration were assayed. The difference in enzyme activity between equilibrated and unequilibrated fractions appeared negligible, indicating that the permeabilization methods, not the buffers, are responsible for the levels of functional enzyme (data not shown).

The specificity for periplasmic enzyme release differs between permeabilization methods. The activity of two control enzymes, succinate dehydrogenase and G6P dehydrogenase, located in the cytoplasmic membrane and cytoplasm, respectively, were determined in each fraction after permeabilization. As Table 1 shows, the permeabilization using microwave radiation, with and without osmotic shock, yielded the highest succinate dehydrogenase activity in the membrane fraction which is consistent with its association with the inner membrane. In contrast, the succinate dehydrogenase activity was highest in the cytoplasmic fraction from the KPi-hexane treatment. This evidence for apparent enzyme-membrane dissociation may be the result of excessive cell lyses from high-powered sonication. The possibility of a destabilizing effect by hexane on the inner membrane, however, cannot be excluded. Table 1 shows that the enzyme activity of G6P dehydrogenase was highest in the cytoplasmic fractions for all permeabilization methods as expected. Higher levels of G6P dehydrogenase activity were seen in the periplasmic and membrane fractions of the microwave method with osmotic shock than the other methods, indicating that cells may have lysed during osmotic shock prior to sonication. This is unexpected given that osmotic shock is a standard method used to selectively release periplasmic enzymes.

The KPi-hexane method does not effectively release L-asparaginase from the periplasm. L-asparaginase activity was determined to be highest in the cytoplasmic fraction obtained from the KPi-hexane method (Table 1). When taken together with the absence of detectable activity in the periplasmic fraction, this suggests enzyme release was only achieved after sonication. Alternatively, this may reflect a higher abundance of the low-affinity
cytoplasmic isozyme, L-asparaginase I, relative to L-asparaginase II (1). Furthermore, Table 1 shows that the highest L-asparaginase activity in the periplasmic fraction was obtained from the microwave method with osmotic shock and implies this to be the more effective method for isolating this enzyme than the solvent treatment. However, due to the formation of an unknown turbid product following the substrate incubation, the effect of omitting osmotic shock from the microwave treatment on L-asparaginase release in this fraction could not be assessed. In addition, the comparable enzyme activities between fractions obtained from the microwave treatment with osmotic shock suggests that the microwave method may be disrupting both the outer and inner membranes, thus, allowing L-asparaginase to diffuse and disperse into other subcellular compartments.

The specific activity of L-asparaginase was higher in the microwave fractions than the KPi-hexane method. The microwave methods yielded higher specific activity for L-asparaginase in all successfully assayed fractions compared to the KPi-hexane method (Table 2). This indicates that the microwave method preserved L-asparaginase activity to a greater extent.

The difference in L-asparaginase activity between the periplasmic fractions of microwave-osmotic shock and KPi-hexane-treated cells is seen with overnight substrate incubation. To determine the effect of increasing incubation times with the assay substrate, L-asparaginase, on assay results, the L-asparaginase activity of the periplasmic fraction taken from the microwave-osmotic shock and KPi-hexane methods were reassessed with overnight incubation. Both fractions gave higher activity values relative to the blank (data not shown). Moreover, the microwave fraction exhibited an enzyme activity that was approximately three-fold higher than the KPi-hexane fraction, a difference that could not be detected previously with the 30 minute incubation.

**DISCUSSION**

L-asparaginase II was chosen as the periplasmic enzyme of interest as it has tumor-inhibitory properties and is used in therapeutic treatment of leukemia (1). The enzyme acts as a therapeutic by converting free L-asparagine to L-aspartic acid and ammonia, limiting nutritional L-asparagine and inhibiting protein biosynthesis to effectively kill T-lymphoblastic leukemias (1). Originally derived from *E. coli*, L-asparaginase requires high purification to minimize immunogenic and toxic effects in patients. Purification can be enhanced through permeabilization methods that isolate particular cellular compartments, in this case, the periplasmic fraction. In this study, we aimed to compare microwave radiation treatment with and without osmotic shock to a simpler and potentially more specific periplasmic protein isolation method, the KPi-hexane solvent system. To compare the methods,
the total L-asparaginase, cytoplasmic G6P dehydrogenase, and inner membrane-bound succinate dehydrogenase activities in the isolated periplasmic fraction of cells subjected to microwave treatments with and without osmotic shock and KPi-hexane were determined.

Membrane permeabilization was performed on cells grown to log phase since optimal L-asparaginase II expression occurs at this stage of growth (3). However, in addition to the periplasmic L-asparaginase, known as L-asparaginase II, we discovered after comparing the methods, that the cytoplasmic isozyme of L-asparaginase I is also expressed under aerobic conditions albeit at a lower activity than its periplasmic counterpart which is optimally expressed anaerobically (3). Following the evaporation of hexane and the removal of the organic phase in the KPi-hexane treatment, the aqueous phase of cells in which periplasmic enzymes were expected to reside, had insignificant L-asparaginase activity indicating a poor release or lack of L-asparaginase II in the periplasmic fraction. The cytoplasmic fraction isolated with the KPi-hexane solvent system showed surprisingly high L-asparaginase activity relative to the periplasmic and membrane fractions and to the fractions isolated by the microwave-osmotic shock and microwave methods, which is consistent with ineffective periplasmic release or the effect of much higher expression of L-asparaginase I relative to L-asparaginase II. However, the corresponding specific activity also indicates that this may be insignificant with respect to the total protein. While these results suggest that active L-asparaginase in aerobically grown E. coli K-12 MG1655 cells may be successfully isolated by microwave and KPi-hexane solvent permeabilization methods, further investigations are necessary to elucidate which isozymes were isolated to determine the selectivity of these respective methods.

The merit of the KPi-hexane solvent system as a superior method to microwave radiation with or without osmotic shock for the selective isolation of the periplasmic protein L-asparaginase II, could not be determined based on the results from this study since the cells were grown in aerobic conditions which may have limited expression of L-asparaginase II and permitted expression of L-asparaginase I. The highest activity from all three enzymes occurred in the cytoplasmic fraction from the KPi-hexane method, which was not expected for the L-asparaginase or succinate dehydrogenase enzymes as they do not reside in the cytoplasm. The high activity of the latter in the cytoplasmic fraction was likely a result of over-sonicating the cells and redistributing succinate dehydrogenase from the membrane fraction to a general soluble fraction. However, since all the assays conducted were relatively non-specific, potential sources of error that may have been introduced into the measurements include interfering signals produced by reactive enzymes such as aminotransferase enzymes in the L-asparaginase assay or naturally occurring chromophores such as malate in the succinate dehydrogenase assay.

The apparent ineffective permeabilization achieved with the KPi-hexane method in isolating L-asparaginase II from the periplasmic fraction may be a consequence of the aerobic conditions in which this isozyme may not have been highly expressed such that the activity detected in all fractions may have been derived from L-asparaginase I (3). This is further compounded by the fact that the substrate concentration used in the assays was well above 0.1 mM, the threshold concentration below which L-asparaginase I activity is limited (3). Since the concentration of 0.01 M L-asparagine used in the assay exceeded the K_M values of 3.55 mM and 0.115 µM for L-asparaginase I and L-asparaginase II, respectively, the cytoplasmic isozyme may have been able to react despite its low affinity (9). The percent hexane for the solvent system as suggested by Geckil et al. for L-asparaginase isolation from Enterobacter aerogenes and Pseudomonas aeruginosa may have also been a contributing factor if it was insufficient to penetrate and disrupt the bacterial outer membrane of E. coli K-12 MG1655 (6).

** TABLE 2. Specific activity of L-asparaginase in equilibrated subcellular fractions of differentially permeabilized log-phase E. coli K-12 MG1655 cells.**

<table>
<thead>
<tr>
<th>Permeabilization Method</th>
<th>Subcellular Fraction</th>
<th>L-asparaginase Specific Activity (milliunits/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microwave-osmotic shock</td>
<td>Periplasmic</td>
<td>3.43</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic</td>
<td>3.87</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>5.68</td>
</tr>
<tr>
<td>Microwave</td>
<td>Periplasmic</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic</td>
<td>4.33</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>3.62</td>
</tr>
<tr>
<td>KPi-hexane</td>
<td>Periplasmic</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>0.17</td>
</tr>
</tbody>
</table>

** specific activity was not determined due to the appearance of a grey turbid solution after incubation with L-asparagine
This data suggests that with aerobic growth, the microwave method using osmotic shock resulted in higher L-asparaginase activity and retained higher specific activity in the periplasmic, cytosolic, and membrane fractions compared to the other permeabilization methods. Further experimentation is required to study the periplasmic L-asparaginase II enzyme in the absence of the cytoplasmic isozyme to gain a better understanding of the permeabilization methods.

FUTURE DIRECTIONS

In this study, one of the main issues was that it was unknown whether the L-asparaginase activity measured from the three isolated cell fractions was derived from L-asparaginase I or L-asparaginase II. We used aerobic growth, which has not been reported in the literature as an effective growth condition for L-asparaginase II expression from \textit{E. coli} MG1655. Therefore, it is suggested that this experiment be repeated with several changes to compare the membrane permeabilization methods for the periplasmic release of L-asparaginase II. To enrich the expression of periplasmic L-asparaginase II by up to 1000-fold and to limit the interference from L-asparaginase I activity, the \textit{E. coli} culture should be grown in anaerobic conditions and the L-asparagine concentration in the assay should be limited to 0.1 mM (3,4). Limiting the substrate concentration will create conditions to favour the detection of L-asparaginase II activity based on its greater substrate affinity, provided that enough substrate is available for measurable changes in enzyme activity to be monitored. Lastly, the various subcellular fractions analyzed here should also be assayed for an alternate periplasmic enzyme such as alkaline phosphatase to verify whether the KPi-hexane treatment facilitates selective release of periplasmic proteins as corroborating trends in the assay results would identify the method and not improper L-asparaginase expression patterns, as the cause for the observations.

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