Formation of \(N\)-Ethylmaleimide (NEM)-Glutathione Conjugate and \(N\)-Ethylmaleamic Acid Revealed by Mass Spectral Characterization of Intracellular and Extracellular Microbial Metabolites of NEM

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Microbial communities in activated sludge usually undergo different physiological and structural modifications upon exposure to toxic chemicals (9, 11, 12). Monitoring the formation of metabolites in response to environmental perturbations could provide valuable insights into a biological system’s physiology. Furthermore, characterization of the intracellular metabolites and identification of reaction intermediates are critical for understanding metabolic pathways that are key parts of the detoxification process. In this study, the metabolic products formed during the biotransformation of a toxic compound, \(N\)-ethylmaleimide (NEM), in a complex microbial consortium in activated sludge was monitored using liquid chromatography-ion trap mass spectrometry (LC-IT-MS). NEM has been shown to cause deflocculation of biomass flocs, resulting in elevated effluent soluble chemical oxygen demand in activated sludge systems (3, 4, 5). Several studies have reported the involvement of glutathione (GSH) in the detoxification of NEM in pure microbial cultures (10, 14) and proposed that an \(N\)-ethylsuccinimido-S-glutathione (ESG) adduct was formed (Fig. 1A) as the intermediate. The fate of ESG is of great interest because this adduct is a strong activator of the KefB and KefC potassium efflux systems in microorganisms (6, 7, 8). Attempts to elucidate the mechanism of ESG breakdown using nuclear magnetic resonance spectroscopy have not been successful (10).

In this study, the extracellular and intracellular metabolites formed during microbial degradation of NEM were characterized using LC-IT-MS. Samples of mixed liquor from the activated sludge of a municipal wastewater treatment plant (Amherst, NY) were used in this study. Preliminary experiments were performed in batch reactors spiked with 0.8 mM NEM to determine the molecular weights of the metabolites produced during the biodegradation of NEM. Samples from these experiments were collected at several time points and were analyzed by LC-IT-MS under scan mode (the conditions used are described below). The newly formed molecular ions observed during microbial degradation of NEM were chosen as target ions for selected ion monitoring and MS-MS analysis in subsequent experiments.

In the actual experiments, two bioreactors were prepared; the first bioreactor served as the control, while the second bioreactor was spiked with 0.8 mM NEM. Samples (30 ml) were collected from each bioreactor at several time points (15 and 30 min and 1, 2, 3, 5, 12, 24, 36, and 48 h). Additional 30-ml aliquots were collected from the second (NEM-treated) bioreactor. One of the aliquots was spiked with sodium azide (0.1% wt/vol), while the other aliquot was immediately frozen in a flask by submerging the flask halfway in an acetone-dry ice mixture. This latter sample was subjected to a freeze-thaw process three times (thawing was achieved by submerging the flask halfway in a 37°C water bath) to disrupt the microbial community. The newly formed metabolites were subsequently monitored by LC-IT-MS.

FIG. 1. Formation of ESG and proposed fragmentation of ESG and formation of N-OPPA and \(N\)-ethylmaleamic acid. (A) Reaction of GSH with NEM to form ESG. (B) Fragmentation of ESG, producing N-OPPA and \(N\)-ethylmaleamic acid.
cells and release the intracellular intermediates into the solution. All four aliquots were centrifuged (5 min at 3,400 \( \times \) g), filtered (0.45-\( \mu \)m nitrocellulose filter), and analyzed by LC-IT-MS under MS-MS mode.

All samples were analyzed using an LCQ Advantage ion trap mass spectrometer connected to a Surveyor LC system (Thermo Finnigan, San Jose, CA) with a reversed-phase Thermo Hypersil-Keystone (Bellefonte, PA) BetaBasic C\(_{18}\) column (length, 100 mm; inside diameter, 2.1 mm; particle size, 3 \( \mu \)m). A gradient mobile phase was used, starting with 5% acetonitrile and 95% water (with 0.3% formic acid) (held for 1 min) and using a linear gradient to obtain a final composition of 95% acetonitrile and 5% water (with 0.3% formic acid) within 15 min. The latter composition was maintained for an additional 2 min before the mobile phase was returned to the initial conditions. The flow rate was 200 \( \mu \)l min\(^{-1}\), the column temperature was 30°C, and the full loop injection volume was 20 \( \mu \)l. The LC-IT-MS system was equipped with electrospray ionization and was operated in positive ionization mode. The capillary temperature was 200°C, the capillary voltage was 10 V, and the spray voltage was 4.5 kV for all applications. Nitrogen was used as sheath gas at a flow rate of 20 \( \mu \)l min\(^{-1}\), and helium gas was used to induce dissociation of selected ions using 48% normalized collision energy.

Analysis of the soluble fraction of the activated sludge after exposure to NEM revealed the presence of a distinct metabolite (\( m/z \) 144; retention time, \( \sim \)10 min), as shown in the total ion chromatograms (TIC) in Fig. 2. The TIC indicated by lines A and B in Fig. 2, which were acquired under scan mode, are the TIC for the soluble fraction of the control and the NEM-treated sample, respectively, prepared by using centrifugation alone. The TIC indicated by line C is the TIC for the NEM-treated sample prepared by first adding azide to quench further microbial activity, followed by centrifugation; this TIC also showed the presence of an additional metabolite at \( m/z \) 187 (retention time, \( \sim \)8 min). This suggests that the addition of azide resulted in metabolite leakage and hence release of the \( m/z \) 187 compound into the solution.

The metabolite at 10 min was subjected to MS-MS fragmentation and was identified as N-ethylmaleamic acid ([M+H]\(^+\) 144), which has been reported to be a product of the GSH conversion of NEM by \textit{Escherichia coli} (10). Excretion of N-ethylmaleamic acid rather than expulsion of the GSH conjugate has been proposed as an economical solution for \textit{E. coli} to retain GSH for further rounds of detoxification (10). The MS-MS spectrum of the \( m/z \) 144 compound (not shown) had a base peak (\( m/z \) 126) representing loss of water (50% relative abundance) and a minor ion (1% relative abundance) at \( m/z \) 100 representing loss of CO\(_2\). This fragmentation pattern (loss of CO\(_2\)) is typical of compounds containing carboxylic acids (1). The MS-MS fragmentation of the \( m/z \) 187 metabolite \( \text{[N-}(2\text{-oxoethyl})\text{-2,2-(propionylamino)propanamide (N-OPPA)\]}\) is shown in Fig. 3, which shows the fragment ions at \( m/z \) 159 and \( m/z \) 88. Figure 1B shows the proposed formation of the ESG

![FIG. 2. Chromatograms of (line A) a control sample, (line B) an NEM-shocked sample, and (line C) an NEM-shocked sample treated with azide after 1 h. The peak at 10 min was not found in control samples. The peak at around 8 min was found only in NEM-treated samples after addition of azide.](http://aem.asm.org/)

![FIG. 3. MS-MS spectra of the \( m/z \) 187 compound corresponding to N-OPPA in the inset, indicating fragmentation that results in \( m/z \) 159 (line a) and \( m/z \) 88 (line b).](http://aem.asm.org/)
breakdown products, N-OPPA and N-ethylmaleamic acid. It appears that addition of azide to the activated sludge mixed liquor resulted in leakage of intracellular metabolites from the cell. The release of intracellular metabolites after addition of azide is not surprising because the ionic strength of the medium was drastically changed upon the addition of azide (final concentration, 0.1% [wt/vol]). It is known that bacterial cells require a compatible ionic strength of the medium to maintain their integrity (2).

To verify that N-OPPA is indeed an intracellular metabolite released during cell lysis, the NEM-treated samples were subjected to a freeze-thaw procedure (13). The results revealed the presence of m/z 144, m/z 187, and m/z 433 ions (Fig. 4) corresponding to N-ethylmaleamic acid, N-OPPA, and intact ESG, respectively. The identities of these metabolites were established based on the MS-MS fragmentation patterns of the respective molecular ions ([M+H]⁺). MS-MS analysis of the in vitro glutathione S-transferase-catalyzed reaction between NEM and GSH, performed to demonstrate the formation of the m/z 433 ion, produced identical molecular ions with the same retention time as the putative ESG in the samples subjected to the freeze-thaw procedure. The immediate increase in the N-ethylmaleamic acid level in the NEM-treated activated sludge suggests that N-ethylmaleamic acid is a stable extracellular metabolite (Fig. 5A). However, some amount of N-ethylmaleamic acid was not released, as indicated in the increase in the concentration when the cells were lysed (Fig. 5B). The relative amount of N-OPPA in lysed cells tended to decrease with time, suggesting that this intermediate is not stable.

Concluding remarks. This study demonstrated that LC-IT-MS can be used to monitor the formation of N-ethylmaleamic acid during the detoxification of NEM by complex mixtures of microorganisms in activated sludge without extensive sample preparation steps. MS-MS characterization of extracellular and intracellular metabolites provided direct evidence of the
formation of the NEM-GSH conjugate (ESG) and its rapid conversion to a nontoxic metabolite, N-ethylmaleamic acid, which was released into the soluble fraction. The results of this study suggest that GSH conjugation is a key process in the detoxification of NEM and that the levels of GSH in the cells are conserved by retaining the GSH adducts of reactive metabolites in the intracellular fluid, with release of only the nontoxic metabolites in the medium. Lastly, this study demonstrates the potential to directly monitor extracellular detoxification products as biomarkers for the presence of a toxic chemical load in wastewater treatment plants using sensitive and selective instrumentation, such as LC-IT-MS.

REFERENCES


