Monitoring of haloalkane dehalogenase reaction by using pH optode: screening of specificity and evaluation of kinetic constants

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Motivation:

Haloalkane dehalogenases (EC 3.8.1.5) are enzymes able to remove halogen from halogenated aliphatic compounds by a hydrolytic replacement, forming three products, alcohol, halid ion and proton. Because the hydrolytic dehalogenation is associated with the production of H^+, application of pH optode for sensitive monitoring of haloalkane dehalogenase activity is very promissing. The pH optode will be tested for detection of pH changes during reaction of haloalkane dehalogenase LinB with set of different halogenated compounds: 1-chlorobutane; 1-chlorohexane; 1-bromohexane; 1,2-dichloroethane; 1,2-dibromoethane; bis(2-chloroethyl)ether; bromocyclohexane; chlorobenzene; 1,1,2-trichloroethylene. The kinetic constants and specificity data obtained by pH optode monitoring will be evaluated and compared with simultaneous gas chromatography analysis (GC) of the reaction mixtures.

Methods:

Sample preparation and pH optode measurement
The reaction vial with stir bar was filed with 4 ml of HEPES buffer pH 7.0 and closed by screw-cap with polytetrafluoroethylene/silicone septum (no head space was present in the reaction vial). Substrate (1 μl) was added to the vial and dissolved in reaction buffer. The sensitivity of pH optode was tested with two phosphate buffers with pH 6.4 and 6.5 (ΔpH = 0.1), before each measurement. Calibrated pH optode was dipped to substrate solution in the reaction vial. Enzymatic reaction was initiated by addition of haloalkane dehalogenase LinB to a final concentration 0.035 mg/ml (0.1 μM), when the signal of pH optode was equilibrated. The reaction was monitored by using GC additionaly to the monitoring of pH changes. The enzymatic reaction was sampled at 0, 5, 10, 20, 30, 40, 60, 90, 120, 150 and 180 min by withdrawing 200 μl of reaction mixture and stopped by mixing with 400 μl of methanol. Samples were automaticaly analysed by GC Trace 2000 (Finnigen, USA) equipped with a flame ionisation detector and a capillary column DB-FFAP 30 m x 0.25 mm x 0.25 μm (J&W Scientific, USA).

Evaluation of kinetic data
The enzymatic reactions were monitored by using pH optode and raw data were recorded as a change of pH optode potential in time. These progress curves were integrated and the ratio related total change of potential to the total amount of converted substrate was calculated. The ratio was used for transformation of the raw data describing the change of substrate concentration in time. Both gas chromatography and transformed pH optode progress curves were fitted with single exponential model and used for evaluation of kinetic constant by using Origin 6.1. (OriginLab, USA). The specificity data were evaluated from slopes of pH optode signal decrease. In both, GC and pH optode measurement, the relative activity was calculated and compared. The activities were related to 1-chlorobutane (considered as 100%).

Results and Discussion:

Measurement of haloalkane dehalogenase kinetics

Enzymatic reaction was started by addition of haloalkane dehalogenase LinB to the reaction mixture containing 1-chlorobutane (Figure 1) and 1,1,2-trichloroethylene (Figure 2). The production of H^+, associated with the hydrolytic dehalogenation, was observed as a decrease of pH optode signal. The enzymatic conversion was monitored by using pH optode and the results were compared with GC measurement. Data from pH optode measurement were transformed to dependence of the substrate concentration on time (Figure 1B). The kinetic constants were then calculated from transformed progress curves. The unique values of k_{cat} and K_m could not be calculated from the data because the substrate concentration did not saturate the enzymatic reaction. A first order equation was fitted to the kinetic data. The first order rate constant for 1-chlorobutane decay 0.03142 ± 0.00001 min^{-1} and k_{cat}/K_m ratio 5.2 x 10^2 $s^{-1}.mM^{-1}$ have been estimated. The pH optode measurement provide lower kinetic constant compared to kinetic constant obtained by GC (first order rate constant 0.045 ± 0.001 min^{-1} and k_{cat}/K_m ratio 7.5 x 10^2 $s^{-1}.mM^{-1}$). This underestimation of kinetic constant can be explained by a delay of optode response on actual change of pH during reaction. The delay in pH optode signal equilibration (10 min) was observed when the pH was changed from 6.4 and 6.5 during calibration. There is also a discrepancy between pH optode measurement and GC measurement of 1,1,2-trichloroethylene in reaction with LinB. The response of pH optode in this reaction was similar to the response observed during conversion of 1-chlorobutane (Figure 2A, 3). The GC measurement did not confirm significant change in 1,1,2-trichloroethylene concentration in the reaction mixture (Figure 2B). Both pH optode and GC measurement of 1,1,2-trichloroethylene with LinB were repeated and substrate conversion was not detected (Figure 14). The discrepancy between three similar experiments was not explained. The instability of pH optode signal was observed when the reaction mixture was sampled (Figures 2A). The system was disturbed by insertion of needle, most probably by unwanted contact of needle with pH optode.

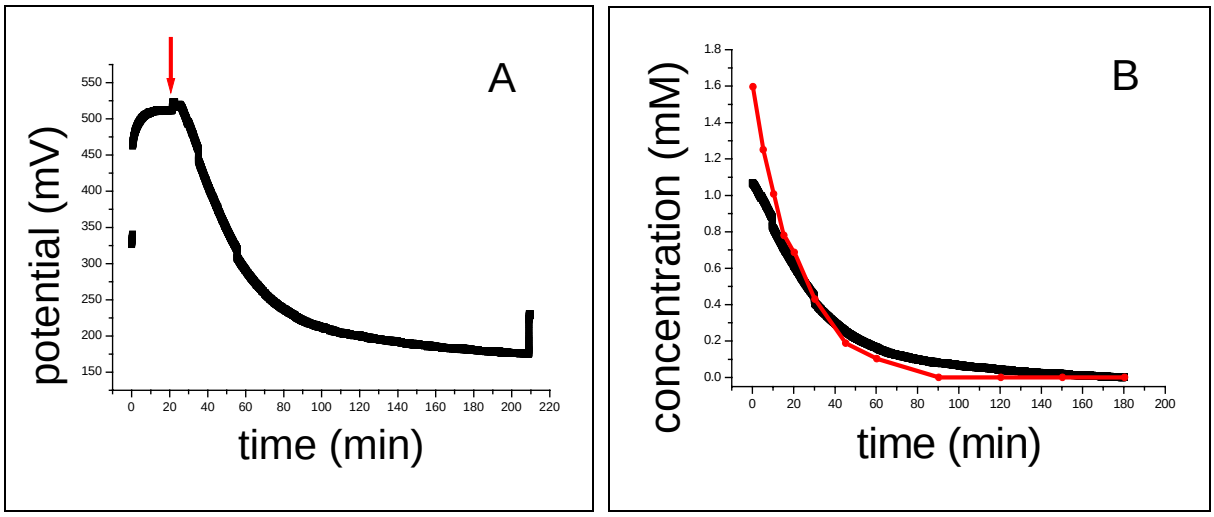

Figure 1. Conversion of 1-chlorobutane (0.222 mg/ml = 2.4 mM) by LinB (0.035 mg/ml = 0.0001 mM): a) recording of pH optode measurement; b) comparison of progress curves of LinB reaction with 1-chlorobutane measured by pH optode (black line) and simultaneous monitoring of substrate concentration by GC (red line). Enzymatic reaction starts at time 0 min or at the point marked with red arrow indicating injection of enzyme. Reported concentrations are those in reaction mixtures and are valid for all figures.

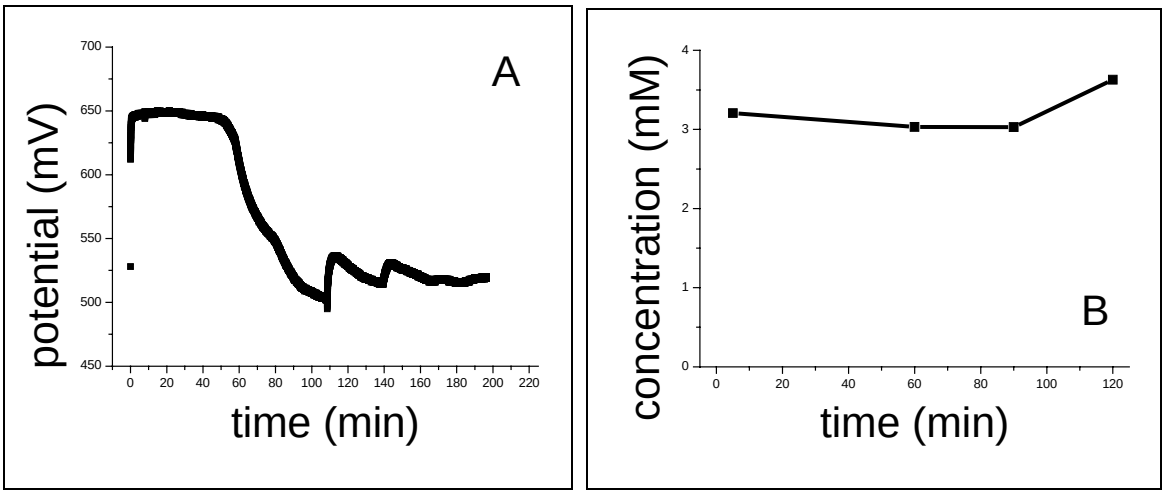

Figure 2. Monitoring of 1,1,2-trichloroethylene (0.365 mg/ml = 2.78 mM) incubation with LinB: a) pH optode signal; b) data from GC analysis.

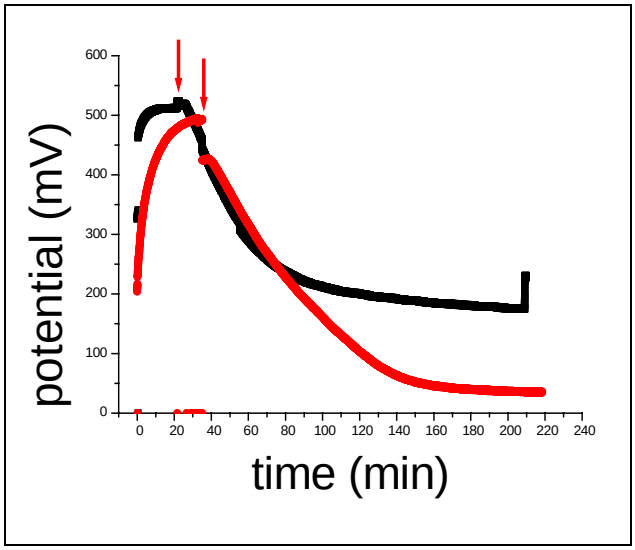

Figure 3. Monitoring of 1-chlorobutane (0.222 mg/ml = 2.4 mM); black line and 1,1,2-trichloroethylene (1.1 mg/ml = 8.37mM); red line incubation with LinB.

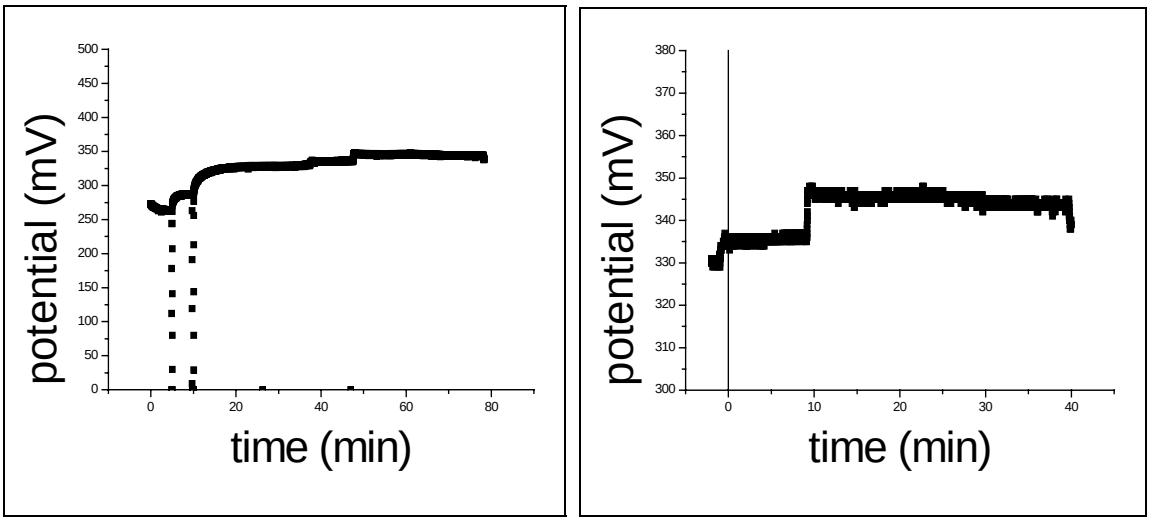

Figure 4. Addition of albumine (0.035 mg/ml) to 1-chlorobutane solution (0.2215 mg/ml = 2.39 mM).

Screening of haloalkane dehalogenase specificity

Haloalkane dehalogenase LinB reaction was measured with set of different halogenated compounds: 1 chlorobutane; 1-chlorohexane; 1-bromohexane; 1,2-dichloroethane; 1,2-dibromoethane; bis(2-chloroethyl)ether; bromocyclohexane; chlorobenzene; 1,1,2-trichloroethylene by pH optode and GC (Figures 5 to 15). The positive enzymatic activity was observed for 1-chlorobutane; 1-chlorohexane; 1-bromohexane; 1,2-dibromoethane; bis(2-chloroethyl)ether and bromocyclohexane, which were converted by LinB, whereas significant enzymatic activity of LinB with 1,2-dichloroethane; chlorobenzene and 1,1,2-trichloroethylene was not detected (Table 1). Conversion of 1,2-dibromoethane was the fastest in both, pH optode and GC measurement. The problem with instability of measurement system at the moment of sample withdrawing was observed during measurement of 1-chlorohexane; 1,2-dichloroethane; bromocyclohexane and chlorobenzene (Figures 7, 9, 12 and 13). The results of relative activity evaluated by pH monitoring correlated well with the result obtained from GC measurement according to Pearson correlation r = 0.90 and Spearman rank order correlation coefficient R = 0.81.

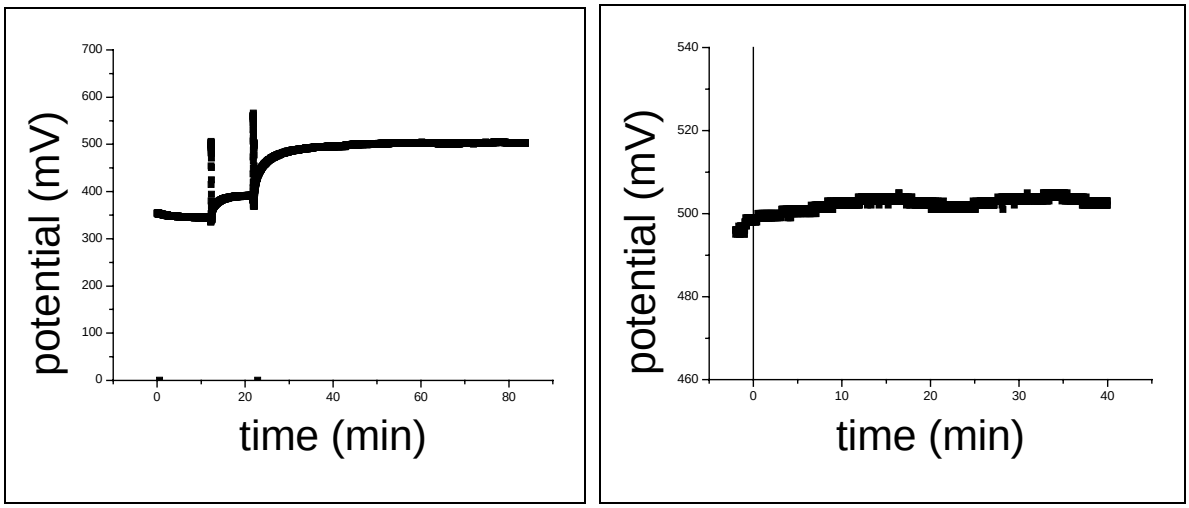

Figure 5. LinB injected to HEPES buffer without substrate (negative control).

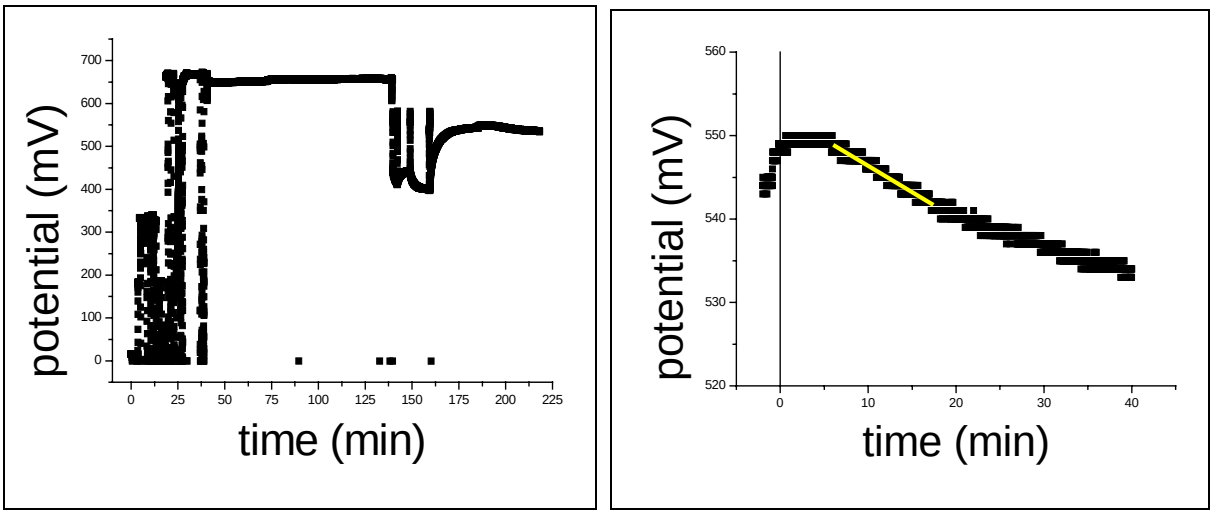

Figure 6. Conversion of 1-chlorobutane (0.2215 mg/ml = 2.39 mM) by LinB; yellow line corresponds to slope.

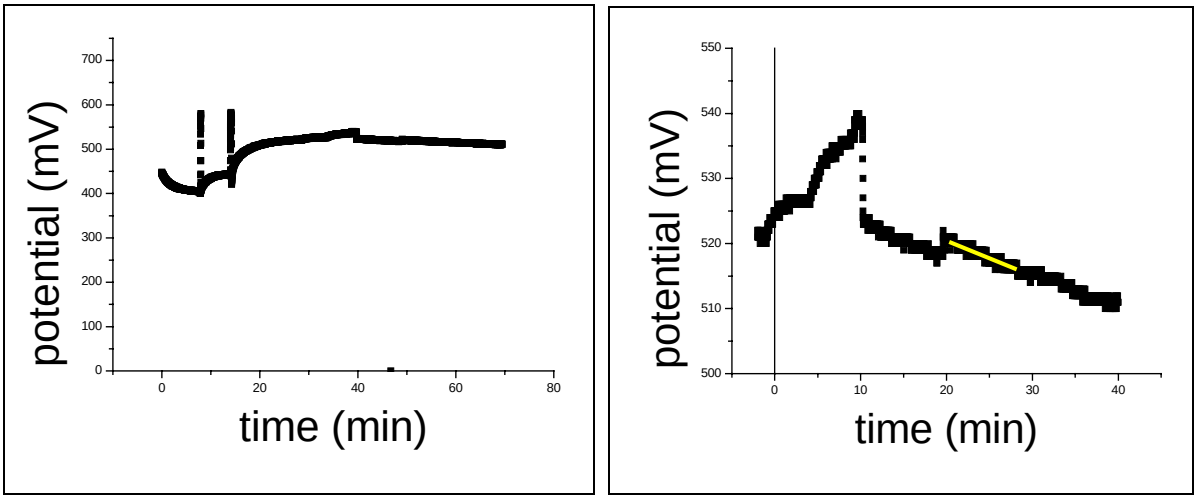

Figure 7. Conversion of 1-chlorohexane (0.2198 mg/ml = 1.82 mM) by LinB; yellow line corresponds to slope.

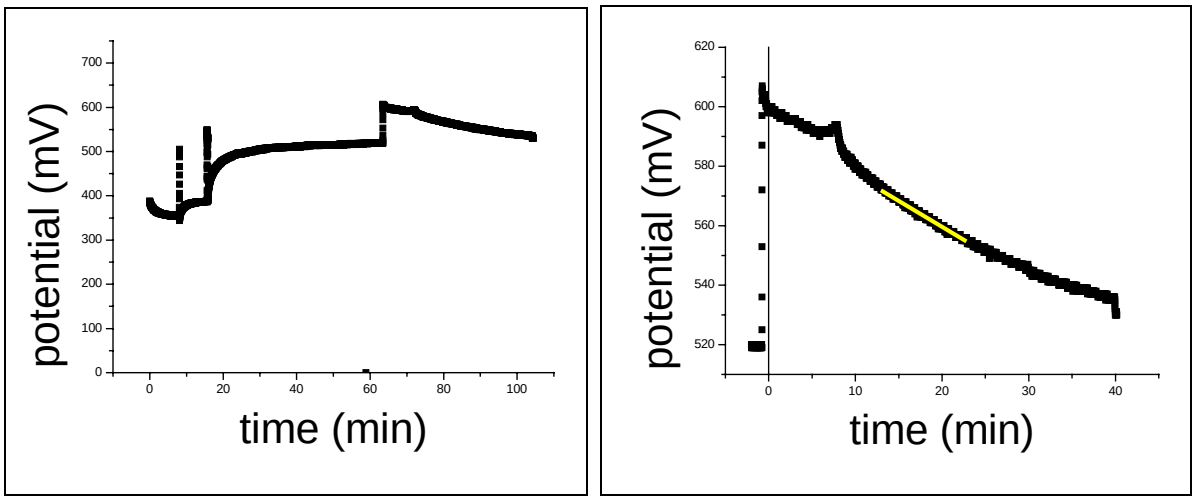

Figure 8. Conversion of 1-bromohexane (0.294 mg/ml = 1.78 mM) by LinB; yellow line corresponds to slope.

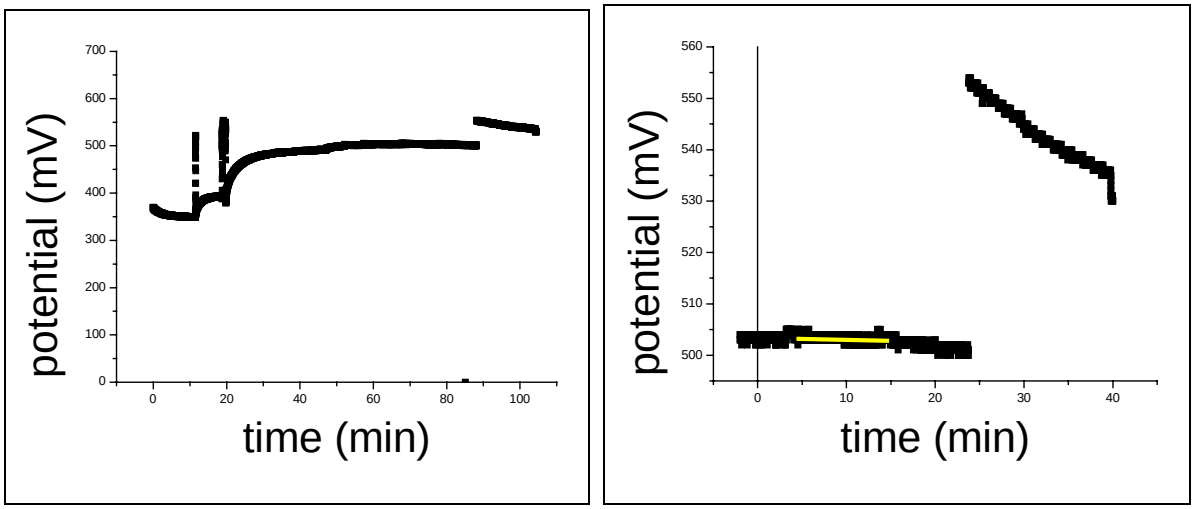

Figure 9. Conversion of 1,2-dichloroethane (0.314 mg/ml = 3.17 mM) by LinB; yellow line corresponds to slope.

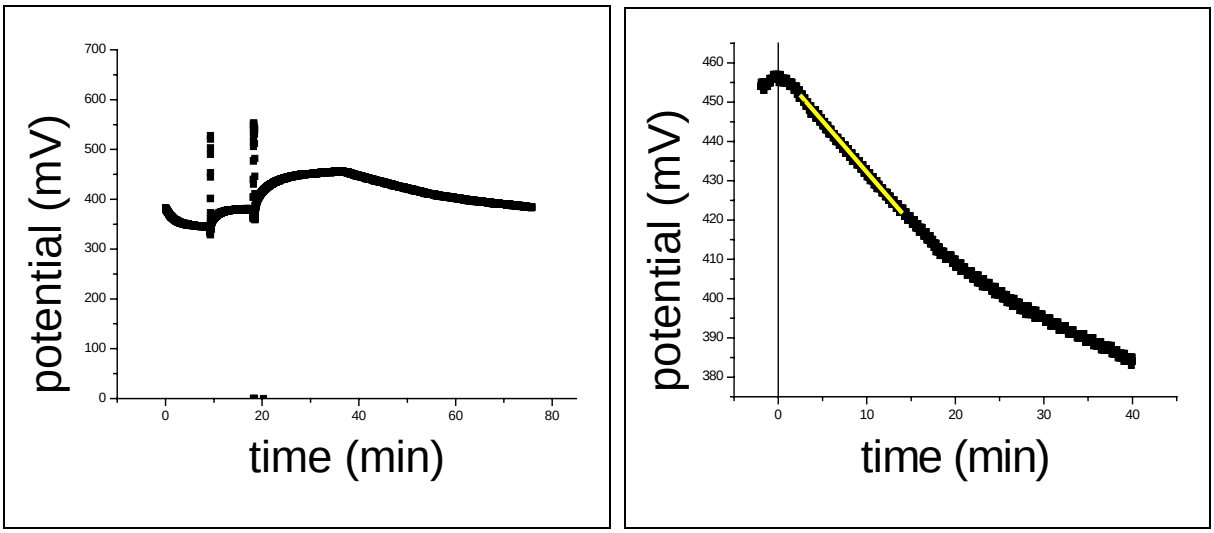

Figure 10. Conversion of 1,2-dibromoethane (0.545 mg/ml = 2.90 mM) by LinB; yellow line corresponds to slope.

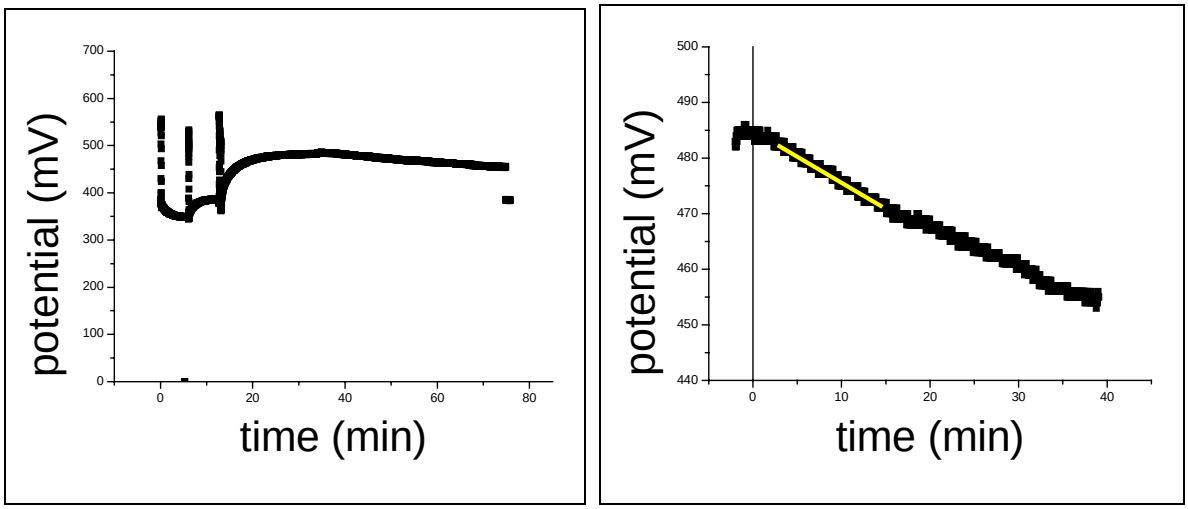

Figure 11. Conversion of bis(2-chloroethyl)ether (0.305 mg/ml = 2.13 mM) by LinB; yelow line corresponds to slope.

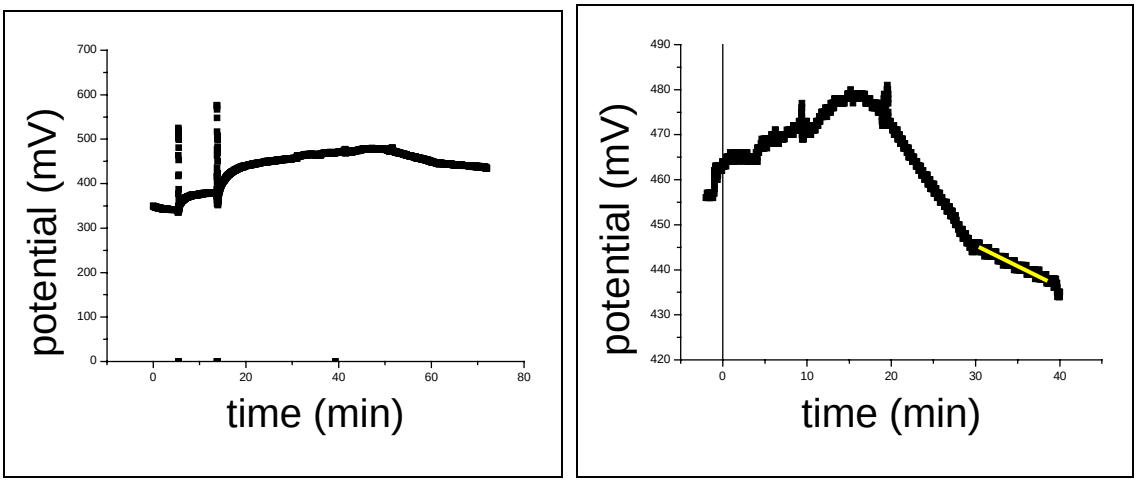

Figure 12. Conversion of bromocyclohexane (0.331 mg/ml = 2.03 mM) by LinB; yellow line corresponds to slope.

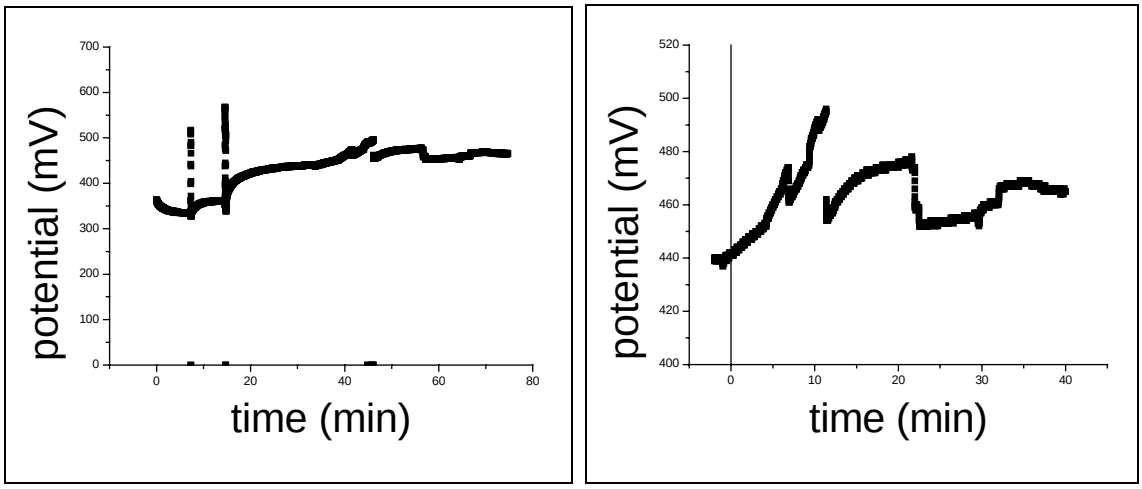

Figure 13. Incubation of chlorobenzene (0.277 mg/ml = 2.46 mM) in presence of LinB.

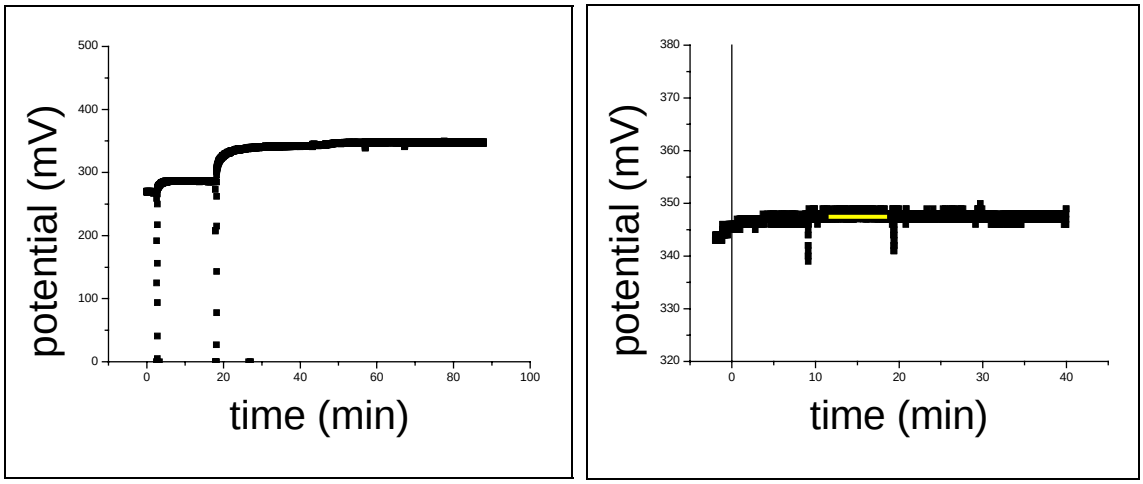

Figure 14. Incubation of 1,1,2-trichloroethylene (0.366 mg/ml = 2.79 mM) in presence of LinB; yellow line corresponds to slope.

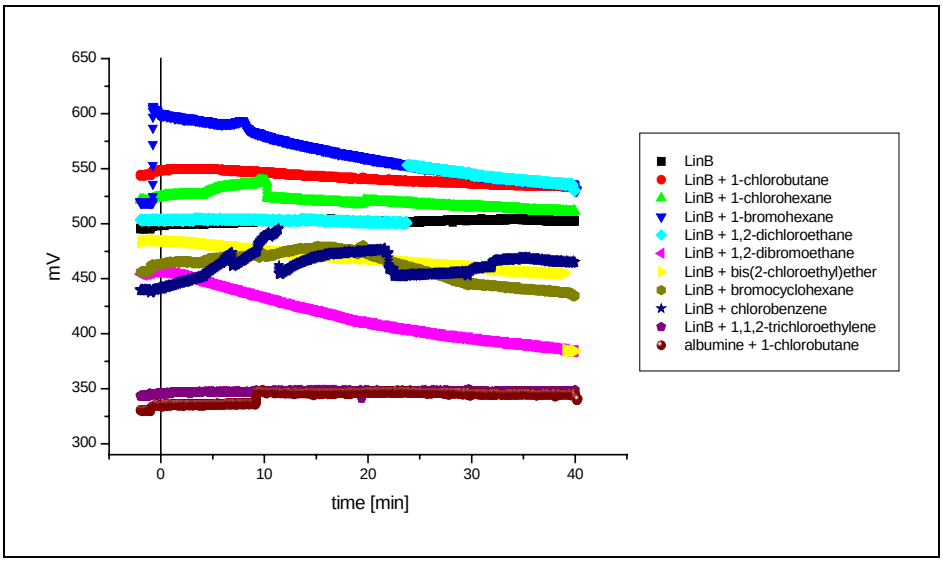

Figure 15. Summary of the pH optode signals obtained during the incubation of LinB with 1-chlorobutane; 1-chlorohexane; 1-bromohexane; 1,2-dichloroethane; 1,2-dibromoethane; bis(2-chloroethyl)ether; bromocyclohexane; chlorobenzene; 1,1,2-trichloroethylene (summary).

Table 1. Activity of LinB towards selected substrates. The total change of potential for 40 min enzymatic reaction was compared with calibration (two phosphate buffers with ΔpH = 0.1) and transformed to ΔpH. Slope pH optode values correspond to the slopes (yellow lines) in Figures 5 to 14.

substrate	ΔpH	relative activity ΔpH	slope pH optode	relative activity pH optode	slope GC	relative activity GC
1-chlorobutane	0.0341	100%	-0.6348	100%	-0.0456	100%
1-chlorohexane	0.0368	108%	-0.5596	88%	-0.0522	114%
1-bromohexane	0.17576	515%	-1.6924	267%	-0.0672	147%
1,2-dichloroethane	0	0%	-0.0821	13%	0	0%
1,2-dibromoethane	0.2057	603%	-2.5421	400%	-0.2156	473%
bis(2-chloroethyl)ether	0.0744	218%	-0.9616	151%	-0.0151	33%
bromocyclohexane	0.0667	196%	-0.8848	139%	-0.0302	66%
chlorobenzene	0	0%	0	0%	0	0%
1,1,2-trichloroethylene	-0.0117	-34%	0.0098	-2%	0	0%

Conclusions:

The enzymatic conversion of halogenated compounds was successfully monitored by using pH optode and the results were compared with GC measurement. The pH optode measurement provide lower kinetic constant compared to kinetic constant obtained by GC. This underestimation of kinetic constant is caused by a 10 min delay of optode response on actual change of pH during reaction. Even though, correlation between pH monitoring and GC measurement was statistically significant. Data from pH optode measurements could be distort by penetrated septum in the reaction vial and substrate evaporation from the reaction mixture. The instability of pH optode signal was observed when the reaction mixture was sampled. The discrepancy between pH optode measurement and GC measurement of 1,1,2-trichloroethylene in reaction with LinB was not explained.

Evaluation of pH optode use for monitoring of enzymatic reaction

Advantages:
1) Continuos, non-destructive measurement
2) High amount of data points
3) Relatively inexpensive and highly sensitive technique

Disadvantages:
1) Differences in pH of titrant and reaction buffer
2) Disturbances of signal during sampling
3) Delay in optode response
4) Preparation of pH optode is time consuming and not standardized
5) False positive signal

Outlook:

Development of a special measurement cell keeping stable conditions (temperature control, stirring, closed cell which prevent of substrate evaporation etc.) will be very suitable for other measurement as well as standardization of pH optode preparation.