Calorimetric Methods for Measuring Stability and Reusability of Membrane Immobilized Enzymes

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Abstract: The aim of this work is to develop calorimetric methods for characterizing the activity and stability of membrane immobilized enzymes. Invertase immobilized on a nylon-6 nanofiber membrane is used as a test case. The stability of both immobilized and free invertase activity was measured by spectrophotometry and isothermal titration calorimetry (ITC). Differential scanning calorimetry was used to measure the thermal stability of the structure and areal concentration of invertase on the membrane. This is the 1st demonstration that ITC can be used to determine activity and stability of an enzyme immobilized on a membrane. ITC and spectrophotometry show maximum activity of free and immobilized invertase at pH 4.5 and 45 to 55 °C. ITC determination of the activity as a function of temperature over an 8-h period shows a similar decline of activity of both free and immobilized invertase at 55 °C.

Keywords: DSC, electrospinning, enzyme immobilization, invertase, invert sugar, isothermal titration calorimetry (ITC)

Practical Application: Enzyme-catalyzed reactions occur in mild and environmentally friendly conditions, but are usually too costly to use in food manufacturing. When free enzymes are used, they are used once and replaced for each reaction, but enzymes immobilized on a solid support can be reused and have the additional advantage of being removed from the product. In this study, new calorimetric methods that are universally applicable to characterizing immobilized enzymes are used to determine the activity, stability, and reusability of invertase immobilized on a nanofiber support.

Introduction

Use of immobilized enzymes in the food industry is not a recent innovation (Sheldon 2007; Hanefeld and others 2009; Sassolas and others 2012; Homaei and others 2013). Enzyme membrane reactors (EMRs) are used to produce a wide array of products including detergents, food (wine, fruit juice, oils and fats, starch, and sugar), animal feed, alcoholic beverages, fine chemicals, leather, textiles, pulp, and paper (Giorno and Drioli 2000). EMRs have many applications in pharmaceuticals, environmental treatments (Chakraborty and others 2016), and potential applications in green chemistry (Jochems and others 2011). The main advantage of EMRs is the ability to separate and reuse active enzyme, thus minimizing the amount of costly enzyme used per reaction (Jochems and others 2011). In addition, EMRs can be used in continuous processes to increase productivity and can be used to conduct multiphase reactions. However, current EMR technology is limited by inefficiency during immobilization of the enzyme onto the membrane, leakage and deactivation of the enzyme, and loss of enzyme cofactors (Chakraborty and others 2016). For commercial applications, immobilized enzymes must be stable through multiple uses, and therefore stable against temperature, pH, mechanical stress, salt concentration, and surfactants as necessary for a given reaction.

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The purpose of this work is to develop new methods for characterizing the activity and stability of membrane bound enzymes. As a test case, we chose invertase immobilized on a nylon-6 nanofiber membrane (NFM; Scampicchio and others 2012) because of the ready availability of invertase and because the system is potentially useful in the food industry. Invertase catalyzes the hydrolysis of sucrose into an equimolar mixture of glucose and fructose called invert sugar that is used to increase sweetness and extend the shelf-life of food products (Kotwal and Shankar 2009). Invert sugar is currently synthesized on an industrial scale using acid hydrolysis, an inefficient process that creates a syrup with undesirable byproducts that alter the color and are carcinogenic (Marquez and others 2008). Use of an enzyme-catalyzed reaction would decrease the undesirable secondary products and potentially increase the efficiency of the reaction, but enzyme cost and retention of enzyme in the product has precluded use of invertase.

In this work, the activity and stability of immobilized invertase was compared with the properties of free invertase. The activity of both free and immobilized invertase were measured by isothermal titration calorimetry (ITC) and a traditional spectrophotometric method. The ITC methods are generally applicable to characterizing the activity of EMRs with other enzymes. A differential scanning calorimetric (DSC) method for determining thermal stability and enzyme concentration on membranes is also described.

The most common method for determining enzyme activity is spectrophotometry (UV-VIS or fluorimetry), which has limited application because it often requires a modified substrate or secondary reaction and cannot be used in opaque media. In contrast, ITC is a universal and direct method for determining the kinetics of enzyme-catalyzed processes that can be used with suspensions, opaque media, and solids (Fotticchia and others 2014). Because ITC measures the heat rate from the enzyme-catalyzed reaction

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directly, modified substrates and secondary reactions are not required. ITC is thus universally applicable to quantify the activity of enzymes. However, access to the reaction vessel in ITCs that are appropriate for use where available enzyme is limited is through a small diameter tube making it difficult to insert and remove an enzyme immobilized on a membrane, and therefore ITC has not previously been considered applicable for activity determinations on membrane-immobilized enzymes. The methods devised in this work provide a solution to this problem. Also, previous determinations of enzyme concentrations bound to membranes have relied on determinations of enzyme activities, thus losing information on the effect of immobilization on specific activity. This paper reports a physical method, DSC, for determining the concentration of enzyme bound to a membrane that also provides information on the thermal stability of enzyme structure.

Materials and Methods

Enzyme and chemicals

Invertase (EC 3.2.1.26; β-D-fructofuranosidase) from baker's yeast (*Saccharomyces cerevisiae*) grade VII, >300 units/mg solid (Sigma-Aldrich, St. Louis, Milano, Mo., U.S.A.) was used without any additional purification. Nylon-6 polymer pellets and formic acid 98% were purchased from Sigma-Aldrich, Italy. Sodium tetraborate (99%), *p*-hydroxybenzoic acid hydrazide (PAHBAH; 97%), and polyethylenimine (PEI), branched average molecular weight (MW) approximately 25000 by light scattering (LS), and average molecular number (MN) approximately 10000 by Gel Permeation Chromatography (GPC) were purchased from Sigma-Aldrich. Glutaraldehyde 25% solution was purchased from Calbiochem, San Diego, CA, U.S.A., and sucrose crystalline, American Chemical Society (ACS) grade, was purchased from Fisher Chemical, Hampton, NH, U.S.A.

Electrospinning nylon-6 for nanofiber membrane

Electrospinning was done at room temperature in a lab hood (Lemma and others 2015). A polymer solution of nylon-6 (23%, w/w) in 95% formic acid/water in a 5 mL syringe with a 0.5-mm internal diameter stainless steel needle was pumped at 0.02 mL/min (Holliston, Mass., U.S.A.) onto a stationary, stainless steel collector plate 11 cm from the needle tip. A 22 kV voltage (Spellman CZE SL150, Hauppauge, N.Y., U.S.A.) was applied between the needle and collector plate, causing a Taylor cone to form by inducing repulsive electrostatic forces that exceeded surface tension. The polymer solution elongates and the fiber diameter decreases as the solvent evaporates. The nanofiber membrane obtained was composed of randomly distributed long nanofibers with diameters ranging from 0.2 to 0.5 μm.

Enzyme immobilization

The invertase immobilized NFMs were prepared fresh for each reaction unless otherwise indicated. Briefly, for the ITC and spectrophotometric reactions, 0.25 cm^2 squares were cut from the NFM and placed in 500 μL of a 0.045 mg/mL solution of invertase and were immobilized as previously described by Amaya-Delgado and others (2006). The effectiveness factor (F_ε) (Ahmad and others 2001; Fatarella and others 2014) is a measure of the efficiency of immobilization and represents the retained activity after immobilization.

$$F_\varepsilon\,(\%) = 100 \times A_i/(A_0 - A_e) \quad (1)$$

A_i is the activity of the immobilized enzyme, A_0 is the activity of the free enzyme solution added for immobilization, and A_e is the activity remaining in the free enzyme solution after immobilization. A_i, A_0, and A_e were determined by the spectrophotometric method (Lever 1972) described later. The effectiveness factor of the NFM-immobilized invertase used in this work was 83%, which agrees with the work of Ahmad and others (2001) for invertase immobilized on lectin (82%). In addition, 2 NFM preparations were made for MC-DSC analysis, one from 5 mg/mL invertase and one from 10 mg/mL invertase. These membranes were too concentrated to be used in activity assays, and thus are only used to determine invertase thermal and areal concentrations using MC-DSC.

Invertase activity assays

A spectrophotometric method that utilizes the reaction of PAHBAH in an alkaline solution with glucose and fructose to produce yellow anions (Lever 1972) was used to measure invertase activity. The yellow anion products were measured spectrophotometrically at 380 nm (microplate reader Fluo Star Optima from BMG Labtech Inc., Cary, N.C., U.S.A.). The rate of yellow anion formation was used to determine enzyme activity.

ITC measurements of invertase activity were made with a NanoITC LV with a 180 μL reaction vessel (TA Instruments, Lindon, Utah, U.S.A.). To determine the activity of both the immobilized and free invertase, the invertase was loaded into the ITC reaction vessel and 4 μL of a 0.84 M solution of sucrose was injected with a stirrer speed of 350 rpm. The concentration of sucrose in the reaction vial at the start of each reaction was 19 mM. A buffer-only sample was used to determine the heat and time of mixing the sucrose into the buffer. Four consecutive single injections of 4 μL of 0.84 M sucrose were made into the same 0.0815 mg/mL invertase or approximately equivalent amount of immobilized invertase activity at 2 h intervals. Because the calorimeter is an overflow system, that is, added titrant displaces the same volume of reactant solution in the reaction vessel, the concentrations of reactants were corrected for this effect. Note that free enzyme concentration must be corrected, but immobilized is not.

The titrant and solution in the reaction vessel were prepared in 0.1 M acetate buffer. The nylon-6 NFM was cut into 0.5 × 0.5 cm squares and added and removed from the ITC with a 500 μL Hamilton syringe fitted with a 12 inch, 22-gauge, and blunt needle. The membrane squares were held on the end of the needle by the vacuum applied with the syringe, inserted through the 2.6 mm opening to the reaction vessel, and released. After the piece of membrane was in place, the reaction vessel was filled with buffer. The membrane pieces were removed from the reaction vessel by reversing the insertion method. On occasion the NFM could not be removed from the ITC and 88% formic acid was added to the reaction vial to dissolve the NFM.

Differential scanning calorimetry

The relative thermal stabilities of immobilized and free invertase were determined in duplicate in a multi-cell differential scanning calorimetry (DSC) (MC-DSC; TA Instruments). The MC-DSC has 3 sample cells and one reference cell with removable, reusable, 1 mL ampules sealed with an O-ring and screw-on lid. Temperature scans were done at 2 °C/min with 6 mg of free invertase or with 0.85 × 0.85 cm squares of NFM-immobilized invertase in 0.6 mL of 0.1 M acetate buffer at pH 4.5. A 2nd scan of the same sample with now-denatured enzyme was subtracted from the 1st scan to correct for the membrane heat capacity and any

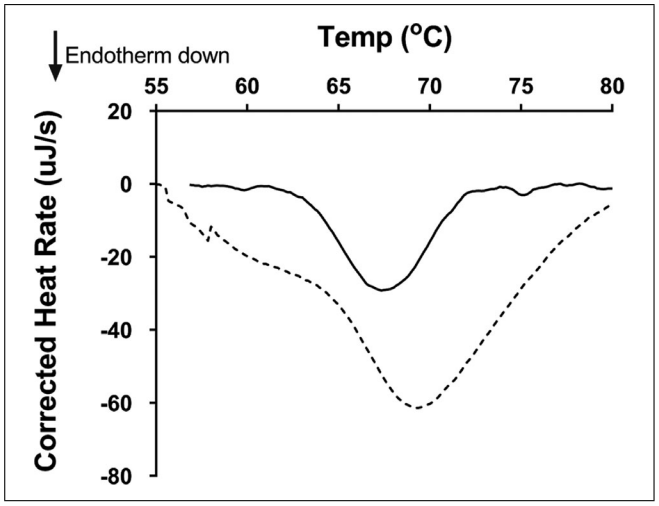

Figure 1–Thermograms of invertase in 0.6 mL of 0.1 M sodium acetate buffer at pH 4.5 scanned from 55 to 80 °C at 2 °C/min in a Multi-Cell DSC (MC-DSC). Black line, 6 mg of free invertase; dashed line, invertase immobilized in Nylon-6 NFM. Data were baseline corrected by subtraction of a 2nd scan of the same sample and the integration baseline. The peak temperature of free and immobilized invertase are 67.3 ± 0.5 and 68.8 ± 0.6 °C, respectively. The integration baselines are shown as dotted lines.

phase transitions in the membrane. The ratio of the total endothermic heat effects produced by thermal denaturation of free and bound enzyme was used to estimate the amount of enzyme on the membrane by assuming the same heat per mg of enzyme. The high sensitivity and relatively large volume of the ampules (1 mL) in the MC-DSC that could accommodate the large amount of membrane required to obtain a good signal make the determination of the temperature and heat of thermal denaturation of immobilized enzymes possible.

Results and Discussion

Thermal stability of free and immobilized invertase

Figure 1 shows DSC curves for free invertase and immobilized invertase from a 10 mg/mL solution. Immobilization of invertase increases the thermal denaturation temperature from a T_{max} of 67.3 ± 0.5 to 68.8 ± 0.6 °C (uncertainties are total estimates from duplicate runs). The observed increase in thermal stability agrees with the work of Amaya-Delgado and others (2006) Integration of the area of the denaturation peak for the free invertase gave 724 ± 14 μJ/mg for the heat of denaturation of the free enzyme in solution. Assuming the same value applies to the immobilized invertase and dividing the peak area (μJ) obtained with membrane bound invertase by 724 μJ/mg gives 19.4 ± 0.4 and 41.2 ± 0.8 mg/cm^2 for the 5 and 10 mg/mL preparations, respectively. The MC-DSC is thus advantageous for determining both thermal stability and areal concentrations of immobilized enzymes. However, because DSC measures the heat effect of conversion of the secondary and tertiary structure into a random coil, a process that occurs at a higher temperature than enzyme activity, DSC measurements do not translate directly into stability of enzymatic activity at lower temperatures.

Temperature and pH effects on immobilized invertase activity

Figure 2 shows an example data set of a single injection of sucrose into free enzyme, immobilized enzyme, blank buffer, and

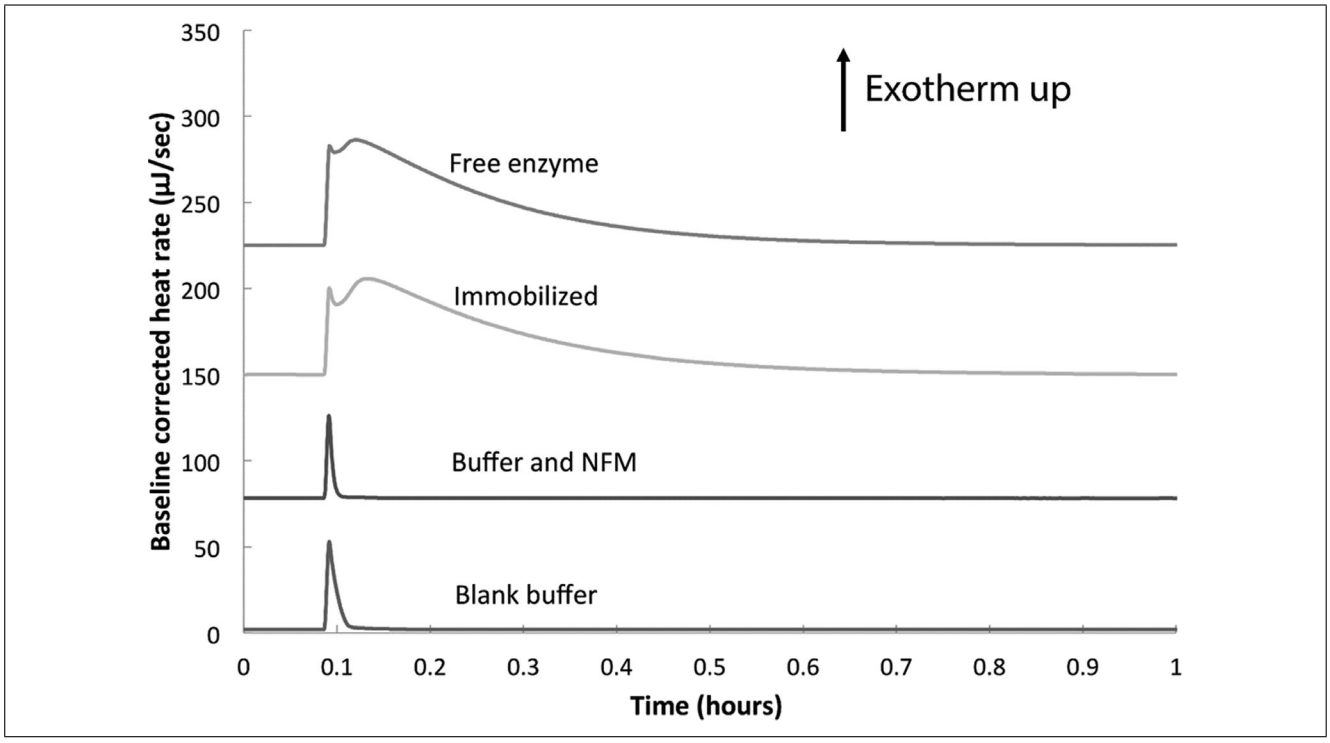

Figure 2–ITC heat rate at 55 °C and pH 4.5 from a single injection of 3.98 μL of 0.84 M sucrose in 0.1 M sodium acetate buffer into 0.1 M sodium acetate buffer, into buffer with blank membrane, into invertase immobilized on nylon-6 NFM, and into free invertase at 8.15 μg/mL. For clarity, the curves have been shifted up by 2, 75, 150, and 225 μJ/s for the buffer, blank membrane, immobilized and free enzyme, respectively. The 1st h of the reaction is shown.

Table 1–Enthalpy of free and immobilized invertase.

Injection no.	Blank buffer (mJ)	Immobilized enzyme (mJ)	Free enzyme (mJ)
1	2.12	44.5	44.4
2	2.22	49.5	47.4
3	2.16	47.7	47.6
4	2.36	45.8	47.4
Average	2.22	46.9	46.7
Standard deviation	±0.10	±2.2	±1.5
$\Delta_{rxn}H$ (kJ/mole) @55 °C		−13.88 ± 0.65 −13.15 (Goldberg and others 1989)	−13.83 ± 0.46 −13.15 (Goldberg and others 1989)

buffer with an enzyme-free NFM in the ITC. The curve describes the rate of the heat production as a function of time. Two methods for determining enzyme activity with ITC have been reported, multiple injection (Todd and Gomez 2001) and single injection (Transtrum and others 2015). In the multiple injection method, reaction rate is measured after each injection of an additional amount of substrate into an enzyme solution under conditions that produce a near steady state, that is, a near constant heat rate after each injection. Preliminary trials showed the steady-state condition cannot be obtained with the invertase preparations used in this study because a rapid decrease of the heat rate occurred after each injection (data not shown). Therefore, a single injection method was applied (Demarse and others 2013), despite the need to inject very concentrated sucrose solution which can be slow to mix in ITC cells because of the high density compared with the buffer solution. However, the injections into blank buffer show that mixing of the concentrated sucrose titrant is complete within 3 min which occurs before the maximum peak rate of the enzyme-catalyzed reaction is reached. The similarity of the peak shapes with free and immobilized enzyme show the membrane does not interfere with stirring or mixing in the 180 μL reaction vessel. The enthalpy changes in Table 1 further show that the calorimeter is preforming properly (Goldberg and others 1989).

For the purposes of this paper, we are only interested in relative activities, that is, between free and immobilized invertase activity, and because a true maximum rate could not be obtained, and therefore the Michaelis–Menton constants, k_{cat} and K_m, could not be determined, relative activity was estimated from the ITC data by fitting the 1st 500 s of the data after the peak to obtain the rate of sucrose depletion. This rate was then divided by the mg of protein in the calorimeter vessel to give specific activity.

Figure 3 shows the relative activities of free and immobilized invertase measured by both spectrophotometry and ITC at temperatures from 25 to 65 °C in increments of 10 °C. In agreement with ITC measurements, the spectrophotometric results show the maximum activity of free enzyme is at 45 to 55 °C. The temperature response for free and immobilized enzyme is the same within a method, but significantly differs between methods at both lower and higher temperatures. The spectrophotometric results show higher activity than the ITC results at 25 and 35 °C. ITC shows no activity at 65 °C whereas the spectrophotometric results show decreased but substantial activity at 65 °C. The different results from the 2 methods are caused by the 40 min delay required to stabilize the ITC signal before an injection. However, ITC measures the realistic stability of invertase activity at a given temperature. Because the indicator reaction used with the spectrophotometric method measures the activity immediately after mixing the solutions, the spectrophotometric method gives an unrealistically high estimate of the stability under a given condition.

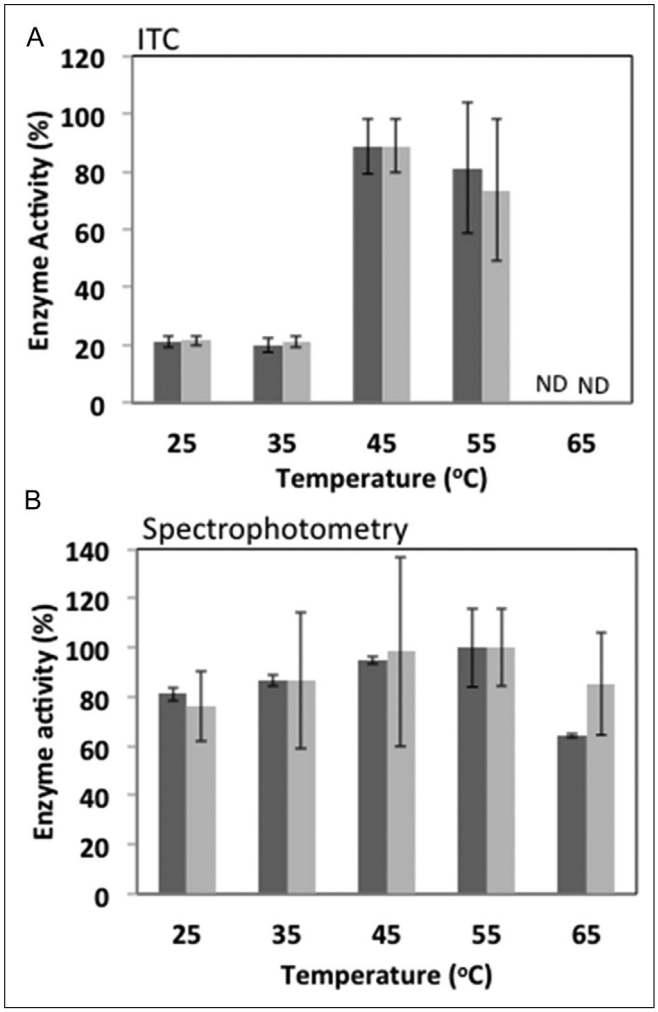

Figure 3–Relative enzyme activity in 0.1 M sodium acetate buffer at pH 4.5 from ITC and spectrophotometric measurements. Free invertase activity is shown with dark bars and immobilized activity with light bars.

The invertase temperature dependence shown in Figure 3 demonstrated that the maximal activity occurred between 45 and 55 °C. Consequently, 55 °C was selected to determine the pH dependence of the reaction. Figure 4 shows the activity of free and immobilized invertase measured by both spectrophotometry and ITC at pHs from 4 to 6. Although the trends with pH are opposite, ITC and spectroscopic measurements both show the maximum activity of free enzyme is at pH 4.5 to 5.5 in agreement with the work of Kotwal and Shankar (2009) and Ahmad and others (Ahmad and others 2001; Kotwal and Shankar 2009). The 2 methods gave different results for immobilized enzyme activity. The spectrophotometric method found immobilized enzyme activity at pH from 4 to 6, but ITC only found activity at pH 4.5. The difference between the results on immobilized enzyme are likely caused by the 40 min delay required to stabilize the ITC signal before an injection, whereas measurements with the spectrophotometric method begin immediately. Because of the immediacy, spectrophotometric measurements can indicate an overly optimistic estimate of enzyme stability.

The data in Figure 3 and 4 establish that ITC is a viable substitute for spectrophotometric assays of membrane bound enzyme

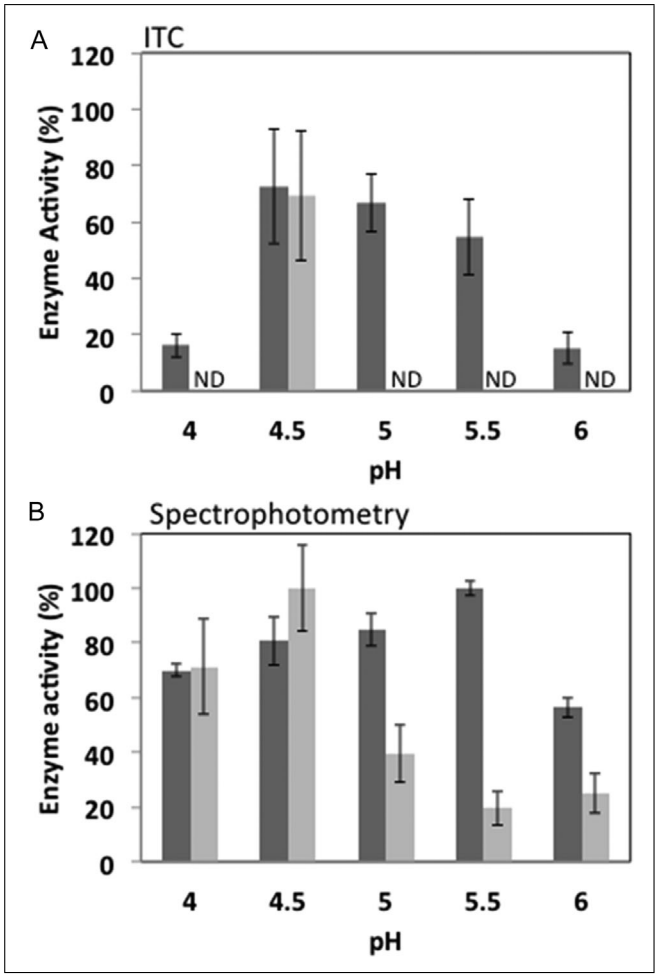

Figure 4–Relative enzyme activity at 55 °C in 0.1 M sodium acetate buffer as functions of pH from ITC and spectrophotometric measurements; free invertase activity (dark bars) and immobilized activity (light bars).

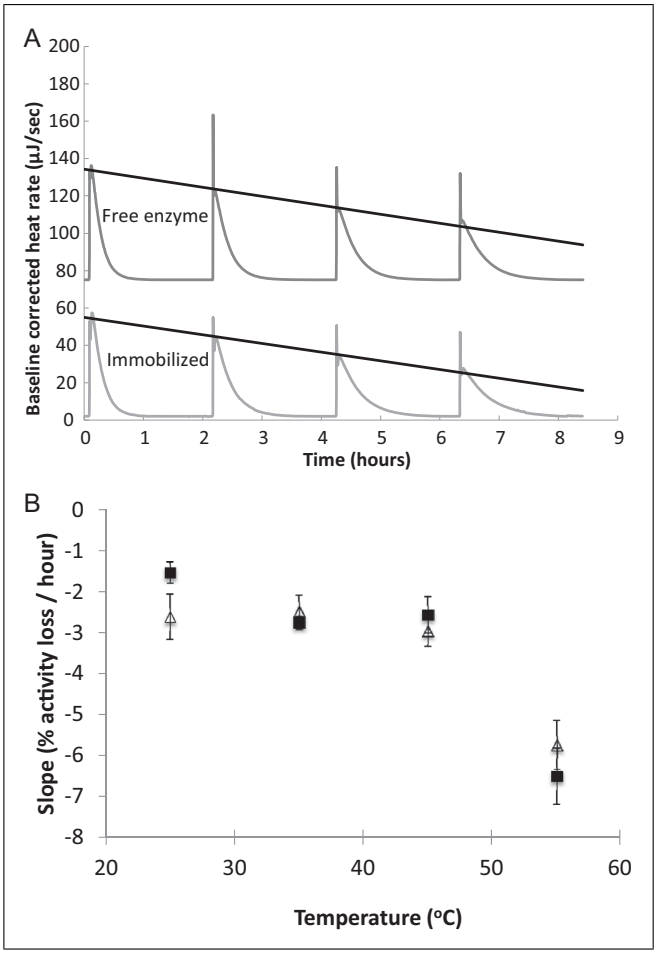

Figure 5–(A) ITC heat rate at 55 °C and pH 4.5 from 4 successive injections at 2 h intervals of 3.98 μL of 0.84 M sucrose in 0.1 M sodium acetate buffer into invertase immobilized on nylon-6 NFM, and into free invertase at 8.15 μg/mL. For clarity, the curves have been shifted up by 2 and 75 μJ/s for the immobilized and free enzyme, respectively. (B) Thermal stability of the activity of free invertase (Δ) and immobilized invertase (■) at 25, 35, 45, and 55 °C given as the slopes of the lines similar to the line shown in (A) fitted to the decreasing peak heights of 4 consecutive injections into the same sample at 2 h intervals at each temperature.

activities. The advantages of ITC are that it is not dependent on a secondary reaction or on the transparency of the media and is a universal detector since nearly all reactions produce heat at sufficiently high rates to be easily measured. Reactions catalyzed by immobilized or novel enzymes, which can be difficult to characterize by other means, can thus be measured by ITC.

Enzymatic stability of invertase immobilized on nylon-6 membrane

Unlike free invertase, the immobilized enzyme can be reused multiple times, and for commercial use, immobilized enzymes must be stable over multiple uses. Because the ITC system is automated, the stability of enzyme activity over lengthy time periods is easily determined by ITC. In this work, the stability of enzymatic activity over time as a function of temperature was estimated from the peak heights of 4 consecutive single injections of sucrose into free and immobilized invertase in the ITC at 55 °C shown in Figure 5A. The peak height is proportional to maximal enzymatic activity, and assuming no product inhibition, the slope of a plot of peak height versus time gives the enzyme stability at each temperature. Figure 5B demonstrates the effect of temperature on enzyme stability at 25, 35, 45, and 55 °C by comparing the slope of the peak heights for the 4 sequential injections of sucrose at each temperature. The smaller slopes a 25, 35, and 45 °C indicate more enzyme stability, whereas the larger slope at 55 °C indicates less enzyme stability. The area under the curve for each injection represents the total heat for the reaction. Glucose and fructose concentrations grow from 0.019 M after the first injection to 0.07 M after the 4th injection, but the similarity of the rate of decay with time within a set indicates there is no significant product inhibition under the conditions of these measurements. Also, Table 1 shows that the reaction goes to completion between injections. Further, product inhibition is not expected to have a major effect on the temperature dependence of the slope. In addition, the inhibition constant (K_i) for glucose and fructose on invertase are approximately 0.4 M and approximately 0.2 M, respectively (Dent and others 2009). The final concentration of products after all 4 injections is less than one 4th of the K_i for glucose and half the K_i for fructose, and is therefore unlikely to have a significant inhibitory effect. Figure 5B shows the immobilized enzyme may be slightly more stable than the free enzyme at 25 °C, but there is no significant difference between free and immobilized enzyme at 35, 45, and 55 °C. No significant activity was observed with either free or immobilized enzyme at 65 °C. In summary, both Figure 3

and 4 show that invertase activity is relatively stable up to 45 °C, but rapidly degrades at higher temperatures. Further, there is no significant difference in the thermal stability of the activity of free and immobilized invertase.

To determine the stability of the invertase on the NFM with spectrophotometry, 5 membranes with immobilized invertase were used for 0, 15, 16, 19, and 21 uses. A use was defined as exposing the membrane to 20 min of reaction conditions (55 °C, 0.1 M sodium acetate, pH 4.5 with no sucrose present). Five membranes were used because each membrane was discarded after it was used in the activity assay. Between uses, the NFM with immobilized invertase was stored at 4 °C in 0.05 M sodium acetate buffer at pH 5.5. After 15 uses, activity decreased by 70%, and by 21 total uses, activity decreased by 90%. The loss in activity was linear with number of uses, that is, the activity decreased by 4.7% per use.

Comparison of the ITC method (Figure 5) and the spectrophotometric method (see previous paragraph) for determining the stability of immobilized invertase demonstrates that ITC has several advantages. The ITC method provided continuous monitoring of enzyme activity and the process is completely automated, thus requiring less time than the spectrophotometric method. Further, the ITC measurements were all made on the same membrane unlike the spectrophotometric method.

Both methods demonstrated that the immobilized invertase lost significant activity over multiple uses. To increase stability, additional modifications, such as PEGylation of invertase (Moreno-Perez and others 2016), would be required to further stabilize activity if this preparation is to ultimately result in a viable EMR for commercial use.

Conclusion

This study demonstrates that enzyme activity of membrane-bound enzymes can be measured by low volume ITC as functions of time and temperature. The concentration of membrane bound enzyme can be measured by large-volume DSC. Invertase immobilized on nylon-6 nanofiber membrane may potentially be useful in commercial applications, although further modification is necessary to increase stability.

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Authors' Contributions

M. Mason performed the ITC and spectrophotometric experiments, analyzed data and wrote the first draft of the manuscript. M. Scampicchio helped with the writing, C. Quinn performed the MC-DSC experiments, and M. Tanstrum assisted with analysis of the ITC data. N. Baker performed experiments. L. Hansen designed experiments and helped write the manuscript. J. Kenealey designed experiments, analyzed data, and aided with the writing of the manuscript.

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