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A thermogravimetric method for studying the kinetics of enzyme catalysed reactions that produce a volatile co-product

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Abstract

A general method, using a thermogravimetric balance, has been developed for determining the rate of enzyme reactions that involve loss of a volatile co-product in the presence of an involatile product, the method requiring only small amounts of both substrate and enzyme. Following our interest in enzyme mediated polyester formation, the method was developed using the *Candida antarctica* lipase B catalysed reaction of various diols with adipic acid or monoethyl adipate (MEAA) to form polyesters and water or ethanol, respectively. The reactions were shown to be first order in enzyme concentration. 1,4-Butanediol was found to be too volatile for accurate rate determination, but the volatilities of 1,6-hexanediol and poly-tetramethylene diol (PTMEG 650) were sufficiently low for reaction rates to be determined below 80 °C for 1,6-hexanediol and 140 °C with PTMEG 650. Although *Candida antarctica* lipase B is a thermozyme it does show significant degradation of reaction rate above 130 °C. Arrhenius parameters for the reaction of 1,6-hexanediol with adipic acid and MEAA confirm that the enzyme has a distinct preference for reaction with ester rather than carboxylic acid groups.

Keywords: Thermogravimetric analysis; Enzyme kinetics; *Candida antarctica* lipase B

1. Introduction

Thermogravimetric analysis (TGA) techniques have a number of advantages that are relevant to the study of any enzyme mediated reaction where a weight loss or gain may be expected. They use small sample weights (10–40 mg), especially beneficial when enzymes are unavailable in large quantities; the temperature can be controlled more accurately than by almost any other technique and most importantly for rate studies, the chosen reaction temperature can be attained very quickly.

Enzymatic reactions are usually characterised by their initial reaction rate, thus if substantial reaction occurs while the sample is being heated to the desired temperature the results obtained will be inaccurate.

TGA measurements are usually made under a dry, inert atmosphere with an accurately controlled gas flow. This is particularly useful, as the volatile co-products may be removed by entrainment, thus enhancing the forward reaction.

One major disadvantage with TGA instruments is that there is no provision for stirring the sample during the reaction; therefore, studies where mass transfer is important cannot be carried out. However, if the enzyme can be readily incorporated into the sample, this is not a major problem, as interest is usually in the pre-diffusion controlled stages of the reaction.

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TGA techniques appeared to be ideally suited to monitoring the synthesis of esters by direct esterification or transesterification as they can produce volatile co-products such as water or an alcohol.

One of the problems specific to enzymatic reactions is that different enzymes contain differing amounts of water and each enzyme requires different amounts of water in order to function [1]. The actual water requirement is usually specified as the thermodynamic water equivalent that gives the optimum activity of the enzyme.

Typically, lipase enzymes require a lower water activity than other enzymes, *Candida antarctica* lipase B has been shown to require very little water, in order to function [2]. Therefore, we were optimistic that residual water in the enzyme would not have a significant effect on our measurements nor would the low water environment have an adverse effect on the activity of the enzyme.

The enzyme catalysed synthesis of polyesters was demonstrated originally using the lipase from *Rhizomucor miehei* [3]. Subsequent work used the immobilised lipase B from *Candida antarctica* (Novozyme 435^{TM}), this was found to be a superior catalyst both in terms of activity and recyclability [4], also the polyesters produced using *Candida antarctica* lipase B were found to have physical properties such as molecular weight distribution, crystallinity and melt viscosity that were significantly different from their analogues produced by conventional high temperature polymerisation.

Candida antarctica lipase B is an enzyme with a remarkable range of activity in diverse biotransformations [5], it had been shown to be a very efficient polyester catalyst without the need for a solvent, which had obvious appeal in an industrial process [6].

The polyesterification reactions of the diols 1,4-butanediol and 1,6-hexanediol and polytetramethylene ether glycol (PTMEG) with adipic acid were used to evaluate the utility of TGA for following such reactions. Furthermore, knowledge of the effects of temperature on the enzymatic synthesis of polyesters would allow us to determine the greatest practicable acceleration feasible by increasing the reaction temperature. In addition, at higher temperatures, the viscosity of the reactants would decrease and we would expect higher molecular weight polymers to result as their viscosity would be less rate limiting.

2. Experimental

The 1,4-butanediol was dried over molecular sieves, 1,6-hexanediol and PTMEG 650 were stored in a desiccator over phosphorus pentoxide and then preheated to 110 °C under a dry nitrogen flow for 5 min in the TGA to remove traces of water. The TGA measurements were carried out in a Shimadzu TGA 50 thermogravimetric balance, which has a sensitivity of ±0.5 μg, a detection limit of 1 μg and can maintain the temperature to within ±0.1 °C.

2.1. General method

The sample (20–25 μl) of diol, or diol + acid, or diol + acid + enzyme was weighed accurately in a standard sized aluminium pan (6 mm diameter × 1.5 mm deep). An accurately controlled flow rate of 50 ml min^{-1} of dry nitrogen was used for all experiments and heating rates were generally 5 °C min^{-1} up to the desired reaction temperature, where the temperature was maintained isothermal (±0.5 °C) until the rate began to "tail off".

2.1.1. Measurement of reaction rates

Although several experimental protocols were evaluated, the preferred method was to prepare an accurately known concentration of the acid in the diol. Approximately 25 mg (accurately weighed on the TGA) of the acid/diol solution was placed in the TGA pan and the sample heated to 50 °C in the TGA oven. The oven was opened and the finely powdered, lyophilised enzyme was added to the solution ensuring that no lumps were present. The weight of the enzyme added was noted and the temperature was raised quickly to the reaction temperature. Reaction started as soon as the temperature reached 55 °C. Weight loss with time was recorded.

3. Results and discussion

Several problems, some general and some specific to the system, have to be considered when using the TGA to follow reaction rates. To obtain consistent, reproducible results for removal of the water of reaction by passing dry nitrogen over the sample, the flow rate had to be maintained within ±2% of the optimum

Table 1
Volatility of 1,4-butanediol, 1,6-hexanediol and PTMEG 650 at relevant temperatures

	Volatility ($\mu g\,min^{-1}$)				
	50 °C	60 °C	70 °C	80 °C	90 °C
1,4-Butanediol	2.5	10.0	16.0		
0.4 M adipic acid in 1,4-butanediol	2.5	9.8			
1,6-Hexanediol	0.0	2.0	4.0	10.5	20.5
0.4 M adipic acid in 1,6-hexanediol	0.0	2.0	4.0		
PTMEG 650	0.0	0.0	0.0	0.0	0.0

$50\,ml\,min^{-1}$ or differences in the rate of removal of water could be observed. The surface area to volume ratio of the sample also affected the rate of removel of water. However, both of these problem were easily solved as the Shimadzu TGA 50 has an accurate gas flow control system and it uses standardised aluminium pans, which are of closely matched size, shape and mass so that the surface area of the sample can be fixed. The sample volume was maintained between 20 and 25 μl, if the sample volume were increased to about 40 μl then the longer diffusion path resulted in a measurable reduction in the rate of removal of water from the sample.

Although the 1,4-butanediol and 1,6-hexanediol substrates have boiling points of 208 and 235 °C, respectively, they still had significant volatility in the flow of nitrogen at 60–70 °C. Significant diol weight loss compared to the weight loss due to water removal had an adverse effect on the accuracy of the method; therefore, the rate of weight loss from dried samples of 1,4-butanediol, 1,6-hexanediol and polytetramethylene diol (PTMEG 650, where 650 represents the average M_r) was determined both from neat samples and from samples containing dissolved adipic acid. The weight losses measured are shown in Table 1. From these results it was concluded that the volatility of 1,4-butanediol was too high, even at 60 °C where reaction begins, for accurate rate determination, thus work continued with the less volatile 1,6-hexanediol and the almost non-volatile PTMEG 650.

These weight loss rates were used as correction factors deducted from the observed weight loss in the relevant experiments.

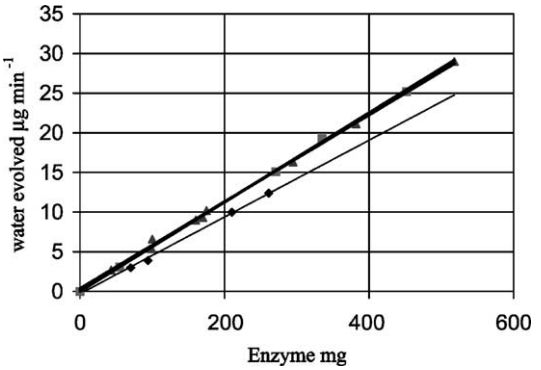

Fig. 1. Effect of enzyme concentration on the rate of reaction of adipic acid with 1,6-hexanediol at 60 °C for acid concentrations of: (■) 1.14, 0.75 mol dm^{-3} and (◆) 0.4 mol dm^{-3}.

The effect of varying the concentration of *Candida antarctica* lipase B on the rate of the polyesterification reaction was determined using 1.14, 0.75 and 0.4 mol dm^{-3} solutions of adipic acid in 1,6-hexanediol at 60 °C. The results are shown in Fig. 1.

From the data in Fig. 1 it becomes obvious that there is a non-linear change of rate with concentration, as would be expected from Michaelis Menten kinetics.

The data for the 1.14 mol dm^{-3} solution of adipic acid was analysed using the Enzfitter curve-fitting program [7]. The Marquardt–Levenberg algorithm was used to obtain the best fit and to give the analysis of variance. The fitted data are shown in Table 2. The curve of best fit to the experimental data was used to give the predicted weight loss.

The analysis of variance gave a correlation coefficient, R^2 of 0.998. On examination of the fitted data it is important to note that the largest percentage residuals (difference between the observed values and the predicted values) are at the lower end of the enzyme concentration. This is understandable because with lower weights of enzyme the overall sensitivity of the instrument is lower and minor oscillations of the balance made it difficult to determine the exact weight of enzyme added. Thus, the optimum weight of enzyme for accurate measurement of the reaction kinetics was between 200 and 500 μg. The correlation coefficients for 0.75 and 0.4 mol dm^{-3} solutions were 0.98 and 0.97, respectively. These results indicate that the reaction rate as measured by water loss is first order with respect to the amount of enzyme present.

Table 2
Statistical analysis of the effect of enzyme concentration on the rate of reaction of 1.14 mol dm^{-3} adipic acid with 1,6-hexanediol at 60 °C

Amount of enzyme (mg)	Observed water loss[a] (μg min^{-1})	Predicted water loss (μg min^{-1})	Residual	Residual (%)
0.043	2.7	2.4346	0.2654	9.83
0.099	5.4	5.5931	−0.1931	−3.58
0.1	6.6	5.6494	0.9506	14.40
0.16	9	9.0181	−0.0181	−0.20
0.17	9.3	9.5781	−0.2781	−2.99
0.175	10	9.8579	0.1421	1.42
0.295	16.3	16.541	−0.2412	−1.48
0.382	21.1	21.348	−0.2483	−1.18
0.518	29	28.799	0.201	0.69

[a] The correction factor for 1,6-hexanediol loss was 2 μg min^{-1}.

The effect of varying temperature on the rate of reaction of adipic acid and MEAA with 1,6-hexanediol, were studied to determine the activation energies of the rate controlling steps. Normally there is a narrow window between the lowest temperature at which activity is observed and the temperature at which degradation of the protein starts to occur, but in the case of *Candida antarctica* lipase B this window is larger than with most other enzymes as it is a thermozyme [8]. However, other factors, such as the melting point of the solutions (∼50 °C), do limit the temperature range over which reaction rates can be observed.

As the reaction is used commercially [9], the preferred system on which to base the rate/temperature studies was the reaction of adipic acid with 1,6-hexanediol. The melting point of the adipic acid solutions in 1,6-hexanediol is approximately 50 °C and no reliable onset of reaction was observed below 58 °C. At temperatures higher than 70 °C the volatility of the 1,6-hexanediol became a problem, for example, the correction factor at 80 °C was 10.5 μg min^{-1}, greater than the typical weight loss per minute due to water formed in the reaction, i.e. 5–10 μg min^{-1}. Such large correction factors render the results unreliable, thus, only rates at 60 and 70 °C were observed, although the errors will be high these give an approximate E_a of 23 kJ mol^{-1}.

The reaction of the half-ester MEAA with 1,6-hexanediol was studied, while the problems associated with the volatility of the diol still applied, the freezing point of the solutions was approximately 40 °C. Also the reactivity of the ester and the M_r of the volatile product, ethanol, were such that meaningful weight losses could be observed. The reaction of a 0.4 mol dm^{-3} solution of MEAA in 1,6-hexanediol was carried out at 50, 55 and 60 °C and the results are presented in Table 3.

Table 3
The reaction of 0.4 mol dm^{-3} MEAA with 1,6-hexanediol between 50 and 60 °C

Temperature (°C)	Enzyme (mg), 100% protein	Weight loss[a] (μg min^{-1})	Rate of reaction (μmol min^{-1} mg^{-1})
50	0.293	12.4	2.35
50	0.298	12.5	2.33
50	0.174	7.6	2.42
55	0.140	6.5	2.58
55	0.187	8.7	2.58
55	0.134	6.5	2.69
60	0.106	5.8	3.04
60	0.271	14.0	2.87
60	0.163	8.3	2.83

[a] Correction factors for loss of 1,6-hexanediol at 50 °C, 0 μg min^{-1}; at 55 °C, 1 μg min^{-1} and at 60 °C, 2 μg min^{-1}.

Table 4
Calculation of activation energy by Arrhenius plot

Temperature (°C)	$1/T$ (K) $\times 10^{-3}$	Rate (μmol min^{-1} mg^{-1})	Rate constant (K)	ln K
50	3.10	2.37	14.8	2.69
55	3.05	2.62	16.4	2.8
60	3.00	2.91	18.2	2.91

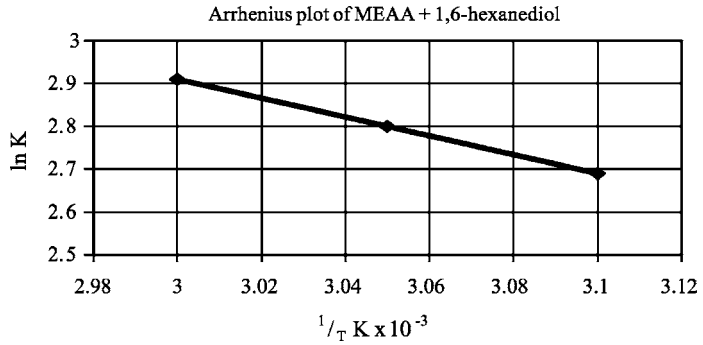

Fig. 2. Arrhenius plot for the reaction of MEAA + 1.6-hexanediol.

These results were plotted as an Arrhenius plot as shown in Table 4 and Fig. 2.

The Arrhenius equation governs the effect of temperature on the rate of a reaction as shown in Eq. (1). Eq. (2) is the equation for a straight line plot, therefore, the activation energy E_a can be calculated from the slope of the graph.

$$\ln K = \ln A - \frac{E_a}{RT} \quad (1)$$

$$\ln K = \ln A - \frac{E_a}{R} \times \frac{1}{T} \quad (2)$$

where K is the rate constant, A the pre-exponential frequency factor, E_a the activation energy, R the gas constant and T is the temperature (in K).

The slope of the plot was 0.22×10^{-3} K, therefore, the activation energy is 18.3 kJ mol^{-1} compared to the activation energy of 23 kJ mol^{-1} for the esterification of the diol by the adipic acid. The only diol that does not have an appreciable weight loss at elevated temperature is the 650 molecular weight polytetramethylene ether diol (PTMEG 650). Therefore, the rate of reaction of a 0.4 mol dm^{-3} solution of adipic acid in PTMEG 650 was determined at 60, 70 and 80 °C.

Table 5
Reaction of 0.4 mol dm^{-3} adipic acid and PTMEG 650 between 60 and 80 °C

Temperature (°C)	Enzyme (mg), 100% protein	Weight loss[a] (μg min^{-1})	Rate of reaction (μmol min^{-1} mg^{-1})
60	0.212	8.1	2.13
60	0.054	2.1	2.22
60	0.139	5.8	2.28
70	0.139	7.1	2.82
70	0.117	5.3	2.70
70	0.155	7.2	2.79
80	0.078	8.3	3.72
80	0.182	10.5	3.30
80	0.108	8.3	4.29

[a] No correction factor required for weight loss of PTMEG 650.

Table 6
Calculation of activation energy by Arrhenius plot

Temperature (°C)	$1/T$ (K) $\times 10^{-3}$	Rate (μmol min^{-1} mg^{-1})	Rate constant (K)	ln K
60	3.0	2.2	13.7	2.6
70	2.9	2.8	17.5	2.9
80	2.8	3.8	23.8	3.2

Because of an earlier observation of declining rates above 80 °C, only the first 10 min of the reactions were considered. The results are given in Table 5.

The results were converted for an Arrhenius plot as shown in Table 6.

The slope of the plot of the above data (Fig. 3) was 3.0×10^{-3} K, giving an activation energy of 24.9 kJ mol^{-1}, reasonably close to the approximate activation energy determined for the reaction of the enzyme in 1,6-hexanediol (23 kJ mol^{-1}).

In all experiments there was a significant stoichiometric excess of hydroxyl over acid and only the initial reaction rates were recorded, therefore we were confident that the reaction we were observing was, in fact, predominantly the acylation of the enzyme. Therefore, the activation energy of the acylation of the enzyme by adipic acid is measured as 24.9 kJ mol^{-1}, whereas that of the acylation by the monoester is 18.3 kJ mol^{-1}. The measurement of the activation energy of the monoester reaction undoubtedly contains a contribution from the acylation of some of the enzyme by the monocarboxylic acid end group, therefore, it is only possible to say that the activation energy for the ester acylation reaction is less than 18.3 kJ mol^{-1}. The activation energy of the transesterification reaction appears to be significantly less than that of the acid acylation reaction; the acylation by the ester will be the preferred reaction. This is as expected because the acylation of the enzyme by the ester involves a nucleophilic substitution with a facile leaving group, whereas acylation by the acid is an acid base reaction that is usually much slower.

Because *Candida antarctica* lipase B is a thermozyme, the activity of the enzyme at high temperatures is of interest for two reasons: it is important to know the upper limits of useful activity for this enzyme and it is necessary that any enzyme remaining in the product at the end of the process be deactivated thermally, as traces of residual enzyme could lead to hydrolysis of the product at room temperature.

The degree of denaturation of the enzyme was determined between 110 and 140 °C with 0.8 mol dm^{-3} adipic acid in PTMEG 650. The enzyme used in these experiments was *Candida antarctica* lipase B supported on acrylic beads, sold as Novozyme 435. The results obtained are shown in Table 7.

It is apparent that the enzyme retains some activity at these elevated temperatures and that 130–140 °C

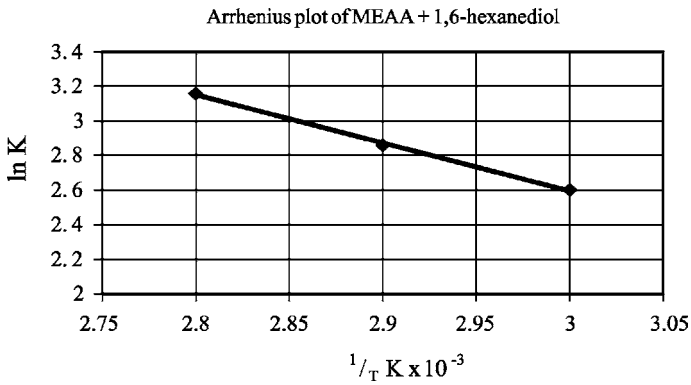

Fig. 3. Arrhenius plot for the reaction of adipic acid and PTMEG 650.

Table 7
The reaction of 0.8 mol dm^{-3} adipic acid with PTMEG 650 at elevated temperatures

Enzyme (mg)	Temperature (°C)	Weight loss[a] (μg min^{-1})	Rate of reaction (μmol min^{-1} mg^{-1})
2.152	110	15.0	0.39
1.892	110	17.0	0.50
1.702	110	18	0.59
1.914	120	28.0	0.81
2.073	120	26.5	0.71
3.322	130	20.9	0.35
1.989	130	10.7	0.3
2.411	140	4.3	0.10
1.884	140	0	0

[a] No correction factor required for weight loss of PTMEG 650.

appears to be the point at which activity is lost. However, these are initial rates, no attempt was made to determine how long the enzyme remained active at these temperatures, or if it could be recycled and remain active after exposure to these temperatures.

4. Conclusions

It has been demonstrated that thermogravimetric analysis is a facile and accurate technique for following enzyme catalysed reactions when a volatile product such as water or ethanol are produced as by-products. In particular, the method is good for measuring accurately the initial rates and energies of activation of the reactions. Because of the small sample size used and the rapid heating to the reaction temperature only a relatively short time of 30 min is required to complete the measurements. Therefore, this technique is ideal for rapid assay of enzyme activity in a laboratory or plant environment.

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