

**GENERATION OF RICINOLEIC ACID FROM CASTOR OIL
USING THE LIPASE FROM GROUND OAT (*AVENA SATIVA* L.) SEEDS
AS A CATALYST**

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SUMMARY

The lipase found in oat seeds acts upon castor oil in organic solvent, yielding approximately 90% ricinoleic acid. Initial velocity is a poor predictor of the time required for complete lipolysis except at low oil concentrations. Equations were developed to estimate kinetic parameters necessary to accurately predict the degree of lipolysis.

INTRODUCTION

Castor oil is derived from the bean of the castor plant, *Ricinus communis* (L.) of the family Euphorbiaceae. In the triglycerides of castor oil, glycerol is esterified at approximately the 90% level by ricinoleic acid, a unique hydroxylated, monounsaturated 18-carbon fatty acid (Naughton, 1979). In industry, the hydroxyl functionality is subject to a variety of reactions such as dehydration, halogenation, alkoxylation, esterification, and sulfation (Naughton, 1974). The amide, amine, and ester carboxylic acid derivatives are uniquely useful for numerous applications which require lipids with a polar functionality (McKenna, 1972).

The splitting of castor oil is difficult to achieve using the traditional high-temperature steam treatment because ricinoleic acid tends to form estolides (polyricinoleic acids) (Lakshminarayana *et al*, 1984); also this method of splitting is very energy intensive. Here it is demonstrated that ground oat seeds can serve as a source of 'immobilized' lipase activity. When castor oil is treated with this oat lipase preparation under appropriate conditions nearly complete splitting can be achieved in an energy efficient manner without detectable estolide formation.

In the prior manuscript (Piazza, this journal) it was demonstrated that several different factors accelerated the lipolysis rate: the oat seeds had to be ground; a hydrocarbon or a chlorofluorocarbon solvent had to be used; the water concentration needed to be carefully controlled, and the temperature had to be raised to 35 °C. Prior to the work described here, the enzymatic splitting of castor oil was achieved only by using the castor bean lipase (Ory *et al*, 1960; Rao *et al*, 1990). In this manuscript the influence of oil concentration upon the reaction rate was investigated, and a kinetic method to treat the data was developed that can be used to predict the degree of reaction and to estimate the Michaelis constant (K_m) and the maximum velocity (V_{max}).

MATERIALS AND METHODS

Chemicals and materials. Castor oil was medicinal grade from Barre-National, Inc. (Baltimore, Maryland). Phenolphthalein was from Baker (Phillipsberg, NJ). All other materials were purchased as described before (Piazza, this journal).

Oil lipolysis. Lipolysis was conducted as described in the prior manuscript (Piazza, this journal). Since castor oil is not very soluble in 2,2,4-trimethylpentane (TMP), the reactions were composed of three phases: solid ground oats, castor oil phase, TMP phase. At the indicated times, the reactions were stopped by extraction with three 50 ml aliquots of a 50:50 (v/v) mixture of acetone and ethanol.

Fatty acid release. Free fatty acid was measured using a titration technique described by Linfield *et al*, 1984. Briefly, a twenty ml aliquot of the combined acetone-ethanol extracts was pipetted into a 50 ml Erlenmeyer flask containing a teflon coated stir bar. Phenolphthalein was added (100 μ l of a 5% (w/v) ethanolic solution), and a calibrated, aqueous 0.1 N NaOH solution was added to the flask until a light pink color was observed. If more than 1.0 ml of the NaOH solution was required for neutralization, the reaction extract was diluted with acetone-ethanol, and the titration was repeated. Blanks were run by extracting the lipolysis reaction mixture immediately after the addition of all of the components.

The accuracy of the data was confirmed by visually monitoring the extent of reaction using thin layer chromatography (TLC). Glass plates (height, 17.5 cm) coated with a 250 micron thick layer of silica gel G (Analtech, Newark, DE) were developed using a three stage system: 1. benzene, diethyl ether, ethyl acetate, acetic acid (72:14:14:0.033 v/v/v/v), development to the top of the plate; 2. hexane, diethyl ether (60:40 v/v), development to the top of the plate; 3. hexane, diethyl ether, formic acid (80:20:2.5 v/v/v), development to 7.5 cm above the origin.

The high acid values obtained after lipolysis preclude significant estolide formation. This conclusion was confirmed by the absence of detectable estolide on two dimensional TLC (Neissner, 1980).

Data analysis. Nonlinear regression analysis of progress curves and other parameters was carried out using the program Abacus which is based upon the Gauss-Newton iterative method. Choices of fits between models and statistical evaluation of fits were as described by Meites, 1979. The development of the equations, a form of linked functional analysis, and their derivation as applied to enzyme kinetics are given elsewhere (Farrell *et al*, 1990). Iterative analysis was carried out until a minimum in the root mean square (RMS) and in the error of the coefficients was achieved. The mathematical expression that describes the kinetics of lipolysis is as follows:

$$P_{obs} = \frac{P_{max} k_1^n t^n}{1 + k_1^n t^n} \quad (1)$$

where P_{obs} is the amount of product observed at time t , P_{max} is the maximum amount of product formed, k_1 is the rate of reaction at one-half P_{max} and n is the cooperativity parameter that is constrained to integer values. Using the values generated from the theoretical fit of equation 1, a rate dP/dt can be generated at any point on the progress curve for which the values of P and/or S are known.

If the curvature seen in the progress curve, as fit with Equation 1, is due only to decrease of substrate with time, then the rate at any point on the curve as determined above is given by:

$$v = \frac{dP}{dt} = \frac{V_{max} k_2 (S_o - P)}{1 + k_2 (S_o - P)} \quad (2)$$

where $(S_o - P)$ is the change in initial amount of substrate S_o minus the level of accumulated of product, P (Orsi and Tipton, 1979). The data generated by Equation 1 were fit with Equation 2 to yield V_{max} and k_2 , the reciprocal of which is equivalent to K_m .

After the analysis of the progress curve as described above is completed, a number of parameters emerge that define the overall design of the lipolytic reaction. Equation 1 yields the maximum amount of product, P_{max} and the reciprocal of k_1 yields the time needed to generate one-half P_{max} or the reaction half time. Additionally, solving Equation 2 yields parameters analogous to those obtained from standard steady state kinetics using initial velocity studies.

RESULTS

Optimal oil level study. As noted in Materials and Methods castor oil is not completely

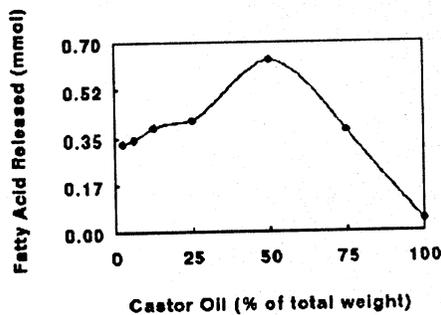


Figure 1. The effect of the castor oil level upon the amount of fatty acid released in one hour by the lipase in oat seeds.

solubilized by TMP. Thus the term 'oil level' will be used, rather than oil concentration. Figure 1 shows the relationship between the castor oil level and the lipolysis rate during the first hour of lipolysis. The optimal lipolysis rate appeared to occur when the amount of castor oil was equal to that of the solvent, TMP. With further increases in the oil level, the lipolysis rate decreased. Lipolysis using only the oil was very slow. The low rate is not due to a limitation in the amount of

water, as there were 0.8 ml or 44 mmol present, a value much greater than the amount of fatty acid formed. (Piazza, this journal).

The time course of lipolysis was followed at a low oil level (oil:oil + TMP; 0.4:16 w/w, Figure 2). Nearly complete lipolysis at this low castor oil level was achieved in 22.5 hours.

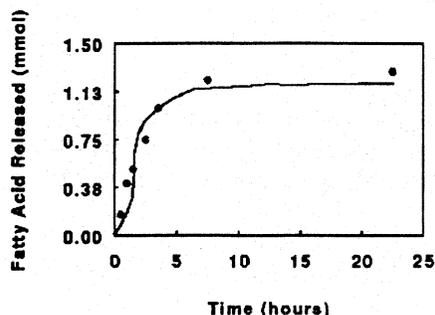


Figure 2. The time course of fatty acid release by the lipase in oat seeds when the level of castor oil is low (oil:oil + TMP; 0.4:16 w/w).

level a greater conversion to fatty acid occurred even though the initial lipolysis rate (Figure 1) was greater at the high oil level. Moreover the half-time for maximum conversion is only 1.77 hours for the low oil level as opposed to 25.7 hours in the high oil sample. The best fit of Equation 1 was obtained with n equal to one for the high oil level reaction, but with n equal to 2 for the low oil reaction. This demonstrates that the splitting of castor oil by oat lipase is a

cooperative process. A possible explanation for cooperativity is that the lipase is activated by fatty acid or possibly the di- and monoglycerides. It is also possible that cooperativity results from physical changes in the ordering of the triglycerides by the products of lipolysis. The data presented here do not provide a definite explanation for the cause of the cooperativity observed in the splitting reaction.

When the time course of lipolysis was followed at a high oil level (oil:oil + TMP; 8:16 w/w), lipolysis was not complete even at 300 hours (Figure 3). The terms 'low' and 'high' will be used in the remainder of this article to refer to the reaction conditions described immediately above.

The reaction progress curves of Figures 2 and 3 were fitted with Equation 1. The coefficients for best fits to the data are given in Table 1. It can be seen that at the low oil

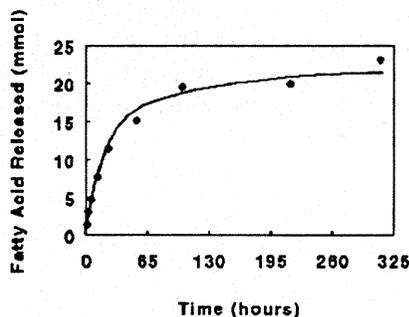


Figure 3. The time course of fatty acid release by the lipase in oat seeds when the level of castor oil is high (oil:oil + TMP; 8:16 w/w).

Table 1. Nonlinear regression analysis of the lipolysis progress curves.

Curve	P_{max} (mmole)	% Conversion	$t_{1/2}$ (hr)	% error	RMS
2	1.26 ± 0.05	97	1.77 ± 0.15	5	0.059
3	23.7 ± 0.71	91	25.7 ± 2.79	3	0.727

By calculating the theoretical rates from Equation 1 using k and P_{max} , the rate at specific

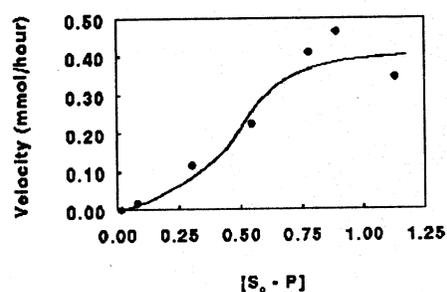


Figure 4. Fit of the time course data found in Figure 2 to Equation 2 and dP/dt values generated from curve fitting of the data to Equation 3.

a substrate level can be obtained. The data for the low oil experiment are plotted in Figure 4 and fitted with Equation 2 to obtain

kinetic parameters: V_{max} equals 0.448 ± 0.05 mmol per hour, and K_m equals 0.465 ± 0.089 mmol per 14.08 g. The latter yields a K_m of 33 mM (1 g = 1.65 cc).

The RMS of this analysis is 0.047 (10% error overall). Compared to other

enzymatically catalyzed reactions, K_m is relatively large. This indicates that the

castor oil glycerides bind to the oat seed lipase poorly. Since the apparent binding constant reflects competition between the enzyme and solvent for the substrate, it might be expected that binding would be poor when an organic substance is used as the solvent instead of water. Nevertheless, in spite of the poor binding, faster and more complete lipolysis was observed using solvent than with a system in which the oil was emulsified in an aqueous buffer (data not shown).

Although as shown in Figure 1, the initial lipolysis rate was faster at the high oil level, this did not compensate for the twenty-fold increase in the amount of oil. A rule of thumb used to estimate the time needed for complete reaction is to compute a pseudo half-time using initial velocity data (Figure 1), assuming that the rate of lipolysis remains constant up to the half time. Then this pseudo half-time is multiplied by ten. Applying this rule to the low oil reaction gives a predicted time for complete lipolysis of 19.8 hours, very close to what was observed in the time course experiment (Figure 2) and within error equivalent to 17.7 ± 1.5 hours from Table 1. With the high oil level, the predicted time for completion from Figure 1 is 204 hours, a much shorter time than what was observed in the time course experiment

(Figure 3) and lower than that computed from Table 1 (257 hr). The disagreement between the initial velocity estimated time, and the actual time required for lipolysis suggests that product inhibition occurred. That oat lipase catalyzed splitting of oil is sensitive to product inhibition was also the conclusion drawn by Lee and Hammond, 1990, when they observed the splitting of soybean oil. The possibility that product inhibition occurred precludes the application of equation 2 to the high oil reaction.

Another way of viewing the differences between lipolysis at the low and the high oil levels is to calculate the enzyme efficiency factor:

$$EEF = \frac{\%P_{\max}}{t_{1/2}} \quad (3)$$

Lipolysis at the low level of oil gave an efficiency factor of 55, while that at the high oil level was only 4. Thus it may be concluded that lipolysis at a low oil level is a more efficient pathway to take. That this would not have been predicted by the initial velocity study (Figure 1) shows the importance of using an analysis of the reaction progress curve in those cases in which complete conversion of starting material to product is the goal.

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