Determination of Nanogram Amounts of Fluoride by Its Inhibition of Lipase Activity Using a Fixed Time Method

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Summary: A fixed time method for the determination of ultratrace amounts of fluoride, based on its inhibition of lipase activity is described. An emulsion of p-nitrophenyl laurate in polyvinylalcohol is used which acts as the substrate for lipase. The assay method is first optimized by studying the effects of various parameters. Kinetics of the enzyme-catalysed reaction and also that of the inhibition process is reported.

Introduction

Lipase, a triacylglycerol hydrolase (EC. 3.1.1.3) is known to catalyse the hydrolysis of triglycerides. This enzyme does not act on water soluble substrates but rather on emulsified substrates[1-3]. This property of lipases distinguishes them from other estereases which can act on water soluble substrates. This is due to the fact that the site of action of lipase is the interface between the oil drops and the aqueous phase, apparently because the active centre of the enzyme is then exposed.

The inhibition of lipase activity by fluoride is the basis of the earliest analytical method based on inhibition. This method dates from 1908, when Amberg and Loevenhart published a method for the parts per million (ppm) of fluoride ions in milk and other materials by inhibition of pigs liver and pancrease esterase[4]. Although the method took 14 hrs to complete, it was remarkably sensitive for its time and stimulated further investigation of such methods. No attempt was made to improve on this work until Linde refined the method[5] and later workers[6] improved it still further so that nanogram (ng, 10^{-9} g) amounts of fluoride could be determined. The method however still suffers from disadvantage of being tedious.

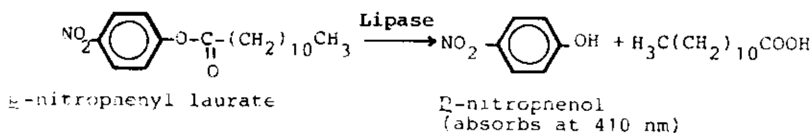
In this paper a "fixed time method" for fluoride determination, based on its inhibition of lipase activity, is described which is very rapid and sensitive. The assay procedure used for lipase involves the hydrolysis of p-nitrophenyl laurate by lipase and measuring spectrophotometrically the rate of formation of p- nitrophenol which absorbs at 410 nm.

Experimental

Lipase (EC. 3.1.1.3, 675 U/mg solid, ex. Candida cylindraceae) was obtained from Sigma London. A stock aqueous solution of the enzyme (44.4 mg/dl) was prepared in deionized water and stored at 4°C. The aqueous enzyme solution at 4°C was stable for months. Barbital buffer (0.1M, BDH Chemical) was used throughout the experiment. p-nitrophenyl laurate was purchased from Sigma London. An emulsion of this substrate was prepared as follows. p-nitrophenyl laurate solution (20mM, 1.0 ml) in acetone was added to a mixture of 8.0 ml of 0.1M barbital buffer (pH 3.8) and 4 ml of 1% polyvinyl alcohol solution, and emulsified. The emulsion was diluted with water to a total volume of 20 ml. The resulting emulsion is stable for 3 days when stored at 4°C but it is preferable to use a fresh emulsion daily. For fluoride 5×10^{-3}M stock solution of AnalaR grade sodium fluoride was prepared in deionized water and used as an inhibitor of lipase. Deionized water from an Elgastate cartridge was used throughout.

All absorbance measurements were carried out on a Unicam SP-8000 UV/visible spectrophotometer. This was supplied with a cell-block which could be maintained at 25°C + 0.1°C with water circulating from a thermostated water bath. 10mm silica cuvettes were used.

The basic procedure followed was decided from the results of a series of experiments to investigate the individual volumes or concentrations in a total 3ml contents of the spectrophotometric cell. 1ml each of barbital buffer (0.1M, pH 8.6) and the substrate emulsion (0.6 ml taken from stock and diluted to 1ml with deionized water) were taken in



the cuvette and the solution mixed thoroughly by covering the mouth of the cuvette with its lid and shaking it well. The cuvette was placed in the thermostated cell block and left to equilibrate for five minutes at 25°C. At zero time 1.0 ml of enzyme solution (0.1 ml of stock enzyme) solution diluted to 1.0 ml with deionized water) was added and the reactants were mixed gently. The progress of the reaction was followed at 410 nm by plotting the absorbance directly on the spectrophotometric chart against a blank which contained substrate and buffer only. In blank the enzyme was replaced by a further 1ml of buffer.

For inhibition studies the conditions used were as follows, which were selected after a great deal of optimization (see later). 50μl of enzyme stock solution and different volumes of inhibitor solution from stock (10,20,30,40,50,100,200 μl) were kept together for 5 min, diluted to 1ml and then introduced into the spectrophotometric cell containing 1ml each of buffer and the substrate emulsion as described above. The progress of the reaction was followed for a fixed period of 5 min directly on the spectrophotometric chart paper relative to the standard response i.e. without the addition of any inhibitor.

Results

Optimization of reaction conditions:

(a) Effect of time on the rate of enzymatic reaction:

Under certain conditions the rate of an enzyme- catalysed reaction remains constant over a certain period of time after which the rate starts to decrease, possibly due to depletion of substrate, or the formation of products that inhibit the enzyme. It is essential therefore to establish the time over which the enzymatic reaction rate is constant, so that it can be easily measured and any change in that rate attributed to the investigated factors.

The absorbance of the reaction mixture in the assay procedure described above was plotted directly on the spectrophotometric chart paper (Fig. 1). A reaction of 5 min was chosen for all further experiments because a reasonable change in absorbance was observed in this time. For rate calculation (i.e. ΔAbs/min), the absorbance was noted after 1 and 4 minutes giving a change in absorbance per 3 minutes, from which then change in absorbance per minute was calculated.

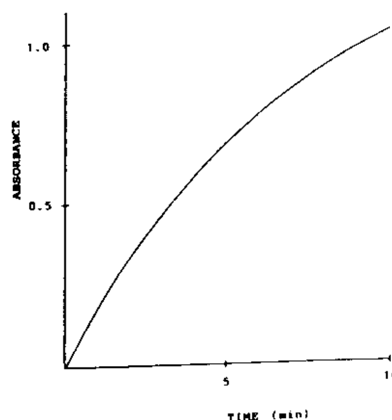


Fig. 1: Curve of absorbance as a function of time for lipase-catalysed hydrolysis of p-nitrophenyl laurate.

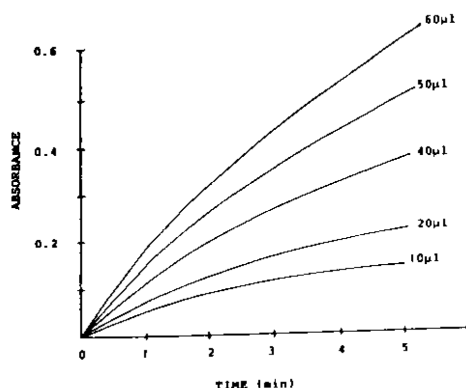


Fig. 2: Absorbance curves obtained for various enzyme concentration.

(b) Effect of Enzyme concentration on the rate of enzyme- catalysed reaction:

Different enzyme concentrations (10-50μl from stock enzyme solution) were assayed under the conditions stated above. At zero time 1 ml of enzyme solution (10μl-50μl diluted to 1ml with deionized water) were introduced into the cuvette containing 1ml each of buffer and substrate equilibrated for 5 minutes at 25°C. The reaction was allowed to proceed for 5 minutes. The absorbance change at 410 nm for each enzyme concentration against a blank was plotted directly on the spectrophotometric chart paper. These absorbance curves obtained are shown in Fig.2.

Rate (change in absorbance perunit time) was calculated for each enzyme concentration. As shown in Fig. 3 a linear increase in rate was observed with an increase in enzyme concentration. 50μl of enzyme was chosen for further experiments as it gave a good absorbance change over the fixed time interval.

(c) Effect of substrate concentration on the rate of enzyme catalysed reaction:

Effect of substrate was investigated to select the suitable substrate concentration for assaying enzyme activity when inhibited by the fluoride. The same conditions as above were used except that different concentrations of substrate were taken using the enzyme concentration fixed (50μl). A calibration graph of rate vs substrate concentration is shown in Fig. 4.

There is a linear increase in rate with increasing substrate concentration in the range 0.1-0.6 mM. A further increase in substrate concentration results in a decrease in rate as is evident from the calibration graph. 0.6mM substrate concentration was therefore chosen for further experiments.

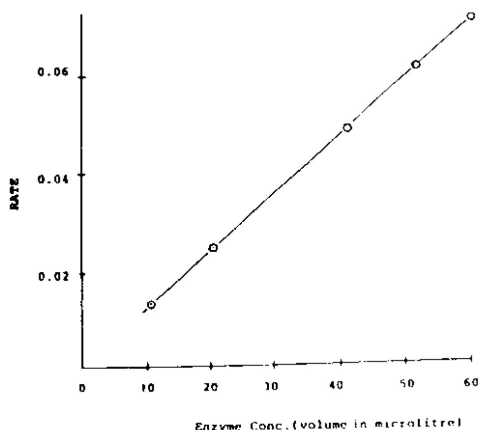


Fig. 3: Calibration graph for the assay of enzyme lipase.

(d) Effect of pH on the activity of lipase catalysed reaction:

The effect of pH on the activity of lipase was investigated by using barbital buffer (0.1M) of varying pH. Rate was calculated for each pH value and a graph plotted rate vs pH, which is shown in Fig. 5. Lipase starts to catalyse the reaction at pH 6.5 and the catalytic activity increases with increasing pH

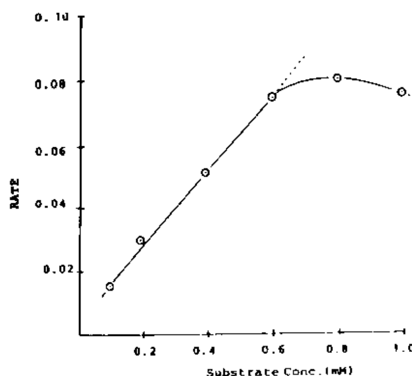


Fig. 4: Calibration graph for substrate (p-nitrophenyl laurate).

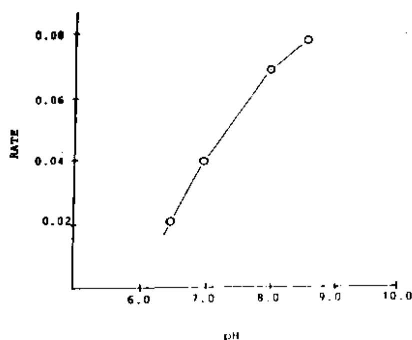


Fig. 5: Effect of pH on the activity of lipase.

upto 8.6. Above this pH the substrate starts self-hydrolysis without the addition of lipase, therefore pH 8.6 was used throughout.

(e) Effect of pre-incubation time on the degree of inhibition.

As far as the effect of incubation time on inhibition is concerned, the enzyme and inhibitor were pre-incubated for 0,5,10 and 20 minutes and then added to the assay mixture. It was observed that at "zero" time the inhibition is negligible but greatest at 5 minutes incubation time. Incubating for longer time did not show any improvement in inhibition, therefore 5 minutes incubation time was used throughout.

Fluoride inhibition of lipase

Enzyme activities were assayed in the presence of different amounts of inhibitor. The absorabance curves obtained directly on the chart re-

corder relative to a standard curve (the one without the addition of inhibitor) are shown in Fig. 6. The absorbance curves clearly indicate that in the range 10-50μl NaF solution, the inhibition increases with an increase in the amount of inhibitor while a further increase in the amount of inhibitor does not show that linear increase in inhibition. As shown in Fig.6 there is no lag in the increase in absorbance when the inhibitor is absent while in the presence of the inhibitor, the curves show a lag in the increase in absorbance. Although in both the cases the contents of the cuvette were thoroughly mixed. Such type of lag in the enzymatic reaction curve is typical of inhibition of the enzyme activity.

Percent inhibition of lipase activity for fluoride ion concentration in the range 10-50μl was calculated from the maximal activities observed in the absence and then presence of fluoride ion by making use of the expression.

$$\% \text{ inhibition} = \frac{(Au - Ai)}{Au} \times 100$$ where

Au = Absorbance resulting from uninhibited reaction.

Ai = Absorbance change when enzyme is inhibited.

Fig. 7 shows plot of % inhibition against fluoride concentration in the range where an inhibition due to fluoride occurred (Fig. 6, 10-50μl). The limit of detection of fluoride ion was found choosing 0.5 ng fluoride ion concentration as this gives the least % inhibition. Using the above conditions enzyme activity was assayed and 9 readings were taken. The standard deviation for n = 9 was calculated, which is +0.035. From a graph of absorbance vs fluoride concentration, 0.035 on the absorbance scale corresponds to 0.05 ng F^-. Therefore the limit of detection of fluoride is twice the standard deviation, i.e. 0.1 ng of F^-.

Kinetics of lipase catalysed reaction

Determination of "Michaelis constant"; using Lineweaver and Burk method[7]. the value of Michaelis constant, "Km" was obtained for the lipase catalysed hydrolysis of p- nitrophenyl laurate. The activity of lipase was assayed with various amounts of substrate. The rate was calculated and 1/rate plotted against 1/[s] on a double reciprocal graph. This is shown in Fig. 8. From the graph the value of Michaelis constant, Km obtained is 1.28mM. This value is comparable with the value

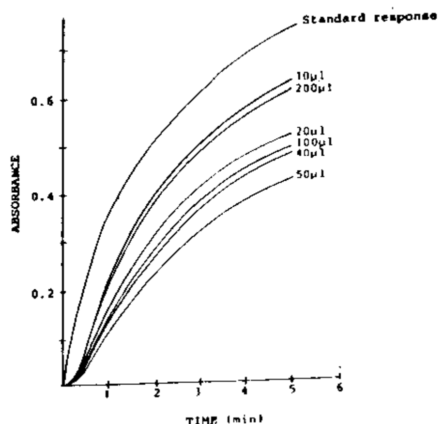


Fig. 6: Absorbance curves obtained directly on a spectrophotometric chart recorder showing the inhibition of lipase by varying amounts of fluoride.

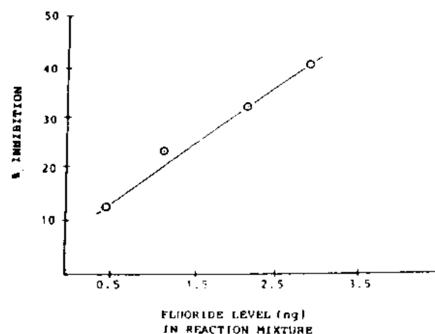


Fig. 7: Plot of % inhibition vs fluoride concentration.

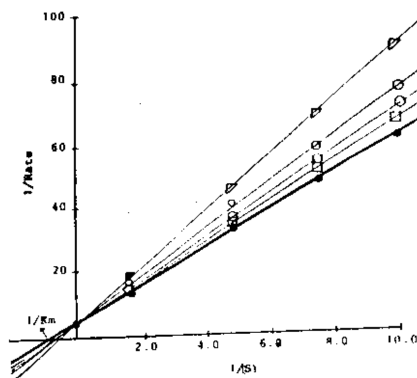


Fig. 8: Lineweaver-Burk Plot for the determination of "Km" and the kinetics of fluoride inhibition of lipase activity.

●-Standard Lineweaver - Burk Plot, □-0.5ng fluoride, O-1.1 ng fluoride, O- 2.2ng fluoride, Δ-2.8ng fluoride.

obtained from human G-200 lipase which is $3.5x10^{-3}$ M using p- nitrophenyl propionate[8] and also the value $3.3x10^{-3}$ using lipase obtained from wheat germ[9].

Type of inhibition:

To find out the type of inhibition whether competitive, uncompetitive or non-competitive, the inhibition process was carried out with various amounts of fluoride. The rate for each one was calculated from the curves obtained.

The data obtained are summarized in Table 1. From the data obtained for each concentration of fluoride, Lineweaver-Burk plots were plotted on a double reciprocal graph which already has the standard Lineweaver-Burk plot (Fig. 8).

From Fig. 8, it is apparent that the value of limiting velocity remains unaltered for all concentrations of fluoride while Km increases as the fluoride concentration is increased, which is characteristic of a competitive inhibition, therefore according to Lineweaver and Burk[7], such a type of inhibition is competitive.

Discussion

Lipases that act on lipid-water interface are difficult to study because of the complex physiological structure of their substrates[9]. No comparable studies have been reported relating to the catalytic efficiency and the mechanism of lipolytic activity of such enzyme with the solubility in aqueous medium of various pseudo substrates like, p-nitrophenyl laurate, acetate, butyrate, propionate i.e. the esters of p-nitrophenol. It has been observed during the course of this investigation that an emulsion of substrate plays an important role in such an enzyme catalysed reaction. The emulsion in turn depends upon the emulsifying agent and the organic solvent in which the substrate is dissolved. The emulsion was first tried with a solution of p- nitrophenyl laurate in dimethylformamide (DMF), but in this case the enzyme showed poor activity. The DMF solvent was then replaced by acetone, in which case the enzyme showed good activity. This suggests the change in activity be associated with the change in the physical state of the substrate molecule, possibly the rearrangement of organic solvent and water molecules surrounding it. Each substrate molecule, depending upon its chemical structure, may need a specific number of organic solvent molecules to form lipophillic and lipophobic layers in order to keep it completely soluble in aqueous medium. In case of substrate assay (Fig. 4) a decrease in rate at some point with increasing substrate concentration may be explained on the basis that since the organic solvent concentration is kept constant, it may not be sufficient to provide enough protection to each substrate molecule, beyond a certain concentration of the substrate. When this situation is reached the substrate molecules start diffusing into the aqueous phase and eventually reach and float onto the surface escaping from attack by the enzyme. The second explanation which may be given and which is observed in enzyme-catalysed reactions is that of inhibition of enzyme by substrate when used in excess[10,11].

Enzyme assay was carried out (Fig. 3) not only to find a suitable enzyme concentration to be used for inhibition studies but also to find out whether in case of such emulsified substrates, the enzyme follows the Michaelis-Menten Kinetics. Linearity of the calibration graph in Fig. 3, showed that it remained proportional to enzyme concentration.

The "fixed time method" for the study of such inhibition processes has proved to be a good analytical approach and can be applied to other inhibition processes too, involving trace metal determinations and to the determination of pesticides[12]. McGaughey and co-Workers[6] have reported an increase in fluoride inhibition of liver esterase as the pH is decreased and that is greatest in the range pH 3-4. But in the present study it was observed that lipases activity below pH 6.5 is completely nil. An attempt was made to find out the effect of pH on inhibition in the range pH 6.5 - 8.6, but the effect remained the same throughout the entire pH range investigated. It is very difficult to comment on it and also on the degree of the extent of inhibition at higher concentration of fluoride.

Since lipases play an important role in the metabolism of the fatty acids they release from triacylglycerols, their Kinetics have been studied recently[13,14]. As with other enzymes, Kinetic data provide the most commonly used method for elucidating their mechanisms, as well as the mechanisms of their inhibitions. The Km value ob-

Table 1: Kinetic Data for fluoride inhibition of lipase

Fluoride stock sol. in υl.	Substrate (emulsion vol) (ml)	Abs/min (rate)	1/Rate	1/[S]
10	0.1	0.015	66.66	10.0
	0.2	0.028	35.70	5.0
	0.4	0.053	18.86	2.5
	0.6	0.070	14.28	1.6
20	0.1	0.014	71.42	10.0
	0.2	0.026	38.46	5.0
	0.4	0.050	20.00	2.5
	0.6	0.063	15.87	1.6
40	0.1	0.013	76.92	10.0
	0.2	0.023	43.47	5.0
	0.4	0.050	20.50	2.5
	0.6	0.060	16.66	1.6
50	0.1	0.011	90.90	10.0
	0.2	0.021	47.61	5.0
	0.4	0.044	22.72	2.5
	0.6	0.056	17.86	1.6

tained for lipase from Candida cylindraceac is comparable with the vlaue obtained for lipase from other sources. As a rule the Km value represents the affinity of the enzyme for the substrate, but here the Km value does not represent the affinity. However as suggested in another report[15], the Km value in case of emulsified substrates does reflect the affinity of enzyme molecule for the emulsion interface and could be considered the enzyme interface dissociation constant, thereby lending a physical concept to the value.

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