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CALORIMETRIC INVESTIGATIONS OF THE LIPASE-CATALYSED HYDROLYSIS OF ACYLATED ACYCLONUCLEOSIDES

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Abstract

Thermal power accompanying the hydrolysis of mono- and bi-acylated acyclonucleosides: (*R,S*)-1-N-(1-acetyl-1-O-acetyl-3-hydroxypropoxymethyl)-thymine (ThL), 1-N-(1,3-di-O-acetyl-propoxymethyl)-thymine (ThAc), and 1-N-(1,3-di-O-acetylpropoxymethyl)-5-fluorouracil (FAc), catalysed by lipase from *Candida Cylindracea* (*Rugosa*) was investigated by isothermal heat conduction microcalorimetry. Changes of the thermal power in time and the total heat effects were determined in long-lasting measurements. Hydrolysis of these compounds was examined also by TLC.

For all three esters the thermal power curves reveal both a relatively intensive and slow heat evolutions. It is suggested that this complexity results from a superposition of: (i) a stereospecific hydrolysis of the ester bond connected to the heterocyclic moiety by a chain endowed with an appropriate chirality, and (ii) a non-specific hydrolysis of another ester bond. A partial chemical degradation of reactants in the buffer was also observed.

Keywords: hydrolysis of acyclonucleoside esters, isothermal heat conduction microcalorimetry, lipase

Introduction

Recently, a great interest in application of high precision isothermal heat conduction microcalorimetry for studying biological and biochemical processes have been noticed. The determination of thermal power *vs*. time $P=P(t)$, is a valuable tool in such studies, because this quantity reflects the progress of reactions [1–3]. Moreover, it can be obtained directly, and in non-invasive manner, i.e. without disturbing the process. The advantages of this technique have encouraged us to its application for monitoring of enzyme-catalysed hydrolysis of some acylated derivatives of thymine (acyclonucleoside esters). DNA nucleoside analogue are particularly important compounds. Namely, among such substances, e.g. uracils, antivirial and anticancer drugs are looked for [4]. We expected that a selective hydrolysis of biacylated derivatives of thymine could lead to monoacylated chiral compounds, and, potentially, to new

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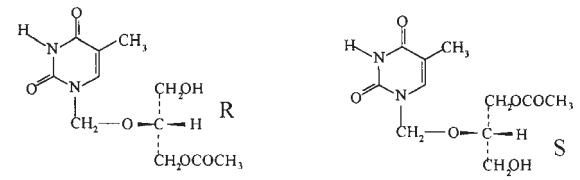
compounds of pharmacological importance. As catalyst, lipase from Candida Cylindracea (Rugosa) was chosen for its stereospecificity in reactions with enantiomers, and of its activity *vs*. one of the two acyl groups in 1,3-diacyl-2-X-glycerol, where 2-*X* denote different substituents [5]. The aim of this work was to investigate the hydrolysis of chosen acyclonucleoside esters catalysed by lipase in a calorimetric way.

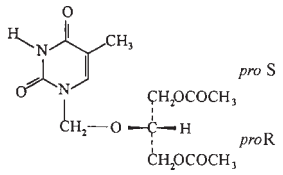
Materials and methods

The following acyclonucleoside esters were synthesized at the Military Academy of Medicine, Łódź: (*R,S*)-1-N-(1-O-acetyl-3-hydroxypropoxymethyl)-thymine (ThL), 1-N-(1,3-di-O-acetylpropoxymethyl)-thymine (ThAc) and 1-N-(1,3-di-O-acetylpropoxymethyl)-5-fluorouracil (FAc) [6, 7]. The structural formulae of these substances are presented in Fig. 1. The lipase extracted from *Candida Cylindracea* (Fluka, No. 62302, *M* ca 67000, activity 30 U mg^{-1}, as declared by the producer) was used.



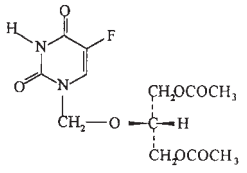
(R,S) - ThL

(R,S)- 1-N-(1-O-acetyl-3-hydroxypropoxymethyl)-thymine



ThAc

1-N-(1,3-di-O-acetylpropoxymethyl)-thymine



FAc

1-N-(1,3-di-O-acetylpropoxymethyl)-5-fluorouracil

Fig. 1 Structural formulae of the esters under study

Lipase, 6.7 mg (ca $1 \cdot 10^{-4}$ mmol), was dissolved in 2.5 mL of Michaelis buffer (pH=7.0) Then a chosen ester, in quantity ca 0.06 mmol, was added. The following systems were studied: ThAc, ThL, and FAc, every one plus lipase in buffer. In addi-

tion, simplified, two-component systems were also examined: ThL, ThAc and lipase, every one plus buffer. The enthalpy of solution of solid lipase in the Michaelis buffer was determined in LKB batch microcalorimeter.

The thermal power determinations were carried out in an isothermal heat conduction microcalorimeter [8], with a vessel of 3 mL in volume, sensitivity 103 μV mW^{-1}, time constant 120 s, and baseline stability 0.5 μV/day. Continuous stirring (60 rpm) was applied during the whole measurement run. The recording of experimental data was started 1 h after the reactants had been mixed, and was followed up to 7 or even 10 days. The evaluation of thermal power P was realised using equations $P=\alpha\Delta(t)$, where $\Delta(t)$ denotes the course of calorimetric signal, and α the heat loss coefficient, equal to 9.7 W V^{-1}. No data smoothing was applied, to save all the information included in the thermokinetic course of the process studied (e.g. a small sharp peak on curve a in Fig. 2, recorded after 7.8 h).

Hydrolysis of the esters was also examined by TLC (Silicagel GF254, chloroform:ethanol=92:8). 1-N-(1,3-dihydroxypropoxymethyl)-thymine was obtained as the final product of hydrolysis of ThL and ThAc. During ThAc hydrolysis, ThL appeared first; in 48 h, the spot of thymine became also clearly visible. ThL enantiomers can not be observed by TLC. Hydrolysis of FAc by TLC was found to give similar results, viz. a product containing one acetyl group appeared quickly, and after 72 h, a 1-N-(1,3-dihydroxypropoxymethyl)-5-fluorouracil spot was clearly visible.

All the measurements were performed at 25°C.

Results and discussion

The results of calorimetric determination are presented in Figs 2–4. All these plots of thermal power *vs*. time (thermokinetics) demonstrate the complexity of processes taking place in the systems under study. Each process is seen to be accompanied by an exothermic heat effect, long-lasting and variable in time.

Figure 2 presents the thermokinetics accompanying the processes in the three-component systems (ester+lipase+buffer). During the first two days, large

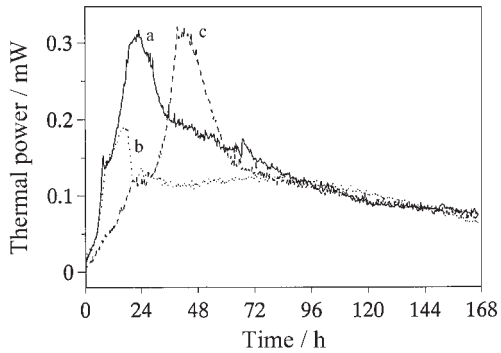


Fig. 2 Thermokinetics of reaction of the ester with lipase in buffer: a – ThAc (solid); b – ThL (dots); c – Fac (dash)

peaks of thermal power appeared; later, the heat evolution is slow. Moreover, in each thermokinetic course the slope of the going-up part of the first peak reveals a drastic change in the rate of heat evolution. The comparison of the first large peaks on curves a and c (for ThAc and FAc), with the maxima observed in the 22nd and 42nd h, respectively, suggests that the fluoro-substitution has a delaying effect on the enzymatic hydrolysis. For all three compounds the value of thermal power after seven days is very similar, ca 0.7 J s^{-1}. This relatively large thermal effect indicates that some process still takes place.

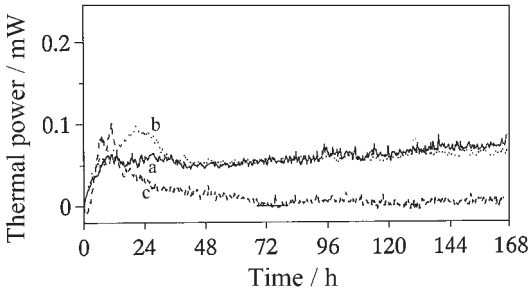


Fig. 3 Thermokinetics of reaction taking place in the mixture of single substrate with buffer: a – ThAc (solid); b – ThL (dots); c – lipase (dash)

The above complexity of thermokinetics cannot be resolved without some additional information. Figure 3 presents the results of the thermal power determinations for the two-component system (single reagent+buffer). Both ThAc (curve a) and ThL (curve b) in the buffer produce long-lasting exothermic heat effects. The thermal power attains the maximum after 12 and 24 h, respectively. Later, in both cases the heat effect is nearly constant in time, and it equals ca 0.7 J s^{-1}. These effects are probably related to a very slow chemical degradation of substrates, which in seven days is still not terminated.

Also the lipase in the buffer (curve c) generates an exothermic heat effect, peaking in 10–12 h of the experiment, and finished after 3 days. The total heat effect, Q, equals 7±0.5 J. Let us point out that the heat effect of solution of lipase in the buffer was found endothermic, and of short duration (ca 2 min). So, it could not be detected in the calorimetric signal (recording started after 1 h). Hence, the thermal effect observed for the lipase in buffer should be due presumably to some transformation of the lipase itself. We have also noticed that the solution of lipase prepared five days prior to its use did not hydrolyse the ester. Therefore, the transformation suggested above is connected with some loss of the enzymatic activity of lipase.

The above results indicate that the complexity of the overall thermokinetics (Fig. 2) results from a superposition of the process of the ester degradation on the main process of lipase-catalysed hydrolysis. So, under assumption of additivity of thermal effects of the two processes, the first step in the data analysis should be the subtraction of the thermal power of the degradation effect from the overall thermal power. For ThAc this operation (curve a in Fig. 2 minus curve a in Fig. 3) results in

the thermal power equal to zero both at the beginning and at the end of the curve (curve a in Fig. 4). This fact indicates that during the first ca 5 h, in the mixture of the ester and lipase in the buffer mainly chemical degradation takes place. Also the non-zero thermal power on the 7th day is due only to this process. It has to be supposed that in the first 5 h the lipase is very likely to achieve the best conformation for catalytic action [9, 10]. However, the heat effect of this possible process of conformation was not detected. According to the assumption taken for subtraction of the effect of degradation, the evaluated difference of the thermal power courses (curve a in Fig. 4) can be related to enzymatic hydrolysis of the two ester bonds existing in ThAc. It can be expected that the hydrolyses of pro–R and pro–S bond (Fig. 1) is accompanied by different thermal power runs. So, the first distinct peak (maximum in ca 24th h of the measurement) seems to be related mainly to the enzyme action *vs*. the ester bond endowed with appropriate chirality. The later part of the curve, with slowly decreasing thermal power, can be attributed to a non-specific hydrolysis of another ester bond. The total heat effect (Q) calculated on the basis of the thermokinetic course equals 42±1 J.

Unfortunately, the system FAc plus buffer was not examined because of lack of the sufficient quantity of the substrate. However, it seems that FAc could undergo similar chemical degradation as ThAc. Let us point out the similarity of the course of thermal power for both esters at the beginning and in the second part of the runs, as well as the similarity of shapes, with a 24 h delay for FAc. That can indicate that the conclusions concerning the superposition of enzyme-catalysed hydrolysis of pro–R and pro–S bonds, and chemical degradation are also valid for FAc.

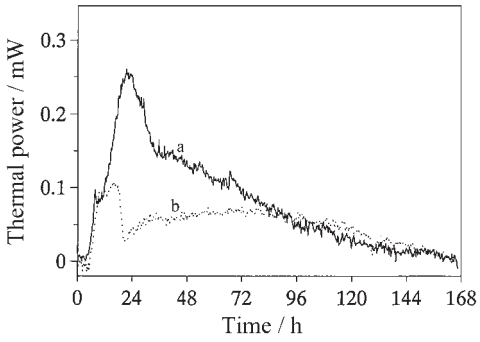


Fig. 4 Thermokinetics of enzymatic hydrolysis of the ester after subtraction of chemical hydrolysis: a – ThAc (solid); b – ThL (dots)

Similarly as for ThAc, for ThL the subtraction of the thermal power related to chemical degradation from the global effect (curves b in Figs 3 and 2, respectively) results (curve b in Fig. 4) in the thermal power close to zero at the beginning and at the end of the experiment. However, curve b in Fig. 4 still describes a complex phenomenon. Two effects can be distinguished: the first with a maximum at ca the 16th h, and a second, broad one with a maximum at ca the 70th h. Let us remind that ThL used

in this study was a racemate (Fig. 1). So, the thermal power presumably results from two simultaneous processes: (i) the stereospecific hydrolysis of the ester bond connected to the heterocyclic moiety by the chain endowed with appropriate chirality, and (ii) a probable non-specific hydrolysis of the enantiomer of ThL with another ester bond, manifested by the slow, long-lasting changes of thermal power. The total heat effect Q equals 26±1 J.

The effect of the partial transformation of lipase (disactivation) is likely to be always consisted in the overall thermal power accompanying the hydrolysis of the esters. Let us notice that the shapes of initial sections of the corrected curves for ThAc and ThL (Fig. 4) and of this for only lipase in buffer (curve c in Fig. 3) are very similar. In particular, the left-hand side slopes of their ascending sections are almost identical. Moreover, for these three cases a minor pre-maximum is noticed, after elapse of ca 8, 9 and 7 h for ThAc, ThL and sole lipase, respectively. However, in contrast to the case discussed earlier, this time the correction of respective curves by subtraction of the thermal power of disactivation of lipase would be not justified. In the present case, the subtraction operation on the total heat effects of the enzymatic hydrolysis of the ester and the lipase degradation seems to be more appropriate. For ThAc such an operation results in the global heat of hydrolysis, specific and non-specific, of two ester bonds equal to 35±1 J (625 kJ mol^{-1}). Analogously, the heat effect of hydrolysis of the two enantiomers in ThL racemate is 19±1 J (311 kJ mol^{-1}). Since the number of moles of ThAc and ThL used in the experiments was the same, the total number of the ester bonds in ThAc was twice as large. Hence, the calculated value can be considered as consistent.

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