J Therm Anal Calorim (2010) 101:715–719
DOI 10.1007/s10973-010-0951-1

Utilization of the IC-calorimeter for study of enzymatic reaction

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CCTA10 Special Issue

Abstract In this article, the monitoring of an enzymatic reaction by means of a miniaturized batch type IC-calorimeter was performed. The aim of this work was focused on an investigation of enthalpy and rate of enzymatic reaction of trypsin with *N*α-benzoyl-L-arginine-*p*-nitroanilide hydrochloride (BApNA). Both the parameters were determined for reactions in different buffers and for varying concentration of enzyme at 37 °C. The rate of reaction decreased with the increasing concentration of enzyme caused by trypsin autolysis.

Keywords IC-calorimeter · Enzyme-catalyzed reaction · Reaction kinetics · Trypsin

Introduction

Due to the universal nature of the heat evolution, related to almost every chemical reaction, the measurement of the evolved heat opens an elegant way for direct studying of elementary biochemical processes. In fact, no sophisticated cascades of reaction steps are necessary to generate signals, which are related to the primary reaction under investigation [1]. In case of numerous applications the classical calorimeters are not suitable for studying the biochemical processes due to their high materials and time consumption. This problem can be solved by using miniaturized calorimeter based on thermopile chips. Such devices are usually called chip- or IC-calorimeters [2] and the necessary amount of the sample is for these calorimeters in the range of μL.

The application of chip calorimeters or generally microcalorimeters is very promising [3, 4]. That way it is possible to investigate small amount of samples and the experiment is much faster in comparison with classical calorimeters, where the transient behaviour is influenced by high thermal masses of both device and reactants. The suitability of the chip calorimeters is strongly dependent on the chip sensor available for the heat energy detection. A great variety of silicon chips exist that are useful for calorimetric purposes. The silicon chips differ in the nature of the membrane, which is carrying the sample, in the structures of applied heater and temperature detection as well as in the arrangements of sample containment [5].

A miniaturized calorimeter with an integrated circuit as the essential part (IC-calorimeter) was applied to study the run of the enzymatic reaction. The silicon chip contains a sensitive thermopile for the temperature measurement and an electrical resistance for the thermal calibration, as parts of the integrated circuit. The calorimetric system is completed by a microlitre syringe for the addition of a second component in batch mode to start the investigated reaction on the chip surface. Information about the thermodynamics and kinetics of the studied reaction can be obtained from the measured heat flow signal of the IC-calorimeter with an extremely low time constant.

Trypsin is one of the important enzymes of digestive tract. The main activity of trypsin within the organism lies in the digestion of proteins inside the small intestine; therefore, trypsin is classified as a proteolytic enzyme. The

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aim of this work was focused on an investigation of enthalpy and rate of enzymatic reaction of trypsin with *N*α-benzoyl-L-arginine-*p*-nitroanilide hydrochloride (BApNA). Both the parameters were determined for reactions in various buffers and for varying concentration of enzyme at 37 °C.

Theory

Device

Miniaturized IC-calorimeter (batch type) was designed for the calorimetric monitoring of reactions in small samples of solutions. Calorimeters with different types of chips (LCM-2506, LCM-2524 and NCM-9924) have been extensively investigated by Lerchner et al. from the TU Bergakademie Freiberg in the collaboration with the Xensor Integration (Delft, Netherlands) company [1, 2, 6]. The calorimeter consists of two axially connected cylindrical aluminium blocks (Fig. 1).

The thermopile chip is located in the centre of the arrangement and the droplet of one reactant is placed in the middle of the chip's surface. The silicon chip is fixed in a conventional integrated circuit within the chip carrier. The ceramic chip carrier is thermally connected to the lower aluminium cylinder by metallic pins. An axial hole in the upper cylinder serves for the mounting of the microsyringe which holds the second reactant before it is added. The wetting ring attached to the top of the interior calorimeter chamber is necessary for faster adjustment of the vapour pressure. This decreases the rate of vaporisation of the liquid sample placed at the surface of the silicon membrane and therefore leads to a faster stabilisation of the signal baseline. An exactly circular gauge with diameter 4 mm is used to ensure an exact positioning of the liquid droplet in the centre of the chip surface (bordered by the sensitive area of the chip). This also helps to increase the reproducibility of the size of the contact area between the droplet and the membrane surface. After placing the first reactant on the chip the calorimeter is closed. Then the microsyringe is filled with the second component and it is put into the hole in the upper block of the calorimeter. Good thermal contact between the blocks, chip, and capillary ensures insignificant temperature differences between both the reactants [7]. About 20 min after preparing the calorimeter, the reaction can by started by emptying the microsyringe. The enthalpy ($\Delta H_r^{37^{\circ}C}$) of studied reaction can be easily evaluated according to Eq. 1 as the ratio of the area of the peak (A) and number of moles (n) and sensitivity of the chip (C) observed when the calorimeter is calibrated [2].

$$\Delta H_r^{37^{\circ}C} = \frac{A}{C \cdot n} \tag{1}$$

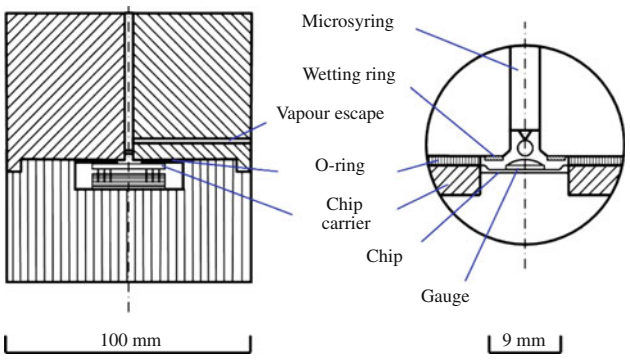

Fig. 1 Scheme of the IC-calorimeter [4]

Enzyme-catalyzed reaction

Enzymes [8] are proteins which act as catalysts in many reactions of biological and biochemical importance. Because of their efficiency, enzymes are effective at very low concentrations. The molecule whose reaction is being studied is called the substrate. The simplest mechanistic scheme describing the action of an enzyme is given in Eq. 2.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{ES} \xrightarrow{k_2} E + P \tag{2}$$

Enzyme reaction consists first of a reversible binding of substrate (S) to an active site of an enzyme (E), which together forms a transient complex of enzyme and substrate (ES). This first reaction step is followed by the dissociation of the complex ES to enzyme and product (P). This second phase is irreversible. The kinetics of the substrate-product transition is in the first place influenced by the substrate concentration. The dependence of the initial reaction rate on the enzyme concentration is linear under the condition of a constant concentration of the substrate and increasing concentration of the enzyme. The dependence of enzyme reaction rate on the substrate concentration can be described by the theory of Michaelis–Menten, Eq. 3 (also denoted as the saturation of enzyme by substrate or as the saturation curve). The equation contains two constant parameters—the maximum reaction rate (v_{max}) and the Michaelis constant k_M. Parameter k_M represents degree of the enzyme's affinity to its substrate. The numeric value of k_M corresponds to the substrate concentration at which the enzymatic reaction rate decreases under $\frac{1}{2}v_{max}$ [9]. This kinetic model is relevant in situations where very simple kinetics can be assumed. More complex models exist for the cases where the assumptions of Michaelis–Menten kinetics are no longer appropriate [10].

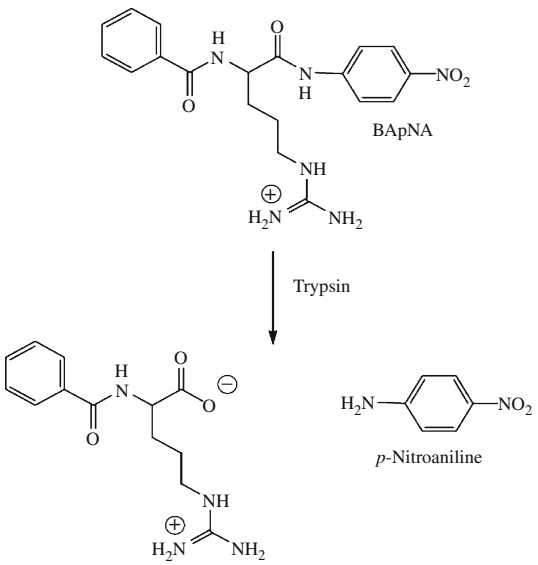

Fig. 2 Enzymatically hydrolysis of substrate *N*α-benzoyl-L-arginine-*p*-nitroanilide hydrochloride

$$v_0 = \frac{v_{\max} \cdot [S]}{[S] + k_M} \tag{3}$$

Main factors influencing the reaction rate are the temperature and concentration of the reactants. Even in the case when the reactants are in optimal concentrations, there are other chemical and physical parameters that can strongly influence the enzyme reaction rate. These are mainly pH, ionic strength, buffer composition, and temperature.

The enzymatic reaction with trypsin studied within the framework of this article is shown in Fig. 2.

Experimental

IC-calorimeter can be used to study solid–liquid or liquid–liquid reactions. One reactant (solid or liquid) is placed on the surface of the chip and the other one (liquid) is put into the microsyringe. After the establishment of thermal equilibrium (approximately 20 min), the reaction was started by addition of a droplet from microsyringe. After the addition of second reactant, the measurement was stopped when the output signal reached the equilibrium state (approximately 10 min). In the case of studied biochemical reaction, the establishment of thermal equilibrium was 60 min before initialization of the reaction. The reaction took less than 2 min and the stabilization of output signal was reached in about 10 min.

Before the series of measurements, the electrical calibration was carried out with the following parameters set: the power resolution 0.1 mW and the time constant 150 s. The sensitivity 2.32 V W^{-1} of the used chip was obtained by the comparison of output and input signals. The details of calibration procedure are described in Ref. [11].

The following were the chemicals used in this experiment. Enzyme: trypsin (EINECS 232-650-8) from bovine pancreas, Sigma-Aldrich; substrate: BApNA (EINECS 244-505-6), Sigma-Aldrich; solvent for substrate: *N*,*N*-dimethylformamide (EINECS 200-697-5), Lach-Ner.

Buffers: (1) 0.1 mol L^{-1} Tris–HCl + 0.025 mol L^{-1} $CaCl_2$ in which Tris (hydroxymethyl) aminomethane (EC 201-064-4), Aldrich; hydrochloric acid 35% p.a (EINECS 231-595-7), Lach-Ner; calcium hydroxide hexahydrate (EINECS 233-140-8), Lach-Ner. (2) 0.1 mol L^{-1} CH_3COONH_4 + NH_4OH in which ammonium acetate, Lachema; ammonium hydroxide minimum 25% p.a. (EINECS 215-647-6), Lach-Ner. (3) 0.1 mol L^{-1} NH_4HCO_3 in which ammonium hydrogen carbonate (EINECS 231-911-5), Penta.

The new solutions of both reactants were always prepared every day. The enzyme was dissolved in the buffer. The substrate BApNA is insoluble in buffers, therefore the solution was prepared by using *N*,*N*-dimethylformamide. *N*,*N*-dimethylformamide was also added into the solution of enzyme, otherwise the large mixing enthalpy of buffer with *N*,*N*-dimethylformamide would mask the enzymatic reaction. The solvent for enzyme and substrate were prepared by mixing equivalent amounts (1:1) of buffer and *N*,*N*-dimethylformamide.

The pH of all buffers was 7.8. The amount and concentration of substrate on the chip was 3 μL and 0.055 mol L^{-1}, respectively. In all experiments, the moisture ring was impregnated with 6 μL of substrate solution. The wetting was necessary for saturation of the vapour chamber. The amount of the enzyme solution in Hamilton microsyringe was 4.4 μL with the concentration varying from 0 to 15 g L^{-1}. Five measurements at 37 °C were performed for each enzyme concentration and buffer.

In the case of IC-calorimeter, the observed reaction reflects as the peak but the blind test has to be subtracted to get real reaction response. The blind test was done under the same conditions as the studied reaction but without one reactant. The blind test for our reaction was done by mixing the solution BApNA + *N*,*N*-dimethylformamide + buffer and solution without enzyme (*N*,*N*-dimethylformamide + buffer). The example of obtained output signal for the reaction and corresponding blind test is given in Fig. 3. The illustration of the peak area evaluation is also shown in the figure. The area A of the reaction can be determined as the difference between the area of enzymatic reaction peak (A'') and blind test peak (A') and is given as follows.

$$A = A'' - A' \tag{4}$$

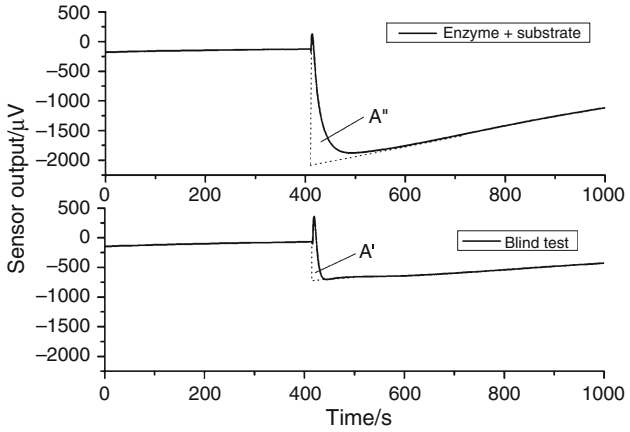

Fig. 3 The time dependence of the output signal of the enzymatic reaction (enzyme + substrate) and the blind test in buffer $CH_3COONH_4 + NH_4OH$. The peak area evaluation is illustrated (A' and A'')

The value of A area represents the reaction enthalpy of the entire system according to Eq. 1.

Results and discussion

The main advantage of using calorimetry for enzyme-catalyzed reactions is the possibility of a direct assay without the requirement of using modified substrates or coupled reactions because the known reaction between trypsin and BApNA was chosen.

Representative example of the measured signals for enzymatic hydrolysis of *N*α-benzoyl-L-arginine-*p*-nitroanilide hydrochloride substrate catalyzed by trypsin is presented in Fig. 4. The obtained curves differ slightly for various types of buffers. For a better comparison, the curves were shifted along the time-axis. The 0 s value in Fig. 4 represents the addition of the second reactant into the reaction scheme (the same time shift is given in Fig. 3).

For the studied reaction system, a significant variation in reactivity in the different buffers was observed. The studied enzyme-catalyzed reaction proceeded fastest in the Tris–HCl + $CaCl_2$ buffer. In both other buffers (NH_4HCO_3 and $CH_3COONH_4 + NH_4OH$), the reaction was considerably slower. Therefore, the evaluation of this curve was more reliable and scatter in the measurements results was low. The reaction progression could be also qualitatively monitored by watching the colouring of the droplet placed on the chip. While the droplet was deep yellow for the TRIS-HCl + $CaCl_2$ buffer, it was only of a light yellow tint for the other two buffers in the corresponding time (the yellow colouring was caused by one of the reaction products *p*-nitroaniline). The different reactivities are for the studied buffers also apparent in Fig. 5, where the parameter K (s^{-1}) is a quantity which is proportional to the rate of response of the enzymatic reaction and is defined as v_{max}/k_M.

One of the aims of this work was to study the dependence of the rate of the enzymatic reaction on the concentration of trypsin in selected buffers. The value of parameter K for reaction in buffer TRIS–HCl + $CaCl_2$ reached the highest value in comparison with other buffers for all the studied concentrations (Fig. 5). The parameter K is proportional to the reaction rate and as was mentioned the rate of enzymatic reaction increases with increasing enzyme concentration. But the results for trypsin are opposite in all three buffers—as is seen in Fig. 5 parameter K decreases with increasing concentration. This could be explained by means of trypsin autolysis. When using a soluble proteolytic enzyme for substrate decomposition it is necessary to take into account the enzyme autolysis. Proteases as trypsin lose activity in time due to their autolytic degradation [12]. Unusual trend of decreasing of

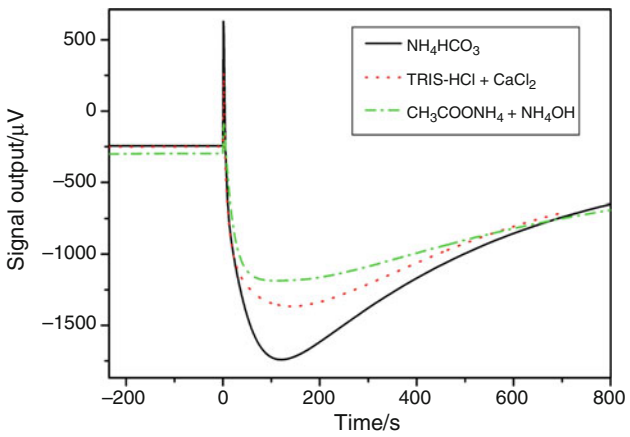

Fig. 4 The illustration of calorimeter output signal for reaction between BApNA and trypsin in different buffers for concentration of enzyme 15 g L^{-1}

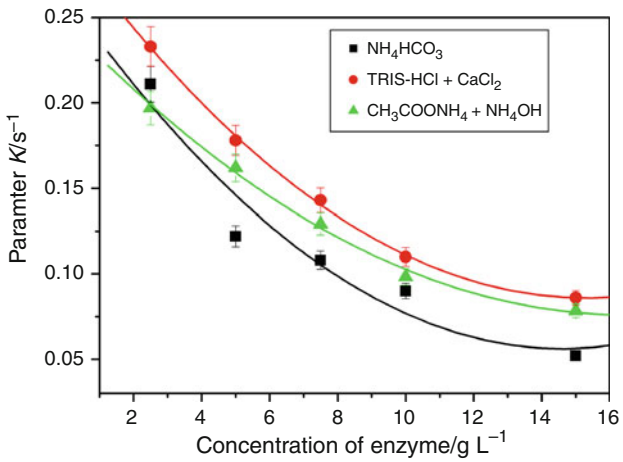

Fig. 5 Dependence of parameter K on concentration of enzyme for enzymatic reaction between trypsin with BApNA in various buffers

Table 1 The reaction enthlapy $\Delta H_r^{37^{\circ}C}$ of enzymatic reaction between trypsin and BApNA in different buffers measured with IC-calorimeter at 37 °C

Reaction system	Buffer	Reaction enthalpy/ kJ mol^{-1}
BApNA + trypsin	NH_4HCO_3	-70.3 ± 1.4
	TRIS–HCl + $CaCl_2$	-13.0 ± 0.4
	CH_3COONH_4 + NH_4OH	-65.0 ± 4.4

the enzymatic concentration in the reaction mixture can be explained by trypsin autolysis. To overcome the decreasing trend, it would be possible to change reaction conditions, e.g. decrease of temperature. This will decrease autolysis but also velocity of substrate transition. Another possibility would be to use an enzyme attached to solid phase. Such immobilized enzyme would be considerably more resistant to autolysis and therefore it could maintain its activity for a prolonged period of time [13, 14].

The enthalpy of reaction was calculated from the peak area of the measured curve after the subtraction of the blind test according to Eqs. 1 and 4. The determined values of $\Delta H_r^{37^{\circ}C}$ show that the buffers differently influence the heat of enzymatic reaction (Table 1).

Conclusions

The suitability of enzymatic reactions of trypsin in different buffers was investigated by using a miniaturized batch type IC-calorimeter. The reaction process was found to be dependent on the surrounding buffer and on the concentration of enzyme. The highest rate of trypsine catalysed reaction was observed in buffer Tris–HCl + $CaCl_2$. The rate of enzymatic reaction decreased with increasing enzyme concentration for all selected buffers. This unusual trend of decreasing of the enzyme concentration in the reaction mixture can be explained by means of trypsin autolysis. The enthalpy of the studied reaction was influenced by applied buffers—the Tris–HCl + $CaCl_2$ buffer resulted in significantly lower value of reaction enthalpy.

Acknowledgements Authors thank to Dr. J. Lerchner and A. Wolf for their help and useful suggestions. The financial support from the Ministry of Education, Youth and Sports of Czech Republic under the project MSM0021627501 is gratefully acknowledged.

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