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Thermochemical investigations of hydrolysis of *p*-nitrophenyl acetate in water–acetontrile mixtures

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Abstract

The α-chymotrypsin-catalyzed hydrolysis of the *p*-nitrophenyl acetate in the solvent mixtures containing from 1.6 up to 10% (v/v) acetonitrile in the presence of aqueous Tris buffer at pH 8.0 was investigated at 298 K by use of an isoperibolic batch calorimeter. A special experimental arrangement of the reaction components for the investigation of the hydrolytically instable substrate was used. Furthermore, the release of *p*-nitrophenol was recorded with an UV–vis-spectrophotometer under comparable conditions. The calorimetric curves consist of two parts. The first part is strongly rising and finished by a break point in the ΔT (time) curve. This first step is dominated by the enzyme-catalyzed reaction. After the break point a slow non-enzymatic process determines the course of the calorimetric curve. The molar enthalpy changes of overall reaction (ester hydrolysis and buffer protonation) of -100 ± 8, -106 ± 5 and -102 ± 5 kJ/mol were evaluated by a combination of the results from the spectrophotometric and calorimetric data for 1.6, 4.0 and 10.0% acetonitrile mixtures, respectively. The obtained results indicate that the enzyme-catalyzed hydrolysis is suitable for quantitative determination of the hydrophobic ester *p*-nitrophenyl acetate in water–acetonitrile mixtures using calorimetric detection.

Keywords: Isoperibolic batch calorimetry; Bovine pancreatic α-chymotrypsin; *p*-Nitrophenyl acetate; Acetonitrile; Reaction enthalpy

1. Introduction

The use of enzyme-catalyzed reactions in water–organic mixtures is highly promising for basic research and biotechnology [1–4]. The application of enzymes in biosensors in organic media is also a topic of continuing interest [5,6]. Such systems have a number of advantages compared to enzymes in aqueous solutions. The most important advantages are the higher solubility of hydrophobic substances (fats, oils, steroids, esters), and the solvent effect on the enzyme-catalyzed reactions [7–10]. Hence the analysis of the thermodynamic and kinetic aspects of biocatalysis in water–organic mixtures appears important.

Since practically all chemical reactions have a nonzero enthalpy change, the heat power accompanying the enzyme-catalyzed reactions in various water–organic mixtures might be a very informative property of the intermolecular processes influencing the activity of enzymes at such unusual conditions. Calorimetry is a reliable method to determine quantitatively this thermokinetic property of a reaction and to obtain information about the reaction rate [11–13]. There are a number of reports on the enthalpy of enzyme-catalyzed hydrolytic reactions in water at different pH value and buffer system [11,14–19]. However, information on the enzymatic reaction enthalpy in the presence of significant concentrations of organic solvents is rather limited. For example, at present time there are only two reports on calorimetric studies of the reactions catalyzed by the archetypical hydrolase α-chymotrypsin (in 3%(v/v) [17] and 3–7% (v/v) [16] ethanol). Experimental data for the enthalpy of enzyme-catalyzed

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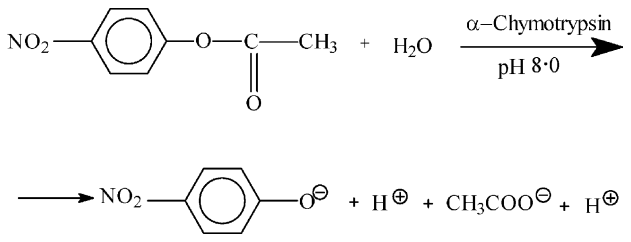

Scheme 1.

reactions in the presence of significant amounts of hydrogen bond accepting organic solvents are not known from publications.

The α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate is a well-documented model reaction for the investigation of the molecular mechanism of enzyme catalysis [20–23]. The use of *p*-nitrophenyl acetate as substrate gives the possibility for the study of both the acylation of the enzyme and the deacylation of the acyl-enzyme. However, no attempts have been undertaken to study the thermal effects at the enzymatic hydrolysis of *p*-nitrophenyl acetate. One of the possible reasons for that is the low solubility of hydrophobic substrate in water.

The aim of the present work is the thermochemical interpretation of the α-chymotrypsin-catalyzed hydrolysis of the hydrophobic substrate *p*-nitrophenyl acetate in the solvent mixtures containing from 1.6 up to 10% (v/v) hydrogen bond accepting acetonitrile in the presence of Tris buffer. The investigated reaction is characterized by the chemical scheme 1 . The used Tris buffer provided the constant pH value and caused an amplifying of the measurable heat exchange by the protonation reaction enthalpy. The buffer protonation reaction: Tris buffer-NH_2 + H^+ → Tris buffer-NH_3^+ occurs to the hydrolysis *p*-nitrophenyl acetate concurrently.

The non-specific nature of heat measurements makes it often difficult to interpret results from calorimetric investigations of complex reaction systems [24]. Therefore, the second aim of the study was to estimate the progress of the hydrolytic reaction using UV–vis-spectrophotometry and to compare these results with the calorimetric data.

2. Experimental

2.1. Materials

Bovine pancreatic α-chymotrypsin (C-4129, essentially salt free; EC 3.4.21.1, specific activity of 52 units/mg of solid) purchased from Sigma. Acetonitrile (HPLC grade, purity 99.9+%, water < 0.02%), *p*-nitrophenyl acetate (N-8130), acetic acid (A-0808), tris[hydroxymethyl]aminomethane (T-1503), tris[hydroxymethyl]aminomethane hydrochloride (T-6666), *p*-nitrophenol (S250583) were also obtained from Sigma. Water used was doubly distilled.

Table 1
Arrangement of the reactants

Type of experiment	Ampoule	Calorimetric cell
Blank	Solid Tris	Solution of Tris–HCl in water–acetonitrile mixtures
Reaction	Solid Tris and α-chymotrypsin powders	Solution of *p*-nitrophenyl acetate and Tris–HCl in water–acetonitrile mixtures
Dissolution	Solid *p*-nitrophenol	Solution of Tris and Tris–HCl in water–acetonitrile mixtures

2.2. Calorimetric measurements

Calorimetric measurements were performed at 298 K using a modified isoperibolic LKB 8700 calorimeter [25]. The calorimeter was calibrated using the Joule effect and tested with dissolution of potassium chloride in water according to the recommendations [26,27].

2.3. Arrangement of reactants

Solutions of different composition (Table 1), consisting mixtures of water–acetonitrile with tris[hydroxymethyl]aminomethane hydrochloride (Tris–HCl) and *p*-nitrophenyl acetate, were prepared directly in the calorimetric cell. The volume in the calorimetric cell was 67 ml. The pH value of solution in the calorimetric cell before the start of the reaction was 4.5 and was measured outside the calorimeter. The stability of *p*-nitrophenyl acetate with regard to hydrolysis was controlled by UV–vis spectrophotometry at 400 nm. No noticeable variation in absorbance at 400 nm was observed for at least 24 h. Acetonitrile concentration in the calorimetric cell was varied from 1.6 to 10.0 % (v/v).

The reaction in the calorimetric cell was initiated by breaking a glass ampoule. In the glass ampoule was a mixture of solid tris[hydroxymethyl]aminomethane (Tris) and chymotrypsin powders. With such an arrangement (Table 1) the chymotrypsin and Tris buffer concentrations in the cell were 1.8×10^{-5} mol/l and 0.033 mol/l, respectively. The resulting pH value of reaction solution was 8.0. This arrangement was necessary because of the instability of the ester in a solution of pH 8.0. The enthalpy change for the calorimetric blank experiment (Table 1) was 17.3 ± 0.5 kJ/mol. This value is in close agreement with the enthalpy change on dissolution of Tris in 0.05 M NaOH at 298 K determined in [28]. Heat evolution for the blank experiment was completed for 1 min and was taken into account for the calculation of the reaction enthalpy.

2.4. Spectrophotometric measurements

The solutions for the reaction were prepared similar as described for the calorimetric experiments. The release of *p*-nitrophenol in dependence of time was measured at 400 nm. An aliquot of 0.2 ml of reaction solution ($C_{substrate}$= 1.5 ×

Table 2
Molar absorptivity of *p*-nitrophenol in Tris buffer, pH 7.0, at 298 K

Added organic solvent	Concentration, % (v/v)	ε_{400} (M^{-1} cm^{-1})
Acetonitrile	1.6	10048
Acetonitrile	4	9515
Acetonitrile	10	8650
None [21]	0	9890
Propanol-2 [21]	1	9830
Propanol-2 [21]	10	9080
Dioxane [21]	1	9790
Dioxane [21]	10	8980

Molar absorptivity of *p*-nitrophenol was determined in 0.4 M Tris buffer, pH 7.0, at 298 K. In the present work Tris buffer concentration was 0.033 M.

10^{-3} mol/l and C_{enzyme} = 0.45 mg/ml) was diluted to 3 ml with an acetonitrile–Tris buffer mixture (the resulting pH value of mixture was 7.0.) and the optical density determined in a Unicam 8625 UV–vis-spectrophotometer.

It was expected that the absorbance of *p*-nitrophenol could be markedly affected by addition of organic solvent largely, because of the decrease in the dielectric constant. Therefore, it was necessary to determine the molar absorptivity of *p*-nitrophenol at pH 7.0 in various solvent mixtures employed. The measured absoptivity values are given in Table 2. Molar absorptivity of *p*-nitrophenol determined [21] are also presented in Table 2. As can be seen, our results are in close agreement with the literature data.

3. Results and discussion

Typical $\Delta T(t)$ curves obtained from the calorimetric measurements of the enzyme-catalyzed reaction are given in the Fig. 1. The curves are corrected for the heat flow using a cooling constant determined in separate calibration experiments. The rise in the $\Delta T(t)$ curves is proportional to the heat power in the calorimetric cell. Typical for the investigated systems

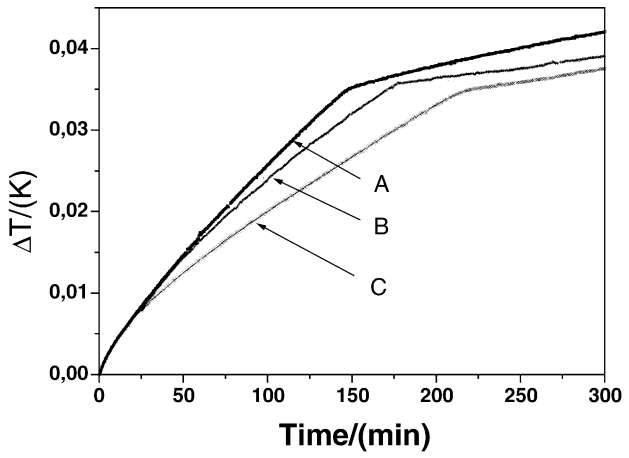

Fig. 1. $\Delta T(t)$ curves for α-chymotypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate in various water–acetonitrile mixtures (A) 1.6% acetonitrile, (B) 4.0% acetonitrile, (C) 10.0% acetonitrile, concentration of enzyme: 0.45 mg/ml. $C_{substrate}$ is 1.5×10^{-3} M.

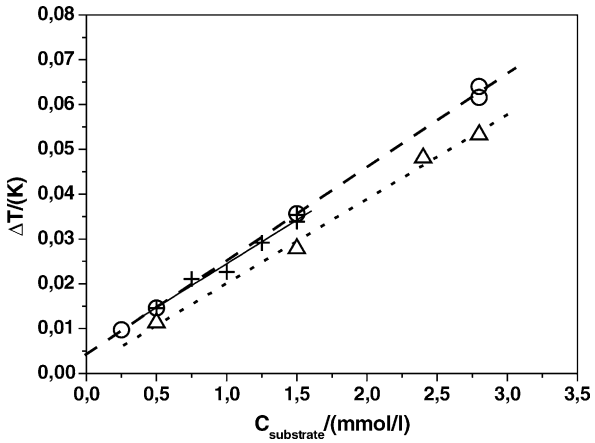

Fig. 2. Correlations between the ΔT values at break point and the initial substrate concentration in water–acetonitrile mixtures. (+) 1.6% acetonitrile. $C_{substrate}$ range is 0.5–1.5 × 10^{-3} M. (◯) 4.0% acetonitrile. $C_{substrate}$ range is 0.25× 10^{-3} M to 2.8 × 10^{-3} M. (Δ) 10.0% acetonitrile. $C_{substrate}$ range is 0.5× 10^{-3} M to 2.8 × 10^{-3} M.

(Fig. 1) is a break point in the calorimetric curve, splitting the course of reactions in the cell into two parts. The break at the end of the strongly rising part in the $\Delta T(t)$ curves indicates the end of a rapid reaction. The second process is much more slower. The second process was not completed for several hours.

At equal substrate concentration, the ΔT value at the break point does not depend essentially on the content of acetonitrile in the solution. As it is seen from the Fig. 1, the ΔT values at break point for 1.5 × 10^{-3} mol/l of *p*-nitrophenyl acetate are 0.0353, 0.0358 and 0.035 K (curves A, B, and C) at 1.6, 4.0 and 10.0% (v/v) acetonitrile, respectively. For a constant substrate concentration the break occurs at a constant ΔT.

On the other hand, the ΔT value at break point depends on the concentration of substrate $C_{substrate}$ linearly, as is shown in Fig. 2. The slope of the linear dependence can be interpreted as the analytical sensitivity of the method. The numerical values for the sensitivity expressed in K l/mol are presented in Table 3. As it is seen from the Table 3, the values of the sensitivity do not depend on the acetonitrile content essentially.

The determination of reaction course of the hydrolysis of *p*-nitrophenyl acetate was the next step of our investigation. The reaction course was quantitatively characterized by the release of *p*-nitrophenol, one of the products of the *p*-nitrophenyl acetate hydrolysis. The release of

Table 3
Thermochemical parameters of the hydrolysis of *p*-nitrophenyl acetate in water –acetonitrile mixtures

Acetonitrile content %(v/v)	Slope (Fig. 2) (K l/mol)	ΔH_R (kJ/mol)	*p*-Nitrophenol yield (%)
1.6	20 ± 1	−100 ± 8	87.5
4.0	20 ± 1	−106 ± 5	86.0
10.0	19 ± 1	−102 ± 5	82.5

Molar reaction enthalpy was calculated using the equation: ΔH_R = slope × C × 100%/yield × V; C, heat capacity of calorimeter from calibration: 300.6 J/K; V, volume: 67 ml.

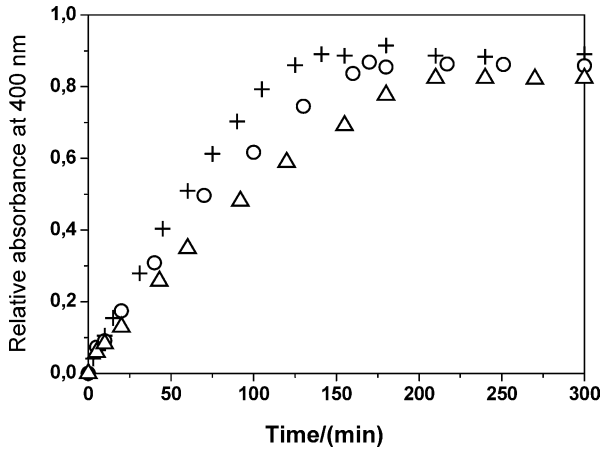

Fig. 3. Progress curves of the *p*-nitrophenol release for α-chymotypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate ($C_{substrate}$ = 1.5 × 10^{-3} M) in various water–acetonitrile mixtures, (+): 1.6% acetonitrile, (◯): 4.0% acetonitrile, (Δ): 10.0% acetonitrile, concentration of enzyme, 0.45 mg/ml.

p-nitrophenol was determined spectrophotometrically at 400 nm. Absorbance at 400 nm in the reaction solution was normalized on the absorbance of the *p*-nitrophenol solution under similar conditions. The values of relative absorbance at 400 nm correspond to the progress of the hydrolysis of *p*-nitrophenyl acetate.

Typical progress curves of the *p*-nitrophenol release are given in Fig. 3. The curves obtained can be subdivided into two parts. Rapid release of *p*-nitrophenol occurs first. A break in the strongly rising part indicates the end of the hydrolytic reaction. No release of *p*-nitrophenol is observed after the break point. The shape of *p*-nitrophenol release curve is in a qualitative agreement with analytical solution of this situation that was described previously [20]. It was shown that the release of *p*-nitrophenol is linear with time after an initial exponential phase. There is also a good agreement between the times of the break point for calorimetric and spectrophotometric data. This result indicates that the break points on calorimetric curves (Fig. 1) correspond to the end of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate. In separate calorimetric and spectrophotometric experiments it was observed that non-enzymatic hydrolysis is a minor contribution to the first part of $\Delta T(t)$ and *p*-nitrophenol progress curves.

It is well known that organic solvents can produce some structural changes in α-chymotrypsin, which alter the catalytic properties drastically [3,29]. They may also influence the active site of enzymes by disrupting the water structure in the vicinity [21]. The end of the α-chymotrypsin-catalyzed hydrolysis depends strongly on the acetonitrile concentration (Figs. 1 and 3). This fact reflects the inhibitory effect of organic solvent on the rate of enzymatic hydrolysis of *p*-nitrophenyl acetate.

The value of *p*-nitrophenol yield does not depend significantly on the content of acetonitrile in the concentration interval studied (Fig. 3). One can see from the Fig. 3, the yield of *p*-nitrophenol is 87.5, 86.0 and 82.5% at acetonitrile concentration of 1.6, 4.0 and 10.0% (v/v), respectively.

Further we attempted to select the contribution of the first rapid reaction from the second process one. The following calculation can be performed:

$$\Delta T = \Delta T_1 + \Delta T_2 \quad (1)$$

ΔT_1 corresponds to ΔT of the enzymatic process.

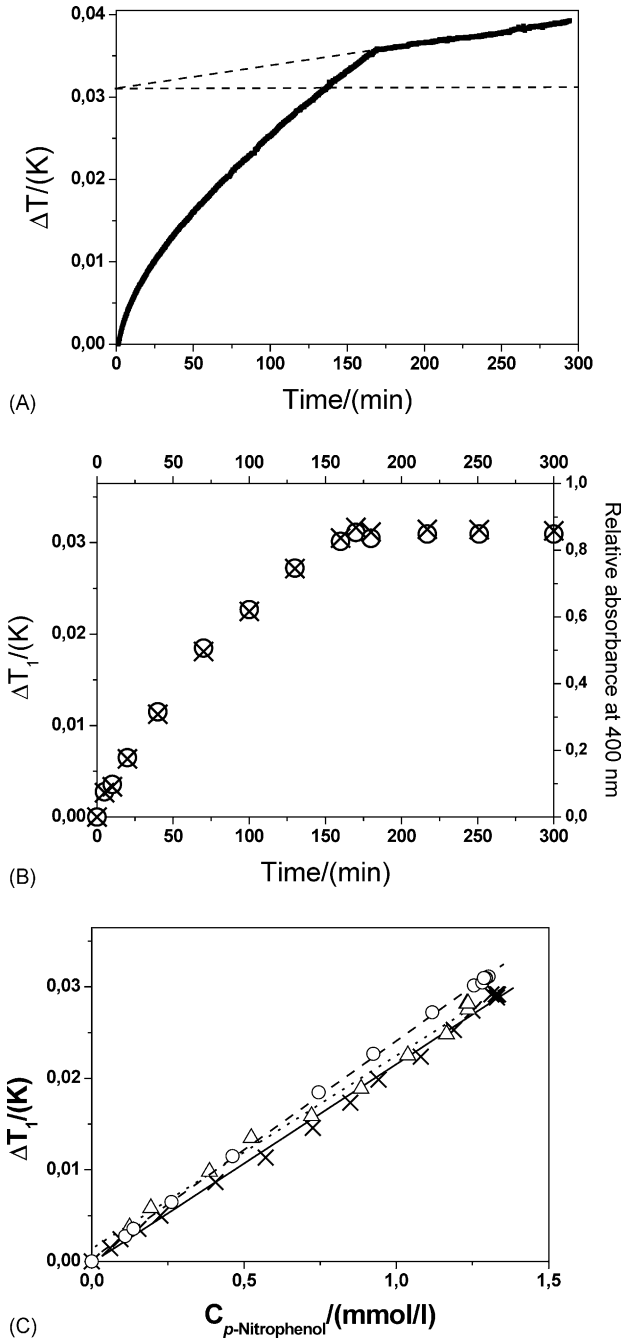

Fig. 4. $C_{substrate}$ = 1.5 × 10^{-3} M,. acetonitrile concentration: 4.0%. (A) Scheme describing the procedure for selection of the first rapid reaction contribution to the total $\Delta T(t)$ curve from the second slow reaction one. (B) $\Delta T_1(t)$ curve and progress curve (◯) of *p*-nitrophenol release (X). (C) Correlation between calorimetric and spectroscopic data: (Δ): 1.6% acetonitrile; (◯): 4.0% acetonitrile; (X): 10.0% acetonitrile.

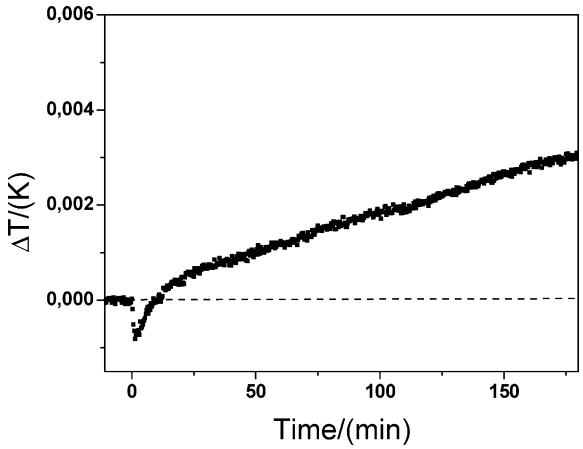

Fig. 5. $\Delta T(t)$ curve observed on the dissolution of solid *p*-nitrophenol in 4% acetonitrile mixtures at pH 8.0 (0.033 M Tris buffer) and 298 K.

ΔT_2 corresponds to ΔT of the non-enzymatic process.

$$\Delta T_2 = \mathbf{a} \times t, \quad (2)$$

a can be estimated from the linear slope of the calorimetric curves (Fig. 1) after 200 min.

Schema of the selection is presented in the Fig. 4A for 4.0% acetonitrile. Selected contribution of the first reaction ($\Delta T_1 = \Delta T - \Delta T_2$) was compared with the progress curve of release of *p*-nitrophenol in the Fig. 4B. Good agreement is observed between calorimetric and spectrophotometric data. The ΔT_1 values for the first reaction contribution are in good linear correlation with the release of *p*-nitrophenol (Fig. 4C).

The slow second process may be interpreted as a result of the instability of *p*-nitrophenol probably due to polymerization reactions [30,31]. As proof we carried out a special calorimetric experiment. The arrangement of compounds for this experiment is given in Table 1. Fig. 5 shows the $\Delta T(t)$ curve for the dissolution of solid *p*-nitrophenol in Tris buffer (pH 8.0). Slow exothermic process was observed for several hours after fast endothermic dissolution of solid *p*-nitrophenol.

It is well known that the overall reaction enthalpy change for ester hydrolysis (ΔH_R) is dominated by the enthalpy change corresponding to the buffer protonation [12,16,17,19]. The hydrolysis of *p*-nitrophenyl acetate gives two acidic products: acetic acid and *p*-nitrophenol (Scheme 1). Therefore, we expected −94.6 kJ/mol for the reaction enthalpy change in comparison with the value of protonation enthalpy of Tris with HCl (−47.3 kJ/mol [32]). If we compare this value with the ΔH_R values presented in Table 3, we can conclude that a main contribution to the measured molar enthalpy change of the α-chymotrypsin-catalyzed hydrolytic reaction is the protonation of primary amine tris[hydroxymethyl]aminomethane.

The obtained results illustrate that ester hydrolysis in the presence of Tris buffer is a good example for demonstrating the possibilities of chemical amplification by protonation processes in calorimetry. For example, the reaction enthalpy for ester (N-acetyl-L-tyrosine ethyl ester) hydrolysis with Tris buffer protonation correction is only −1.1 kJ/mol [17].

4. Conclusions

The combination of calorimetric data for the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate with spectrophotometric results on the release of one of the final products of this reaction provides a useful tool for investigating the hydrolysis of *p*-nitrophenyl acetate in water–acetonitrile mixtures. The observed break point in the calorimetric curves can be considered as a result of two parallel reactions. The first process is determined by the enzyme-catalyzed ester hydrolysis. The second slow process is expected to be a result of the instability of *p*-nitrophenol in water–organic mixtures. The molar enthalpies of overall reaction (ester hydrolysis and buffer protonation) in water–acetonitrile mixtures can be evaluated from the combination of spectrophotometric and calorimetric data. A main contribution to the molar enthalpy change of the overall reaction is given by the protonation of primary amine tris[hydroxymethyl]aminomethane. There is no detectable influence of the organic solvent on the overall reaction enthalpy. The organic solvent influences only the rate of the enzymatic hydrolysis. The obtained results indicate that the α-chymotrypsin-catalyzed hydrolysis is suitable for quantitative determination of hydrophobic ester *p*-nitrophenyl acetate in water–acetonitrile mixtures by use of calorimetric detection.

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