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Cosolvent and pressure effects on enzyme-catalysed hydrolysis reactions

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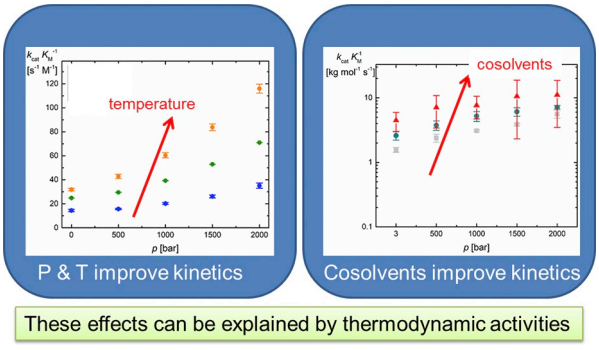
HIGHLIGHTS

- Combined pressure & cosolvent effects on hydrolysis of SPNA and HPNA were measured.
- Kinetic constants K_M and k_{cat} depend strongly on cosolvent, pressure and temperature.
- Combination of DMSO, high pressure and high temperature are beneficial for kinetics.
- Also TMAO increase the baro-stability of the enzyme α-CT.
- Activity coefficients explain the observed influences on kinetics.

GRAPHICAL ABSTRACT



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ABSTRACT

Thermodynamics and kinetics of biochemical reactions depend not only on temperature, but also on pressure and on the presence of cosolvents in the reaction medium. Understanding their effects on biochemical processes is a crucial step towards the design and optimization of industrially relevant enzymatic reactions. Such reactions typically do not take place in pure water. Cosolvents might be present as they are either required as stabilizer, as solubilizer, or in their function to overcome thermodynamic or kinetic limitations. Further, a vast number of enzymes has been found to be piezophilic or at least pressure-tolerant, meaning that nature has adapted them to high-pressure conditions.

In this manuscript, we review existing data and we additionally present some new data on the combined cosolvent and pressure influence on the kinetics of biochemical reactions. In particular, we focus on cosolvent and pressure effects on Michaelis constants and catalytic constants of α-CT-catalysed peptide hydrolysis reactions. Two different substrates were considered in this work, *N*-succinyl-L-phenylalanine-*p*-nitroanilide and *H*-phenylalanine-*p*-nitroanilide. Urea, trimethyl-*N*-amine oxide, and dimethyl sulfoxide have been under investigation as these cosolvents are often applied in technical as well as in demonstrator systems. Pressure effects have been studied from ambient pressure up to 2 kbar. The existing literature data and the new data show that pressure and cosolvents must not be treated as independent effects. Non-additive interactions on a molecular level lead to a partially compensatory effect of cosolvents and pressure on the kinetic parameters of the hydrolysis reactions considered.

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1. Introduction and aim of this work

A powerful tool to obtain novel insights into the mechanism of enzyme-catalysed reactions and to modulate their efficiency is to use extreme solution conditions, such as high temperature or high pressure [1,2] and/or to vary the solvent by adding particular cosolvents. Since catalysis by enzymes takes place stepwise (binding to the substrate, chemical conversion and product release), these variables can influence both equilibrium constants and rate constants in different ways. Also, technically, the use of extreme conditions might be a valuable tool for the design and optimization of reactions. While enzyme-catalysed reactions enable catalysis under mild reaction conditions, application is often limited by the low stability of the enzyme. The resulting narrow process window might cause low reaction rates, which in turn might lead enzyme-catalysed routes appear unattractive for industrial applications compared to chemical synthesis. A way to overcome these limitations is the use of high pressure and of addition of cosolvents to the reaction medium in order to stabilize the enzyme, to increase the reaction yield and to accelerate the reaction rate. The aim of this manuscript is to highlight the influence of pressure and of cosolvents on the kinetics of enzyme-catalysed reactions. Existing literature data are used for this purpose, but also new experimental data are presented that underline the influence of combined pressure and cosolvent effects on enzyme kinetics.

2. Effect of cosolvent on enzyme kinetics

Reaction kinetic data of enzyme-catalysed reactions might very strongly depend on the reaction medium, and thus, on the kind and concentration of the cosolvents present. Thus, using cosolvents is a powerful means to influence reaction rates and to broaden the process window in industrial biotechnological production. The addition of a cosolvent can positively influence the reaction equilibrium [3] and the reaction kinetics [4] and additionally stabilize the enzyme against denaturation [5]. In this work, we focus on the use of high pressure and of cosolvents on the reaction kinetics. A good overview of kinetic constants, namely the Michaelis constant, K_M, and catalytic constant, k_{cat}, can be found in the BioModels® database [6–8] that relies on experimentally determined parameters from BRENDA database, SABIO-RK, and STRENDA database. Reaction kinetics are valid only for the catalyst (the enzyme) under consideration. Thus, enzymes that just differ by a few amino acids in their primary structure (e.g. ADH 200 and ADH 270) [9] might show already a completely different kinetic profile. The cross-dependencies become even more complex if cosolvents are present in the medium. Such influences can be well described by determination and quantification of K_M and k_{cat}, which are defined by evaluating classical Michaelis-Menten kinetics [10] which describes the binding of an enzyme E to a substrate S to form an enzyme-substrate complex ES before finally releasing one or more products P. The cosolvent can interact directly with the enzyme, the substrate and/or the enzyme substrate complex and thereby interfere with the reaction mechanism and thus with the reaction kinetics [11,12]. Cosolvents that are discussed in the present work do neither act as substrate, activator nor inhibitor towards the enzyme. That is, cosolvents only interact by non-covalent solvent-mediated molecular interactions without affecting the active centre of the enzyme or modifying the structure of the protein.

In the literature, cosolvent effects on the reaction kinetics are explained on the one hand by direct molecular interactions between the cosolvent and the enzyme. Especially interactions between cosolvent and the active site(s) of the enzyme were studied at an atomistic level using molecular dynamics simulations. Such methods account for direct interactions between (co-)solvent molecules and single amino acids of the enzyme's active site [13–15]. On the other hand, enzyme-independent cosolvent/substrate interactions have been found to be the main cause for the observed cosolvent influence on K_M [9,16–20]. These works are based on the application of thermodynamic substrate activities instead of substrate concentrations in order to evaluate kinetic Michaelis-Menten (or Lineweaver-Burk) [21] plots. The required activity coefficients are accessible using thermodynamic models such as excess Gibbs energy models (e.g., the eNRTL model [22], the Debye-Hückel extended UNIQUAC equation [23], the Pitzer model [24]) or equations of state (such as the electrolyte Perturbed-Chain Statistical Associating Fluid Theory [25]). Such models have shown to be especially suited for biomolecular charged systems and are thus appropriate means for enzyme-catalysed reactions [26]. Further, molecular simulations are increasingly applied successfully to model activity coefficients in biomolecular systems [27]. The assumption of enzyme-independent molecular interactions between the substrate and the cosolvent that were proven in these works [9,16–20] led to a successful new approach for non-empirical quantifications and explanations of cosolvent effects on K_M. This new development led to a huge step in biocatalysis as it directly points to the possibility to design the reaction medium by physically-based methods. Regarding quantitative data, non-empirical predictions of cosolvent influences on k_{cat} are not yet established. However, some works have already observed cosolvent effects on k_{cat}. Okochi et al. [28] studied the influence of ionic liquids on the reaction rate of 3α-hydroxysteroid dehydrogenase and reported an increase in k_{cat}. Affleck et al. [29] and Faulds et al. [30] found a significant influence of organic (co)solvents on k_{cat} of enzyme-catalysed reactions. A nonlinear behaviour was observed for k_{cat} over a wide range of concentrations of organic solvents. The authors assumed that interactions within the active site of the enzyme are the reason behind their results, but quantification or modelling was not provided to support the hypothesis. Smith and Canady [31] pointed to the importance of accounting for the thermodynamic activity of the enzyme in order to determine cosolvent influences on k_{cat}. However, a thermodynamic model for the prediction of cosolvent influences on k_{cat} has not yet been presented in the literature so far. Determining activity coefficients of enzymes might still be a challenge [32] although some approaches exist in the literature [32,34]. Nevertheless, such activity coefficients are required for the prediction of the activity-based values of k_{cat}.

3. Effect of high pressure on enzyme kinetics

In the following, the focus is on the effects of high hydrostatic pressure (HHP) on the rate constants of enzymes. Pressure-induced changes in the rate of enzyme-catalysed reactions can be rationalized through either direct changes in the structure of the enzyme (like a higher conformational flexibility or selection of a different conformational sub-state of the enzyme's native fold), changes in the reaction mechanism, e.g. by an alteration in the rate-limiting step and/or changes in the solvent properties that effect the enzyme structure or the rate-limiting step, or by changes of the thermodynamic activities (via the activity coefficients) of the reacting species involved by cosolvents. In general, the pressure effects on a rate constant, k, can be described by the EYRING-equation [2]:

$$\frac{\mathrm{d}\ \ln(k/k_0)}{\mathrm{d}p} = -\frac{\Delta V^{\neq}}{RT} \tag{1}$$

where p denotes pressure, T temperature, R is the ideal gas constant, $\Delta V^{\neq}$ the molar activation volume, and k_0 the rate constant at a reference pressure p_0.

The activation volume, $\Delta V^{\neq}$, describes the volume difference between the transition state and the reactants, which contains contributions from solvation as well as intrinsic and steric factors. These factors determine the sign and the magnitude of the activation volume [35–37]. A negative value of $\Delta V^{\neq}$ indicates an acceleration of the catalysis upon a pressure increase. Decrease of void volume or associative processes, including bond formation in the rate-determining step, and especially the hydration of charged and polar amino acids (electrostriction effects) in the transition state can be responsible for a

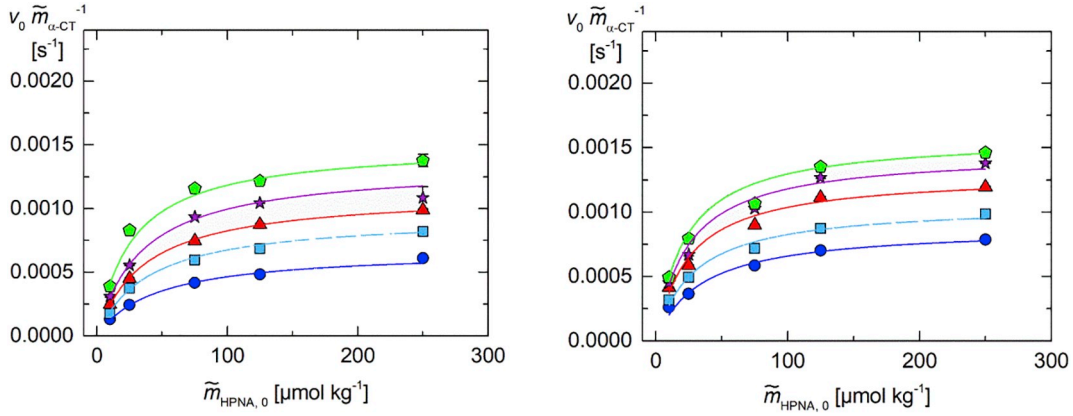

Fig. 1. Michaelis-Menten plots of the HP*p*NA hydrolysis catalysed by α-CT at different pressures at T = 30 °C in Tris buffer solution (at pH = 8) in the absence of cosolvents (left) and in the presence of 0.5 mol kg^{-1} TMAO (right). Circles: 3 bar, squares: 500 bar, triangles: 1000 bar, stars: 1500 bar, pentagons: 2000 bar. Data from this work.

negative contribution to $\Delta V^{\neq}$. The opposite sign points to a deceleration of the enzyme reaction, which results, for example, from a relaxation of solvation due to charge neutralization or an increase of void volume (packing defects) [38].

In recent years, high-pressure technology has expanded, particularly in food industry and pharmaceutics. The latter one uses HHP, for example, to optimize enzyme-catalysed synthesis of high added-value products such as antibiotics, pharmacological peptides and carbohydrates [2]. From an industrial point of view, a way to obtain higher yield and reduce product costs is crucial. To this end, fundamental studies using HHP as a novel parameter may help optimize enzymatic reactions. For fast kinetics studies, the high-pressure stopped-flow (HPSF) technology offers several advantages over other trigger mechanisms [39]: pressure propagates rapidly (typically on the μs timescale) and pressure-jumps can be performed bidirectionally if the enzyme reaction is reversible in the pressure range covered. In HPSF technology, the two reactant syringes and the rapid-mixing chamber are arranged inside a stainless steel pressure vessel. The pressure vessel has an integral thermostated jacket and a temperature monitor. The observation cell has sapphire observation windows, which provide direct coupling between the sample cell and optical fibres. With both UV/Vis-absorption and fluorescence measurement capabilities, the system has an empirical dead time of about 10 ms and allows stopped-flow determinations to be made at pressures up to 2000 bar.

For several enzyme classes [2], a stabilization of the enzyme and acceleration of the reaction by high pressure has been reported, such as for the proteolysis of chymotrypsin with ubiquitin as substrate [40]. The opposite effect has been shown, for example, for the oxidation of Amplex red by horseradish peroxidase, the pressure inhibition being due to a positive activation volume [41]. Further, HHP has also been found to induce a biphasic pressure behaviour, such as in the case of the oxidation of benzyl alcohol by yeast alcohol dehydrogenase [42].

Most of the research carried out in the area of high-pressure enzymology investigated pressure effects on the function of enzymes obtained from organisms living under atmospheric pressure, i.e. from mesophiles. It would be also interesting to reveal how proteins from deep-sea organisms (piezophiles) have adapted to high-pressure conditions. Such studies are still very scarce [41,42]. Although there are meanwhile a number of studies reporting on pressure effects on enzymatic reactions, decoding of the atomic-level mechanism behind substrate binding and the reaction itself at high-pressure conditions remains still a major challenge. In this respect, some advances have been made in recent years for small enzyme models using molecular dynamics (MD) simulations and quantum mechanics/molecular mechanics (QM/MM) free energy simulations, such as for the Zn-finger hydrolase [43].

Here, we discuss exemplarily the catalytic activity of α-chymotrypsin (α-CT) at different pressures and temperatures. The serine protease hydrolyses peptide bonds, in this study a model peptide called *N*-succinyl-L-phenylalanine-*p*-nitroanilide (SP*p*NA) forming the product *p*-nitroaniline. Our data demonstrate the promising potential of using both temperature and pressure modulation to enhance the enzyme efficiency. Next to globular proteins, also short amyloid-forming peptides, which might be good candidates for prebiotic enzyme families (e.g. for γ-carbonic anhydrase activity in the presence of zinc) reveal a similar enhancement of enzymatic activity in combination with high hydrostatic pressure and temperature [44]. An additional positive effect on the kinetic parameters has been shown to be feasible in the presence of particular osmolytes, organic solvents or macromolecular crowders [45–47].

4. Combined pressure and cosolvent effects: hydrolysis of HP*p*NA

Using the high-pressure stopped-flow (HPSF) system we studied the activity of α-chymotrypsin in the pressure range from 1 to 2000 bar and at three temperatures (20, 30 and 40 °C) in the absence and presence of cosolvents. The measurement procedure along with the calibration and analysis by spectrophotometry is explained in detail in our recent works [19,48]. Further, a brief introduction to the method is given in the appendix. Two different substrates were under investigation, SP*p*NA as well as *H*-phenylalanine-*p*-nitroanilide (HP*p*NA), which were hydrolysed by α-CT. The results for SP*p*NA hydrolysis are discussed using data [19] and compared in the following to the results for HP*p*NA hydrolysis using new data presented in this work.

The kinetics of HP*p*NA hydrolysis is illustrated in Fig. 1. As expected, with increasing initial HP*p*NA molality, the normalized reaction rates increase at constant pressure. Further, the reaction rates also increase with increasing pressure. The experimental data can be described using Michaelis-Menten kinetics.

Fig. 1 compares also the kinetics of HP*p*NA hydrolysis at neat conditions and in the presence of 0.5 mol kg^{-1} TMAO. It can be seen that the initial rate of the system with TMAO is greater than for the neat reaction system. This behaviour is qualitatively similar up to pressures of 1500 bar. The higher the pressure, the lower the beneficial influence of TMAO, however. This effect may be due to the excluded volume effect TMAO generally imposes on the protein-substrate system, which might promote preferential binding of the substrate to the active site of the enzyme, and this effect of stabilization by TMAO is obviously damped with increasing pressure.

Fig. 2 shows the catalytic constant, k_{cat}, and the catalytic efficiency, k_{cat}/K_M, for the hydrolysis of HP*p*NA in the buffered neat system (grey), at 0.5 mol kg^{-1} TMAO (dark cyan) and at 2.2 wt.-% DMSO (red) in the

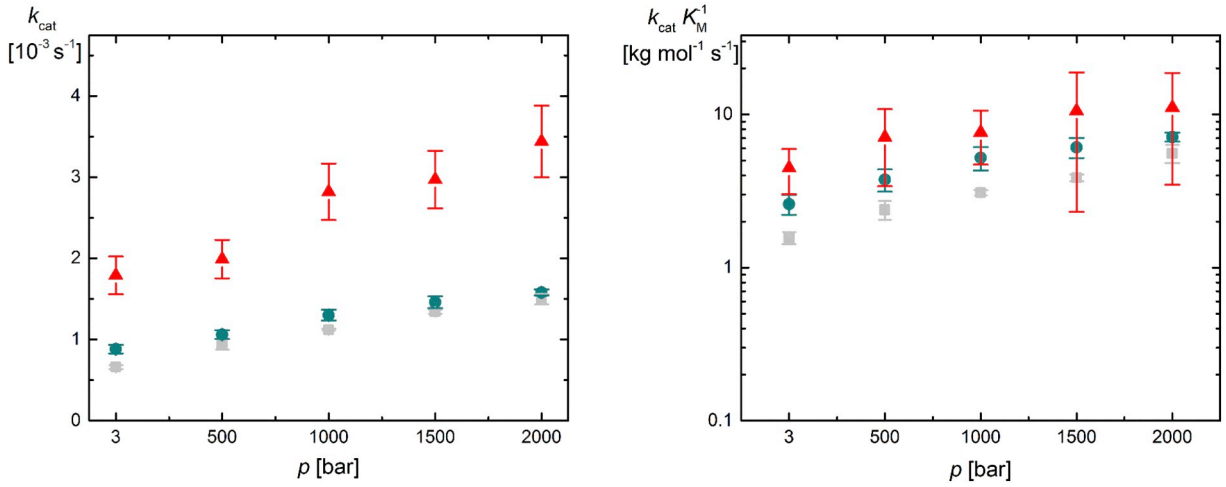

Fig. 2. Catalytic constant k_{cat} and catalytic efficiency k_{cat}/K_M for the hydrolysis of HP*p*NA in the buffered neat system (squares), at 0.5 mol kg^{-1} TMAO (circles) and at 2.2 wt.-% DMSO (triangles) in the system as function of pressure at T = 30 °C in Tris buffer solution (at pH = 8). Data from this work.

system as function of pressure at T = 30 °C in Tris buffer solution (at pH = 8). Independent of the pressure, both, TMAO and DMSO have a positive effect on the catalytic efficiency of HP*p*NA hydrolysis. The cosolvents apparently mainly affect the catalytic constant, while the K_M values upon addition of DMSO remain unchanged compared to the neat reactions within experimental uncertainty, and the K_M values slightly decrease upon addition of TMAO (results not shown graphically).

5. Combined pressure and cosolvent effects: hydrolysis of SP*p*NA

In general, it is known that TMAO at low concentrations has a very small influence on kinetic parameters for peptide hydrolyses [46,48]. Nevertheless, the kinetic profile for HP*p*NA hydrolysis is different from those of the other works [46,48]. For example, we discuss the hydrolysis of SP*p*NA by α-CT. Fig. 3 displays the pressure and temperature dependent Michaelis-Menten plots for this reaction. The initial rate, v_0, increases with increasing substrate concentration, and approaches the maximum value at 8 mM of SP*p*NA, where the enzyme is almost saturated with substrate. Compared to HP*p*NA, up to 1.5 times lower K_M values are found for SP*p*NA, which becomes obvious by comparing Figs. 1 and 3. This may indicate that there is less affinity of the enzyme for the substrate HP*p*NA than for SP*p*NA. Even more dramatic is the fact that k_{cat} is about 4.5 times higher for the investigated SP*p*NA hydrolysis than for the hydrolysis of HP*p*NA. This indicates that the enzyme has a higher catalytic efficiency for longer-side-chain peptides compound than for shorter-side-chain peptides such as HP*p*NA.

In both cases, at higher temperature and pressure, an enhancement of the enzyme activity is shown by the higher reaction rates observed. The v_0-values rise by a factor of four upon a temperature increase from 20 to 40 °C, and, upon increasing the pressure from 1 to 2000 bar, v_0 increases twofold. Fig. 4 highlights the pressure and temperature dependence of the Michaelis constant, of the turnover number, and of the catalytic efficiency. The Michaelis constant is decreasing with increasing pressure, so it can be reasoned that high pressure enhances the affinity of the substrate to the enzyme. The turnover number of α-CT increases markedly with increasing pressure. As a consequence, the catalytic efficiency increases threefold when the pressure rises from 1 to 2000 bar at 20 °C. Regarding the results of the temperature effects on the kinetic parameters, we observe that the k_{cat}-values and k_{cat}/K_M-values increase by a factor of two, respectively three, upon a temperature increase from 20 to 40 °C at 1 bar, while the K_M increases, i.e. the affinity of the substrate is reduced.

At all three temperatures, α-CT exhibits a negative activation volume, $\Delta V^{\neq}$, whereby the order of magnitude of $\Delta V^{\neq}$ is very small (about $-6.5\ cm^3\ mol^{-1}$ at 20 and 30 °C, and about $-8.5\ cm^3\ mol^{-1}$ at 40 °C). The negative value indicates a smaller compression of the

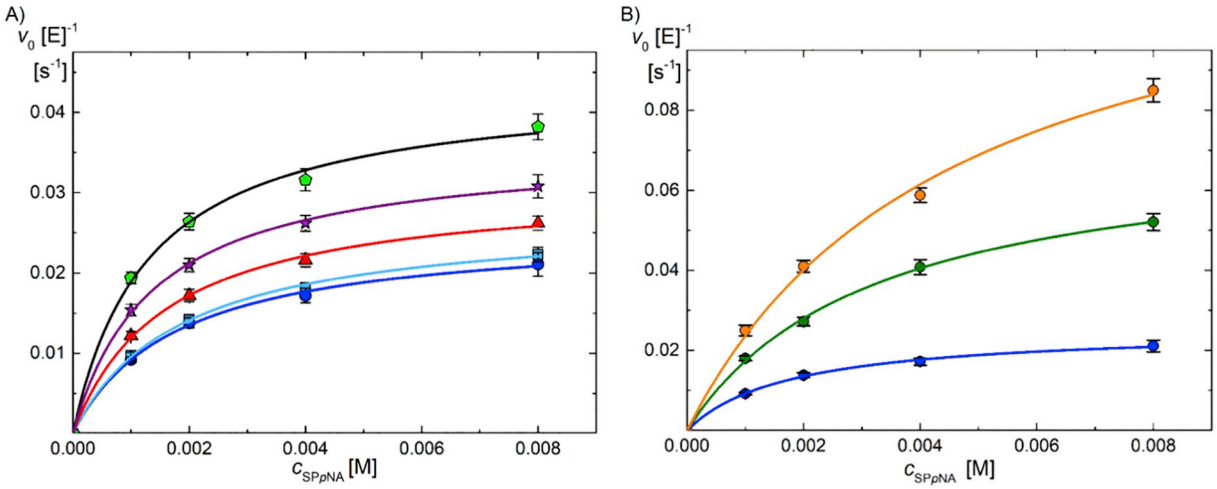

Fig. 3. Michaelis-Menten plots of the SP*p*NA hydrolysis catalysed by α-CT (8 μM) at different pressures at T = 20 °C (A) and temperatures (B) in Tris buffer solution (at pH = 8). A) Circles: 1 bar, squares: 500 bar, triangles: 1000 bar, stars: 1500 bar, pentagons: 2000 bar, B) 20 °C (lower data series), 30 °C (middle data series) and 40 °C (upper data series). Data from [19].

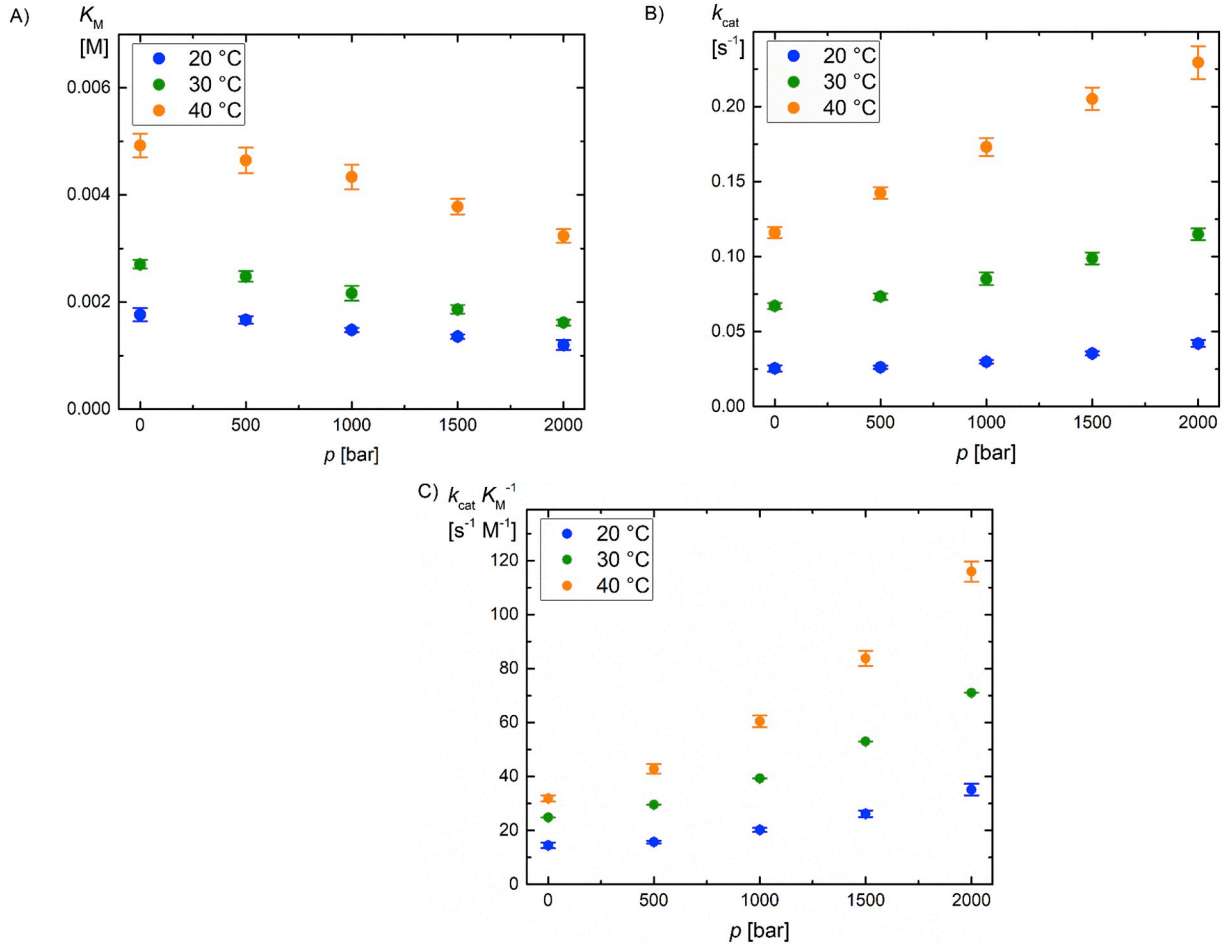

Fig. 4. Pressure dependence of the kinetic parameters for the catalysed hydrolysis of SP*p*NA by α-CT at different temperatures: A) Michaelis constant, K_M, B) turnover number, k_{cat}, and C) catalytic efficiency, k_{cat}/K_M. Data from [19].

enzyme-substrate complex (ES) compared to the transition state ($ES^{\neq}$), which can be explained by a decrease of void volume and/or by a higher hydration density around the charged and polar amino acid residues (electrostriction effect) in the transition state [49]. This effect is most significant for the highest temperature measured, indicated by the most negative $\Delta V^{\neq}$ value. For the hydrolysis of HP*p*NA, α-CT exhibits also a negative activation volume, which is approximately in a similar range as for the substrate SP*p*NA and previous works [46,48–51].

A pressure-induced enhancement of the activity is associated with a reduction of the activation Gibbs energy, $\Delta G^{\neq}$. Exemplary, at 2000 bar and 40 °C, $\Delta G^{\neq}$ is merely reduced by $-1.7\ kJ\ mol^{-1}$. In the pressure range from 1 to 2000 bar the activation energy, E_a, is nearly constant. The E_a-value of about $60\ kJ\ mol^{-1}$ is typical for the catalytic reaction of α-CT with low-molecular weight substrates [44].

Recently, researchers have started expressing reaction kinetics of enzyme-catalysed reactions in terms of thermodynamic activity-based properties. Access to thermodynamic activities allows formulating activity-based Michaelis-Menten equations, which in turn yield activity-based Michaelis constants and even activity-based catalytic constants. Such constants are independent of the cosolvent. The advantage of such activity-based expressions is twofold. On the one hand, it allows for predictive screening of potentially beneficial cosolvents, i.e. cosolvents that favour the reaction kinetics by increasing k_{cat} or/and decreasing K_M. This might be useful to bypass cosolvent screening from the still empirical and experimentally based methods applied so far in order to decrease time and costs for the development and optimization of biocatalytic processes. On the other hand, molecular interactions between the substrate and the components in the reaction mixture can be expressed as thermodynamic activity in terms of the activity coefficient of the substrate. Thus, the effects of cosolvent and pressure on substrate-solvent and enzyme-solvent interactions can be quantified and optimized. Please note that in this approach we assume that cosolvents do neither act as substrate, activator nor inhibitor towards the enzyme. The addition of the cosolvent does not change the structure of the enzyme (e.g., by modifying the catalytic pocket), it changes the activity of the solutes, only, which is sufficient to explain the experimental data obtained.

In the literature, molecular models on different scale have been used to access molecular interactions in multi-component biosystems containing cosolvents. Among such methods, molecular simulation has been applied to quantify interactions between cosolvent and the catalytic center of enzymes [13,14]. However, recent results using molecular simulation [20] showed that cosolvent-enzyme interactions may not be able to explain the observed cosolvent influence on Michaelis constants. Also other groups focused on relating observed cosolvent effects on Michaelis constants with enzyme-independent molecular interactions between cosolvent and substrate [9,16–19]. Among these methods, an engineering-based approach based on PC-SAFT has been developed and applied to several enzyme-catalysed reactions. First, this activity-based approach has been used to predict the cosolvent influence on Michaelis constants of the reduction of acetophenone by alcohol dehydrogenases [9,18]. Similarly, Michaelis constants were predicted for the hydrolysis of SP*p*NA [19]. Activity-based and concentration-based Michaelis constants were found to differ by a factor of about 39, which was explained by the high activity coefficient

of the substrate SP*p*NA in water. This highlights the importance of considering substrate-related interactions in the reaction mixture. For both, α-CT and ADH reactions under investigation, quantitative agreement between predictions and experimental data were obtained [9,18,19]. In all these works, the enzyme was not considered for predicting the cosolvent effect on the Michaelis constants. That is, the interactions between cosolvent and substrates are solely responsible for the experimentally observed effect of the cosolvent on the Michaelis constants. Based on these results, direct interactions between cosolvent and enzyme need not be invoked to describe the experimental data, i.e., the reason for the cosolvent effect on the Michaelis constants is the direct interaction between substrate and cosolvent. In contrast, enzyme-cosolvent interactions can affect catalytic constants, and a framework is currently been developed to predict the catalytic constant as function of pressure and cosolvent. Finally, it should be mentioned that such activity-based approaches are only valid given that inhibition does not take place and given that the enzyme is stable under the applied cosolvent and pressure conditions.

6. Conclusions

Many enzyme-catalysed reactions are dismissed on laboratory scale due to unfavourable reaction kinetics or low product yield. One of the main reasons is that such processes can only run under a narrow process window as the catalyst (the enzyme) usually dictates temperature or pH ranges that do not cause instabilities, precipitation, inactivation etc., of the enzyme. A way to overcome narrow process windows is the addition of chemically inert cosolvents to the reaction system and the use of high pressure. Such strategies have already often been applied for enzyme catalysis due to their beneficial influence on the reaction kinetics and enzyme stability.

The results discussed in this work demonstrate that the kinetics of the hydrolysis of peptides catalysed by α-CT displays an enhanced performance at high pressure. This has been shown for two different reactions, the hydrolysis of the peptides SP*p*NA and HP*p*NA at pH 8 for temperatures between 20 °C and 40 °C. In addition, the high-pressure effect appears to stabilize the enzyme against thermal inactivation as evidenced by a higher initial rate. Hence, both pressure and temperature can favourably be utilized to accelerate such enzyme-catalysed reactions. Furthermore, the cosolvent study reveals an additional positive effect on the turnover number of the reactions considered. The presence of TMAO enhances the thermo-baro-stability of α-CT, which is consistent with earlier studies. Overall, the effects of pressure, temperature and cosolvent addition (e.g., osmolytes) can be used to efficiently optimize the catalytic properties of enzymes. Furthermore, thermodynamic models, such as PC-SAFT, help to improve the understanding of the reaction. Such models are required to establish an activity-based expression of the kinetic equations. This allows predicting the influence of combined pressure and cosolvent effects on reaction kinetics on a physical basis. Further, activity-based expressions allow distinguishing between substrate-cosolvent and enzyme-cosolvent effects, which can be even considered as function of pressure, and help to understand effects of pressure and cosolvents on a fundamental basis. In the future, this understanding will help us to design applications in the biotechnology, food and pharmaceutical industry, where enzyme catalysis plays a crucial role.

Acknowledgements

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Appendix

A.1. Experimental section

The chemicals used for kinetic studies, their supplier and purity are given in Table A1. All chemicals were used without further treatment.

Table A1
Chemicals used in this work.

Name	CAS-number	Purity	Manufacturer
α-Chymotrypsin	9004-07-3	–	AppliChem GmbH
H-phenylalanine-p-nitroanilide	2360-97-6	> 99%	Bachem AG
Trimethylamine N-oxide dihydrate	62,637–93-8	> 99%	Fluka
Dimethyl sulfoxide	67–68-5	> 99.9%	Merck Millipore
Trizma hydrochloride	1185-53-1	> 99%	Sigma-Aldrich
Trizma base	77–86-1	> 99.9%	Sigma-Aldrich

In this work 0.1 mM Tris-HCl buffer solution was used consisting of Trizma hydrochloride and Trizma base. This buffer solution was set to pH 8 at 30 °C.

The compositions of the mixed solutions in the high-pressure stopped-flow cell as well as the measuring conditions are given in Table A2.

Table A2
Compositions of solutions for kinetic studies and measuring conditions.

System	T [°C]	p [bar]	pH	$\widetilde{m}_{buffer}$ [mmol/kg]	$\widetilde{m}_{HPNA}$ [μmol/kg]	$\widetilde{m}_{\alpha-CT}$ [μmol/kg]
Neat	30	3, 500, 1000, 1500, 2000	8	0.1	10, 25, 75, 125, 250	20
0.5 m TMAO	30	3, 500, 1000, 1500, 2000	8	0.1	10, 25, 75, 125, 250	20
0.28 m DMSO	30	3, 500, 1000, 1500, 2000	8	0.1	10, 25, 75, 125, 250	20

All kinetic studies presented in Figs. 1–2 were conducted at least twice with independent stock solution for each set of measurements. This allows giving a meaningful uncertainty value of the experimental data. The experimentally determined initial reaction rate showed very high accuracy. The error bars were calculated from the standard deviation of the independent experiments.

In order to determine the kinetic constants, curve fitting was done using the data analysis software OriginLab. Thus, the non-linear representation

was used to evaluate the enzyme kinetic data. The curve function according to Michaelis-Menten equation is thereby obtained by adjusting K_M and k_{cat} to experimental data points in the diagram while minimizing the error squares according to the Levenberg-Marquardt algorithm. Consequently, the error bars in Figs. 1–2 result from the accuracy of the adjustment of the Michaelis-Menten plot. The error bars of the kinetic constants do consider also the uncertainty of the experimental measurements (especially the uncertainty caused by biological-duplicate measurements).

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