Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Molecular Catalysis B: Enzymatic 39 (2006) 124–127
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Equilibrium shifts in enzyme reactions at high pressure

M.E. Bruins *, A.E.M. Janssen, R.M. Boom

Food and Bioprocess Engineering Group, Department of Agrotechnology and Food Sciences, Wageningen University, P.O. Box 8129, 6700 EV, Wageningen, The Netherlands

Available online 9 March 2006

Abstract

The effect of pressure on the equilibrium of a reaction was studied. Theoretical equilibrium constants and product concentrations have been calculated at elevated pressures. The theory is illustrated with an example of L-malate synthesis catalyzed by a fumarase. To study shifts in the equilibrium relatively low pressures can be applied (50–200 MPa), but our calculations show that for process optimisation much higher pressures (up to 1000 MPa) have to be used.

At these higher pressures, more stable enzymes are needed. We performed experiments with the hyperthermophilic β-glycosidase from *Pyrococcus furiosus* as a catalyst. Oligosaccharides were synthesized from glucose in an equilibrium reaction at pressures from 0.1 to 500 MPa. The enzyme remained active at 500 MPa. The equilibrium of the reaction was influenced by pressure and shifted towards the hydrolysis side, decreasing final oligosaccharide concentrations with increasing pressure. This pressure dependence of the final product concentration and the equilibrium constant could be described with a positive reaction volume of 2.4 mol/cm^3.

Keywords: Reaction volume; *Pyrococcus furiosus*; Glucosidase; Fumarase; Enzyme stability

1. Introduction

1.1. Enzymes and high pressure

Unlike temperature and pH, pressure is often neglected as a fundamental process parameter in enzyme reactions. High pressure is not yet used to increase yields in synthetic biocatalytic processes. The main reason is enzyme instability at elevated pressures. Therefore, studies on high-pressure enzymatic reactions have focussed on the behaviour of the structure of the enzyme and its (un)folding under pressure. Reviews of Gross and Jaenicke [1] and Heremans and Smeller [2] show the effect of pressure on structure, function and dynamics of proteins. Less attention has been given to enzyme kinetics. The work that has been done on enzyme kinetics is mostly limited to initial rate measurements.

There are several reasons why the exploration of pressure as a fundamental system parameter is of interest. The kinetics and equilibria of enzymatic reactions may differ significantly when carried out under high pressure, and in some instances the selectivity of the reaction may be influenced. In particular use of high pressure for the enzymatic synthesis of pharmacological peptides, antibiotics, carbohydrates, polyols and sweeteners would be worth exploring.

In this paper, we focus on the effect of pressure on the equilibrium of a reaction.

1.2. High-pressure enzymatic equilibrium reactions

In literature only a few enzyme-catalyzed reactions of which the equilibrium was determined under pressure are reported. In some cases, the reaction volume was determined as part of a volume profile analysis.

The only synthesis reaction that was reported to increase the equilibrium constant by the application of pressure was the conversion of fumarate and water to L-malate [3,4]. We used data from this work in the theory section of this article to verify calculations and to discuss some problems and possibilities for enzyme-catalyzed equilibrium reactions under pressure.

The group of Kunugi has studied protease catalyzed peptide synthesis [5,6]. Pressure, however, did not increase peptide yields in equilibrium reactions. Condensation of Fua-Gly and Leu-NH_2 showed a decrease in yield at elevated pressures. They did find a six-fold increase in peptide yield at 200 MPa as compared to ambient pressure for a kinetically controlled peptide

* Corresponding author. Tel.: +31 317 485096; fax: +31 317 482237.
E-mail address: Marieke.Bruins@wur.nl (M.E. Bruins).

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.01.033

formation from Cbz-Asp and Phe-Ome, where a higher catalytic activity due to pressure overcomes the negative equilibrium shift.

The catalysis of the hydration of CO_2 and the dehydration of HCO_3^- by carbonic anhydrase is an example of an enzyme catalyzed equilibrium reaction in which charged groups are being formed or neutralized. Reaction volumes for ionization are in the order of $-20\,cm^3/mol$ [7,8]. This specific reaction has a reaction volume for hydrolysis of $-26\,cm^3/mol$ [9]. Thus CO_2 synthesis is decreased under pressure. In this research the determination of the reaction volume was part of a volume profile analysis.

The conversions of sucrose by invertase and dextransucrase were also described by volume profiles [10]. Here, the reaction volumes were determined by density measurements of the reaction solution in time. Both reactions showed a negative reaction volume for the conversion of sucrose to glucose and fructose ($-7.3\,cm^3/mol$ by invertase) and to dextran and fructose ($-4.1\,cm^3/mol$ by dextransucrase). This latter reaction is a transglycosilation and may be referred to as an oligosaccharide synthesis reaction. Dextran hydrolysis by dextranase is also studied in this article, but the reaction volume for the complete conversion of dextran to glucose was not determined.

In an article by Morild [11] about the volume changes of ethanol and NAD^+ in the alcohol dehydrogenase catalyzed reaction to acetaldehyde + NADH, the reaction volume of $-7\,cm^3/mol$ was only referred to as being from equilibrium studies at elevated pressures, which unfortunately remained unpublished.

1.3. Hyperthermophilic enzymes at high-pressure

In all studies mentioned in the previous section, pressure did not exceed 200 MPa for equilibrium measurements. This is enough for mechanistic studies, but for real improvement in product yields higher pressures have to be applied. This will be explained further in our calculations in Section 2.2.

To study enzyme catalyzed equilibria under pressure long reaction times are necessary and therefore enzymes are needed that are not easily inactivated by pressure. Usually enzymes withstand pressures up to 400 MPa [12]. Some monomeric enzymes can even withstand higher pressures. Enzymes obtained from thermophilic organisms were found to be stabilized by pressure [13]. Usually, thermostable proteins are piezostable as well, although exceptions have been reported [14]. With the ongoing exploration of our natural resources, more and more thermo- and piezostable enzymes become available. This will enable high-pressure research on biocatalytic processes that is no longer limited by enzyme (in)stability.

2. Theory

2.1. The equilibrium constant as a function of pressure

For any chemical reaction, the equilibrium constant (K) is related to the Gibbs free energy (ΔG) by:

$$\Delta G = -RT \ln K \tag{1}$$

in which R is the gas constant and T is the absolute temperature (in K). The pressure (P in MPa) dependence of the Gibbs free energy is given by:

$$\left(\frac{\partial \Delta G}{\partial P}\right)_T = \Delta V \tag{2}$$

in which ΔV is the reaction volume change of the system in cm^3/mol that is equal to the difference of the partial molar volumes of products and reactants:

$$\Delta V = \Delta V_{\text{products}} - \Delta V_{\text{reactants}} \tag{3}$$

These partial molar volumes include both intrinsic (van der Waals volumes) and solvational (contraction of the solvation shell and change in volume of the cavities) constituents. In this article we used ΔV as a constant that is independent of pressure. The combination of Eqs. (1) and (2) results in:

$$\left(\frac{\partial \ln K}{\partial P}\right)_T = \frac{-\Delta V}{RT} \tag{4}$$

in which the gas constant R equals $8.3145\,cm^3\,MPa\,K^{-1}\,mol^{-1}$. This equation implicates that the application of pressure to a system in equilibrium will force the equilibrium to the state with the smallest volume. Thus a negative reaction volume shifts the equilibrium to the product side of the reaction.

2.2. Calculations

Literature [7,12] shows that for the formation of chemical bonds negative reaction volumes around $-10\,cm^3/mol$ are found. Eq. (4) can be used to calculate hypothetical yields for a synthetic equilibrium reaction:

$$A + B \leftrightarrow C \qquad \text{for which } K = \frac{[C]}{([A][B])} \tag{5}$$

Fig. 1a depicts the change in equilibrium constant that applies to all reactions with a reaction volume of $-10\,cm^3/mol$. It shows the exponential change of the equilibrium constant with pressure. Using Eq. (5), the equilibrium constant can be translated to a product concentration. In our calculations we assumed equimolar quantities of A and B. In Fig. 1b, the concentration of product C as a function of pressure is shown. When pressure is applied to a reaction with a relatively unfavourable equilibrium that yields a low product concentration of only a few percentages at atmospheric pressure, major improvements can be made (lower line in Fig. 1b). At a relatively low pressure of 200 MPa the final product concentration will already be more than doubled. Higher pressures can further increase product concentrations to a 4.6-fold increase at 400 MPa and a 22-fold increase at 1000 MPa. Thus, synthesis reactions that are hardly detectable at ambient pressure may have much higher conversions at these elevated pressures. In reactions that already have higher yields at ambient pressure, pressure can be used to increase the product concentration to almost 100% (upper line in Fig. 1b), avoiding the need for the removal of excess substrate for product purification.

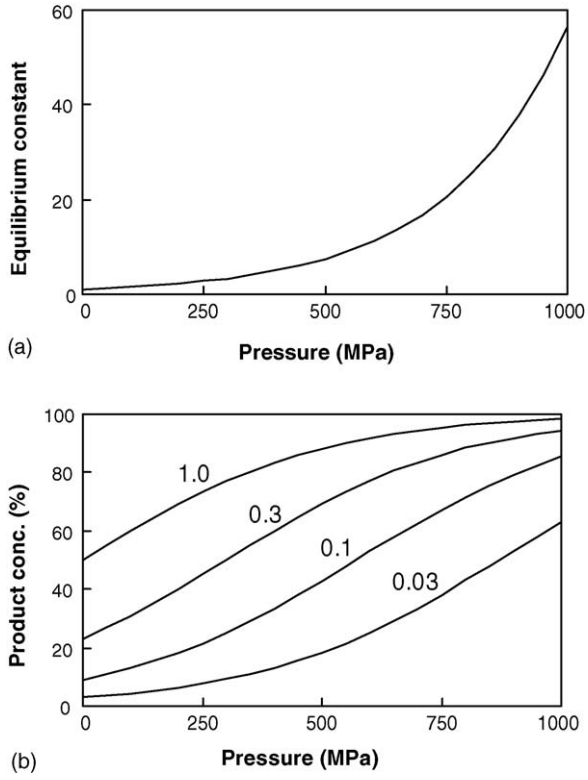

Fig. 1. (a) Improvement of the equilibrium constant at different pressures. K at 0.1 MPa = 1; T = 298 K; $\Delta V = -10\,cm^3/mol$. (b) Increase in product concentration at equilibrium as a function of pressure for a condensation of A + B → C; T = 298 K; $\Delta V = -10\,cm^3/mol$; from top to bottom line: K at 0.1 MPa = 1.0, 0.3, 0.1 and 0.03.

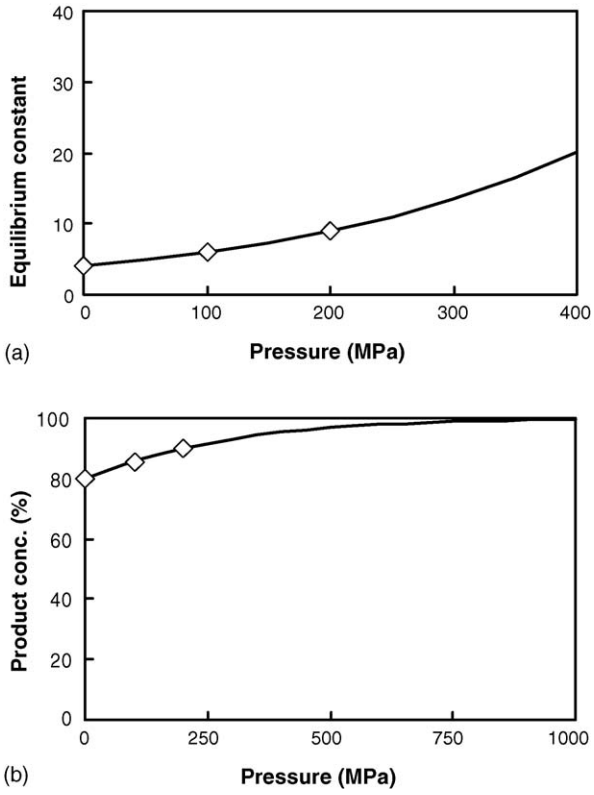

Fig. 2. (a) Improvement of the equilibrium constant at different pressures for the fumarase catalyzed conversion of fumarate and water to L-malate; line fitted with $\Delta V = -10.0\,cm^3/mol$ and T = 298 K. Data as measured by Andersen and Broe [3]. (b) Increase in product concentration at equilibrium as a function of pressure for the fumarase catalyzed conversion of fumarate and water to L-malate; data calculated from measurements by Andersen and Broe [3]; line fitted with $\Delta V = -10.0\,cm^3/mol$ and T = 298 K.

2.3. The condensation of fumarate to L-malate

The only synthesis reaction that was successfully improved by the application of pressure was the conversion of fumarate and water to L-malate [3,4]. We used the equilibria data as measured by Andersen and Broe to fit a reaction volume (see Fig. 2a). The equilibrium constant was related to the product concentration via K = [L-malate]/[fumarate]. From this we can calculate the product concentration at different pressures, as shown in Fig. 2b. The data were further used to extrapolate to much higher pressures than the measured ones. Theoretically, a 100% yield can be obtained at 1000 MPa. Unfortunately, this reaction has a positive activation volume, which means that an increase in pressure slows the reaction rate. Already at 300 MPa, conversion of the substrate was too slow to be measured [3], so the practical application of this reaction is limited.

3. Experimental

3.1. Materials

β-Glycosidase from *Pyrococcus furiosus* was kindly provided to us by van der Oost from the Laboratory of Microbiology at Wageningen University. The enzyme was dissolved in 20 mM Tris buffer pH 8.0. For the experiments a 0.05 M Tris buffer, titrated with 1.0 M NaOH to pH 8.0, was used.

3.2. HPLC analysis of sugars

Samples from the reaction mixture were centrifuged at 13,000 rpm, diluted and analyzed on HPLC using a RSO Oligosaccharide Column (Phenomenex, Amstelveen, The Netherlands) at 80 °C. The column was eluted with Milli-Q water at a flow rate of 0.3 ml min^{-1}. The eluent was monitored with a refractive index detector. It was assumed that the response was independent on the degree of polymerization. Glucose, disaccharides, trisaccharides and tetrasaccharides were detected and measured as percentages of total sugar on weight base.

3.3. High-pressure enzymatic synthesis of oligosaccharides

Glucose condensation was studied at 60 °C at varying pressures from 0.1 up to 500 MPa. The reaction solution consisted of 0.95 g buffer, 0.25 ml enzyme solution and 0.8 g glucose. The reaction mixture was put in polyethylene bags and pressurized in a multi vessel high-pressure apparatus (Resato FPU 100-50, Resato International B.V., Roden, The Netherlands) that was kindly made available to us by Agrotechnology and Food Innovations of Wageningen University and Research Centre. The pressure build-up rate was 5 MPa/s. The pressure medium used was glycol. The reaction mixtures were incubated for different

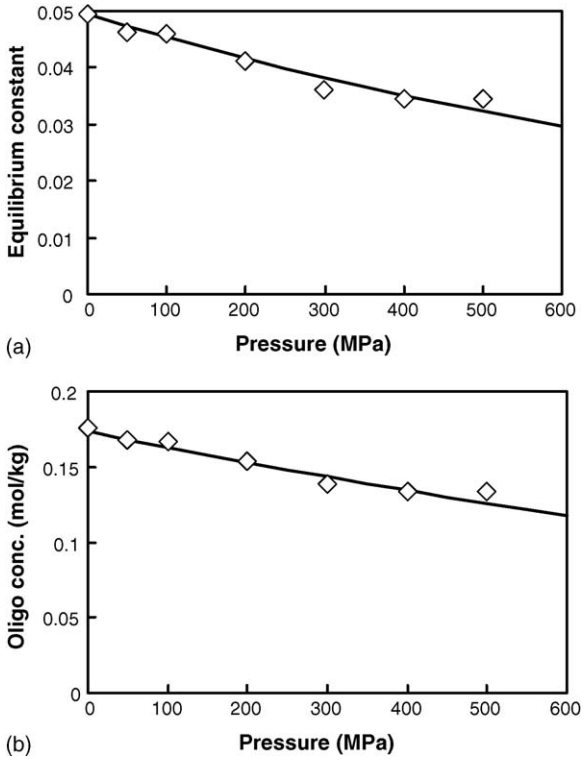

Fig. 3. (a) Decrease of the equilibrium constant at different pressures for the conversion of glucose to oligosaccharides catalyzed by β-glycosidase from *P. furiosus*; line fitted with $\Delta V = 2.4\,cm^3/mol$ and $T = 333\,K$. (b) Decrease in product concentration at equilibrium as a function of pressure for the conversion of glucose to oligosaccharides catalyzed by β-glycosidase from *P. furiosus*; line fitted with $\Delta V = 2.4\,cm^3/mol$ and $T = 333\,K$.

times to check whether equilibrium was reached. After depressurizing, 50 μl sample was added to 400 μl Milli-Q and 50 μl 1.0 M H_2SO_4 on ice to inactivate the enzyme. After 15 min, 50 μl 1.0 M NaOH was added to neutralize the sample.

4. Results and discussion

4.1. High-pressure enzymatic synthesis of oligosaccharides

The experiments on gluco–oligosaccharide synthesis, catalyzed by the hyperthermophilic β-glycosidase from *P. furiosus* showed a decrease in oligosaccharide concentration with pressure (Fig. 3b). The oligosaccharide mixture consisted mainly of disaccharides, but trisaccharides and tetrasaccharides were also formed. In all cases equilibrium was reached within 72 h. We did not investigate the exact reaction rate, but we did not encounter the problem of Andersen and Broe [3], where the speed of the reaction decreased to almost zero at 300 MPa.

From the final sugar concentrations the equilibrium constants were calculated and plotted as a function of pressure in Fig. 3a. The pressure dependence of the equilibrium constant could be described with a positive reaction volume of $2.4\,mol/cm^3$. This reaction volume was also used to fit the product concentrations as a function of pressure.

The synthesis of oligosaccharides from glucose is a condensation reaction in which water and a disaccharide are formed. This means that the reaction acts as a double displacement reaction. Therefore the reaction volume is closer to zero than to $-10\,cm^3/mol$ as it is for reactions with only bond formation. The positive value means that hydrolysis is preferred over synthesis when pressure is increased. Interestingly, this was also the case in the condensation of Fua-Gly and Leu-NH_2 for peptide synthesis as described by Kunugi [5,6].

5. Conclusion

Pressure is an important parameter in synthesis reactions that influences the equilibrium of a reaction. To study this effect, relatively low pressures can be applied (up to 200 MPa) at which enzyme inactivation is not yet important. Our calculations show that for process optimisation, much higher pressures (up to 1000 MPa) have to be used. At these high pressures, more stable enzymes are needed. We showed that with the use of a thermophilic enzyme, higher pressures can be applied.

We will continue to study equilibrium reactions under pressure at our laboratory, taking advantage of the ongoing discovery of more stable enzymes.

Acknowledgements

The authors gratefully acknowledge Ariette Matser for enabling us to perform high-pressure experiments at Agrotechnology and Food Innovations (WUR), John van der Oost from the laboratory of Microbiology (WUR) for donating the β-Glycosidase from *P. furiosus* and Tim Baks for critically reading the manuscript.

References

[1] M. Gross, R. Jaenicke, Eur. J. Biochem. 221 (1994) 617.
[2] K. Heremans, L. Smeller, Biochim. Biophys. Acta 1386 (1998) 353.
[3] B. Andersen, P.E. Broe, Acta Chem. Scand. 26 (1972) 3691.
[4] P. Butz, K.O. Greulich, H. Ludwig, Biochemistry 27 (1988) 1556.
[5] S. Kunugi, K. Tanabe, K. Yamashita, Y. Morikawa, T. Ito, T. Kondoh, K. Hirata, A. Nomura, Bull. Chem. Soc. Jpn. 62 (1989) 514.
[6] S. Kunugi, Prog. Polym. Sci. 18 (1993) 805.
[7] T. Asano, J. Lenoble, Chem. Rev. 78 (1978) 407.
[8] Y. Kitamura, T. Itoh, J. Solution Chem. 16 (1987) 715.
[9] X. Zhang, C.D. Hubbard, R. van Eldik, J. Phys. Chem. 100 (1996) 9161.
[10] H. Ludwig, K.O. Greulich, Biophys. Chem. 8 (1978) 163.
[11] E. Morild, J. Phys. Chem. 81 (1977) 1162.
[12] V.V. Mozhaev, K. Heremans, J. Frank, P. Masson, C. Balny, Trends Biotechnol. 12 (1994) 493.
[13] D.J. Hei, D.S. Clark, Appl. Environ. Microbiol. 60 (1994) 932.
[14] E. Mombelli, E. Shehi, P. Fusi, P. Tortora, Biochim. Biophys. Acta 1595 (2002) 392.