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Increased susceptibility of β-glucosidase from the hyperthermophile *Pyrococcus furiosus* to thermal inactivation at higher pressures

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The stability of β-glucosidase from the hyperthermophile *Pyrococcus furiosus* was studied as a function of pressure, temperature and pH. The conformational stability was monitored using FTIR spectroscopy, and the functional enzyme stability was monitored by inactivation studies. The enzyme proved to be highly piezostable and thermostable, with an unfolding pressure of 800 MPa at 85 °C. The tentative pressure–temperature stability diagram indicates that this enzyme is stabilized against thermal unfolding at low pressures. The activity measurements showed a two-step inactivation mechanism due to pressure that was most pronounced at lower temperatures. The first part of this inactivation took place at pressures below 300 MPa and was not visible as a conformational transition. The second transition in activity was concomitant with the conformational transition. An increase in pH from 5.5 to 6.5 was found to have a stabilizing effect.

β-Glucosidases catalyse the hydrolysis of β-*O*-glucosidic bonds with broad substrate specificity [1]. The β-glucosidase from the hyperthermophile *Pyrococcus furiosus* is one of the most thermostable enzymes known to date. It has a high kinetic stability, with a half-life of 85 h at 100 °C and maximal activity between 102 and 105 °C at pH 5.0 [2]. This high thermal stability presumably originates from its tetrameric structure, which has been observed for all hyperthermophilic members of family 1 β-glucosidases, whereas mesophilic and thermophilic family 1 enzymes are mainly active as monomers or dimers [3]. The structure of the β-glucosidase is a tetramer with four identical 58 kDa subunits [2]. Each subunit consists of a single domain of 472 amino acids, with 18 α-helices and 16 β-strands. The centre of the monomer is formed by a $(\beta\alpha)_8$-barrel or TIM-barrel, a fold that has been observed for all family 1 glycosyl hydrolases. The sequence and structure of the β-glucosidase from *P. furiosus* resemble those of the β-glucosidase of *Sulfolobus solfataricus*. They share 53% and 56% sequence identity at the amino acid and the DNA level, respectively; they also have a similar catalytic mechanism and substrate specificity. However, the molecular basis of the high thermostability appears to be different. A biochemical comparison suggested that the β-glucosidase from *P. furiosus* is mainly stabilized by hydrophobic interactions, whereas salt bridge interactions are crucial for the stability of the β-glucosidase from *S. solfataricus* [1].

In this study, we explored the stability of β-glucosidase from *P. furiosus* at different temperatures and

Abbreviation
DAC, diamond anvil cell.

pressures. Previous studies have focused on the thermal stability, revealing that the secondary structure of the enzyme remains intact up to the upper limit of the investigated temperature range (99 °C) [1,4] To our knowledge the pressure stability of this protein has not been investigated so far, although a previous pressure study of *S. solfataricus* β-glucosidase up to 250 MPa found that this enzyme is highly piezostable, with a half-life of 91 h at 60 °C and 250 MPa. This seems to confirm the notion that thermostable proteins are usually also very piezostable [5–8]. Knowledge of the pressure stability of an enzyme is of practical importance. In previous research, we studied the use of pressure as a tool to increase the product concentration in equilibrium reactions. To study shifts in the equilibrium, relatively low pressures can be applied (50–200 MPa), but our calculations showed that for process optimization, much higher pressures (up to 1000 MPa) have to be used. This illustrates the need for more pressure-stable enzymes. The β-glucosidase from *P. furiosus* was previously used to modify oligosaccharide yields under pressure, where it remained sufficiently active at 500 MPa [9].

In this work, we continued our study of the stability of the hyperthermophilic β-glycosidase from *P. furiosus*. To assess the stability, we monitored the changes in secondary structure with FTIR spectroscopy and enzyme inactivation. For practical applications, enzyme activity is the most important parameter, but the loss of structure can be measured over a wider range of temperature and pressure. Very often, enzyme inactivation that is due to a small change in the active site is coupled to a conformational change in the protein. In addition to pressure and temperature, the chemical composition of the protein solution (pH, salts) will also influence the stability of the enzyme. In particular, the effect of pH is also considered here, as the pH of the solvent is both pressure and temperature dependent [10]. On the basis of our data, we present a tentative pressure–temperature phase diagram, which reflects the pressure–temperature conditions in which the enzyme is active.

Results and Discussion

The influence of constant pressure on enzyme inactivation

A solution of β-glucosidase of *P. furiosus* was pressurized up to the desired pressure, temperature equilibrated, and subsequently kept at constant pressure for 1 h. The increase in enzyme inactivation after 1 h (A_{70}) was measured and compared to the blank, which had only been pressurized and equilibrated for temperature changes (A_{10}). The results at 25 °C and pH 6.0 are depicted as squares in Fig. 1.

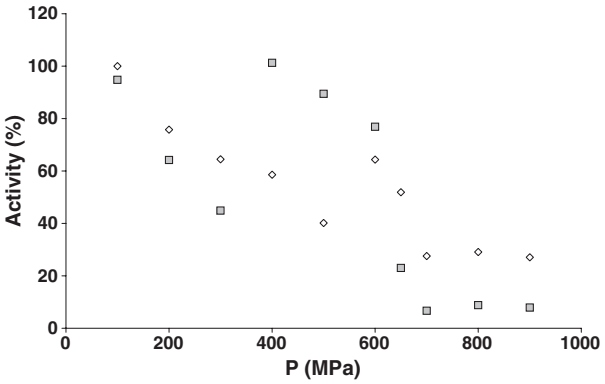

Fig. 1. Influence of pressure on the enzyme activity at constant pressure (A_{70}/A_{10}) (▣) and under pressurization (A_{10}/A_0) (◇) of *P. furiosus* β-glucosidase at 25 °C and pH 6.0.

The results show increased enzyme inactivation at pressures from 100 to 300 MPa. At a constant pressure of 400 MPa, no enzyme inactivation occurred. When a higher constant pressure was used, enzyme inactivation increased again. The same trend was also visible at 40 and 60 °C, when no enzyme inactivation was measured after 1 h at 400 MPa. It can be concluded that keeping the enzyme solution at 100 or 400 MPa does not result in any loss of activity as compared to the activity immediately after pressurization. In these cases, the inactivation equilibrium was reached within 10 min. For some other samples, the situation was less clear, as there was a difference in activity after 10 and 70 min. Here, the enzyme was still inactivated in time.

Figure 1 also shows the extent of the inactivation during pressurization and temperature equilibration. This inactivation was considerable, suggesting fast inactivation, as half of the enzyme was already inactivated after pressurization and equilibration at 400 MPa. We therefore plotted the enzyme activity as a function of pressure when compared to the unpressurized sample. This is illustrated in Fig. 2A for $T = 25$ °C. The difference between Figs 1 and 2A is the inclusion of the pressurization time in Fig 2A. Activity (A_{70}) is compared to the untreated blank (A_0) instead of to the pressurized blank (A_{10}). From the difference between the two figures, one can see that a large part of the inactivation already occurred in the first 10 min of the experiment. This inactivation was not due to a temperature rise during pressurization. The β-glucosidase from *P. furiosus* does not become inactivated after weeks of storage at temperatures below 60 °C at atmospheric pressure [11], and it can

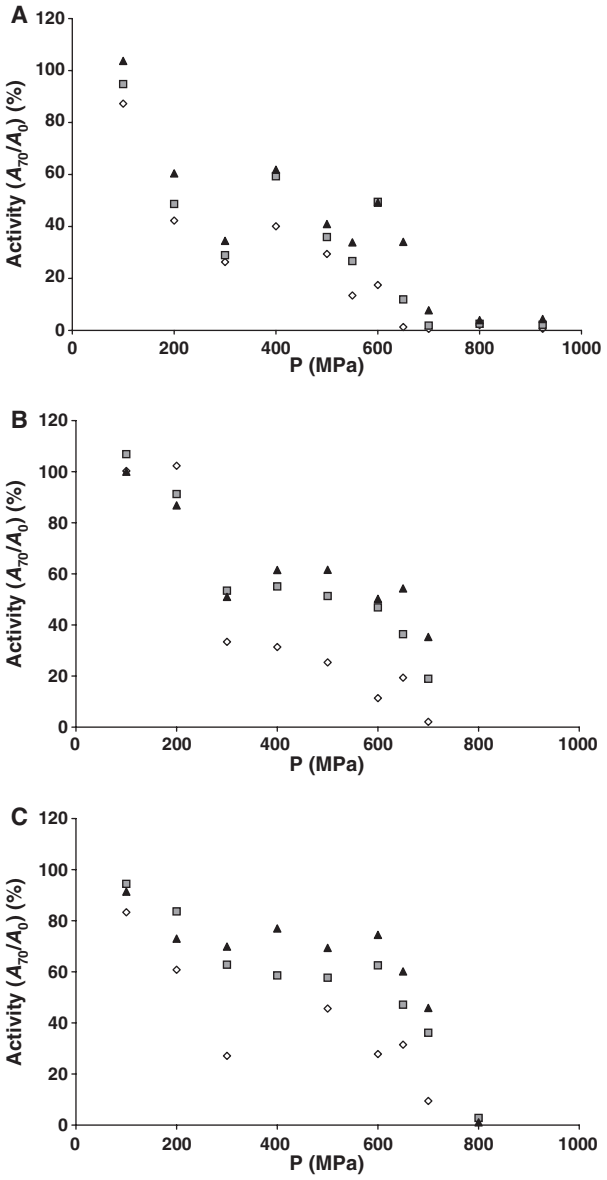

Fig. 2. Influence of pressure on the enzyme activity (A_{70}/A_0) of the β-glucosidase after 70 min of pressure treatment including pressurization. Temperatures used were 25 °C (A), 40 °C (B) and 60 °C (C) at pH 5.5 (◇), pH 6.0 (■) and pH 6.5 (▲).

be concluded that a higher temperature helps to stabilize the enzyme against pressure denaturation. Therefore, when fast denaturation occurs, as is the case in our experiments, enzyme stability should be compared to that of unpressurized samples.

The influence of pressure treatment on enzyme inactivation

The inactivation of β-glucosidase was measured after pressure release as a function of the incubation temperature, pH and pressure. Activity measurements were compared to those of untreated sample. A significant part of the inactivation took place at pressures ≤ 300 MPa (Fig. 2). At higher pressures, a plateau could be seen between 300 and 600 MPa, where no further enzyme inactivation occurred, suggesting the existence of a pressure intermediate. Pressure intermediates have also been reported for other proteins, e.g. lysozyme, ribonuclease, α-lactalbumin, apomyoglobin [12], tropomyosin [13] or synthetic proteins [14]. Hydrostatic pressure is increasingly being used in the study of protein folding, misfolding, aggregation and transitions. In comparison with other methods of denaturation, such as temperature or chemical agents, pressure induces more subtle changes in protein conformation, allowing the stabilization of partially folded states that are often not significantly populated under more drastic conditions [15]. The inactivation plateau that indicates an intermediate state was less clear at 25 °C. At pressures above 600 MPa, inactivation increased again. Complete inactivation occurred between 700 and 800 MPa.

Several of the samples from this experiment were also loaded on a native gel to detect possible dissociation or aggregation of the protein. On the gel (Fig. 3), one band was found for all samples; this band probably corresponded to the native enzyme. The samples that were treated at 600 and 700 MPa also showed a second band with lower mobility. This protein with higher molecular mass could be an aggregated form of the enzyme. Aggregation may therefore be a cause of inactivation at higher pressures. No dissociation into subunits was observed. One similar example from the literature showed that inactivation of the dimeric almond β-glucosidase was not a result of unfolding, dissociation or aggregation of the intact enzyme [16].

Influence of temperature on enzyme inactivation by pressure treatment

The influence of temperature on the inactivation of pressure-treated samples can be seen when comparing Fig. 2A–C, which show results obtained at different temperatures. Clearly, the enzyme is more pressure stable at higher temperatures. Comparison of the measurements made at pH 6.0 shows that after pressure treatment at 25 °C, 36% activity was left at 500 MPa, at 40 °C the same activity was still present at 650 MPa, and at 60 °C, 36% residual enzyme activity was found at 700 MPa. The maximum temperature for pressure stabilization may very well not have been reached; however, we were not able to use the high-pressure equipment at higher temperatures.

At 60 °C, the β-glucosidase from *Pyrococcus* is very piezostable as compared to other β-glucosidases. Almond β-glucosidase has a residual activity of only 20% after 1 h at 200 MPa and 60 °C [16]. The thermophilic β-glucosidase from *S. solfataricus* is more piezostable at 60 °C, with a 50% inactivation at 250 MPa and 60 °C [16]. Under these conditions, the residual enzyme activity of the *Pyrococcus* β-glucosidase is estimated to be about 70%, making it the most piezostable of these three enzymes at higher temperatures. At lower temperatures, however, pressure-assisted cold-induced changes in the structure cause denaturation and make the enzyme less stable, but still comparable to, for example, the almond β-glucosidase or the β-galactosidase from *Escherichia coli* [17].

Influence of pH on enzyme inactivation by pressure treatment

The pressure–temperature dependence of the pH of the Mes buffer (pH 6.0) was calculated in the relevant range for the enzyme inactivation experiments, using the equation of Elyanov & Hamann [18]. The temperature dependence of this buffer is -0.011 ΔpH unit·°C^{-1} [19], and the reaction volume (ΔV_0) is 3.9 cm^3·mol^{-1} [20]. At higher temperatures, the pH will decrease, and at higher pressures, it will decrease. The pH varies from 5.6 to 6.3 in the pressure–temperature plane of measurements for the inactivation studies when starting with a buffer of pH 6.0 at ambient conditions (for a graph of the pH of Mes buffer plotted as a function of pressure and temperature, see [10]).

From Fig. 2, we can conclude that the enzyme is more pressure stable at higher pH values over the whole pressure and temperature range used in the inactivation experiments. This is in agreement with previous inactivation measurements at atmospheric pressure and 95 °C [10]. Here, measurements were conducted as a function of time. A decrease in pH of 0.5 units caused the enzyme inactivation constant to increase by a factor of 2–3.

Temperature dependence of the FTIR spectra of β-glucosidase

FTIR spectroscopy was used to follow the thermally induced changes in the secondary structure of β-glucosidase from *P. furiosus*. As previous reports suggested that β-glucosidase unfolds at temperatures > 100 °C (at 0.1 MPa) [1,4], the heat denaturation was investigated using the variable-temperature cell, where a low pressure was applied to keep water in the liquid state. Figure 4 shows the effect of temperature on the deconvoluted amide I′ band (1600–1700 cm^{-1}), which is the conformationally most sensitive vibrational mode. At 25 °C, two peaks at 1654 and 1636 cm^{-1}, indicative of α-helix and β-sheet structures, respectively, can be observed [4]. As temperature increases, the native peaks disappear, and concomitantly one can observe the appearance of two peaks at 1618 and 1683 cm^{-1}, which are typical of the formation of an intermolecular antiparallel β-sheet aggregate [21].

The thermal stability of β-glucosidase was assessed by plotting the temperature dependence of the peak intensity at 1618 cm^{-1} (Fig. 4B). The melting point of the enzyme was estimated to be ∼ 122 °C at 50 MPa in Tris (pH 7.5), which is in close agreement with the value of 108 °C found in a sodium phosphate buffer (pH 6) at 0.3 MPa by differential scanning calorimetry [22]. Clearly, this enzyme from a hyperthermophile is more stable than those from mesophiles [16,17]. The downward trend above 127 °C is indicative of the dissociation of the aggregates at higher temperatures, as observed previously in the case of myoglobin and lysozyme [21,23].

Thermal stability up to 80 °C was also investigated at 200 and 400 MPa. Under these conditions, thermal unfolding could not be observed.

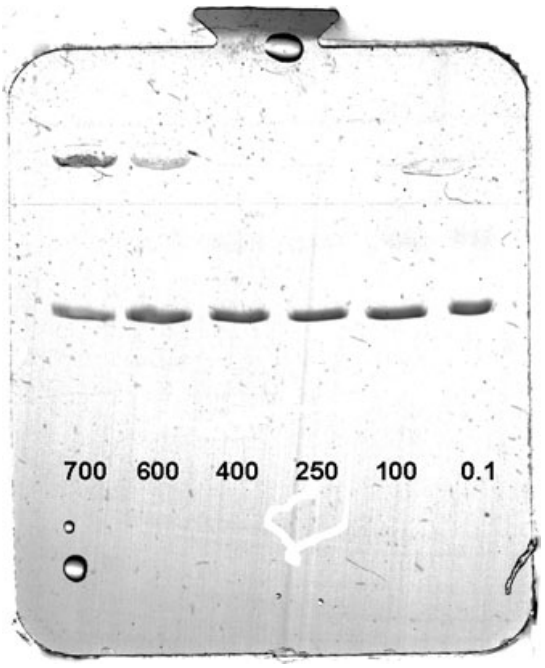

Fig. 3. Native gel electrophoresis of the pressure-treated enzyme. The pressure in MPa is given below the lanes.

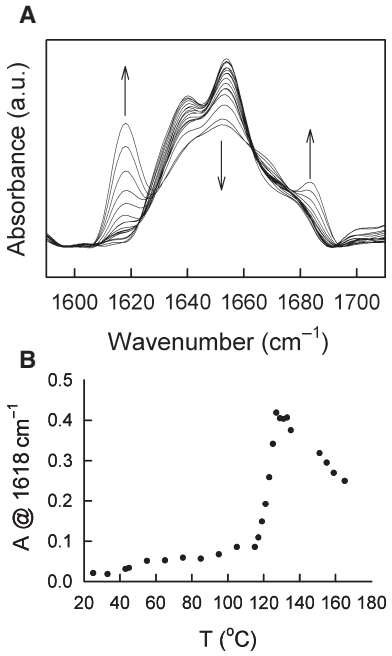

Fig. 4. The effect of increasing temperature from 25 °C up to 127 °C on the normalized deconvoluted amide I′ band of β-glucosidase at atmospheric pressure (A) with ΔA_{1618} at several temperatures (B). The arrows indicate the direction of the temperature-induced changes.

Pressure dependence of the FTIR spectra of β-glucosidase

To determine whether pressure inactivation is correlated with a conformational change, the secondary structure of β-glucosidase was also monitored by FTIR spectroscopy during compression. Figure 5 illustrates the conformational changes observed at different temperatures. The loss of the intensity at 1654 cm^{-1} is accompanied by an increase in absorbance around 1621 cm^{-1}. The latter peak can be attributed to the pressure-induced solvation of α-helices [24]. In addition, the band at $\sim$ 1.0 GPa in Fig. 5C does not resemble the broad, featureless band typical of an unfolded protein. Taken together, these observations suggest that the unfolding at the level of the secondary structure is incomplete, with the pressure-unfolded state having molten globule-like characteristics. Consistent with previous work, this pressure-unfolded state is highly aggregation prone at high temperatures, as evidenced by the appearance of the spectral bands at 1683 and 1618 cm^{-1} upon decompression (Fig. 5C) [25].

The changes in absorbance at 1654 cm^{-1} have been plotted as a function of pressure at different temperatures (Fig. 6). A cooperative transition can be seen at

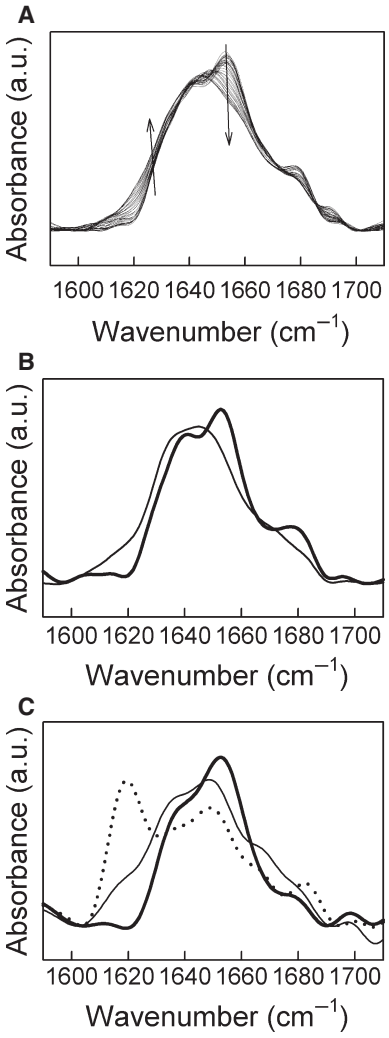

Fig. 5. Effect of pressure on the deconvoluted amide I′ band of β-glucosidase (A) at 10 °C, pressure range from 0.1 to 1.1 GPa, (B) at 30 °C at 0.1 MPa (solid line, bold) and at 740 MPa (solid line) and (C) at 85 °C at 0.1 MPa (solid line, bold), at 1.0 GPa (solid line) and after pressure release (dotted line). The arrows indicate the direction of the pressure-induced changes.

most temperatures. However, at the low and high ends of the temperature range investigated (10–105 °C), the change in absorption was very gradual and no clear transition was measured (Fig. 6A). A reduced cooperativity at low temperature was previously also observed for myoglobin [26]. It most likely reflects the fact that close to the low and high unfolding temperatures, the native state is already more heterogeneous. Hence, it was not possible to determine the pressure midpoint at these temperatures.

On the basis of the above results, a tentative pressure–temperature stability diagram can be drawn (Fig. 7). Note that this graph also includes the points

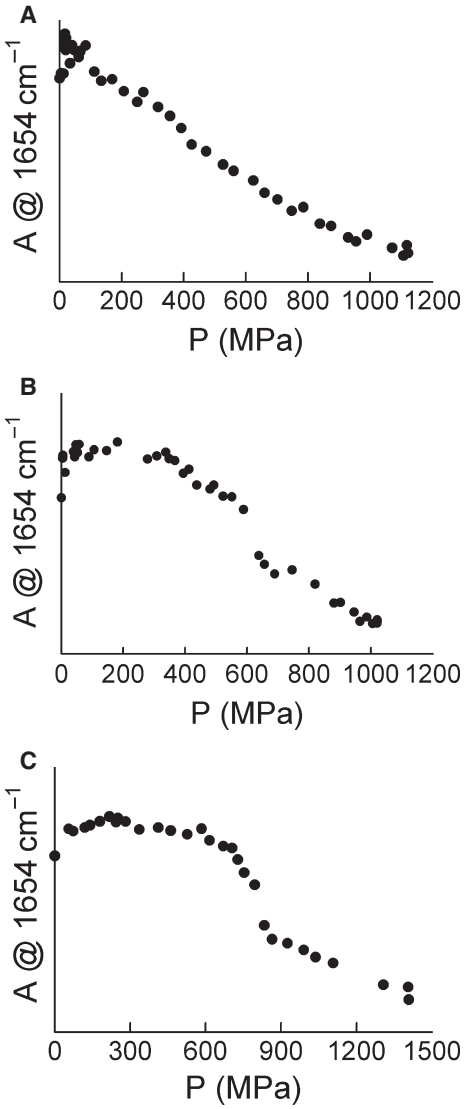

Fig. 6. Absorbance at 1654 cm^{-1} against pressure at (A) 10 °C, (B) 30 °C and (C) 85 °C.

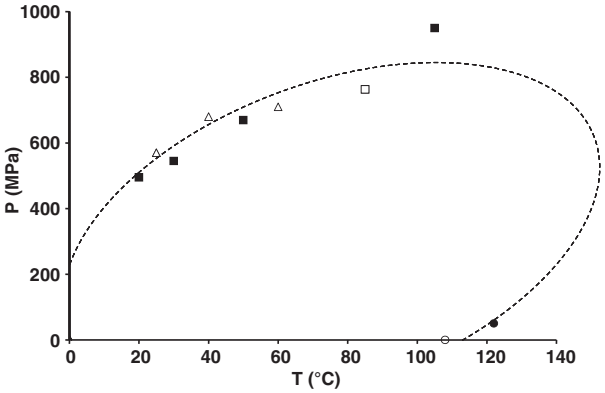

Fig. 7. Pressure–temperature diagram indicating the transition points. (△) Second transition estimated from the inactivation experiments; transition points based on A_{1654} (■) or A_{1618} (●) from the FTIR experiments; ○ corresponds to literature data [22]. Open symbols: measurements at pH 6 in Mes or phosphate buffer. Closed symbols: measurements in Tris buffer at pH 8. The solid line is a guide to the eye, assuming an ellipse.

determined from the inactivation measurements, as well as the melting point at 0.3 MPa taken from Bauer & Kelly [22]. The diagram shows that at low pressures, β-glucosidase is stabilized by pressure against thermal denaturation, which has also been observed for other glucosidases [27]. The enzyme becomes less pressure sensitive as temperature increases, with an optimum at 85 °C. Finally, the pressure at which the protein unfolds coincides with that at which the second transition in the inactivation measurements of Fig. 2 occurs. The absence of a transition at lower pressures suggests that the initial inactivation (100–400 MPa) is not correlated with a change in secondary structure. This is consistent with the finding that at pH 10 and 75 °C, the inactivation of β-glucosidase also does not involve any loss of secondary structure [1].

Conclusions

The stability of the β-glucosidase from the hyperthermophile *P. furiosus* was studied as a function of temperature, pressure and pH. As well as the expected high thermostability, the enzyme proved to be highly piezostable as well. This may be a more general feature of hyperthermophilic enzymes [5,28]. An increase in pH from 5.5 to 6.5 and possibly also higher values was also shown to have a positive effect on the stability of the enzyme.

A biochemical study by Ausili *et al.* on the hyperthermostability of the β-glucosidase from *P. furiosus* suggested that the enzyme is mainly stabilized by hydrophobic interactions, and that it has a very compact protein core with only a few, small internal cavities [4]. The absence of cavities is an important factor contributing to the pressure stability of the enzyme [29].

Another striking feature of *P. furiosus* β-glucosidase is its tetrameric structure, which has been observed for all hyperthermophilic members of family 1 β-glucosidases, whereas mesophilic and thermophilic family 1 enzymes are mainly active as monomers or dimers [3]. In the case of *P. furiosus* β-glucosidase, the subunit interfaces involve fewer electrostatic interactions such as salt bridges and ion pairs than in the case of the enzymes from the hyperthermophiles *S. solfataricus* and *Thermosphaera aggregans* [4]. Electrostatic interactions are known to be very pressure sensitive because

of the large volume change associated with the formation of a free charge [30]. Hence, a reduction in the number of electrostatic interactions would increase the pressure stability of the tetramer. Maintaining the tetrameric structure of β-glucosidase is therefore not only important for its temperature stability, but also contributes to the pressure stability. However, a subtle change in the oligomeric state of the enzyme, not causing dissociation or association, may have led to a lower active state of the enzyme at lower pressures (100–400 MPa). Also, a change in the active site and/or the substrate-binding site may have led to considerable inactivation at lower pressures (100–400 MPa) well before any conformational changes occurred.

Experimental procedures

Enzyme purification

The enzyme was prepared from a lysate of *E. coli* in which the *celB* gene encoding β-glucosidase from *P. furiosus* was cloned and expressed as described previously [31]. Briefly, the cell lysate was heated in order to denature proteins other than the hyperthermostable enzyme, and this was followed by an anion exchange chromatography step for further purification. The enzyme was then dialysed against 5 mM Mes (pH 6.0) and freeze-dried. For activity measurements, the enzyme was redissolved at 0.5 mg·mL^{-1} in 0.1 M Mes buffer (pH 6.0) at 25 °C and 0.1 MPa. FTIR spectroscopy experiments were performed in deuterated 0.1 M Mes buffer (pD 6.0) (measurement at 85 °C) or 0.05 M Tris/DCl buffer (pD 8.0) (all other conformational measurements), leading to final pD values of 6.4 and 7.5, respectively, and final protein contents of 100 and 50 mg·mL^{-1}, respectively.

Enzyme inactivation measurements

β-Glucosidase activity was assayed at atmospheric pressure using *p*-nitrophenyl-β-D-glucopyranoside as an artificial substrate. Ten microlitres of enzyme solution was added to a standard reaction mixture that was equilibrated at 80 °C to make a 1.0 mL solution of 2.0 mM *p*-nitrophenyl-β-D-glucopyranoside in 0.1 M Mes buffer (pH 6.0). The reaction was terminated after 10 min by addition of 1.0 mL of 1.0 M sodium bicarbonate. The increase in absorbance at 420 nm as a result of *p*-nitrophenol formation was measured spectrophotometrically.

Inactivation studies

The enzyme solution was diluted 120-fold in Mes buffer, pH 5.5, 6.0 or 6.5. Four hundred and fifty microlitres of enzyme solution was put in polyethylene bags and pressurized in a laboratory-scale multivessel high-pressure apparatus (Resato FPU 100-50; Resato International B.V., Roden, The Netherlands). The pressure vessels were pre-equilibrated to the desired temperature. A glycol mixture was used as pressure medium. One vessel contained three bags, to ensure similar treatment of samples with a different pH. The pressure was increased gradually (100 MPa·min^{-1}) to minimize any temperature increase due to adiabatic heating, but nevertheless, the temperature increased by 9–10 °C during the pressure build-up. Therefore, an equilibration period was taken into account to allow the temperature to reach its desired value, once the preset pressure was reached. At this point, the valves of the individual vessels were also closed, and the central circuit was decompressed. The total time of pressurization and equilibration was approximately 10 min. After that, at $t = t_{10}$, the first vessel was decompressed. After 1 h (t_{70}), another vessel was individually depressurized. All samples were immediately cooled in ice–water, and enzyme inactivation was measured within a few hours. The measured activities were that of the untreated blank sample (A_0), the pressurized blank sample (A_{10}) and the sample that was kept at a predefined pressure for 1 h (A_{70}).

The inactivation of β-glucosidase from *P. furiosus* was studied at pressures up to 900 MPa, at temperatures of 25, 40 and 60 °C, and at pH 5.5, 6.0 and 6.5.

Gel electrophoresis

To detect possible dissociation or aggregation of the protein, native PAGE was performed with samples prepared at 25 °C and different pressures, as described in the inactivation studies. These samples (A_{70}) were mixed 1 : 1 with electrophoresis buffer (10 mM Tris, pH 6.8, 2.5% bromophenol blue) and applied to the gel (8–25% gradient gel) for 25 min. The proteins on the gel were stained with Coomassie blue.

FTIR spectroscopy

Infrared spectra were recorded on a Bruker IFS66 FTIR spectrometer (Bruker, Karlsruhe, Germany) equipped with a liquid nitrogen-cooled mercury cadmium telluride detector at a nominal resolution of 2 cm^{-1}. Each spectrum is the average of 256 interferograms. Equilibration after pressure increase and measurement of the sample took approximately 10 min, leading to a pressure build-up of 250–300 MPa per hour. The sample compartment was continuously purged with dry air to minimize the spectral contribution of atmospheric water.

The pressure experiments were performed using a diamond anvil cell (DAC) [32]. The pressure stability was measured at various temperatures by adjusting the temperature of the water bath to which the DAC was connected. The sample temperature was monitored using a thermocouple located close to the diamonds. For pressure measurements at temperatures above 85 °C, a modified variable-temperature cell

(Graseby Specac, Orpington, UK) was used. In this set-up, the classic temperature cell is replaced by a DAC.

A baseline correction was performed in the amide I′ region (1600–1700 cm^{-1}), assuming a linear baseline. In order to enhance the component peaks contributing to the amide I′ band, the spectra were treated by Fourier self-deconvolution using the BRUKER software (OS/2 version). The line shape was assumed to be Lorentzian with a half-bandwidth of 21 cm^{-1}, and an enhancement factor k of 1.7 was used.

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