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Effect of soluble additives on enzyme thermo- and/or baro-deactivation

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Abstract

The influence of various physico-chemical parameters on *Kluyveromyces lactis* β-galactosidase stability was investigated. The combined effects of temperature, pressure and polyols as additive, on the enzyme stability at 25 and 45°C were studied, in the pressure range from 0.1 to 400 MPa, and for polyol concentration between 0 and 2 M. At 25°C, the stabilizing effect of polyols against pressure-induced denaturation increased with additive concentration, giving the following ranking of stabilization efficiency: erythritol > xylitol > sorbitol > glycerol > ethylene glycol, erythritol being the most effective agent, whatever the concentration. Moreover, with or without polyols, stabilization against thermal denaturation at 45°C due to the application of moderate pressures (< 150 MPa) was observed, in the range where pressure alone does not denature the enzyme. The stabilizing effect of ethylene glycol, glycerol, xylitol and sorbitol at atmospheric pressure against thermal deactivation was significant and increased with their concentration. However, erythritol, at atmospheric pressure, was a strong destabilising agent at 45°C. But when the pressure was increased, considerable stabilization was observed. Indeed, erythritol was the most effective agent at 2 M and 350 MPa against thermal denaturation at 45°C. The application of high hydrostatic pressure, in the range where pressure alone does not inactivate *K. lactis* β-galactosidase, allows to further increase the level of stabilization achieved at atmospheric pressure and 45°C, owing to polyols.

Keywords: β-Galactosidase; High pressure; Temperature; Polyols; Deactivation; Stabilization

1. Introduction

Kluyveromyces lactis β-galactosidase is, among other things, a biocatalyst of standard usage in the food industry to hydrolyse lactose [1], for the preparation of low-lactose dairy products for people suffering lactose intolerance, and for the preparation of new foods and dairy products [2]. Enzymes, such as β-galactosidase, can be denatured by several physical means such as temperature or pressure. Knowledge of the processes occurring in enzymes under high pressure and temperature is of immediate relevance and importance in biochemistry, because of the various advantages which will arise by carrying out enzymatic reactions in non-conventional conditions, such as changes in enzyme selectivity or increased rates of reaction. The study of the effect of high pressure on proteins has received considerable attention in recent years [3,4]. Detailed studies on the behaviour of proteins and other food constituents under high level of pressure and/or temperature

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may help to find new applications for the food industry [5]. Since high hydrostatic pressure and temperature exert antagonistic effects on weak intra- and intermolecular interactions of proteins, the stability of enzymes will be affected differently by these two parameters [6,7]. The antagonistic effects of pressure and temperature on biochemical systems are a consequence of both Le Chatelier's and the microscopic ordering principles, the latter establishing that increased temperature leads to disorder, increased pressure to order [8]. Hawley [9] formulated this antagonistic behaviour giving phase diagrams for protein denaturation. It has recently been proposed that high hydrostatic pressure may modulate both the activity and stability of several enzymes leading to improved applications for them [10,11]. It has been also shown by Athès et al. [12] that high pressure (>200 MPa) or high temperature (>45°C) can both induce an irreversible inactivation of the β-galactosidases of *Aspergillus oryzae*, *K. lactis* and *Escherichia coli*, but that moderate pressures (50–200 MPa) exerted a protective effect against thermal inactivation for the three β-galactosidases investigated. The behaviour of enzymes under pressure is further complicated by the wide range of experimental parameters that may influence pressure effects, such as temperature, pH, ionic strength and solvent composition [13].

Considering these observations, and in order to further elucidate the mechanisms of the action of pressure and temperature on proteins, we have studied the effects of combined pressure–temperature treatments on *K. lactis* β-galactosidase stability. Moreover, in addition to temperature and pressure treatments, we used polyols as soluble additives, to gain information by perturbing the system, i.e., by varying the physico-chemical properties of the medium. Weemaes et al. [14] have observed that the pressure and/or thermal stability of mushroom polyphenoloxidase can be changed by intrinsic factors such as additives. We have chosen polyols, additives known to enhance enzyme thermostability [15,16]. The main goal of the present work is to study the influence of various physico-chemical parameters on *K. lactis* β-galactosidase stability. To this effect, the combined effects of temperature, pressure and polyols as additives on the enzyme stability were investigated at 25°C and 45°C, in the pressure range from 0.1 to 400 MPa, and for polyol concentration between 0 and 2 M.

2. Experimental

2.1. Materials

2.1.1. Enzyme

K. lactis β-galactosidase (β-D-galactoside galactohydrolase or lactase, EC 3.2.1.23), Maxilact LX-5000, a purified industrial liquid β-galactosidase preparation, was kindly donated by Gist-Brocades (Seclin, France). Its purity was checked by electrophoresis and it was shown to be a dimer (molecular weight of 200,000), composed of two identical subunits. It is a glycoprotein with 45% carbohydrate (w/w) [17]. The protein content of the liquid preparation, determined by the Lowry method [18] with bovine serum albumin (Sigma) as standard, was 54.9 g of protein l^{-1}. The enzyme concentration is expressed as the mass unit of protein per volume unit. In all the experiments, the β-galactosidase was dissolved at 5 g of Maxilact l^{-1}, that is to say, 0.24 g of protein l^{-1}, in potassium phosphate buffer 0.01 M pH 7.3 with or without additives. The phosphate buffer, in spite of its pH variation with pressure, was preferred to the classical Tris buffer because the last strongly inhibits β-galactosidase activity. In addition, the Tris buffer has an additional destabilising effect on the enzyme.

2.1.2. Additives

The five polyols, ethylene glycol, glycerol, erythritol (Prolabo), xylitol (Merck) and sorbitol (Sigma), were used as additives without further purification. All remaining chemicals were of analytical grade.

2.1.3. High pressure device

The experimental apparatus for high hydrostatic pressure experiments was made up of a 700-MPa handscrew pump (Top Industrie, Vaux le Pénil, France) and a home-made thermostated high pressure vessel (internal volume of 2 ml). The pressure cell (0.1–700 MPa) was made of Marval X12 steel surrounded by a mantle through which the thermostating fluid was pumped. The sample was placed in a cylindrical 0.7 ml capacity glass tube which was closed by a parafilm®. The pressure vector was water. Pressure was checked using a pressure gauge (Top Industrie).

2.2. Methods

2.2.1. Combined temperature–pressure treatment in the presence of additives

Samples of enzyme solutions in the 0.7-ml capacity tubes were pressurised in the high pressure vessel thermostated at 25 or 45°C. The time needed to put the sample in the vessel and to pressurise was fixed at 1 min, and it also takes 1 min to depressurise, remove the sample out of the vessel, and rapidly cool it in ice water. Pressure was increased steadily up to the required value, in the range 0.1 to 400 MPa, and maintained for 40 min or different periods of time, for the determination of half-lives without additive. We have verified that the variation of pH induced by the addition of polyols and by an increase in pressure do not themselves affect β-galactosidase stability. For each experiment, control enzyme solutions were kept at the same temperature and atmospheric pressure. The residual activity was expressed as the ratio of the activity of the treated sample to that of the control one.

2.2.2. β-Galactosidase activity assay

The β-galactosidase activity was assayed at 37°C with respect to its artificial substrate *o*-nitrophenyl-β-D-galactopyranoside (oNPG, Sigma). oNPG (2 ml) at 2 g l^{-1} (6.6 mM) in 0.01 M potassium phosphate buffer pH 7.3 was added to 50 μl of enzyme solution and the developed colour was measured for 1 min at 420 nm with a Diode-Array spectrophotometer (Hewlett Packard 8452A, Waldbronn, Germany). The molar extinction coefficient of *o*-nitrophenol under these conditions was 3150 M^{-1} cm^{-1}. Activities were calculated by linear regression of the absorbance vs. reaction time. The irreversibility of β-galactosidase inactivation during the storage time periods after pressure treatment was ascertained (no recovery of activity was observed) and activity was measured four times for each sample. It has been checked that the polyols do not themselves decrease the enzyme stability.

3. Results and discussion

3.1. Influence of polyols on pressure denaturation at 25°C

Choosing the residual activity of enzyme as a criterion for enzyme stability, the effect of different polyols on β-galactosidase barostability at 25°C was investigated. The enzymatic activity after the release of pressure was measured as a function of pressure, in the 0.1–400 MPa range, for a treatment of 40 min at 25°C. Fig. 1

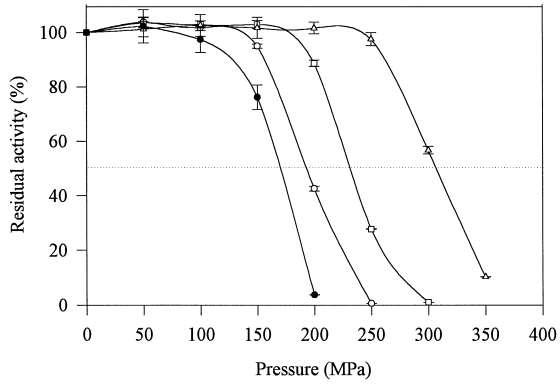

Fig. 1. Residual enzyme activity (measurements taken after return to standard conditions) of *K. lactis* β-galactosidase after 40 min of incubation under pressure, at 25°C in the absence of additive (●) and in the presence of sorbitol 0.5 M (○), 1 M (□) and 2 M (△). Residual enzyme activity is expressed as a percentage of the initial enzyme activity before pressure inactivation.

gives the results obtained in the presence of three concentrations of sorbitol (0.5, 1 and 2 M). Sorbitol has a positive effect on the resistance to pressure deactivation, rising with its concentration. Without additive, a significant decrease in catalytic activity of β-galactosidase took place above 100 MPa. With sorbitol (0.5 M) the decrease was observed at 200 MPa. With sorbitol (2 M), the threshold of this loss of activity is over 300 MPa. Therefore, there is a shift of the inactivation curves towards high pressures when sorbitol concentration varies from 0.5 to 2 M.

The influence of different polyols containing two to six carbon atoms (ethylene glycol (C2), glycerol (C3), erythritol (C4), xylitol (C5) and sorbitol (C6)) on pressure deactivation was further studied. For each polyol, we have obtained the same kind of plot (results not shown), and from these curves, we have estimated the pressures of half deactivation ($P_{1/2}$), defined as the approximate pressure needed to reduce the initial activity to 50% of its value, for a treatment time of 40 min. Table 1 shows these $P_{1/2}$ values obtained for each polyol.

With all these polyols there is a shift to high pressures when increasing concentration, and the shapes of the curves are identical (not shown), as for sorbitol (Fig. 1). From Table 1, we confirm that the stabilizing effect of polyols against pressure denaturation increases with additive concentration, up to a concentration of 2 M. The following ranking of stabilization efficiency of the five polyols is obtained, whatever the concentration.

erythritol > xylitol > sorbitol > glycerol > ethyleneglycol.

The enhancement of the barostability is not proportional to the molecular size of the additive, as is the case in classical thermostability studies with other enzymes [19]. The protective effects of polyols against β-galactosidase pressure denaturation at 25°C have been studied into all the details in a work by Athès and Combes [20].

Table 1
Pressures of half deactivation ($P_{1/2}$ in MPa) of *K. lactis* β-galactosidase, for a 40-min high pressure treatment at 25°C and for different concentrations of polyols

	Ethylene glycol	Glycerol	Erythritol	Xylitol	Sorbitol
0	170	170	170	170	170
0.5 M	180	200	270	220	190
1 M	200	220	310	270	230
2 M	230	270	380	325	310

Each pressure is determined with a maximum standard deviation of 10 MPa.

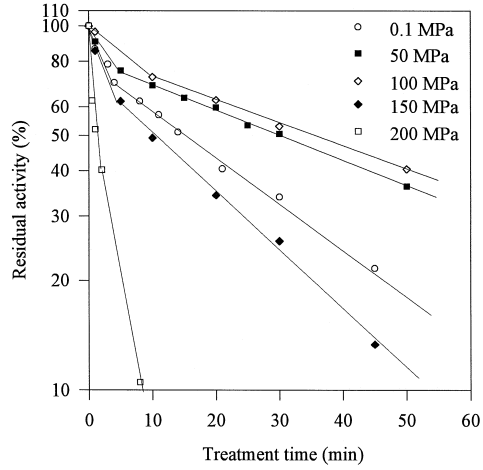

Fig. 2. Influence of pressure on the inactivation kinetics at 45°C of *K. lactis* β-galactosidase. Logarithmic variation of residual enzyme activity (determined with respect to control sample stored at 25°C at atmospheric pressure) as a function of the incubation time. Activity measurements were taken after return to standard conditions.

3.2. Determination of β-galactosidase half-lives at 45°C and different pressures without additives

In contrast to the possibility of denaturing enzymes, a stabilizing effect of high pressure against thermal inactivation has been reported for several enzymes [11], so the influence of pressure on the thermal inactivation kinetics at 45°C of β-galactosidase was investigated. The results obtained are reported in Fig. 2. The logarithmic plot of the residual activities vs.

time indicates biphasic (two slope) kinetics. This kind of plot leads to the estimation of half-lives ($t_{1/2}$: time needed to reduce the initial activity to 50% of its value), directly from the kinetics, by a linear fit to the data represented on a semi-log plot, generally after the inflection of the curves. The biphasic deactivations are not surprising, considering the complexity of an enzyme molecule. Indeed, the fractional rate of decrease in specific activity of *K. lactis* β-galactosidase has been previously shown to follow a biphasic model [21]. The rates of inactivation observed on Fig. 2 at 45°C were strongly dependent on pressure. To estimate the stabilizing effect of pressure against thermal inactivation at moderate pressures, half-lives have been determined as a function of pressure and are reported in Table 2. The observed stabilization appeared to be maximal at 100 MPa, where the half-life at 45°C was 36 min, whereas it was only 15 min at atmospheric pressure and 11 min at 150 MPa. The pressure range of stabilization against thermal denaturation corresponds to the range in which pressure alone does not lead to a significant inactivation. These results are qualitatively consistent with the different effects of pressure vs. temperature on other proteins, which result in a thermodynamic stability diagram presenting an elliptical contour [9,22]. The elliptical outline observed demonstrates that proteins could be successively denatured and reactivated when increasing pressure at a fixed temperature. This could explain the effects of pressures values of 50 and 100 MPa against thermal inactivation of β-galactosidase at 45°C. A similar behaviour was reported for several enzymes [23,24]. It remains unclear whether stabilization against thermal inactivation is a general phenomenon with mesophilic enzymes: high pressure did not exert any protective effect on the *Saccharomyces cerevisiae* α-glycosidase whereas the thermal inactivation at 30°C of carboxypeptidase A from bovine liver was virtually completely prevented at 200 MPa [10]. The stabilizing effect observed with *K. lactis* β-galactosidase would then not be restricted to a limited number of enzymes.

Table 2
Influence of pressure on the half-life ($t_{1/2}$) at 45°C of *K. lactis* β-galactosidase

Pressure (MPa)	0.1	50	100	150	200
$t_{1/2}$ at 45°C (min)	15 ± 1	30 ± 1	36 ± 2	11 ± 2	1 ± 0.5

In a review about pressure effects on enzyme activity and stability at high temperatures, Michels et al. [25] report that pressures greater than about 300 MPa generally cause enzyme inactivation, whereas pressures less than about 200 MPa can either stabilise or destabilise proteins. In the case of *K. lactis* β-galactosidase, the same phenomenon is observed, but because of the specific enzyme stability, there is a significant discrepancy in the pressure range, inactivation occurring from 150 MPa instead of the previously reported value of 300 MPa. Comparison of the data for thermal, high pressure, and combined pressure–temperature denaturation of *K. lactis* β-galactosidase revealed that the thermal stability at 45°C of the enzyme can be enhanced by application of pressures up to 100 MPa.

3.3. Influence of polyols and pressure on thermal denaturation at 45°C

The effect of polyols (ethylene glycol, glycerol, erythritol, xylitol, and sorbitol) on β-galactosidase stability vs. combined temperature–pressure treatments was investigated. The enzymatic activity was measured as a function of pressure, after return to standard conditions, for a treatment of 40 min at 45°C. Fig. 3 gives the results obtained with sorbitol 0.5, 1 and 2 M. At atmospheric pressure, sorbitol has a positive effect on the resistance to thermal denaturation, which is enhanced when the concentration is increased. So, the use of sorbitol enables β-galactosidase to be protected against thermal denaturation at 45°C, without pressure application. When the pressure is increased (up to 150 MPa) a protective effect against thermal denatu-

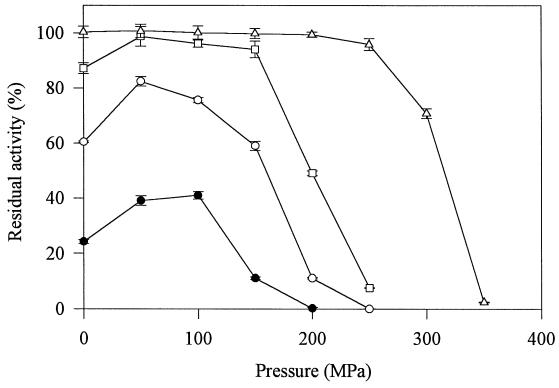

Fig. 3. Residual enzyme activity (measurements taken after return to standard conditions) of *K. lactis* β-galactosidase after 40 min of incubation under pressure, at 45°C in the absence of additive (●) and in the presence of sorbitol 0.5 M (○), 1 M (□) and 2 M (△).

ration was observed with 0.5 and 1 M sorbitol. With sorbitol (2 M), the loss of activity is encountered only beyond 300 MPa. The use of sorbitol enables the thermal and pressure stability to be raised in tandem, and the shape of the pressure stabilization curve with sorbitol is quite similar to the one obtained without additive.

Fig. 4 gives the results obtained with erythritol 0.5, 1 and 2 M. Erythritol, whatever the concentration, exhibits a destabilising effect at atmospheric pressure on β-galactosidase, leading to a complete loss of enzyme activity after a 40-min treatment at 45°C. While this additive was the most effective in preventing pressure deactivation at 25°C, it is a strong destabilising agent when the temperature is increased to 45°C. However, when the pressure increases, in the range where pressure alone does not affect enzyme stability, one may observe an enhancement of enzyme thermal stability at 45°C. The curves obtained shift to higher pressures when erythritol concentration is increased from 0 to 2 M. This shift could be explained by the enhancement of pressure stability when a more concentrated polyol solution is used, a phenomenon already evident from Table 1. Application of pressure in the presence of erythritol leads to a spectacular increase in thermal stability, especially with erythritol (2 M), with a stabilization maximum at 250 MPa. The residual activity reaches a value of 70% while it was almost zero at atmospheric pressure. With erythritol 0.5 M and 1 M, the stabilization maximum occurs at 150 and 200 MPa, respectively. We have thus shown that the maximal stability level achieved at atmospheric pressure with sorbitol and erythritol can be further increased by applying high hydrostatic pressure, up to a critical value at which pressure denaturation of enzyme occurs. As observed in Fig. 2, moderate pressures exert a protective effect against thermal inactivation at 45°C irrespective of the presence or absence of additives for a 40-min treatment, as shown for sorbitol and erythritol in Figs. 3 and 4. Moreover, the range where pressure stabilization occurs varies depending on the polyol used and on its concentration.

Fig. 5 is plotted to compare the different effects of the five polyols. It shows the variation in β-galactosidase residual activity as a function of pressure after a 40-min treatment at 45°C in the presence of polyols at a concentration of 0.5 M (A) or 2 M (B). The use of additives, such as ethylene glycol, glycerol, xylitol and sorbitol, enables β-galactosidase to be protected against thermal denaturation at 45°C, without pressure application. This protective effect of polyols increases with their concentration, from 0.5 to 2

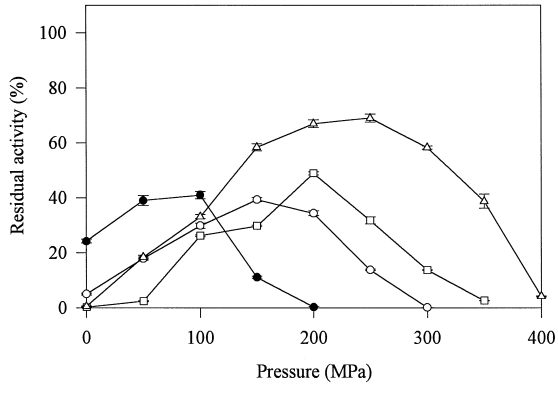

Fig. 4. Residual enzyme activity (measurements taken after return to standard conditions) of *K. lactis* β-galactosidase after 40 min of incubation under pressure, at 45°C in the absence of additive (●) and in the presence of erythritol 0.5 M (○), 1 M (□) and 2 M (△).

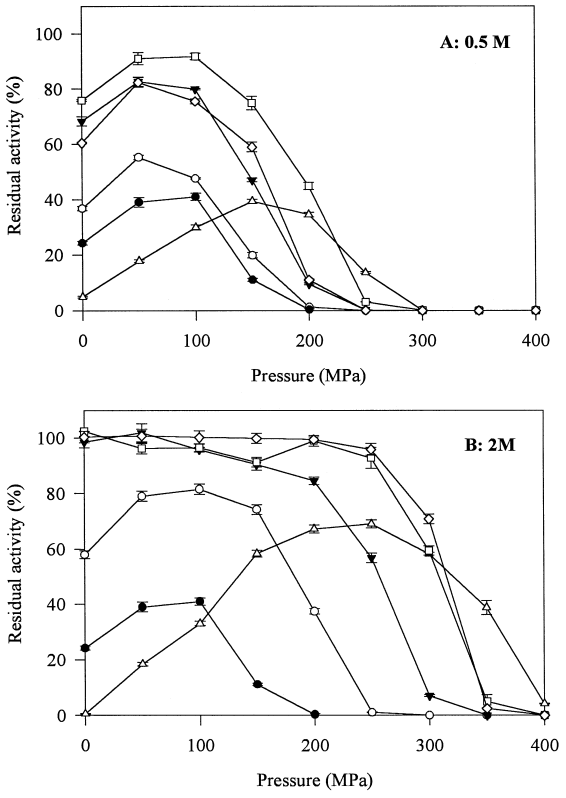

Fig. 5. Residual enzyme activity (measurements taken after return to standard conditions) of *K. lactis* β-galactosidase after 40 min of incubation under pressure, at 45°C in the absence of additive (●) and for a polyol concentration of 0.5 M (A) or 2 M (B): ethylene glycol (○), glycerol (▼), erythritol (△), xylitol (□), and sorbitol (◇).

M. Erythritol is the exception, being a destabilising agent whatever the concentration, at 45°C and atmospheric pressure. In our case, the thermostability does not increase proportionally with the size of the polyols as in classical thermostability studies [26], because of the particular behaviour of erythritol. The behaviour of ethylene glycol, glycerol, xylitol and sorbitol falls within the same kind of pressure stabilization curves. For a 0.5-M concentration (Fig. 5A), these four polyols lead to a stabilization maximum due to pressure between 50 and 100 MPa. At 0.5 M, the use of xylitol offers the best performance, but beyond 150 MPa, pressure and temperature effects are added, as for the three other polyols, leading to denaturation. For a 2-M concentration (Fig. 5B), xylitol and sorbitol allows 100% enzyme recovery of residual activity at atmospheric pressure, and no significant deactivation occurs below 300 MPa. With glycerol, the behaviour is similar up to 150 MPa and the loss of activity occurs beyond 200 MPa. For ethylene glycol, the stabilization maximum due to pressure reaches 80% of residual activity at 100 MPa. Erythritol, for a 2 M solution, is less effective than the other polyols up to 100 MPa, but from 200 to 400 MPa, its stabilizing effect increases and by 350 MPa it is the most effective agent. This is consistent with its action on barostability at 25°C. Indeed, erythritol was the most effective agent against pressure denaturation (Table 1).

The beneficial effect of increased pressure on enzyme thermostability at moderate pressure (where moderate is defined with respect to the denaturing pressure range for *K. lactis* β-galactosidase) is therefore reversed at higher pressures, at a threshold which depends on the additive used and its concentration.

The observed increase in enzyme thermal stability due to pressure and additives could partly be explained by a preservation of the protein hydration at the surface of the native globule, fixing in that way the protein structure. The thermal denaturation of enzyme in an aqueous solution proceeds generally via the exposure of hydrophobic parts of the protein in water [27], that give rise to unfolding, and is certainly accompanied by a loss of a number of essential water molecules, leading to irreversible inactivation due to wrong structural rearrangements. The effect of pressure and polyols seems adverse to this tendency. Indeed, application of pressure may fortify the enzyme hydration shell and is directed toward preferential hydration of the protein [11], through a favourable effect of pressure on solvation of both charged and nonpolar groups [3]. In the same way, the polyol molecules may be preferentially excluded from the surface layer of the protein molecule [28] and the water shell around the enzyme is preserved, that is, the conformation of the protein

becomes more rigid [16]. The osmotic stress created by the presence of polyols is perhaps also the origin of the preservation of the native conformation of our enzyme, through its preferential hydration. Having regard to our results, the action of polyols may be in part specific, because of their particular affinity for the enzyme. In a medium with three components (water–protein–polyol), interactions occurring between the polyol and the protein, leading to the displacement of some water molecules surrounding the protein molecule, will be a function of protein affinity for the additive. The interaction of polyols with the active site of the enzyme has also been considered as one of the factors explaining the stabilizing effect of polyols [19]. In the case of erythritol particularly, which has a high affinity for *K. lactis* β-galactosidase as a competitive inhibitor (data not shown), it can be assumed that the binding of the additive molecule to the surface of the protein requires that water molecules be displaced. Erythritol has a K_I of about 15 mM, more than 10 times lower than the K_I of the other polyols (from 100 to 500 mM), so has the higher affinity for β-galactosidase (found experimentally, data not shown). The preferential binding of erythritol seems to favour the denaturing effect of temperature at atmospheric pressure, while it permits to obtain a stable conformation of enzyme molecule under high hydrostatic pressure. Beyond a pressure threshold, which depends on the polyol used and on its concentration, the effects of pressure and temperature on enzyme activity reinforce each other, leading to a rapid deactivation of the enzyme. Indeed, without stabilizing soluble additives, pressures higher than 150 MPa cause non-reversible and more extensive effects on β-galactosidase.

So, high pressure, up to a certain value, probably enables the unfolding of the enzyme molecule, due to temperature effects, to be avoided and, in common with most of the polyols, to fortify the hydration shell of the protein. The possibility of stabilizing β-galactosidase against thermal inactivation by maintaining it at high pressure and/or in the presence of polyols would be of great interest for biotechnological applications as many industrial processes require elevated temperatures. Moreover, high pressure could be used to carry out new β-galactosidase-catalyzed reactions at higher temperatures.

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