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Effect of small carbohydrates on the catalytic activity of a protease and two glycohydrolases

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Abstract

The effect of sugars and polyols on the kinetic behavior of a protease, thermolysin from *Bacillus thermoproteolyticus*, and two glycohydrolases, pullulanase from *Bacillus* sp. 3183 and inulinase from *Aspergillus niger* was studied. Kinetic parameters, especially the catalytic efficiency (k_{cat}/k_M) of enzymes were determined in buffer and in the presence of small carbohydrate additives. It was shown that the catalytic efficiencies of the studied enzymes depend on both the nature and the concentration of the additive. Thermolysin was found to be more activated by sugars and polyols than pullulanase or inulinase. This implies a specific effect of the additive besides its effect on the enzyme micro-environment.

Activation free energy of the thermolysin catalyzed reaction in the presence of additives was found to be lower than in buffer medium. As temperature was kept constant, the activation energy change is assignable to entropic change. Such an effect, mainly responsible for the activation of the enzyme by the micro-environment, is called *solvactivation*. In turn, the lowering of catalytic efficiency (below the level obtained with buffer) by such additives as glycerol is called *solvinhibition.*

An infrared spectroscopy study of thermolysin in the presence of additives showed that no conformational change is observed and that small carbohydrates contribute to preferential hydration of the enzyme. Raman spectra showed that no direct interaction of thermolysin was observed in the presence of glycerol and that the secondary structure of the enzyme remained unchanged.

It was possible to differentiate the effects of small carbohydrates on the studied enzymes. The specific activating or inhibiting effect seems linked to the effect of these solutes on the water structure.

Keywords: Small carbohydrates; Modified enzymatic reaction medium; Activation energy; Catalytic efficiency; Solvactivation of enzyme; Solvinhibition of enzyme

1. Introduction

Adding highly soluble carbohydrates like polyols or simple sugars to enzymatic reaction media, decreases water activity and modifies the physicochemical properties. These additives were also found relevant as regards the thermal stability of enzymes (Germain, Slagmolen & Crichton, 1989; Asther & Meunier, 1990). Although the subject of thermal stability is well documented, there is no sound interpretation of why and how an enzyme is stabilized by a specific additive. Nevertheless, from these studies, it arises that the increase in the thermal stability of proteins comes from the increasing of the rigidity of their tertiary structure (Germain et al., 1989). The effect of additives on the catalytic behavior of enzymes is relatively less studied than that concerning thermostabilization. Previous works (Larreta-Garde, Xu, Lamy, Mathlouthi & Thomas, 1988; Lamy, Portmann, Mathlouthi & Larreta-Garde, 1990; Inouye, Kuzuya & Tonomura, 1994; Inouye, Lee & Tonomura, 1996; Inagaki, Tadasa & Kayahara, 1994, 1995; Almarsson & Klibanov, 1996) have observed qualitative and quantitative changes in the enzymic activity when additives like salts, sugars, polyols and alcohols are present in reaction medium. These works have been limited to kinetic studies and little molecular information about the mechanism by which the additives modify the catalytic behavior of enzymes was reported.

In some cases, the presence of the additive decreases the catalytic efficiency (k_{cat}/k_M) by decreasing the catalytic constant (k_{cat}) and increasing the Michaelis constant (k_M). This suggests that the additive acts as an inhibitor of the enzymatic reaction. In other cases, where there is an increase in efficiency (k_{cat}/k_M), the additive is considered as an activator. We have recently (Mejri, Pauthe, Larreta-Garde & Mathlouthi, 1998) studied the effect of sugars and polyols on thermolysin activity and found an agreement with Dewar (1986) that these additives act as *solvactivators*

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or *solvinhibitors* depending on their effect on the catalytic efficiency of thermolysin.

We now report a kinetic comparative study on the effect of small carbohydrates on the activities of three hydrolases. This effect depends not only on the sugar and its concentration but also on the enzyme. Investigation at the molecular level was carried out using vibrational spectroscopy. The effect of carbohydrate additives on enzyme hydration and conformation is discussed.

2. Experimental

2.1. Enzymes assay

The enzymes studied are thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus* Rokko, pullulanase (EC 3.2.1.41) from *Bacillus* sp. 3183 and inulinase (EC 3.2.1.7) from *Aspergillus niger*.

2.1.1. Thermolysin activity

The peptidasic activity of thermolysin (Sigma, Protease Type X, 60 U/mg of protein) was measured using *N*-(3-[2-furyl] acryloyl)-Gly-leu amide (Sigma) as substrate, in the presence of sucrose, trehalose, maltose, D-fructose, D-glucose, D-mannose, sorbitol, maltitol, mannitol and glycerol (Sigma). The complete reaction procedure was previously described (Mejri et al., 1998). A nonlinear regression method based on the Michaelis–Menten equation was used to determine thermolysin kinetic parameters.

2.1.2. Pullulanase activity

The activity of pullulanase (Fluka, 1.6 U/mg of protein) was measured on pullulan (Fluka), by determining the amount of released maltotriose using the Somogyi–Nelson method (Nelson, 1944; Somogyi, 1952) for the quantification of reducing sugars.

The reaction was carried out in a 50 mM sodium citrate buffer (pH 5) and in additive modified media at 40°C. Glycerol, sorbitol, maltitol, sucrose and trehalose (Sigma) were used as additives in the reaction media. Kinetic parameters were determined using Lineweaver–Burk plots.

2.1.3. Inulinase activity

The activity of inulinase (Fluka, 17 U/mg of protein) was measured on chicory inulin (Sigma), by determining the amount of released reducing sugars using the Somogyi–Nelson method.

The reaction was carried out in a 10 mM sodium acetate buffer (pH 4.1) and in additive modified media at 37°C. Glycerol, sorbitol, maltitol and trehalose (Sigma) were used as additives in the reaction media and Lineweaver–Burk plots were used to determine inulinase kinetic parameters.

2.2. FT-IR spectra

Infrared spectra (200 scans) of enzymes in buffer (80 mg/ml) and in additive modified media were recorded with a resolution of $4\,cm^{-1}$ using a Nicolet® infrared spectrophotometer (Model 'Impact 410') equipped with ZnSe horizontal attenuated total reflectance (ATR) cell.

2.3. Raman spectra

Raman spectra of thermolysin in buffer (2 mg/ml) and in 5 M (460 g/l) glycerol solution were recorded on a double monochromator PHO (CODERG) spectrometer in the frequency region $500–1800\,cm^{-1}$ (HELLMA cell, $10 \times 10\,mm^2$). An argon ion laser (Coherent Radiation Model Innova) at 800 mW power for 488 nm excitation radiation was used. Data accumulation of 200 independent scans with time averaging was used to obtain the spectra and improve the signal-to-noise ratio. The treated Raman spectra were obtained following the same procedure as described by Pourplanche, Lambert, Berjot, Marx, Chopard, Alix et al. (1994). The deconvolution of the different regions of the spectra into their different composing bands was obtained using the GRAMS 32 software (Galactic Industries Corporation, USA).

The secondary structure of the enzyme was determined by the decomposition of the amide I band (C=O stretching mode of the peptidic bonds, $1630–1700\,cm^{-1}$) according to the method proposed by Alix, Berjot and Marx (1985). The principal component regression (PCR) and the partial least squares (PLS) statistical methods were applied using the PLSplus/IQ™ software (Galactic Industries Corporation, USA).

The region assigned to the skeletal vibrational mode of the polypeptidic backbone ($990–890\,cm^{-1}$), and the bands of the spectra arising from the tyrosine residues (880, $830\,cm^{-1}$) and from the aliphatic side chains (CH_2 at $1465 \pm 20\,cm^{-1}$ and CH_3 at $1450 \pm 20\,cm^{-1}$) constitute strong markers of the protein conformational change.

Thermolysin is a metalloprotease with catalytic zinc chelated to two histidine residues (Colman, Jansonius & Mathews, 1972). Bands arising from such residues were readily identified (1266, 1287 and $1508\,cm^{-1}$) and their environment was thus explored.

2.4. Determination of the activation energy

Arrhenius plots were used to quantify the thermolysin activation energy by additives. Reactions were performed in buffer and in modified media at a range of temperatures varying between 20 and 50°C.

3. Results and discussion

3.1. Kinetic results

To study the kinetic effects of small carbohydrates on the activities of hydrolases, we determined kinetic parameters of enzymes in buffer and additives modified media with a

Table 1
Optimal activating concentration, maximum catalytic efficiency and the corresponding activation percentage for sugars and polyols used in thermolysin activation experiments

Additive	Concentration for maximum activation (g/l)	Maximum efficiency ($mM^{-1}\ s^{-1}$)	Activation (%)
Buffer		5.50	100
Sucrose	340	16.70	300
Trehalose	340	17.17	312
Maltose	450	10.00	180
Glucose	50	12.00	218
Fructose	180	9.00	163
Mannose	220	10.00	180
Sorbitol	150	8.85	160
Mannitol	120	10.20	185
Maltitol	150	13.00	236
Glycerol	40	6.70	120

range of carbohydrate concentrations varying between 0 and 700 g/l depending on the solubility.

Previously, we have shown (Mejri et al., 1998) that the catalytic activity of thermolysin is noticeably enhanced in the presence of sugars and polyols. The degree of solvactivation was found to depend on concentration and co-solvent nature. The effects of small carbohydrates on the catalytic efficiency of thermolysin, pullulanase and inulinase are reported in Tables 1–3, respectively.

Table 1 shows that thermolysin is most activated by sucrose and trehalose at relatively high concentrations (~300% at 350 g/l) and slightly activated by glycerol at low concentration (120% at 40 g/l). In the case of pululanase (Table 2), the activation is lower and the maximum activation coefficient is reached with trehalose and sorbitol (~200%). The optimal activating concentrations are also lower than those observed for thermolysin. On the other hand, inulinase is slightly activated by small carbohydrates at low concentrations (137% in the presence of 37 g/l of sorbitol solution) (see Table 3).

It should be recalled that increase in the concentration of small carbohydrates leads to an inhibiting effect (activity lower than in buffer). It is especially the case for glycerol which was found to decrease thermolysin efficiency for a concentration above 55 g/l (Mejri et al., 1998).

Table 2
Optimal activating concentration, maximum catalytic efficiency and the corresponding activation percentage for sugars and polyols used in pullulanase activation experiments

Additive	Concentration for maximum activation (g/l)	Maximum efficiency ($(g/l)^{-1}\ s^{-1}$)	Activation (%)
Buffer		20.80	100
Sucrose	68	33.75	162
Trehalose	68	44.80	215
Sorbitol	110	45.00	216
Maltitol	137	38.50	185
Glycerol	110	33.00	158

Table 3
Optimal activating concentration, maximum catalytic efficiency and the corresponding activation percentage for sugars and polyols used in inulinase activation experiments

Additive	Concentration for maximum activation (g/l)	Maximum efficiency ($mM^{-1}\ s^{-1}$)	Activation (%)
Buffer		26.00	100.00
Trehalose	68	29.00	111.50
Sorbitol	37	33.00	127.00
Maltitol	68	29.50	113.50
Glycerol	18	35.50	114.60

3.2. Thermodynamic results

Thermolysin was the enzyme with the most modified reaction medium (10 additives). To find the differences between the small carbohydrates added in reaction media, in their effect on free energy of the reaction, thermolysin reaction was chosen.

The activation energy of the thermolysin catalyzed reaction in buffer and in additives modified media was determined using the Arrhenius plot. The reactions were carried out in buffer and in the presence of additives at their optimal activating concentrations. The results are summarized in Table 4. It may be observed that the activation energies obtained in the presence of additives are lower than those obtained in buffer. The presence of small carbohydrates in the reaction medium seems to decrease the energy barrier needed for the reaction to proceed and leads to the activation of the enzyme. The higher the activation percentage, the lower the activation energy. The origin of such a decrease in activation energy is linked to a change in the mobility of water molecules in the medium rather than to the macroscopic parameter of water activity. Indeed, small carbohydrates are known to be water activity depressors, which may have an effect on water molecules availability in the micro-environment of the enzyme. However, sugar concentrations, which give the highest enzyme activation, correspond to calculated water activities near to one for sugar solutions considered as ideal solutions ($a_{W_{id}}$).

3.3. Spectroscopic results

3.3.1. Raman spectra of thermolysin

Raman spectroscopy was used to investigate the effect of glycerol on the secondary structure and the global conformation of thermolysin taken as an example. The Raman spectra of thermolysin in buffer and in 5 M (460 g/l) glycerol solution were collected and treated (Fig. 1). The characteristic vibrational bands were identified and assigned according to the literature (Koenig, 1979; Arrondo, Muga, Castresana & Goni, 1993; Pourplanche et al., 1994). It may

Table 4
Optimal activating concentration, maximum catalytic efficiency and its corresponding activation coefficient (X), activation energy and calculated water activity for sugars and polyols used in thermolysin activation experiments (ND means not determined)

Carbohydrate	Activating concentration (g/l)	Maximum efficiency ($mM^{-1}\ s^{-1}$)	Activation (%)	Activation energy (kJ/mol^{-1})
Buffer		5.50	100	12.03 ± 0.8
Sucrose	340	16.70	300	7.18 ± 0.5
Trehalose	340	17.17	312	ND
Maltose	450	10.00	180	ND
Glucose	50	12.00	218	10.22 ± 0.5
Fructose	180	9.00	163	11.50 ± 0.5
Mannose	220	10.00	180	ND
Sorbitol	150	8.85	160	11.80 ± 0.3
Mannitol	120	10.20	185	ND
Maltitol	150	13.00	236	9.60 ± 0.5
Glycerol	40	6.70	120	11.60 ± 0.5

be recalled that glycerol at such high concentration (5 M) inhibits the activity of thermolysin.

The secondary structure of thermolysin in buffer and in glycerol modified medium was determined by using the decomposition of the amide I band and also analyzing the amide III band. The results are elucidated in Table 5. The data corresponding to the crystallized enzyme was taken as reference (source: Protein Data Bank).

The percentages of secondary structure of the crystallized enzyme differ from those of the hydrated form. However, there is no large difference between the secondary structure in buffer and in glycerol concentrated medium. This stability of the secondary structure was confirmed by the absence of restructuring of the amide III band.

Analysis of the environment of tyrosine residues through variation in the peak intensity ratio I_{850}/I_{830} constitutes a precise indicator for possible conformational changes of the protein (Siamwiza, Lord, Chen, Kakamatsu, Harada, Matsumara et al., 1975). This ratio is equal to 1.41 in buffer and 1.36 for the enzyme in glycerol modified medium. Such variation is considered to be not significant. The bands corresponding to the aliphatic side chains of residues (CH_2 at 1465 cm^{-1} and CH_3 at 1450 cm^{-1}) and the region of the skeletal vibrational mode (990–890 cm^{-1}) showed similar profiles in the two cases, and no shift was observed. All these results confirm the stability of the secondary and global structure of the enzyme when dissolved in buffer or in glycerol medium. The bands corresponding to the

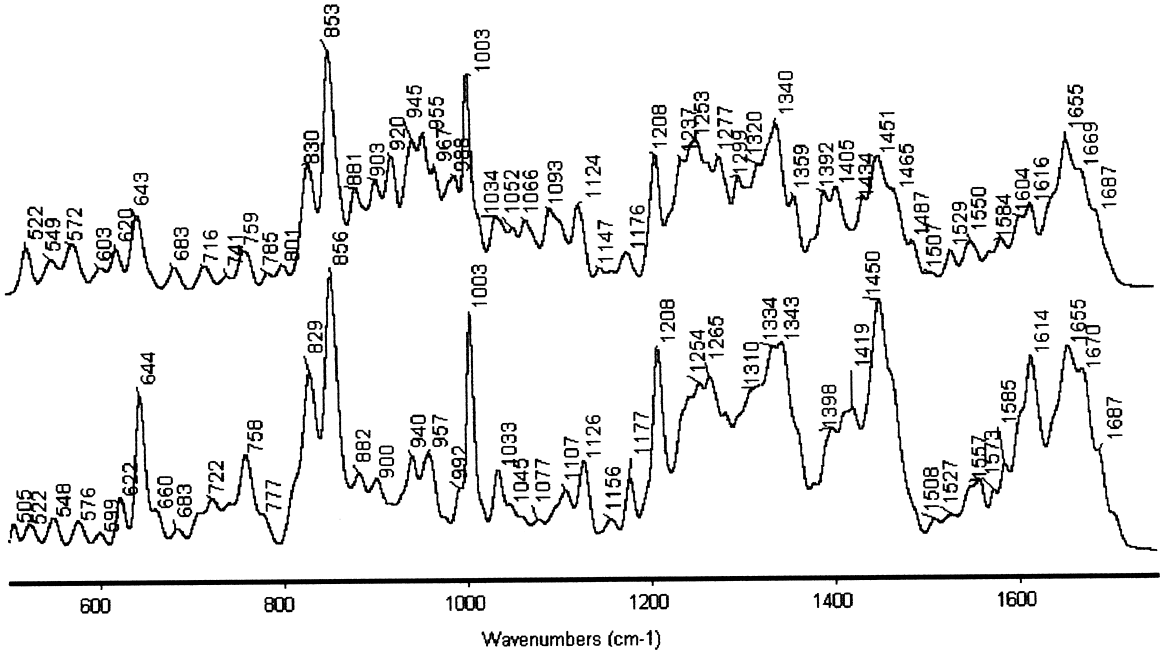

Fig. 1. Raman spectra of thermolysin in the frequency region 1800–500 cm^{-1}: (a) in buffer; and (b) in the presence of glycerol (460 g/l). Enzyme concentration: 2 mg/ml.

Table 5
Secondary structure of thermolysin in the crystallized form (reference), in buffer medium and in glycerol concentrated medium

	α-Helix	β-Sheets	Undefined
Reference (%)	28	42	30
Thermolysin in buffer (%)	42	30	28
Thermolysin in glycerol (%)	41	29	30

residues that chelate the catalytic zinc (at 1266, 1287 and 1508 cm^{-1}) provide information about the structural changes inside the cavity of the catalytic site. These bands remain unchanged and no shift in frequencies is observed. Such results imply that the presence of glycerol induces no structural change in the catalytic site.

It may be considered that the addition of glycerol in the environment of the enzyme does not induce any conformational changes. Therefore, it seems that the quantitative variations of the catalytic activity of the enzyme, induced by the presence of the additive, are not due to structural changes in the enzyme itself. Pourplanche et al. (1994) observed subtle modifications in the secondary and tertiary structures of soybean lipoxygenase induced by the presence of sorbitol. Such modifications were considered to be responsible for the modification of the enzyme specificity.

3.3.2. FT-IR spectra of thermolysin

A complementary infrared spectroscopic study was performed on the hydrolases in the presence of the additives. Fig. 2 represents the spectra of thermolysin in the presence of variable sucrose concentration in the amide vibrational region. Amide I and II infrared bands provide information on the effect of hydration on the protein structure. The intensities of these bands, in the case of thermolysin, in the presence of variable concentration of sucrose and glycerol are represented in Table 6. No appreciable changes of the spectra of pullulanase and inulinase in the presence of glycerol were observed as compared to the enzyme in buffer. These spectra are not reported.

Table 6
Intensities of the Amide I and II bands of thermolysin in the presence of variable concentration of glycerol and sucrose

Thermolysine in	Amide I intensity (1650 cm^{-1})	Amide II intensity (1548 cm^{-1})
Buffer	0.165	0.396
Glycerol (g/l)		
46	0.174	0.411
92	0.181	0.416
184	0.185	0.425
Sucrose (g/l)		
68	0.173	0.395
170	0.178	0.402
342	0.181	0.414
513	0.189	0.421
684	0.213	0.455

The intensities of the IR bands listed in Table 6 seem to increase with increasing additive concentration. This implies a preferential hydration of the enzyme in the presence of the additives (Carpenter & Crowe, 1989). This preferential hydration results from the exclusion of the co-solvents from the immediate domain of the protein in

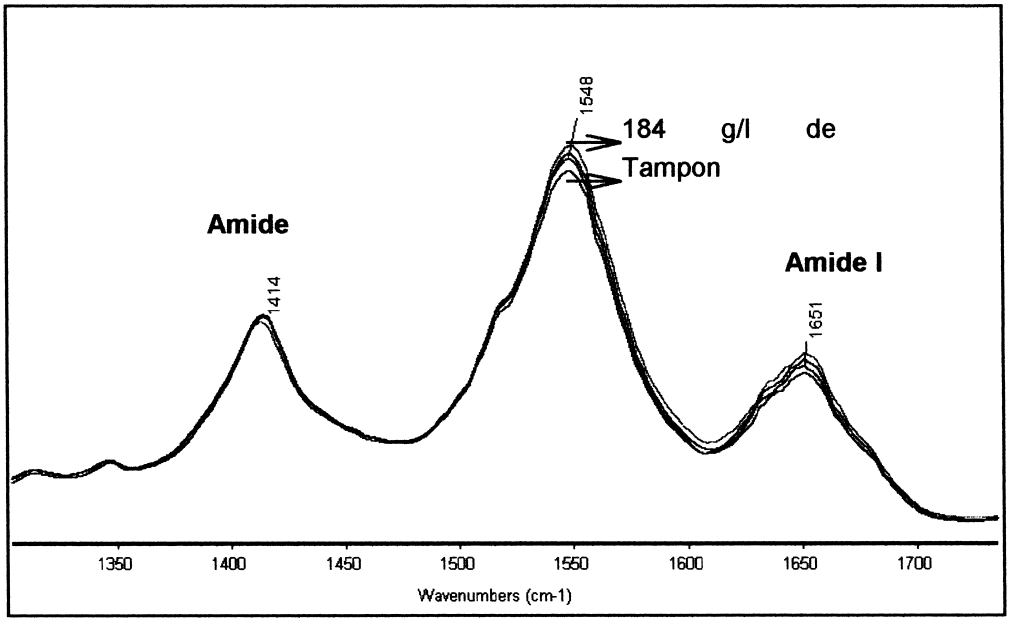

Fig. 2. Infrared spectra of thermolysin in the frequency region 1750–1350 cm^{-1} in the presence of variable sucrose concentration (68, 170, 342 and 684 g/l). Enzyme concentration: 80 mg/ml.

aqueous solution, when they are present at high concentration. The phenomenon is largely studied and has been attributed to the effect of additives on the thermostabilization of enzymes (Arakawa, Bhat & Timasheff, 1990). The authors (Arakawa et al., 1990) explained that the preferential hydration does not always stabilize the native structure of globular proteins. The stabilizing effect was found to depend on the nature of the solvent, on that of the co-solvent and, especially, on the chemical nature of the protein surface, which determine to a major extent the interactions between the protein and the co-solvent. In this study, the phenomena of solvactivation and solvinhibition of enzymes by the solvent have also been found to depend on the nature of the additive and the enzyme, although a preferential hydration of the enzyme was observed. In the case of a protease like thermolysin which acts in a hydrophobic natural medium, the action of small carbohydrates on the hydrophilic character of the reaction medium, and on the water activity and structure could have an important effect on the enzymatic catalysis. However, in the case of pullulanase and inulinase which act naturally in hydrophilic media, they are less sensitive to such changes in the reaction media (maximum activation reached with sorbitol around 200% for pullalanase). On the other hand, small carbohydrates present a structural homology with the products of the reactions catalyzed by glycohydrolases, and they can act as competitive inhibitors.

4. Conclusions

In this work, we have shown that the activity of hydrolases is quantitatively affected by the presence of small carbohydrates. Indeed, the catalytic efficiency of the enzymes depends on the additive present in the reaction medium and varies as a function of its concentration. As a general rule, the catalytic efficiency increases with increasing sugar concentration to reach an optimum, after which it decreases. Glycerol seems to be particularly interesting in this study. This additive has a weak activating effect on the studied enzymes at low concentrations ($\pm$40 g/l) and an inhibiting effect in the largest range of tested concentrations. This is in agreement with the literature (Pourplanche et al., 1994; Ligné, Pauthe, Monti, Gacel & Larreta-Garde, 1997).

The phenomena of activation and inhibition of enzymes by the solvent, called solvactivation and solvinhibition, respectively, have been found to depend not only on the nature and the concentration of the additive but also on the enzyme. Thermolysine was found to be more activated by sugars and polyols than pullulanase or inulinase. This implies a specific effect of polyhydroxylic additives apart from their effect on the enzyme micro-environment.

Activation free energy of the thermolysin-catalyzed reaction in the presence of additives was found to be lower than that in buffer medium. As temperature was maintained constant, the activation energy change seems to be very likely due to an entropic change (i.e. water structure enhancement by additives). Such an effect is mainly responsible for the solvactivation of enzymes by the micro-environment.

Vibrational spectroscopic study showed that the enzymes are preferentially hydrated in the presence of additives and that their conformations remain unchanged. This study permitted us to differentiate the effect of small carbohydrates on the activity of one protease and two glycohydrolases. This specific activating or inhibiting effect seems to be linked to the effect of these solutes on water structure. The relation between enzyme activity and water structure was previously evoked (Drapron 1985; Mathlouthi, Larreta-Garde, Xu & Thomas, 1989). It was shown that, for reactions occurring in carbohydrate solutions, the enzymic reaction proceeds more rapidly the less the water structure is perturbed by the solute.

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