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Water miscible mono alcohols effect on the structural conformation of *Bacillus clausii* GMBAE 42 serine alkaline protease

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ABSTRACT

Proteases are largely employed in biocatalysis. In order to increase the number of their applications it is useful to shed light on the reasons that cause a non-optimal activity of these enzymes when used in inactivating experimental conditions (e.g., in the presence of co-solvent to favor substrate dissolution). To this end the effect of different mono alcohols on the activity and the conformation of alkaline protease from *Bacillus clausii* GMBAE 42 was investigated. We found that the enzyme in the presence of 20–25% of methanol, ethanol, 1-propanol or 2-propanol halves its activity. At the concentration of 10%, all the alcohols caused a slightly more intense far-UV (CD) circular dichroism signal of the protease at around 208 and 220 nm with respect to the protein in only buffer, which suggests an increase of helicity in the secondary structure of the protein. Monitoring the shift of the fluorescence emission of the protease with respect to that of the standard N-acetyl-L-tryptophan-ethyl ester, we suggest that with all the alcohols tested the decrease of activity might be due to the loss of tertiary structure (even though at a lower extents in methanol and ethanol compared to 1-propanol and 2-propanol).

1. Introduction

Proteases are one of the most important groups of industrial enzymes. They have applications as additives in detergents, in the hydrolysis of fibrous proteins to produce biomasses, in the food industry and in different areas of industrial biotechnology. Among this latter application, proteases have extensively been employed for the synthesis of peptides, in the regio-selective acylation of carbohydrates and in the kinetic resolution of racemic mixtures of amino acids and their derivatives, including some neuroprotective glutamic amino acid derivatives very interesting from the pharmaceutical point of view [1,2,3,4]. Nevertheless, the biocatalytic use of proteases is often hindered by the low solubility of compounds in buffers. A strategy to overcome this limitation is the use of co-solvents (e.g., water miscible organic solvents) that added to water favor the solubility of the hydrophobic substrates. On the other hand, in the presence of organic solvents often the enzymatic activity is inhibited and the biocatalyst is subjected to a number of factors that can alter its native structure and function [5,6]. Indeed, also for proteases that have been proved to be active in neat organic solvents there is a strict correlation between catalytic activity and conformational structure of the enzyme [7].

Recently we isolated an alkaline protease from *Bacillus clausii* GMBAE 42 (Protease P42) that is rather stable at high pH values and in the presence of some detergents [8]. However there is little or no report in literature about the effect of water miscible mono alcohols on the conformational structure and activity of the enzyme. In this paper, we are reporting the effect of mono alcohols on activity and conformational structure of Protease P42 by using spectrophotometric techniques as fluorescence spectroscopy and circular dicroism (CD).

2. Experimental

2.1. Chemicals

Chemicals used in the cultivation of the microorganism were supplied by Oxoid Ltd (Hampshire, England) and Merck AG

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(Darmstadt, Germany). All other chemicals used were of HPLC grade and obtained either from Merck AG (Darmstadt, Germany) or Sigma Chem. Ltd. (St Louis, USA).

2.2. Alkaline protease purification

The alkaline protease of *B. clausii* GMBAE 42 was purified according to the procedure described by Kazan et al. [8]. After 72 h cultivation, the culture medium was centrifuged (11,000 *g*, 30 min, 0–4 °C) to remove *B. clausii* cells. The dissolved proteins in the supernatant were precipitated by the addition of solid ammonium sulphate to 75% saturation. The precipitate was collected by centrifugation (15,000 *g*, 30 min, 0–4 °C), dissolved in 50 mM glycine–NaOH buffer, pH 10.5, dialyzed against the same buffer, and applied to a DEAE-cellulose column (25 × 2.5 cm diameter). Elution was carried out with the same buffer at a 15 ml h^{-1} flow rate. The alkaline protease activity eluted as the first major protein peak. Fractions with a specific activity greater than 3,000 U mg^{-1} were collected, pooled and used for further studies.

2.3. Determination of alkaline protease activity

The modified method described by Takami et al. [9] was used for alkaline protease activity. Enzyme solution (0.5 mL) in 0.0013 mg/mL concentration was mixed with 2.5 ml 50 mM glycine–NaOH (pH 10.5) buffer containing 0.6% casein as a substrate, and incubated for 20 min at 30 °C. The reaction was stopped by addition of 2.5 ml of a solution containing 0.11 M trichloro acetic acid, 0.22 M sodium acetate and 0.33 M acetic acid. The mixture was allowed to stand at 30 °C for 20 min and then filtered to remove precipitate. The absorbance was measured at 280 nm. One unit of activity was defined as the amount of enzyme able to produce 1 μg tyrosine in 1 min under assay conditions. All activity assays were carried out independently five times, and the results were calculated as mean values. The standard errors of mean values were lower than 4%.

Ethanol, methanol, propanol and 2-propanol were used to investigate the effect of mono alcohols on protease P42 activity. 0.05 ml of suitably diluted enzyme solution (specific activity: 4174 U mg^{-1}, protein concentration: 0.0013 mg ml^{-1}) was mixed with 0.450 ml glycine–NaOH buffer (pH 10.5) containing different percentages of mono alcohols (10–60%) and these mixtures were incubated for 2 h at 30 °C. After incubation, the residual proteolitic activity was measured in all samples as described above and stability is expressed as the remaining proteolytic activity relative to control without any solvent (100%).

2.4. Fluorescence measurements

Intrinsic fluorescence emission spectra of protease in water/organic solvent mixtures were recorded with a Jasco FP–550 spectrofluorimeter over the 270–400 nm range, with excitation at 295 nm to minimize the emission arising from tyrosine residues. The protein concentration of protease was, in all cases, 0.02 mg/mL and the concentration of *N*-acetyl-L-tryptophan ethyl ester (*N*-Ac-Trp-O-Et) was 0.01 mg/mL. All spectra were recorded at 25 °C after 2 h from the sample preparation.

2.5. Circular dichroism measurements

Far-UV circular dichroism spectra of protease in water and in water containing 10% or 50% of mono alcohols were recorded with a Jasco 600 spectropolarimeter of which the optical system was continuously purged with nitrogen, at temperature of 25 °C over the 200–250 nm range (conditions of measurements: one accumulation, scan speed 10 nm/min, response 4 s). Protease concentration was 0.2 mg/mL and the optical path 0.1 cm. All the spectra were corrected by subtraction of the blank spectrum and smoothed using Jasco Spectra Analysis software (method: means-movement, convolution width 21).

3. Results and discussion

3.1. Effect of the organic solvent on the protease hydrolytic activity

All the mono alcohols tested in the present study, the activity decreased as a function of the alcohol percentage. The concentration of alcohol that halves the hydrolytic activity (C_{50}) was extrapolated from Fig. 1 and resulted 19 (2.5 mol/l), 23 (3 mol/l), 24 (4.1 mol/l), 28% (6.9 mol/l) for 1-propanol, 2-propanol, ethanol and methanol, respectively. An analogous variation of C_{50} as a function of the alcohol was observed for α-chymotripsin, being C_{50} 3.6, 4.4, 6.2 and 8 mol/l for 1-propanol, 2-propanol, ethanol and methanol, respectively [10]. Joshi et al. [11] studied the effect of methanol, ethanol and 2-propanol on *B. cereus* MTCC 6840 alkaline protease and 52, 72 and 52% of MTCC 6840 alkaline protease activity was remained at 50% alcohol concentrations for 2 h incubation at 4 °C. Comparing these results with those we have found with protease P42 it appears that this latter enzyme is less stable in the presence of mono alcohols compared to MTCC 6840 alkaline protease and to α-chymotripsin.

In order to shed light on the reasons activity of protease P42 decreased as a function of alcohol concentration, we monitored by circular dichroism (CD) and fluorescence the effects of the alcohols on the enzyme conformation.

3.2. Fluorescence spectroscopy

Protease P42 shows fluorescence spectrum when excited with a radiation at 295 nm suggesting the presence of at least one tryptophan (Trp) residue in the protein molecule (Fig. 2). On the other hand, it is known that chymotrypsin-like serine protease, including protease P42 [8], usually have in their amino acid sequence at least one tryptophan residue.

The maximum emission wavelength (λ_{max}) of a fluorophore depends on the environmental polarity around the molecule. Proteins containing Trp residues and submitted to a solvent composition change might undergo a variation of the environment surrounding the Trp residues because of the change in the solvent polarity and/or as a consequence of conformational changes of the

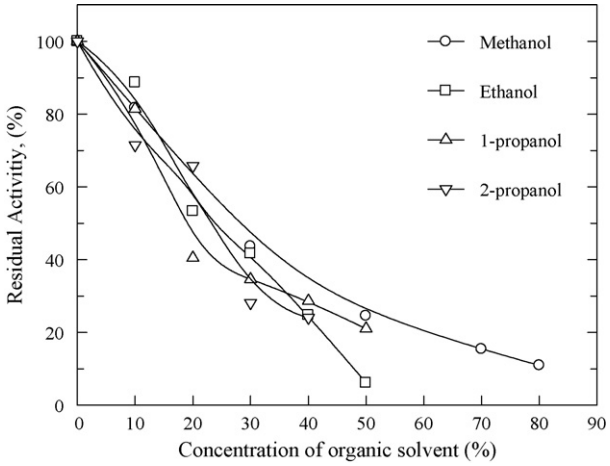

Fig. 1. Residual hydrolytic activity (%) of protease P42 with casein in methanol/water, ethanol/water, 1-propanol/water, and 2-propanol/water mixtures.

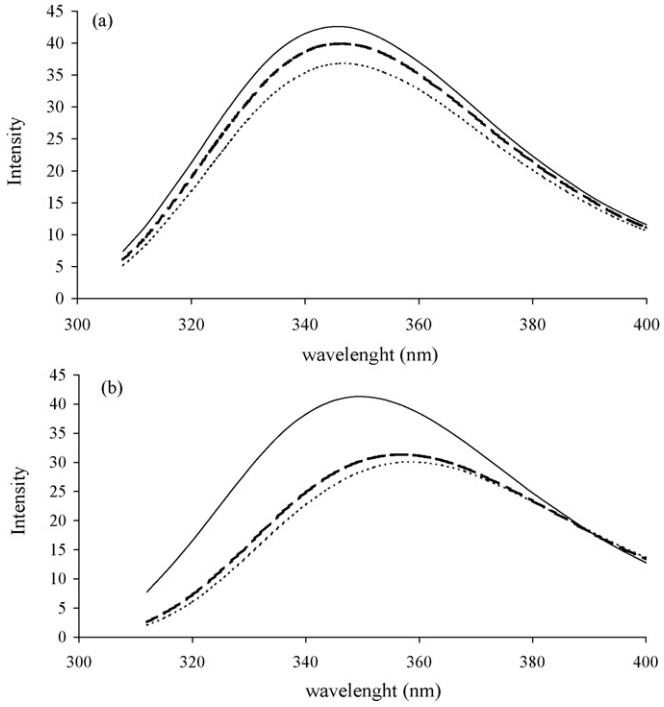

Fig. 2. Fluorescence spectra of Protease P42 (a) and of N-Ac-Trp-OEt (b) in buffer (dashed and thin) and in buffer containing 10% (dashed and thick) or 50% (continuous line) 1-propanol. A similar trend was observed in methanol/water, ethanol/water and 2-propanol water mixtures.

protein. In order to evaluate how different water/alcohol mixtures affected the conformation of protease P42 fluorescence data were analyzed in terms of $\Delta\lambda_{max}$ of emission (Fig. 3a) [12]. A decrease of the $\Delta\lambda_{max}$ upon increase of the percentage of alcohol points

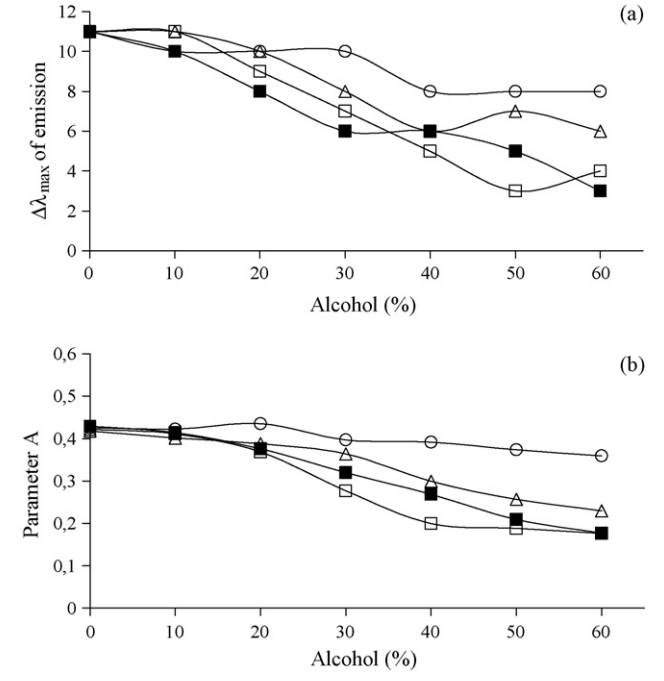

Fig. 3. Difference of emission wavelength ($\Delta\lambda_{em}$) and parameter A of protease P42 in alcohol/water mixtures. Alcohol was methanol (circles), ethanol (triangles), 2-proapanol (black squares) and 1-propanol (white squares). $\Delta\lambda_{em}$ is the difference between λ_{max} of emission of the standard *N*-Ac-Trp-O-Et minus λ_{max} of the protein. Parameter A is defined as: (IF_{325}/IF_{365})protein $- (IF_{325}/IF_{365})_{N\text{-Ac-Trp-OEt}}$. IF is intensity of fluorescence.

Table 1
Correlation between $\Delta\lambda_{em}$ and residual enzyme activity (v).

Solvent	Relative polarity[a]	a[b] (residual activity/$\Delta\lambda_{em}$)	b[b]	r
Methanol	0.762	23.6	−167.9	0.74
Ethanol	0.654	15.3	−82.0	0.84
1-Propanol	0.617	15.0	−78.0	0.83
2-Propanol	0.546	12.6	−43.6	0.91

[a] Data were obtained from [15].
[b] Parameters a and b and r (correlation coefficient calculated by a least-squares method) refer to the equation $v = a^*\Delta\lambda_{em} + b$ as described in Fig. 4.

out a loss of tertiary structure (e.g., if $\Delta\lambda_{max}$ is 0, the protein is completely unfolded exposing all the tryptophan residues to the solvent). We found that $\Delta\lambda_{max}$ diminishes as a function of the alcohol percentage suggesting that the alcohols cause a variation of the tertiary structure. The effect appears correlated also to the alcohol relative polarity being the $\Delta\lambda_{max}$ decrease in 1-PrOH > 2-PrOH > EtOH > MeOH (Fig. 3a). The unfolding might start from a protein swelling caused by the interaction between the protein and the hydrophobic solvent [12].

The structural modification of an enzyme alters its catalytic activity. Thus, a correlation between the activity of protease P42 and a variation emission wavelength for the enzyme is expected. However, we found only a modest correlation between the decrease of $\Delta\lambda_{max}$ and that of the residual activity if the whole range of alcohol concentration (0–60%) was considered, especially in the case of methanol (Table 1). In fact, if 40% 1-propanol and 2-propanol caused an activity loss of 80% and a change of $\Delta\lambda_{max}$ from 11 to 3, 60% methanol and ethanol caused almost complete loss of activity but a much less marked variation of $\Delta\lambda_{max}$ that passed from 11 to 8.

An analogous trend is observed if the fluorescence spectra are compared in terms of ratios of fluorescence intensities measured at 325 and 365 nm (Parameter A, Fig. 3b), that is, taking into account the overall shape of the fluorescence spectrum. Indeed, with 60% alcohol parameter A decreased from about 0.42 to 0.36, 0.23, 0.18 and 0.18 for methanol, ethanol, 1-propanol and 2-propanol, respectively.

In spite of the fact that the correlation coefficient between variation of $\Delta\lambda_{max}$ and residual activity, the slopes (a values in Table 1) of linear regression shown in Fig. 4 can give us an indication of how pronounced are the conformational changes that cause a loss of activity. The highest a value observed for methanol, suggests that a marked decrease of activity is caused by small conformational changes. As the relative polarity of the alcohol decreases, larger variations of the tertiary structure occurs for the same loss of activity.

3.3. Circular dichroism

In the far-UV CD spectra of protease P42 recorded in buffer (Fig. 5) two minima at around 220 and 208 nm, which are typical of α-helix secondary structure, can be distinguished. However, the broadness of the band (likely due to the presence of a signal at 217 nm) might indicate the presence of some β-sheets too. On the other hand, it is known that most of serine hydrolyses have a typical α/β-folding [13].

Interestingly, it has to be noted that with 10% of alcohol there is a small increment of the band at around 208 nm that suggests a small variations of the enzyme secondary structure (e.g. it might concern fragments of the protein after that major variations of the tertiary structure have occurred). Similar increments have also been reported by other research groups with cytochrome c in the presence of methanol and ascribed to an increment of the protein helicity [14]. However, this small changes in the secondary

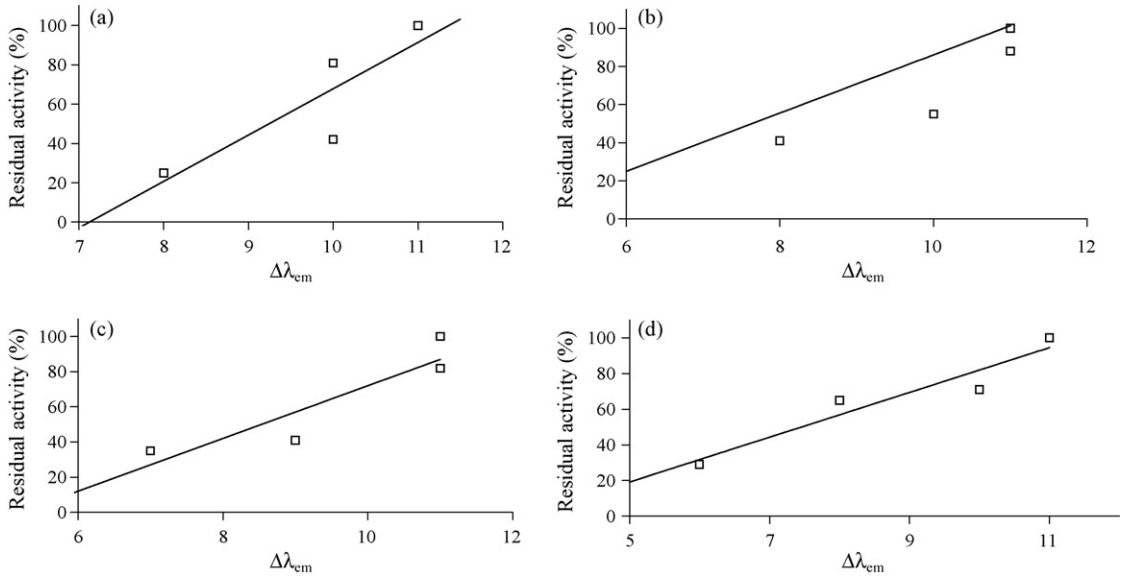

Fig. 4. Correlation between $\Delta\lambda_{max}$ of emission and residual activity (%) measured for protease P42 in methanol (a), ethanol (b), 1-propanol (c) and 2-propanol (d). $\Delta\lambda_{max}$ of emission and residual activity (v) values from Figs. 1 and 3a (points up to 30% of alcohol) were fitted to the equation $v = a^*\Delta\lambda_{em} + b$ (Kijima et al. [12]).

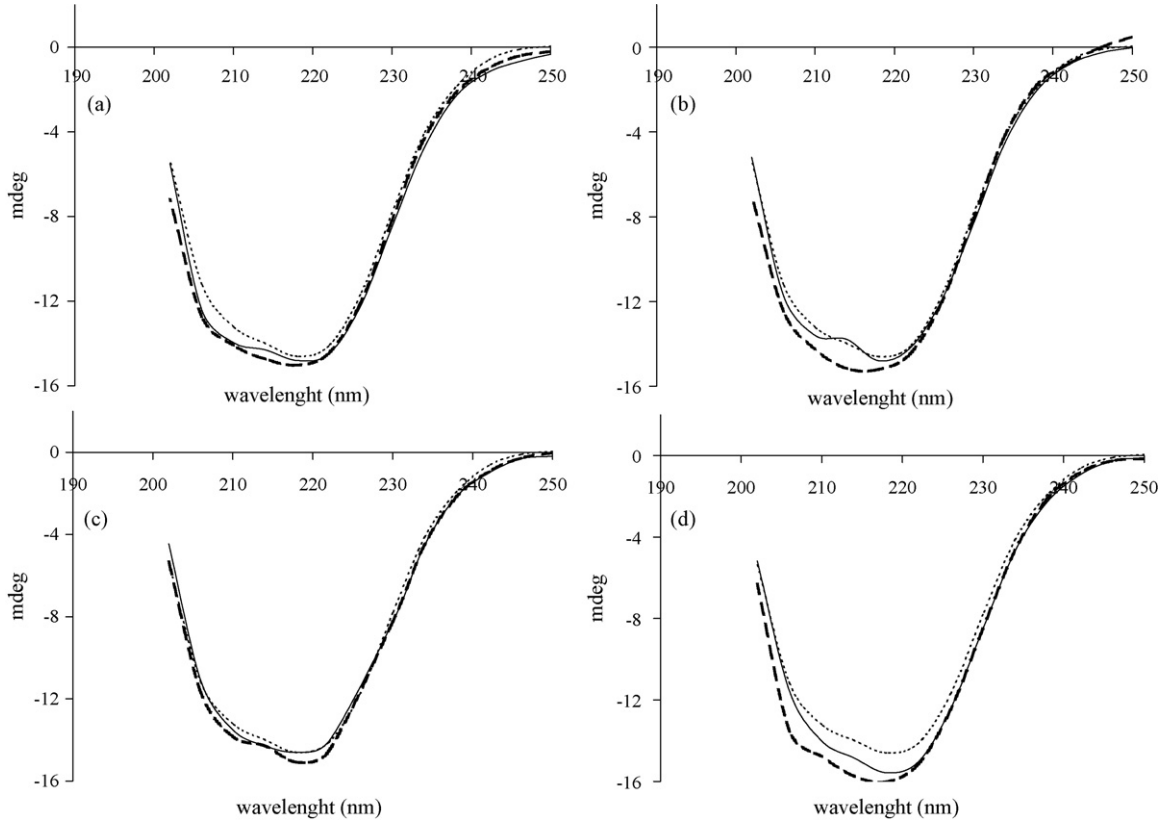

Fig. 5. Circular dichroism spectra of protease P42 in different alcohol/water mixtures: in buffer (dashed and thin), in buffer containing 10% (dashed and thick) or 50% (continuous line) alcohol. The alcohol was methanol (a), ethanol (b), 1-propanol (c), 2-propanol (d).

structure of the enzyme is associated with a 20–30% decrease of catalytic activity (Fig. 1). Nevertheless, the fact that with 50% of alcohol CD spectra of protease P42 has shape and intensity at 220 nm comparable to that in only buffer suggests that the enzyme secondary structure is stable. These data also agree with those reported by Ogino et al. [5] that observed a higher stability of the enzyme secondary structure of subtilisin from *Bacillus licheniformis* and of PST-01 protease from *Pseudomonas aeruginosa* in the presence of methanol than when in the absence of it.

4. Conclusions

The results obtained in this work showed that methanol, ethanol, 1-propanol and 2-propanol negatively affect the hydrolytic activity of protease P42. Nevertheless, the fact that with 20% of

organic solvent the enzyme preserves about half of the activity measured in only buffer indicates that this enzyme can be employed in biocatalytic applications (e.g. hydrolysis of low water miscible substrates) that require a co-solvent to increase substrate solubility.

It is worth pointing out that, even a minimal or partial loss of the tertiary structure (as in the case of methanol that showed a lower decrease of the $\Delta\lambda_{em}$ compared to the other alcohols) might be responsible for an almost complete loss of activity. However, even though the study has shown that the loss of the enzyme tertiary structure can play a crucial role to the decrease of activity, it cannot be excluded that the loss of activity is due to (minor) changes of the secondary structure or other inhibitory effects that concern the catalytic mechanism.

Finally the results obtained through this study highlighted that the unfolding process is strongly depended on the solvent relative polarity. Therefore, efforts (e.g. chemical modifications and/or protein engineering) that aim to improve the enzyme stability in water/organic solvent mixtures might be different according to the organic solvent.

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