International Journal of Biological Macromolecules 49 (2011) 652–656

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Effects of trehalose and sorbitol on the activity and structure of *Pseudomonas cepacia* lipase: Spectroscopic insight

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ARTICLE INFO

Article history:
Received 17 February 2011
Received in revised form 23 June 2011
Accepted 25 June 2011
Available online 1 July 2011

Keywords:
Pseudomonas cepacia lipase
Surface plasmon resonance
Osmolyte
Circular dichroism
Catalytic activity
Intrinsic fluorescence

ABSTRACT

The stability of enzymes with no reduction in their catalytic activity still remains a critical issue in industrial applications. Naturally occurring osmolytes are commonly used as protein stabilizers. In this study we have investigated the effects of sorbitol and trehalose on the structural stability and activity of *Pseudomonas cepacia* lipase (PCL), using UV–visible, circular dichroism (CD) and fluorescence spectroscopy. Surface plasmon resonance (SPR) technique was used to trace changes in the refractive index and dielectric constant of the environment. The results revealed that catalytic activity and intrinsic fluorescence intensity of PCL increased in the presence of both osmolytes. Far-UV CD spectra indicated that the protein has undergone some conformational changes upon interacting with these osmolytes. Increasing the concentration of sorbitol led to changes in the refractive index and consequently the dielectric constant of environment; whereas in the case of trehalose, such changes were not significant. Unfavorable interactions of trehalose with protein surface induced higher preferential exclusion from the enzyme–water interface than that of sorbitol. Results of this report could give further insights about the stabilization mechanism of osmolytes.

1. Introduction

It is well known that stabilizing osmolytes affect the stability and catalytic activity of different proteins [1–4]. Apart from increasing the stability and catalytic activity, these osmolytes do not change the structure of proteins. Such advantage could encourage the use of stabilizing additives in a variety of industrial applications [1–3,5–8]. A few general schemes about the stabilization mechanism of these osmolytes have been proposed so far, which arise from their preferential exclusion from the surface of protein, and the property of increasing surface tension of water in their vicinity [1–4]. Varieties of proteins are known to interact with osmolytes molecules in different ways, where the interactions depend on the physicochemical properties of the proteins. According to previous reports, the stabilizing effects of osmolytes depend on the nature of the osmolytes [3]. Spectroscopic techniques such as fluorescence and circular dichroism have shown that osmolytes do not induce noticeable alterations in the secondary and tertiary structures of the native proteins. It has been also proved that such compounds are inert at the protein surface but are capable of affecting the solvent molecules in the vicinity of protein [6–10]. In this light, powerful tools are required to detect changes in solvent molecular arrangement; amongst which, surface plasmon resonance (SPR) is a surface-sensitive technique which detects changes of dielectric constant induced by the molecular adsorption onto a noble metal film. The sensing mechanism of SPR method is based on the measurement of small changes of refractive index that occur in response to the analyte binding, to or near the surface of a noble metal thin film (Au, Ag, Cu) [11–13]. Surface plasmon resonance has been widely used in biomolecular interaction studies, including determination of affinity constants and kinetic binding parameter. Sensitivities of dipole resonance position to the refractive index are found to be only dependent upon the wavelength of the resonance, the medium and dielectric properties of the metal [14–18]. Considering the sensitivity of SPR to the surrounding medium, it would be possible to study the effect of osmolytes on the proteins via this powerful technique.

Pseudomonas cepacia lipase (PCL) is a microbial lipase which has been widely used as organic solvents, stereo- and regio-selective modificators of pharmaceutical compounds and food products in the industry [19–22]. Since PCL has a variety of industrial applications, enhancing its activity and stability remains a critical issue in

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doi:10.1016/j.ijbiomac.2011.06.025

the context of biotechnology. Several methods have been employed to increase the activity and stability of enzymes, including chemical modification, immobilization, protein engineering of the enzyme, mutagenesis and utilization of stabilizing additives [10,23,24]. This report investigates the effect of trehalose and sorbitol on the structure and activity of PCL as a model enzyme. To address how the osmolytes can affect the structure of the enzyme, we have studied the individual effects of trehalose and sorbitol on the structure of PCL by circular dichroism (CD) and fluorescence techniques. The activity of enzyme was also measured in the presence of two mentioned osmolytes. In order to trace the refractive index and dielectric constant alterations upon the addition of osmolytes, microenvironment of the enzyme (PCL) has been studied by means of SPR technique.

2. Materials and methods

2.1. Materials

The lipase from *P. cepacia* was obtained from Sigma Chemical Co. (St. Louis, USA). Nominal specific lipase activity was 49 U/mg of protein. 2-Propanol, Triton X-100, sorbitol, trehalose, and Tris–HCl were purchased from Sigma. Para-nitrophenyl palmitate (pNPP), iso-butanol and sodium chloride were provided from Merck (Germany). All chemicals used in this study were of analytical grade. Quartz triple-distilled water was used for all experiments.

2.2. Enzyme dialysis

Prior to all experiments, PCL solution should be dialyzed, since there is high concentration of detergent and other salts. $CaCl_2$ is one of the most unfavorable salts in the solution, which aggregates the enzyme molecules and misrepresents the spectroscopic data. Dialysis was carried out once, for a period of 24 h, at 4 °C, in 1 l of dialysis buffer (Tris–HCl 50 mM, pH 8), and constant stirring at 120–200 rpm. The ratio of sample volume to buffer volume was 1:500.

2.3. PCL activity measurement and determination of protein concentration

Hydrolytic activity of protein was measured with emulsified pNPP according to Pencreac'h et al. [23]. One volume of pNPP in 2-propanol (2 mg/ml) was mixed with 9 volume of 50 mM Tris–HCl buffer, pH 8.0, containing Triton X-100, 0.4% (w/v). Then, 1 mL of the mixture (at 25 °C for 2 min) incubated in a quartz cuvette of UV–visible spectrophotometer, S-2100 (Scinco, Seoul, Korea). The reaction starts by the addition of 20 μl of enzyme solution at an appropriate dilution in 50 mM Tris–HCl buffer, pH 8. Absorbance difference was monitored at 410 nm for 2 min. Having the molar extinction coefficient value of *p*-nitrophenol ($15\ cm^{-1}\ mM^{-1}$), slope of the absorbance versus time curve gives the reaction rate. One unit of enzyme activity corresponds to release of 1 μmol *p*-nitrophenol per minute. Concentration of the protein was measured by Bradford assay, using bovine serum albumin as a standard [25].

2.4. PCL activity measurement in the presence of osmolytes

Stock solutions of sorbitol and trehalose (1.2 M) were prepared in 50 mM Tris, pH 8. After filtering, the stock solutions were mixed with appropriate proportion of the same buffer to give a final osmolyte concentration, ranging from 0 to 1 M, and final protein concentration of 0.2 mg/ml. For kinetic studies, 0.2 mg/ml of the enzyme was incubated with different concentrations of osmolytes in the assay medium. One volume of pNPP in 2-propanol (2 mg/ml) was mixed with 9 volume of the osmolytes solution. Activity of the enzyme was determined using pNPP as substrate for enzyme.

2.5. Fluorescence spectroscopy study

Tertiary structural alterations of proteins in the presence of different concentrations of osmolytes were monitored by fluorescence spectroscopy. The buffer used in these equilibrium measurements was 50 mM Tris, pH 8, at ambient temperature, with final protein concentration of 20 μg/ml. Fluorescence intensity of PCL with different concentrations of osmolyte was measured on LS 55 spectrometer (Perkin Elmer, Langen, Germany). Samples were excited at 290 nm, and the emission spectra were recorded between 300 and 400 nm. Both excitation and emission slits were set at 5 nm [3,26].

2.6. Circular dichroism spectroscopy study

Interaction of a chiral molecule with polarized light is very specific and has proved to be an important technique for studying macromolecular structures. With the help of CD spectropolarimeter, any conformational changes that might be induced in the structure of macromolecules could be assessed [27,28].

CD spectra were taken in the far-UV region (190–260 nm) by J-715 spectropolarimeter (Jasco, Tokyo, Japan). For far-UV CD characterization concentration of the PCL was adjusted at 0.2 mg/ml and osmolyte concentration ranged from 0 to 1 M. The final spectra were obtained by subtracting the buffer from the original protein spectra. Data was smoothed and analyzed by J-715 software, using fast Fourier-transform to prevent data distortion. The molar ellipticity values were obtained at 1-nm intervals using the equation: $[\theta] = (\theta \times 100MRW/cl)$; where c is the sample concentration, l is the length of the cuvette cell, and MRW represents "mean amino acid residue weight", i.e. 102 for PCL. The ellipticity, θ, is measured in terms of degree at wavelength of λ. Finally molar ellipticity could be reported accordingly (degree $cm^2\ dmol^{-1}$).

2.7. Surface plasmon resonance measurement

Surface plasmon resonance technique was used to determine the interaction of PCL with two osmolytes, i.e. sorbitol and trehalose. The SPR peaks of two dimensional arrays of Au nanoparticles on the glass substrate were investigated in the presence of PCL and various concentrations of each osmolyte mixture. Then SPR absorption spectra of UV–visible spectrometer, Stellar.net (Florida, USA) from 2 mm diameter optical fiber that transfers a non-polarized light beam (400–850 nm) through samples to a CCD detector was measured. The corresponding SPR wavelength of Au nanoparticles was obtained under exposure to different mixtures of PCL and osmolyte. The same glass slides were used as the reference (without gold nanoparticles) [12]. The buffer used in this equilibrium measurement was 50 mM Tris, pH 8 at 25 °C with final PCL concentration of 20 μg/ml. Solutions of trehalose and sorbitol with different concentrations (0–1 M) were mixed with the enzyme, and enough time was given to reach equilibrium. SPR of gold nanoparticles was recorded immediately after the addition of osmolytes.

3. Results and discussion

3.1. Lipase hydrolytic activity

Results revealed that hydrolytic activity of PCL increased in the presence of sorbitol and trehalose (0.2–1 M), each osmolyte being investigated individually. Increasing the concentration of individual osmolytes led to further increase of PCL activity (Fig. 1).