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Designing of nitrile hydratase from alkaline protease using quanidine hydrochloride and cobalt metal ion

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ABSTRACT

Alkaline protease (22.5 kDa) from *Bacillus licheniformis* was denatured using 3–6 M Guanidine hydrochloride followed by renaturation with cobalt metal ion in buffer solution (pH 7.5) in order to design cobalt dependent nitrile hydratase AP-*x*MGdn-Co, where *x* = 3, 4, 5 and 6. The native alkaline protease showed a 20% residual molar ellipticity at 212 nm while on treatment with Gdn-HCl, it got reduced to 12% due to nearly complete collapse of the globular structure of the protein. The observed circular dichroism spectra of the renatured proteases with Co metal ion are different from the native enzyme, because those molecules contain many metal chelating sites through its coordinating amino acid residues. The introduction of cobalt centered new active site was evidenced by the fluorescence spectroscopy (appearance of a new peak at 360 nm) and ICP analysis (0.0476–0.0553 mg/L). The specific activities of the modified enzymes were measured using the selective catalytic hydrolysis of 3-cyanopyridine to nicotinamide. The highest specific activity of renatured enzymes (AP-*x*MGdn-Co) were obtained at optimum pH of 7.5 and temperature of 50 °C, due to the proper co-ordination of amino acid residue with cobalt metal ion and the appropriate arrangement of α-helix, β-strand, turn and unordered segment in the globular structure.

1. Introduction

Nitrile hydratases (NHases) are bacterial enzymes that catalyze the hydration of nitriles to the corresponding amides. NHases contain non-heme iron or non corrinoid cobalt as their active sites. Two of the NHase enzymes obtained from *Chlororaphis B23* and from *Rhodococcus rhodochrous J1* are used for industrial production of acrylamide and nicotinamide [1]. However, the wild strains still have disadvantages such as poor thermo-stability, buffer ion inactivation, and the accumulation of the by-products [2]. In order to overcome these drawbacks, the heterogeneous cloning and expression of NHase in different recombinant strains have been attempted [3–5]. Fe-type NHases require light energy to activate the chemical reaction and preferentially hydrate small aliphatic nitriles [6–8]. Co-type enzyme exhibits high affinity for aromatic and hetero aromatic nitriles [9–11]. Although the ionic radii of Fe^{3+} (0.64 Å) and Co^{3+} (0.63 Å) are similar, the amino acid residues around their metal center are unlikely conserved [12].

Bacterial alkaline proteases are the important industrial enzymes which exhibit high activity at optimal pH and temperature with broad substrate specificity. There has been a renewed interest in the molecular study of replacement/insertion of metal ion in these enzymes [13]. In this present work, designing of nitrile hydratase from alkaline protease enzyme is discussed with reference to catalytic conversion of 3-cyanopyridine to nicotinamide. Reversible denaturation induced by guanidine hydrochloride at different molar was done to have different unfolded state of proteins [14–19]. Similar experiments have been reported with the enzymes, alkaline phosphatase and carbonic anhydrase [20,21].

2. Experimental

2.1. Materials

Guanidine hydrochloride (Gdn-HCl, 99%, A.R.), Cobalt (III) fluoride (99%, A.R.), 3-Cyanopyridine (98%, A.R.), Nicotinic acid (98%, A.R.), Nicotinamide (98%, A.R.), Na_2HPO_4 (99%, A.R.), $NaH_2PO_4{\cdot}2H_2O$ (99% A.R.), Nitrile hydratase recombinant from *E. coli*, were purchased from Sigma–Aldrich (USA). Alkaline Protease (EC3.4.21.62) from *Bacillus licheniformis* was donated by Genencor

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International, The Netherlands. Fast spin dialyzer 2000 μl chamber, Cellulose acetate dialysis membrane MWCO = 1000 Da, were purchased from Harvard apparatus, Holliston, MA. Acetonitrile HPLC grade (98%, A.R.), Phosphoric acid (85%, A.R.) and Hydrochloric acid (35.4%, A.R.) were purchased from s.d. Fine Chemical, India. These chemicals were used for enzyme modification and their activities measurements.

2.2. Introduction of cobalt centered new active site

1 ml of Guanidine hydrochloride (3–6 M) was added to 1 ml of alkaline protease (12 mg) and mixed well. This solution was kept for 30 min at room temperature. To this solution, 0.5 ml of CoF_3(0.3 mM) solution was added and the resultant solution was transferred into a double side fast spin dialyzer chamber having cellulose acetate membrane (MWCO = 1000 Da). This was dialyzed in 12 ml CoF_3 and 100 ml sodium phosphate buffer (0.1 M, pH 7.5) twice and then dialyzed with sodium phosphate buffer four times. This was finally transferred into a 5 ml vessel and kept at ~4 °C for 10 h in a refrigerator to achieve rearranged globular structure. The native alkaline protease and 3–6 M Gdn-HCl denatured enzymes are designated as AP-Blank, AP-3MGdn, AP-4MGdn, AP-5MGdn and AP-6MGdn respectively. The above denatured enzymes are renatured in presence of cobalt metal ion and designated as AP-3MGdn-Co, AP-4MGdn-Co, AP-5MGdn-Co and AP-6MGdn-Co respectively. The creation of cobalt centered new active site is shown in Fig. 1.

2.3. Fluorescence spectroscopy

The absorbance change due to the coordination of Co metal ion with peptide was measured using renatured enzyme solution (2 mg/mL) with reference to the native alkaline protease in the range of 200–400 nm using a UV–VIS–NIR scanning spectrophotometer (CARY, Varian 500 SCAN) to obtain excitation wavelength (spectra not shown). The same concentration of cobalt active site centered novel enzyme solution was excited at 285 nm and the fluorescence spectra was collected in the range of 290–450 nm using model Fluorolog Horiba Jobin Yvon spectrofluorimeter at room temperature to measure the absorbance shift due to the cobalt metal ion in the protein complex.

2.4. Inductive coupled plasma (ICP) analysis

Alkaline protease is a calcium containing enzyme in its globular structure. The amount of calcium present in the native enzyme (AP-Blank) was obtained with the instrument having calcium detection limit of above 0.01 mg/L. The native enzyme was denatured using 3–6 M Gdn-HCl and then renatured by dialyzing in phosphate buffer with cobalt metal ion for the reason to check the ability to replace (calcium) or insertion of cobalt metal ions in native protease structure. The total amount of cobalt metal ion present in the new active site created enzyme solutions was determined in the detection range of 0.007 mg/L using Inductively Coupled Plasma Optical Emission Spectrometry (ICP; Perkin Elmer, Optima 3300 RL).

2.5. Circular Dichroism (CD) spectroscopy

Circular Dichroism (CD) spectra were measured at 25 °C in 190 and 280 nm range using a JASCO- 815 UV–Vis spectropolarimeter, equipped with a thermostat, in a 3-mL cuvette with 1-cm path length. Protein concentration was 0.1 mg/mL. Samples were scanned after 30 min incubation with the denaturant (3–6 M Gdn-HCl) in 0.1 M sodium phosphate buffer at pH of 7.5. Each spectrum was corrected for baseline. The molar mean residue ellipticity at wavelength λ, $[\Theta]_\lambda{}^{25\,^\circ C}$ in degrees centimeter^2 decimole^{-1}, was obtained from

$$[\Theta]_\lambda^{25^\circ C} = \frac{\theta(\mathrm{MRW})}{10\,lc}$$

where θ = experimentally observed ellipticity in degrees at wavelength λ, l = path length in centimeters, c = protein concentration in grams per milliliter and MRW = mean residue weight of protein (=112), calculated from the amino acid composition of alkaline protease.

The secondary structure of denatured alkaline protease in 3–6 M Gdn-HCl and the renatured alkaline protease in presence of cobalt metal ion were assigned using the Define Secondary Structure of Proteins (DSSP) program [22]. The secondary structural components was classified into six types: regular α-helix (αR), distorted α-helix (αD), regular β-strand (βR), distorted β-strand (βD), turn, and unordered structure [23]. The secondary structure fractions of the globular proteins from the CD spectrum of the denatured and renatured alkaline protease were analyzed using the reference set 4 in the member of the DICHROWEB online server (http://dichroweb.cryst.bbk.ac.uk/). Two methods for analyzing protein CD spectra were used, as implemented in computer programs SELCON3 and CONTIN/LL. The detailed descriptions of these methods are available in the literature [24].

2.6. Activity assay of the enzyme

The resultant modified alkaline protease (~12 mg) was made into 4 ml enzyme solution using sodium phosphate buffer (0.1 M, pH 7.5) and then used in all kinetic studies. Nitrile hydratase enzyme activity was measured by incubating with 0.53–4.7 mg/ml of 3-cyanopyridine for exactly 6 h at 50 °C. The reaction was stopped by adding 0.1 ml of 1 M HCl solution and the precipitate was removed by centrifugation. The hydrolysis product nicotinamide from 3-cyanopyridine was measured by using JASCO LC-2000 plus system equipped with a HiQ Sil C18Hs reverse-phase column (4.6 mm × 250 mm; KYA Technologies Corporation, Tokyo, Japan) at a flow rate of 1.0 ml/min at 30 °C, with the following solvent system: acetonitrile-10 mM NaH_2PO_4–H_3PO_4 buffer (pH 2.8), 1:4 (v/v) [25].

2.7. Optimum temperature, pH and stability studies

The effect of temperature on the nitrile hydratase enzyme activity was observed by incubation with 1.56 mg/ml of 3-cyanopyridine in 0.1 M phosphate buffer solution having a pH 7.5 at temperatures ranging from 30 to 60 °C, and alternatively incubated at a fixed temperature of 50 °C at pH ranging from 6 to 9 for determining the effect of pH. The stability was studied by measuring the specific activity of natural nitrile hydratase (1 mg) or AP-Co-4MGdn-HCl (~12 mg) enzyme was made into 4 ml enzyme solution using sodium phosphate buffer (0.1 M, pH 7.5) followed by incubation with 1.56 mg/ml of 3-cyanopyridine at temperature of 50 °C and pH of 7.5 by holding for different time intervals. The reaction was stopped by adding 0.1 ml of 1 M HCl solution and the precipitate was removed by centrifugation. The hydrolysis product of nicotinamide from 3-cyanopyridine was determined as described earlier (Section 2.6).

3. Results and discussion

3.1. Secondary structural component from CD spectra

The circular dichroism (CD) spectra of the native, Gdn-HCl denatured alkaline proteases exhibit characteristic peaks in the UV