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A spectroscopic and thermal stability study on the interaction between putrescine and bovine trypsin

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ABSTRACT

The interaction of putrescine with bovine trypsin was investigated using steady state thermal stability, intrinsic fluorescence, UV–vis spectroscopy, far and near- UV circular dichroism and kinetic techniques, as well as molecular docking. The Stern-Volmer quenching constants for the trypsin- putrescine complex were calculated revealing that putrescine interacted with trypsin *via* the static fluorescence quenching. The enthalpy and entropy change values and the molecular docking technique revealed that hydrogen bonds and van der Waals forces play a major role in the binding process. Upon putrescine conjugation, the V_{max} value and the k_{cat}/K_m values of the enzyme was increased. The results of UV absorbance, circular dichroism and fluorescence techniques demonstrated that the micro environmental changes in trypsin were induced by the binding of putrescine, leading to changes in its secondary structure. The thermal stability of trypsin- putrescine complex was enhanced more significantly, as compared to that of the native trypsin. The increased thermal stability of trypsin- putrescine complex might be due to the lower surface hydrophobicity and the higher hydrogen bond formation after putrescine modification, as reflected in the increase of UV absorbance and the quenching of fluorescence spectra. It was concluded that the binding of putrescine changed trypsin structure and function.

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1. Introduction

A considerable interest has been focused on studying the interaction of stabilizers with organic and biological molecules, promising to be an exciting field of basic and applied research [1]. Putrescine (1,4 diaminobutane) is an aliphatic diamine belonging to the group of biogenic amines with a low-molecular-weight nitrogenous base. The basic amino groups carry a positive charge at the physiological pH of 7.4, making them appropriate for a wide range of functions in different cell types. Putrescine is found in decomposed animal materials, different kinds of foodstuffs, viruses, semen and all cell types [2]. This polyamine shows many physiological functions and is a precursor in the synthesis of other polyamines (spermine and spermidine) and the formation of carcinogenic *N*-nitrosamines. Putrescine, due to its polycationic nature, interacts with negatively charged molecules such as proteins, phospholipids, DNA and RNA [3]. The importance of negatively charged, aromatic and polar residues in relation to the interaction of polyamines with proteins has been confirmed [4]. The charge distribution is important for molecular recognition and the hydrophobic polymethylene backbone of them confers structural flexibility [5].

The effects of putrescine in decreasing membrane fluidity and increasing the resistance to fragmentation can be due to the stabilization of the membrane skeleton and deletion of the free radicals as luminal growth factors for intestinal maturation and growth [6,7]. It can play a significant role in the prevention of food allergies [8]. Putrescine also has direct effects on several ion channels and receptors, resulting in the regulation of Ca^{2+}, Na^{+} and K^{+} homeostasis [9]. Polyamines have the kosmotropic properties [10,11]. Kosmotropes cause water molecules to favorably interact, thereby stabilizing the intramolecular interactions in macromolecules such as proteins [12].

Bovine trypsin (T8003, E.C. 3.4.21.4), a serine protease, is a soluble globular protein that cleaves peptide bonds at the carboxyl-terminal end of lysine, arginine and ornithine residues, which are

Abbreviations: CD, circular dichroism; BAEE, N_α-benzoyl-L-arginine ethyl aster.

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widely employed in biomedicine, biotechnology and food industry, as well as in protein analysis [13,14]. Trypsin consists of a single chain polypeptide of 223 amino acid residues. X-ray crystallography of trypsin indicates that it has two nearly equal size domains. It has six antiparallel strands making a β-sheet unit through a network of hydrogen bonds [15]. The amino acid sequence of trypsin is cross-linked by six disulfide bridges. The active site amino acid residues of trypsin include His 46 and Ser 183 [16]. It has an isoelectric point, pI, of around 10.5 [15].

To this date, there is not any report showing the effect of putrescine on the stability and conformation of trypsin. In addition, the explanation of enhanced stability, in terms of conformational change in trypsin, has not been provided. Thus, the aim of this study was to investigate the effect of putrescine on the stability and conformation of trypsin, and to discuss the stability improvement of the modified trypsin in terms of the conformational change.

2. Materials and methods

2.1. Materials

Trypsin from bovine pancreas (catalogue no. T8003) was obtained freeze – dried from Sigma and used without further purification. Tris–HCl buffer, N_α-benzoyl-L-arginine ethyl aster (a substrate used as a probe for the activity of trypsin, BAEE, catalogue no. B 4500) and putrescine were also bought from Sigma Aldrich Co. All trypsin solutions were made in the 50 mM Tris–HCl buffer at the pH 8.

2.2. Thermal stability assays

The thermal stability of free enzyme and enzyme- putrescine complexes was investigated at different temperatures using a Pharmacia 4000 UV–vis spectrophotometer equipped with an external thermostat. According to the method developed by Zhang et al., thermal stability of trypsin (0.1 mg/mL) was estimated by incubating enzyme solutions in buffer for 10 min, at 280 nm and pH 8 [17]. The absorbance changes were recorded at 280 nm in the temperature range of 293 to 373 K, as well as the heating rate 1 K/min. The absorption data were plotted as a function of temperature.

2.3. Fluorescence spectroscopy measurements

Fluorescence spectroscopy was used to monitor the binding of putrescine to the protein molecules and the tertiary structure changes in trypsin induced by putrescine. Steady-state fluorescence spectra of trypsin were measured by exciting 280 nm with scanning between 300 and 450 nm, at 298 and 308 K. The intrinsic fluorescence of enzyme was recorded using a Shimadzu RF-5301 fluorescence spectrophotometer with a temperature adjustable cell holder. A 1-cm quarts cuvette was used for these studies. Emission and excitation slits were set to 3 and 5 nm, respectively. The structural changes of trypsin (0.1 mg/mL) were monitored in the absence and presence of different concentrations of putrescine (0–7 mM). The mixture of trypsin with putrescine was incubated for 10 min to form the protein-putrescine complex before the measurement of the fluorescence intensity. To obtain the binding parameters, data were analyzed by the modified Stern-Volmer equation [18–20].

2.4. Circular dichroism (CD) measurements

The UV-CD studies were performed by an Aviv model 215 Spectropolarimeter (Lakewood, New Jersey, USA), using quartz cells of 1 mm and 10 mm path length for the Far-UV (200–260 nm) and Near-UV (260–320 nm) regions, respectively. Dry nitrogen gas was supplied to purify the equipment before and during the course of the measurements. The data was expressed as the mean residue ellipticity $[\theta]$ in degree cm^2 d mol^{-1}, defined as $[\theta] = 100\,\theta_\lambda\, MRW/cl$, where θ_λ is the observed ellipticity in degrees, *MRW* is the mean molecular weight of amino acid residue, *c* is the trypsin concentration in mg/mL, and *l* is the length of the light path in cm [21]. CD samples were prepared with a concentration of trypsin (0.8 mg/mL for far UV-CD, and 1.6 mg/mL for near UV-CD) and different concentrations of putrescine (0, 2 and 4 mM). The suspension trypsin, Tris–HCl buffer and putrescine were allowed to equilibrate for 15 min at the room temperature. All spectra were also subtracted with putrescine solutions and smoothed. The different secondary structures were performed by the CDNN program, version 2.1.0.223, using a network trained with 33 complex spectra at the reference set.

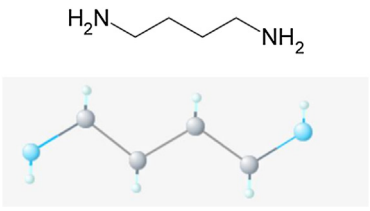

Fig. 1. Structure of putrescine.

2.5. UV absorption spectra analysis

The Pharmacia 4000 UV–vis spectrophotometer (Japan) was applied to record the UV–vis absorbance spectra over the wavelength of 260–310 nm. The quarts cells used for these studies were 1 cm in length. The spectrophotometer was baseline with the Tris–HCl buffer as the relevant blank. The same volumes of putrescine (1–4 mM) and trypsin at 0.1 mg/mL were equilibrated for 15 min at 37 °C and then the spectra of the enzyme were obtained at λ_{280}. The resulting absorbance change in the absence and presence of putrescine was plotted *versus* putrescine concentration.

2.6. Enzymatic activity assay

In order to investigate the effects of putrescine on the enzyme activity, the catalytic activity of trypsin was determined by UV–vis spectrophotometer at pH 8.0 and the temperature of 37 °C. Various concentrations of putrescine were incubated with trypsin (25 μg/mL) in a 50 mM Tris–HCl buffer solution (pH 8) for 15 min and the reaction mixture was used to monitor the change in absorbance at 253 nm and 37 °C after the addition of BAEE [22]. All assays were repeated at least 3 times. The kinetic parameters (K_m, V_{max}, k_{cat} and k_{cat}/K_m) were determined from the data of trypsin activity against a range of BAEE concentrations, based on the equation of Lineweaver-Burk.

2.7. Molecular docking studies

Molecular docking studies of trypsin and putrescine were performed using AutoDock 4.0 software package [23]. The crystal structure of trypsin was downloaded from the Protein Data Bank (PDB) (trypsin accession number: 2PTN). The geometry of putrescine was modeled by HyperChem program [24] and minimized by the molecular mechanics method *via* MM^+ force field (Fig. 1). Trypsin was considered as rigid and putrescine as fully flexible during docking. During molecular calculation studies, all water molecules were removed from the original Protein Data Bank file. Furthermore, Kollman united atom type charges, essential hydro-

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