Chemosphere 185 (2017) 1056–1062

Contents lists available at ScienceDirect

Chemosphere

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

Probing the binding properties of dicyandiamide with pepsin by spectroscopy and docking methods

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HIGHLIGHTS

- Interaction of pepsin with dicyandiamide were examined.
- Dicyandiamide binding affects the native structure of pepsin.
- Binding is moderate and dicyandiamide mainly binds at hydrophobic cavity of pepsin.

ARTICLE INFO

Article history:
Received 7 December 2016
Received in revised form
20 July 2017
Accepted 21 July 2017
Available online 22 July 2017

Handling Editor: I. Cousins

Keywords:
Dicyandiamide
Fluorescence
Molecular modeling
Pepsin

ABSTRACT

Dicyandiamide (DCD), considered to be a nitrification inhibitor, poses threat to human's health with exposure from milk, infant formula and other food products. In this work, DCD was investigated for its binding reaction with pepsin using spectroscopy and docking methods. Fluorescence experiments indicated DCD quenched the fluorescence of pepsin through a static process. Thermodynamic analysis of the binding data ($\Delta H^0 = -21.72$ kJ mol^{-1} and $\Delta S^0 = 17.61$ J mol^{-1} K^{-1}) suggested the involvement of hydrophobic and hydrogen bonding in the complex formation. The pepsin interacted with DCD at a hydrophobic cavity, leading to a conformational changes in the pepsin, as revealed from UV–vis absorption, Fourier transform infrared, the time-resolved fluorescence, three-dimensional fluorescence and circular dichroism spectral results.

1. Introduction

A nitrification inhibitor (dicyandiamide, DCD, structure was shown in Fig. 1A) was widely applied as a product in agriculture for promoting the growth of pastures where cows graze (Zaman et al., 2009). However, cows eating contaminated grass may result in the production of milk with DCD residues and cause pollution of the downstream products (MacMahon et al., 2012). On the other hand, DCD is a nitrogen-rich compound that was deliberately added to a number of different types of animal- and human-food sources to artificially enhance apparent protein content. Recently, Abernethy et al. provided rapid and effective approaches to combat economically motivated adulteration in protein-containing products (Abernethy and Higgs, 2013). Inoue et al. also reported a HILIC–MS/MS method for determination of DCD in powdered milk, and the DCD contamination in one sample was detected and quantified at 79.1 ± 1.2 ng/g (ppb) powder (Inoue et al., 2014.). The mouse studies reported that the DCD LD50 mortality dosage was 5000 mg/kg, and its acute toxicological properties were similar to those of melamine (Liu et al., 2016). Investigation of the toxicology suggested that high doses of DCD are considered toxic to humans and can result in some diseases, such as methemoglobinemia and eczema (Yasuhara et al., 1997). In 2012, the presence of DCD in powdered milk has been reported in New Zealand, and caused panic (Lucas, 2013). Many consumers become overly sensitive about the contamination of DCD in dairy products, especially infant formula. DCD can be transferred to the body of human being via the food cycle. Therefore, DCD residue in different types of animal- and human-food sources has become a potential risk to human health (Ge et al., 2016; Liu et al., 2015).

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http://dx.doi.org/10.1016/j.chemosphere.2017.07.115
0045-6535/

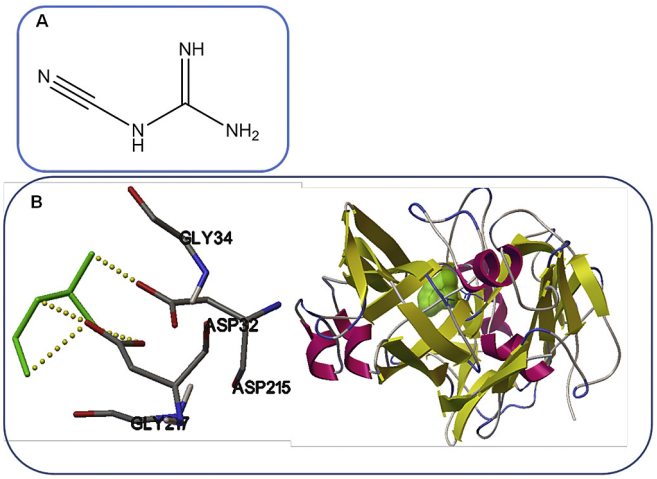

Fig. 1. The chemical structure of DCD (A); Molecular docking poses of DCD with pepsin (B). The structure of DCD is represented using stick model. The hydrogen bond is rendered in yellow dotted line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Pepsin, a critical digestive enzyme, is responsible for the most of digestive activities in the stomach (Gole et al., 2000). Pepsin consists of 326 amino acids. It is a multi-tryptophan protein that contains five Trp residues (Trp39, Trp141,Trp181, Trp190, and Trp300). The five Trp residues can be used as intrinsic fluorophores to provide information about the pepsin–ligand interaction and ligand-induced conformational change (Jin et al., 2008). During the process of digestion, it has effects of food digestion by cleaving peptide bonds between hydrophobic and preferably aromatic amino acids (Kageyama, 2002). In the meantime, pepsin can interact with ingredients of the food, and the activity of pepsin may be affected. When DCD enter into human stomach, the pepsin may be the directly binding targets and affect some micro-environmental and conformational changes in pepsin. The previous studies revealed that excess intake of some compounds can induce certain toxic effects of chemicals on pepsin. Wang et al. described the binding mechanism of phthalate plasticizers with digestive proteases (Wang and Zhang, 2013). Huang et al. investigated the binding properties of a kind of new oral taking antibiotic with pepsin (Huang et al., 2010). Knowledge of interaction mechanisms between small molecules and pepsin are very important to evaluate the toxicity of these small molecules that enter the stomach through food or drug.

Therefore, in this study, we have investigated the binding mechanism and determined the binding constants, the numbers of binding site of DCD with pepsin by means of spectroscopic techniques and molecular modeling method. These investigations will be helpful for realizing the transportation and distribution of DCD in *vivo* at molecular level, which are helpful to determine its toxicological effects.

2. Materials and methods

2.1. Materials

Pepsin (from porcine stomach mucosa, Purified Enzymes, ≥3000 NFU/mg) was purchased from Sangon Company (Shanghai, China) and used without further purification. All other reagents were of analytical grade, obtained from commercial sources. DCD was purchased from J&K Scientific Ltd. (Beijing, China) and the physicochemical properties of DCD were shown in Table 1. Pepsin solution (3.0×10^{-4} M) was prepared in pH 2.00 citric acid buffer solution. The solution of DCD (1.0×10^{-3} M) was prepared in water. During experiment, water was purified with a Millli-Q purification system. All of pH values were measured with a pHS-3C acidity meter (Leici, Shanghai, China).

2.2. Methods

2.2.1. Molecular docking

The binding interaction of DCD with pepsin was simulated by molecular docking using Autodock 4.2 (Morris et al., 2009). The native structure of pepsin was taken from Protein Data Bank having PDB ID 5PEP (Cooper et al., 1990). All water molecules were removed. The polar hydrogen and the Gasteiger charges were added at the beginning of docking study. The geometry of DCD was subsequently optimized to minimal energy. Grid maps were generated with 0.375 Å spacing using a grid box of 80–80–80 Å. That Lamarckian genetic algorithm (LGA) implemented in Autodock was applied to calculate the possible conformation of the DCD that bound with pepsin (Lv et al., 2013). During docking process, a maximum of 10 conformers were considered for DCD. The docking conformation with the lowest binding free energy was used for further analysis.

2.2.2. Fluorescence measurements

The fluorescence spectra were studied using a FP-6500 spectrofluorometer (JASCO, Japan). The excitation wavelength was 280 nm and both the slit widths of excitation and emission were set at 5 nm. The fluorescence spectrum of the buffer was subtracted and the inner-filter effect was eliminated following a reported method (Lakowicz, 2007):

$$F_{cor} = F_{obs} \times e^{(A_{ex}+A_{em})/2} \quad (1)$$

F_{cor} and F_{obs} are the corrected and observed fluorescence intensities, respectively, A_{ex} and A_{em} are the absorbance values of the system at excitation and emission wavelengths, respectively. The data of fluorescence intensities used corrected data in this study. The quenching rate constant values of DCD-pepein were calculated using Stern-Volmer equation.

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{sv}[Q] \quad (2)$$

In the Eq. (2), F_0 and F are the fluorescence intensities of pepsin in the absence and presence of DCD, K_q is the quenching rate constant of the biomolecule, t_0 is the average lifetime of the fluorophore in the absence of the quencher as 1×10^{-8} s (Lakowicz and Weber, 1973), K_{SV} is the Stern–Volmer quenching constant, and [Q] is the concentration of DCD.

In addition, binding constants (K) and binding sites (n) were obtained using the following equation (Huang et al., 2001; Abdelhameed et al., 2017):

$$\log \frac{F_0 - F}{F} = \log K + n \log[Q] \quad (3)$$

The change in Gibbs free energy was calculated by van't Hoff equation.

$$InK = -\Delta H^0 / RT + \Delta S^0 / R \quad (4)$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (5)$$

K, T, R are the binding constants corresponding to various temperatures, the temperature (298 K, 303 K and 310 K) and gas constant (8.3145 J mol^{-1} K^{-1}), respectively.