International Journal of Biological Macromolecules 68 (2014) 50–59

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Structural functional and folding scenario of an anti platelet and thrombolytic enzyme crinumin

Kunwar Awaneesh Singh [b], Sanjay Singh [b], Medicherla V. Jagannadham [a,*]

[a] Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi, UP 221 005, India
[b] Department of Pharmaceutics, Indian Institute of Technology, Banaras Hindu University, Varanasi, UP 221 005, India

ARTICLE INFO

Article history:
Received 30 January 2014
Received in revised form 29 March 2014
Accepted 2 April 2014
Available online 13 April 2014

Keywords:
Crinumin
Thrombolytic
Anti-platelet
Protein folding

ABSTRACT

A folding pattern, conformational stability and therapeutic role of a protein helps in developing a suitable drug. Crinumin, a thrombolytic and anti platelet agent, has been studied for its functional and conformational properties by equilibrium unfolding methods. The crinumin belongs to $\alpha+\beta$ class of protein and exhibits a non native structure and two molten globule states at different conditions. Two domains in the molecular structure of the protein with altered stability are present that unfold sequentially. The enzyme maintains activity as well as structural integrity even in adverse conditions. These observations provide an understanding of protein folding as well as facilitate the development of a potential drug.

1. Introduction

A unique three-dimensional structure of a protein is responsible for its specific function which is achieved through protein folding. Still, it is one of the big challenges for the scientist of protein chemistry to understand the exact mechanism of protein folding. Studies towards unwrapping the secret facts of such challenges are not just beneficial for theoretical point of view, but also help in better understanding the molecular basis of protein stability. In addition to this, knowledge of the mechanisms involved in the three-dimensional structure acquisition, can be employed to design a protein with desired properties for biotechnological and pharmacological applications.

The folding of a protein, mainly depends on its amino acid sequence and cellular environment in which the polypeptide chain is present [1]. Preceding reports in this direction illustrated that the polypeptide chain undergoes various conformational states during folding. Characterization of these intermediate conformational states would be an attractive approach to elucidate the folding mechanism because such conformational states might mimic the *in vivo* protein-folding pathway [2–9]. Insight into these intermediate states would also help us to figure out when and how a variety of forces come into action to achieve functional three-dimensional structures of the proteins. In this way all the conformational states should be analyzed with respect to their structure and function, but rapid formation of these intermediate species makes it difficult for better analysis by conventional stopped-flow experiments [10]. This problem can be overcome by focusing on the structural studies of intermediates generated at equilibrium. This is achievable by removing protein-bound metal ions or prosthetic groups as well as by dissolving the protein in acid solution or in the presence of moderate concentration of denaturants [2,11]. These intermediates are well occupied and stable, thus gives an opportunity for their detailed characterization.

Among the common equilibrium intermediates reported for many proteins, the 'molten globule' (MG) state is the best characterized form. Molten globule states are believed to provide information on early events in the folding process. Therefore, structural and functional analysis of the molten globule state is crucial for understanding the mechanism of protein folding.

A number of reports which emphasized their study specifically on folded forms of proteins are available, but researches focusing on non-native states of various structural types are still limited. In this respect, further studies about different conformational states, generated during the unfolding and refolding of multi domain proteins are needed to understand the principles governing the folding pathways.

Though plant proteases are one of the important classes of protein, but not much work was reported which deals with its general

* Corresponding authors. Tel.: +91 542 2367936; fax: +91 542 2367568.
E-mail addresses: kawaneesh@gmail.com (K.A. Singh), jvm@bhu.ac.in (M.V. Jagannadham).

http://dx.doi.org/10.1016/j.ijbiomac.2014.04.002
0141-8130/

folding aspects. Various studies have been performed in our laboratory to understand the folding behavior of various novel plant proteases such as ervatamins (A, B and C), procerain, heynein and cryptolepain. Ervatamins, procerain and heynein are plant cysteine proteases belonging to the α+β structural class while cryptolepain is a serine protease but predominantly β-sheet protein [12–15]. Study of another mechanistic class of proteases is essential to understand the folding of plant proteases in detail. Keeping this in view, conformational studies have been carried out on crinumin in solution. Crinumin, a novel glycosylated serine protease purified in our laboratory from the latex of a medicinally important plant *Crinum asiaticum*.

Crinumin is a glycoprotein and predominantly α+β protein with molecular weight of 67.7 kDa. The molecular structure of the protein contains 13 Tryptophan and 24 Tyrosine amino acid residues. Extensive biochemical studies have been established with its numerous novelties [16]. Crinumin can dissolve human blood clot as well as inhibit the formation of new clots [17]. These properties make the present work significant and relevant effort towards the development of a potential new therapeutic agent, for the treatment and prevention of thromboembolic diseases. Detail mapping of protein conformational changes offers significant information in reference to drug designing. In this regard the enzyme is crystallized by hanging drop method followed by structure determination [18].

Present work describes the biophysical characterization of crinumin to understand its structural, functional and folding-unfolding behavior. The output of this work will help in developing the appropriate drug for the treatment of thromboembolic disease as well as a model system for comparative biophysical study of other proteins.

2. Experimental procedures

2.1. Materials

Urea, ANS and GuHCl were purchased from Sigma Chemicals. The refractive index was used for the determination of concentrations of urea and GuHCl solutions [19]. All other used chemicals were commercially available and of analytical grade with high purity. All the solutions were prepared in double distilled water and the samples for spectroscopic measurements were centrifuged and filtered through 0.45 μm filters. The exact concentration of the protein and pH was estimated prior to the experiments.

2.2. Protein purification

In the development of the protocol for purification of crinumin, different chromatographies including size exclusion chromatography are explored. The finalized protocol of purification of the protein which is highly reproducible in term of activity and yield is reported. The enzyme was purified from the latex of *C. asiaticum* with the help of cation-exchange chromatography [16]. Latex was collected into 10 mM acetate buffer pH 5.0 from the plant via incisions on the midrib of their leaves. After storage at −20 °C for 36 h, it was thawed at room temperature and centrifuged at 15,000 × *g* (Sorvall RC-5C Plus) for 30 min to remove insoluble materials. Supernatant was loaded on a CM-Sepharose column (GE Healthcare) and bound protein was eluted with a NaCl gradient in the same buffer. Protein content (by absorbance at 280 nm) and proteolytic activity (described below) of the fractions was measured. Similarly homogeneity of the protein in the fraction was judged by SDS-PAGE. The fractions with activity and higher homogeneity were pooled and concentrated by membrane filtration using Amicon concentrator with YM10 membrane. The clear solution of crinumin was stored at 4 °C for further use.

2.3. Activity assay

The proteolytic activity of protein was measured by using casein as a substrate, as described previously with minor modification [20]. An aliquot of crinumin was incubated at 37 °C for 15 min in a final volume of 500 μl 50 mM Tris–HCl buffer pH 7.5. In the reaction mixture 0.5 ml casein solution (1%) was added and the reaction was allowed to proceed for 90 min. The reaction was terminated by the addition of 0.5 ml 10% TCA and the precipitate was removed by centrifugation. Peptides in the supernatant were estimated from the absorbance at 280 nm and the activity was calculated by using the definition. One unit of enzyme activity was defined as the amount of enzyme that gives rise to an increase of one unit of absorbance at 280 nm/min of digestion under the given assay conditions. The specific activity is the number of units of activity per milligram of protein.

2.4. Absorbance measurements

Absorbance measurements were carried out with the help of Beckman DU-640B spectrophotometer equipped with a constant temperature cell holder. Protein concentration for all absorbance measurements was in between 1 and 2 mg/ml. Absorbance spectra were recorded between 240 and 320 nm.

2.5. Spectropolarimetry

A spectropolarimeter (Jasco J 500A) calibrated with ammonium (+)-10-camphorsulfonate was used for the circular dichroism (CD) measurements. The temperature of the cell holder was controlled with the help of Julabo F 25 water bath. Conformational changes in the tertiary structure were observed in the region between 260 and 320 nm with a protein concentration of 36 μM. The cuvette with a path length of 10 mm was used. Changes in the secondary structure of the protein were monitored in the region between 200 and 260 nm with a protein concentration of 6 μM in a cuvette of 1 mm path length. Mean residue ellipticities were calculated (after subtracting appropriate blanks) using the formula

$$[\theta] = \frac{\theta_{obs} \times MRW}{10cl}$$

where θ_{obs} is observed ellipticity in degrees, MRW is mean residue weight (1 1 0), *c* is concentration of protein (g/ml) and l is the path length in centimeters [21]. Used sensitivities were 1 and 2 m°/cm for far UV and near UV measurements, respectively.

2.6. Fluorescence spectroscopy

PerkinElmer LS-50B spectrofluorimeter with a constant temperature cell holder was used for fluorescence measurements and the temperature was controlled by Julabo F 25 water bath. Tryptophan was selectively excited at 292 nm while for both the tryptophan and tyrosine fluorescence of the protease, the excitation wavelength of 278 nm was used. The emission was recorded between 300 and 400 nm with 10 and 5 nm slit widths for excitation and emission, respectively. The protein concentration used for all fluorescence measurements was 2 μM.

2.7. pH denaturation

pH-induced denaturation of crinumin was performed by using 50 mM buffers of KCl–HCl (pH 0.5–1.5), Gly–HCl (pH 2.0–3.5), sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–8.0), Tris–HCl (pH 8.5–10.5), and Gly–NaOH (pH 11.0–12.5). The desired concentration of protein from a stock solution was mixed into the appropriate buffer and the solution was incubated for 24 h at 25 °C.