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Improvement of proteolytic and oxidative stability of Chondroitinase ABC I by cosolvents

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ABSTRACT

Recently, utilization of the enzyme Chondroitinase ABC I (cABC I) has received considerable attention in treatment of spinal cord injury. cABC I removes chondroitin sulfate proteoglycans which are inhibitory to axon growth and enhances nerve regeneration. Therefore, determination of cABC I resistance to proteolysis and oxidation provides valuable information for optimizing its clinical application. In this work, proteolytic stability of cABC I to trypsin and chymotrypsin as well as its oxidative resistance to H_2O_2 was measured. Moreover, the effect of cosolvents glycerol, sorbitol and trehalose on cABC I proteolytic and oxidative stability was determined. The results indicated that cABC I is highly susceptible to proteolysis and oxidation. Comparison of proteolytic patterns demonstrated a high degree of similarity which confirmed the exposure of specific regions of cABC I to proteolysis. However, proteolytic degradation was significantly reduced in the presence of cosolvents. In addition, cosolvents decreased the rate of both cABC I proteolytic and oxidative inactivation. Notably, the degree of stabilization provided by these cosolvents varied greatly. These findings indicated the high potential of cosolvents in protein stabilization to proteolysis and oxidative inactivation.

1. Introduction

Chondroitinase ABC I (cABC I, EC 4.2.2.4) is a galactosaminoglycan (GAG) degrading lyase commonly produced by the bacterium *Proteus vulgaris*. cABC I has been characterized extensively in terms of its enzymatic activity and substrate specificity. The enzyme processes a variety of GAGs including chondroitin 4-sulfate (C4S), chondroitin 6-sulfate (C6S) and dermatan sulfate (DS) via a β-elimination reaction. These compounds which are composed of disaccharide repeat units of uronic acid linked to N-acetyl-D-galactosamine constitute side branches of proteoglycans [1–4].

It is becoming increasingly evident that GAGs which predominantly reside in the extracellular matrix hinder axon regeneration after spinal cord injury (SCI). Moreover, the inhibition of GAGs promotes neural plasticity and functional recovery after acute SCI. Therefore, administration of cABC I to manipulate GAGs has recently received much attention as a potential treatment strategy to increase neural regeneration [5–7].

However, intrathecal delivery of cABC I to the injured tissue faces some challenges, most notably enzyme stability. A recent study examining thermal stability of cABC I showed a significant decrease in cABC I activity kept at body temperature [8]. In order to improve the stability of an enzyme, several methods including chemical and genetic modifications, manipulation of solvent through utilization of cosolvents and immobilization of enzymes can be employed [9]. Accordingly, we have recently established two strategies to enhance cABC I thermal stability. In the first study, we have explored cosolvent mediated thermostabilization approach and have demonstrated that cosolvents such as glycerol, sorbitol and trehalose increase thermal stability of cABC I [10]. In the other investigation, we have made use of enzyme engineering to improve cABC I thermostability [11]. Conceivably, application of these approaches in vivo will be valuable for optimizing the efficacy of cABC I as a therapeutic preparation.

Another point worth considering for clinical application of proteins is their resistance to proteolytic digestion. Generally, proteolytic stability of proteins is determined by regions that are accessible and flexible enough for a cleavage. Therefore, even natively folded proteins may contain protease sensitive regions [12]. Clearly, proteins are rapidly degraded after unfold-

Abbreviations: cABC I, Chondroitinase ABC I; C6S, chondroitin-6-sulfate; C4S, chondroitin-4-sulfate; DS, dermatan sulfate; GAGs, galactosaminoglycans; SCI, spinal cord injury.

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ing which indicates a correlation between their proteolytic and thermal stability [13]. In addition to the elements of secondary structure, cleavage of peptide bonds is sterically impeded by post-translational modifications and the presence of disulfide bonds [12]. However, analysis of cABC I structure doesn't reveal any of these features [14]. From these findings as well as data from cABC I thermal stability analysis, it is expected that cABC I will be highly susceptible to proteolysis which remains to be investigated.

In addition to thermal and proteolytic inactivation of proteins, another source of proteins instability is their oxidation and subsequent inactivation or denaturation. Generally, oxidative modification of residues within proteins can be mediated by a variety of endogenous and exogenous systems such as oxidases, hydrogen peroxide, superoxide, and metal-catalyzed oxidation [15]. Although all residues are susceptible to oxidation, their susceptibility varies greatly. In particular, methionine and aromatic amino acid residues are primarily oxidized upon exposure of proteins to oxidants. Modification of these residues can severely decrease the conformational stability of proteins and, as a result, a reduction in protein thermal stability [16]. Thus, there might be a close correlation between oxidative and themostability of proteins [17]. Hence, it is plausible that the enhancement of oxidative stability of cABC I might lead to an improvement in its thermostability and vice versa.

Cosolvents are widely used to increase the stability of biological macromolecules in solution. The general observations are that these compounds increase thermal unfolding temperature, prevent loss of enzymatic activity and inhibit irreversible protein aggregation [18,19]. The putative mechanism underlying these beneficial effects has been primarily described on the basis of preferential exclusion of cosolvents from protein surface [20,21]. Recent works from Bolen and coworkers have revealed that preferential exclusion is achieved due to unfavorable peptide backbone-cosolvent interactions. Therefore, greater unfavorable interaction on unfolding results in a shift towards the folded state and consequently improvement in protein stability [22,23].

In order to determine the effect of cosolvents on proteolytic and oxidative stability of cABC I, this study was conducted. Here, we investigated the susceptibility of cABC I to trypsin, chymotrypsin and H_2O_2. Then, we extended our study to the effect of trehalose, sorbitol and glycerol on tryptolytic digestion and oxidative inactivation of cABC I. Interestingly, the results showed that these cosolvents decreased the rate of cABC I inactivation in the presence of trypsin, chymotrypsin and H_2O_2.

2. Materials and methods

2.1. Chemicals

Ni-NTA agarose was purchased from Qiagen (USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was provided by Takara (Japan). Protein ladder was obtained from EURx (Poland). Trypsin, chymotrypsin, chondritin 4-sulfate (C4S) and other chemicals with analytical grade purity were supplied by Sigma-Aldrich (USA).

2.2. Recombinant expression and protein purification of cABC I

Recombinant cABC I protein was obtained using the expression plasmid pET28 transformed into *E. coli* BL21 as previously described [10]. Briefly, 500 ml of Luria-Bertani (LB) medium supplemented with kanamycin (0.05 mg/ml) was inoculated with pre-cultivated medium of transformants and incubated at 37 °C. The culture was induced with 0.7 mM IPTG in mid-log phase and incubated at 27 °C for 6 h. Then the cells were harvested by centrifugation (3500*g*, 10 min, 4 °C). After resuspension of the cell pellet in lysis buffer (50 mM potassium phosphate, 300 mM NaCl, 5 mM imidazole, 1 mM phenylmethanesulfonyl fluoride (PMSF); pH 6.8), the cells were disrupted by sonication. The soluble proteins were subsequently isolated by centrifugation (15,500*g*, 20 min, 4 °C) and loaded on Ni-NTA column. Then proteins were eluted with increasing concentrations of imidazole in 50 mM phosphate buffer, pH 6.8. The purity of enzyme was assessed by SDS/PAGE using 12.5% (w/v) Bis/Tris gel and subsequent coomassie brilliant blue staining. Protein concentration was also measured according to the method of Bradford [24].

2.3. Proteolytic degradation of cABC I

The sensitivity of cABC I to chymotrypsin and trypsin was measured at 25 and 37 °C with final concentrations of 400, 2, 1 μg/ml of highly purified cABC I, chymotrypsin and trypsin respectively (E/S ratio 1:200 and 1:400). The reactions were performed in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$. After defined time intervals, samples were withdrawn and immediately mixed with 5 μl of 10 mM PMSF to stop the reaction. Then samples were boiled in sample buffer and separated by SDS/PAGE. In order to determine the percent of cABC I remained after protease digestion, densitometry analysis was performed using ImageJ software.

To evaluate the effect of cosolvents on tryptolytic stability of cABC I, the above experiment was carried out in the presence of glycerol (20%), sorbitol and trehalose (1 M). Then tryptolytic degradation of cABC I was determined at indicated time intervals. The concentration of these cosolvents was selected based on our previous study and was used for further measurements as well.

2.4. Proteolytic inactivation of cABC I

In order to determine proteolytic inactivation of cABC I, aliquots of enzyme incubated with trypsin (E/S ratio 1:400) and chymotrypsin (E/S ratio 1:200) were taken at indicated time intervals. Then cABC I activity was measured in 50 mM phosphate buffer, pH 6.8 at 25 °C. The measurements were performed at 232 nm using C4S as cABC I substrate. Then remained activity was revealed as percentage of the intact enzyme.

Proteolytic stability of cABC I was also investigated by enzyme incubation with trypsin (E/S ratio 1:400) in the presence of glycerol (20%), sorbitol and trehalose (1 M). Then the time course of inactivation was followed by measuring the remained activity of cABC I.

2.5. Oxidative inactivation of cABC I

To investigate the oxidative stability of cABC I, purified enzyme (0.1 mg/ml) was incubated with different concentrations of fresh H_2O_2 (1–100 mM) in phosphate buffer, pH 6.8 at 25 °C. Samples were taken at indicated time intervals and immediately added into assay buffer to quench the remaining H_2O_2. The remained activity was measured under standard conditions of the assay and expressed as a percent of non-treated enzyme activity.

To determine the effect of cosolvents on cABC I oxidative stability, incubation of cABC I with H_2O_2 (1 mM) was performed in phosphate buffer, pH 6.8 supplemented with glycerol (20%), sorbitol and trehalose (1 M) at 25 °C. Then remained activity was measured as described above.

3. Results and discussion

3.1. Proteolytic degradation of cABC I

Limited proteolysis is a useful experimental approach to study conformational stability of a protein. Here, we investigated the effect of unspecific proteases trypsin and chymotrypsin on cABC