Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

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

Improving the catalytic efficiency of Fibrinolytic enzyme from *Serratia marcescens* subsp. *sakuensis* by chemical modification

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ARTICLE INFO

Keywords:
Amino acid specific
Chemical modification
Circular dichroism spectrum
Fibrinolytic
Fibrinogenolytic
Kinetic studies

ABSTRACT

Microbial fibrinolytic enzymes have gained increased attention due to their potential to prevent or cure cardiovascular diseases. Promising natural enzymes are often modified to improve/enhance the kinetic constants. Hence an attempt was made to chemically modify the fibrinolytic enzyme produced by marine *Serratia marcescens* subsp. *sakuensis* using amino acid specific modifiers. The aim was to enhance the kinetic constants and gather information on the vital amino acid residues involved in the catalysis. Modification of cysteine, histidine, tryptophan and serine residues resulted in drastic reduction in fibrinolytic activity indicating their presence in the active site. Modification of carboxylate residues resulted in a 19-fold increase in specific activity suggesting their presence in the catalytic site. Interestingly, ratio of fibrinolytic to fibrinogenolytic activity of the modified enzyme did not change significantly. There was a 507-fold reduction in K_m value after chemical modification and due to that, 219-fold enhancement of catalytic efficiency was evidenced. Circular dichroism spectrum analysis of the modified and native enzyme revealed changes in α- helix and ß-sheet conformation of the enzyme. Furthermore, the modified enzyme was more responsive to the presence of most of the metal ions tested.

1. Introduction

Microbial fibrinolytic enzymes have gained increased attention in the research community recently due to their potential to prevent or cure cardiovascular diseases. These enzymes catalyze the breakdown of fibrin present within the blood clot [1]. In a healthy human body, two enzymes namely thrombin (3.4.21.5) and plasmin (3.4.21.7) maintain a constant state of equilibrium between blood coagulation and fibrinolysis. However, due to several medical conditions, this equilibrium is disturbed resulting in thrombosis [2]. Cardiovascular diseases such as coronary artery disease (CAD), venous thromboembolism (VTE) and ischemic strokes have thrombosis in common [2]. Under these conditions, fibrinolytic enzymes can be administered as drugs for the dissolution of the blood clot, restoring uniform blood flow within the blood vessels. Apart from fibrinolytic enzymes, plasminogen activators (tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) and bacterial type plasminogen activator-like streptokinase (SK)) and oral anticoagulants (heparin, warfarin, dabigatran and rivaroxaban) are already in use [3,4]. Despite the availability of these varied fibrinolytic agents, there is an ongoing effort to find new fibrinolytic agents due to the demerits of existing ones namely, inter and intrapatient variability of effective dose, food and drug interactions, intra-cranial haemorrhage, short half-lives, lower fibrin specificity, allergic reactions and high production costs. There is an ongoing effort to find new fibrinolytic enzymes from microbial sources, medicinal mushrooms, earthworms and snakes [5].

Natural enzymes are modified frequently to improve/enhance the specificity of the enzyme towards the target protein making it more effective and to understand its amino acid composition to further understand its therapeutic role. Chemical modification is not site-directed, instead particular amino acid residues/groups are targeted using specific reagents [6,7]. Nevertheless, it is possible to overcome problems of site-directed modification and risk of inappropriate folding of the enzyme during genetic modification [6,8]. Chemical modification of biocatalysts can alter affinity, specificity or stability, which in turn makes it superior to its native form [9,10]. Chemical modification technique is often employed for the identification of key amino acid residues and for enhancing the functionality of the enzymes [11–15]. In addition, chemical modification of enzymes can be also used to improve enzyme immobilization [16,17].

In the present work, chemical modification of the fibrinolytic enzyme produced by *Serratia marcescens* subsp. *sakuensis* was attempted

Abbreviations: CAD, coronary artery disease; VTE, venous thromboembolism; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; SK, streptokinase; DEPC, Diethylpyrocarbonate; DTNB, 5, 5-Dithio-bis-(2-nitrobenzoic) acid; EDAC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; NBS, N-bromosuccinimide; PMSF, Phenylmethanesulfonyl fluoride

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https://doi.org/10.1016/j.procbio.2018.06.015
Received 14 March 2018; Received in revised form 15 June 2018; Accepted 16 June 2018

using certain amino acid specific modifiers. The chemically modified enzyme was then evaluated for its specificity towards both fibrin and fibrinogen substrate. Kinetic parameters for the modified enzyme was then studied using fibrin substrate in order to better understand the catalytic mechanism of the enzyme, the rate of reaction and the factors that affect this rate. The secondary structure of the modified fibrinolytic enzyme was determined and compared with that of the native unmodified enzyme.

2. Materials and methods

2.1. Materials

Fibrin, fibrinogen of human origin, DEPC (Diethylpyrocarbonate), NBS (N-bromosuccinimide) and PMSF (Phenylmethanesulfonyl fluoride) were purchased from Sigma-aldrich (India). DTNB (5, 5-Dithio-bis-(2-nitrobenzoic) acid) and EDAC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) were procured from Hi-media. All other chemicals and reagents used were of analytical grade.

The source of fibrinolytic enzyme for this study was *Serratia marcescens* subsp. *sakuensis* (KU296189.1) which was isolated and studied earlier in our lab [18]. This marine isolate has been preserved on starch casein agar slants at 4 °C with periodic sub-culturing and glycerol stocks of the microorganism have been maintained for long-term use. Fibrinolytic enzyme was produced by culturing *Serratia marcescens* subsp. *sakuensis* in the medium comprising yeast extract (12% w/v), soya peptone (1.25% w/v), NH_4Cl (4% w/v) and $CaCO_3$ (0.075% w/v) for 32 h at 37 °C, 150 rpm [18]. The fermentation broth was centrifuged at 12,000 × *g* for 15 min to obtain the cell-free crude enzyme solution, which was then subjected to ammonium sulphate precipitation (40% saturation) followed by dialysis against 20 mM sodium phosphate buffer of pH 7. The dialyzed enzyme sample was loaded onto ENrich SEC 650 column in fast protein liquid chromatography (FPLC) system (BIO RAD NGC Chromatography). Active fractions of the purified enzyme were collected, pooled together and lyophilized [5]. This purified enzyme was further used for chemical modification.

2.2. Fibrinolytic activity assay

Fibrinolytic enzyme activity was estimated by the method described by Agrebi et al. [1] using fibrin as substrate. The purified enzyme solution was mixed with equal volume of substrate solution (prepared by dissolving 10 mg of fibrin in 100 mL of 0.1 M glycine–NaOH buffer pH 9.0) and incubated at 55 °C for 15 min. The reaction was terminated by adding 1 mL of 0.2 M trichloroacetic acid (TCA) solution and was allowed to stand at room temperature for 15 min. The contents were centrifuged at 10,000 × *g* for 15 min at 4 °C to separate the precipitate. Absorbance of the supernatant was read at 280 nm against a suitable blank. Standard graph was constructed by varying the concentrations of tyrosine (0–100 μg/mL) and its absorbance being measured at 280 nm. One unit of fibrinolytic enzyme activity was expressed as 1 μg of tyrosine liberated per min under standard assay conditions.

2.3. Chemical modification of fibrinolytic enzyme

Fibrinolytic enzyme was subjected to modification by different modifiers that target specific amino acid side chains. The modifiers used were DTNB (5, 5-Dithio-bis-(2-nitrobenzoic) acid), NBS (N-bromosuccinimide), EDAC (Ethyl-3-(3-dimethylaminopropyl) carbodiimide), DEPC (Diethylpyrocarbonate) and PMSF (Phenylmethanesulfonyl fluoride) which acts upon cysteine, tryptophan, carboxylates, histidine and serine side chains respectively. All modification reactions were carried out in duplicates. The modification of amino acid residues was carried out as per the established protocols as described below. Control was maintained with distilled water instead of enzyme solution. Upon incubation, samples were subjected to dialysis overnight against 20 mM phosphate buffer (pH 7.0). The samples were then tested for the fibrinolytic activity under standard assay conditions and results were expressed in terms of percentage relative activity.

2.4. Modification of cysteine residues

Modification of cysteine residues was performed using DTNB according to the method described by Hu and Guo [14]. 50 mM DTNB stock solution was prepared in 100 mM phosphate buffer (pH 8.0). Different concentrations of DTNB (10 mM, 15 mM, 20 mM and 30 mM) were taken to investigate its effect on the fibrinolytic activity. Equal volumes of DTNB and purified enzyme sample were added to the test tube used and incubated at 37 °C in 20 mM phosphate buffer (pH 8.0) for 2 h.

2.5. Modification of tryptophan residues

For modification of tryptophan residues, NBS was used and the reaction was carried out according to the method of Hu and Guo [14]. A stock solution of 5 mM NBS was prepared in 100 mM phosphate buffer (pH 8.0). The concentration of NBS was varied (0.01 mM, 0.05 mM, 1 mM) and equal volumes of NBS and purified enzyme sample was added and incubated at 37 °C in 20 mM phosphate buffer (pH 8.0) for 2 h.

2.6. Modification of histidine residues

Modification of histidine residues by DEPC was performed by the method described by Hu and Guo [14]. Stock solution of 30 mM DEPC was prepared in 99.9% (v/v) ethanol. Different concentrations of DEPC (1 mM and 30 mM) were taken for the modification reaction. Equal volumes of purified enzyme sample and DEPC was added and incubated at 37 °C in 20 mM phosphate buffer (pH 8.0) for 2 h.

2.7. Modification of serine residues

The serine residues were modified using PMSF according to the method described by Hu and Guo [14]. Stock solution of 30 mM PMSF was prepared in 99.9% (v/v) ethanol. Different concentrations of PMSF (1 mM and 30 mM) were taken and the modification reaction was carried out at 37 °C in 20 mM phosphate buffer (pH 8.0) for 2 h containing equal volumes of purified enzyme solution and PMSF.

2.8. Modification of carboxylate residues

The carboxylates residues such as glutamate and aspartate were modified using EDAC according to the method of Hu and Guo [14]. A stock solution of 100 mM EDAC was prepared in 99.9% (v/v) ethanol. Concentration of EDAC (0.5–100 mM) was varied and used for the modification reaction. Equal volumes of enzyme sample and EDAC was added and incubated at 37 °C in 10 mM potassium acid phthalate-NaOH buffer (pH 4.1) for 2 h.

2.9. Fibrinogenolytic activity assay

Fibrinogenolytic activity was determined according to the method described by Wang et al. [19] with minor modifications. In brief, substrate solution was prepared by mixing 10 mg of fibrinogen in 50 mL of 0.9% (w/v) NaCl solution. One ml of the substrate solution was mixed with 0.5 mL of 245 mM phosphate buffer (pH 7.0) and incubated at 37 °C for 5 min. As described in our previous report [18] since our aim was to estimate the fibrinogenolytic activity, addition of thrombin was avoided. One ml of the enzyme solution was added to the reaction mixture, followed by mixing for 5 min at 30 °C. The reaction was stopped by adding 1 mL of 0.2 M TCA solution. This reaction mixture was centrifuged at 10,000 × *g* for 10 min at 4 °C and the absorbance of

the supernatant was read at 280 nm. One unit of fibrinogenolytic enzyme activity is expressed as 0.01 per min increase in absorbance at 280 nm.

2.10. Protein concentration

Protein concentration was determined using standard Lowry's method [20] wherein, bovine serum albumin (BSA) was used as standard and the absorbance was read at 660 nm.

2.11. Kinetic parameters

Different concentrations of the fibrin substrate (0.004 - 0.03 mg/mL) were used to determine the initial reaction rates. The assay was conducted under optimum conditions (pH 7.0 and 55 °C) [5]. The Michaelis-Menten constant (K_m) and maximum rate of reaction (V_{max}) values were determined according to Lineweaver-Burk double reciprocal plot upon plotting the respective $1/[S_0]$ and $1/[V_0]$ values for the fibrin substrate [21]. Subsequently, the K_{cat} and the catalytic efficiency (K_{cat}/K_m) of the enzyme with fibrin substrate were also calculated.

2.12. CD (Circular Dichroism) spectra

CD analysis for native and modified enzyme was performed using Jasco J-715 spectropolarimeter at Indian Institute of Science, Bangalore, India. The analysis was carried out using a cell of 1 mm path length with wavelength range 190–260 nm. CD spectra recorded in the wavelength region were an average of three scans at a scan speed of 50 nm/min, a response time of 4 s and corrected by subtracting the appropriate blank runs of 20 mM sodium phosphate buffer (pH 7.0). CD values (mdeg) obtained were aligned with increasing wavelength (nm) and were plotted on a graph. Secondary structure percentage predictions were made using K2D2 software.

2.13. Effect of metal ions on modified enzyme

The concentration of metal ions chosen were similar to that present in human blood as described in our previous report [5]. The chemically modified fibrinolytic enzyme (with 2.5 mM EDAC) was incubated at 37 °C for 1 h with metal ions such as Cu^{2+}, Mn^{2+}, Zn^{2+}, Li^{+}, Co^{2+}, Pb^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, K^{+} and Na^{+} at their respective concentrations as mentioned in the Table 3. Samples were then subjected to fibrinolytic assay and enzyme activity was expressed in terms of residual activity (%). Activity of the enzyme in absence of metal ions was taken as 100%.

3. Results and discussion

3.1. Modification of cysteine residues

With increasing concentration of DTNB the fibrinolytic activity decreased and an enzyme activity of 7.22% was obtained with 30 mM DTNB (Fig. 1A). According to Rudyk and Eaton [22] the thiol side chain [-SH] present in the cysteine residues of enzyme acts as a nucleophile thereby reducing DTNB into a mixed protein disulfide and 5-Thio-2-NitroBenzoicacid (TNB). Thus, results obtained indicate the need for intact cysteine residues for fibrinolytic activity as these cysteine residues are a part of the catalytic site. The results obtained concur from the findings reported by Park et al. [23] and Uchikoba et al. [24], wherein the addition of DTNB to the protease resulted in enzyme activity loss.

3.2. Modification of tryptophan residues

As shown in Fig. 1B it was observed that with increasing concentration of NBS the fibrinolytic enzyme activity decreased. 0.1 mM NBS resulted in complete loss of enzyme activity. Results obtained suggest that tryptophan residues play an important role in the fibrinolytic enzyme catalysis and are a part of the active site of the enzyme. Similar results were reported by Chang et al. [25] wherein the fibrinolytic activity was found to be reduced to 1.5% with 0.5 mM NBS. They had stated in their report that NBS plays the role of a protein-oxidizing agent that particularly oxidizes tryptophan residues to produce oxindole derivative.

3.3. Modification of histidine residues

As observed in Fig. 1A, at both high and low concentrations of DEPC, the fibrinolytic activity decreases considerably. Hnizda et al. [26] had quoted that higher concentrations of DEPC leads to the formation of products such as formyl-biscarbethoxy histidine and urethanecarbethoxy histidine which results in disruption of the imidazole ring that forms the active group of histidine, thereby rendering it unfit for catalytic action of the enzyme. Thus, results obtained indicate the requirement of intact histidine residues for fibrinolytic enzyme catalysis as they are a part of the catalytic site of the enzyme. These results are comparable to the findings reported by Chang et al. [25], wherein, 85% loss in the enzyme activity was observed on modification with 2.5 mM DEPC.

3.4. Modification of serine residues

PMSF at both higher (1 mM) and lower (30 mM) concentration had an inhibitory effect on the fibrinolytic activity (Fig. 1A). Fibrinolytic enzyme activity was found to reduce to 2.23% with 30 mM PMSF when compared to that in the control (Fig. 1A). Results obtained suggest the presence of serine residues at the active site of the enzyme and thereby plays a role in the catalytic activity of the enzyme. These findings concur with the results reported in our previous study, where the fibrinolytic enzyme was found to be a serine metalloprotease in nature [5]. These results are similar to the results reported by Agrebi et al. [1], Chang et al. [25], Ju et al. [27] and Mahajan et al. [28]. Han et al. [29] had outlined the mode of action of PMSF, it is said to react with hydroxyl group (−OH) present in the enzyme to produce o-benzyl sulfonyl-serine.

3.5. Modification of carboxylate residues

Different concentrations of EDAC (0–100 mM) was taken for the modification experiment. Upon modification with 2.5 mM EDAC (optimal concentration) the specific enzyme activity increased significantly from 377.88 U/mg to 7226.30 U/mg (Fig. 2). Results obtained suggest the presence of carboxylate residues such as aspartic and/or glutamic residues at the catalytic site of the fibrinolytic enzyme. According to Wen et al. [11] and Hu and Guo [14], EDAC modifies the carboxylate residues by reacting with their side chains as well as with the C-terminal residues. Liu et al. [30] had reported that EDAC is a carbodiimide that reacts with the carboxyl (−COOH) group to form O-acylisourea intermediate, this intermediate shows high reactivity towards amine and thus leads to formation of amide bond between two protein molecules. At 2.5 mM concentration, there might be sufficient EDAC molecules to react with the enzyme thereby, forming an amide bond network among the enzyme molecules (Fig. 2). Occurrence of fibrinolysis depends on factors such as the structure of fibrin substrate and availability of specific domains on the enzyme molecule for enzyme-substrate interaction. The amide bond network formed upon modification of the enzyme with EDAC might provide more domains for specific interaction with the substrate, making it more accessible for the enzyme-substrate complex to be formed, which could be the possible reason for enhancement in the fibrinolytic activity observed in this study. It was reported by Mosesson [31] and Weisel [32] that fibrin

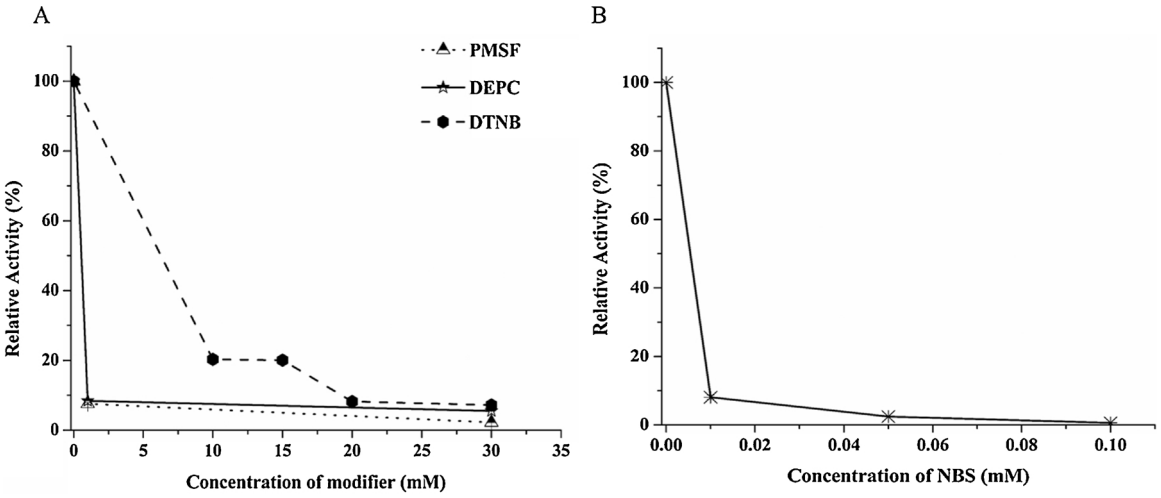

Fig. 1. Comparison of the effect of modifiers on enzyme activity (A) Effect of PMSF, DEPC and DTNB on fibrinolytic activity (B) Effect of NBS on fibrinolytic activity. Bars represent standard deviation (n = 2). The error bars were smaller than the markers used.

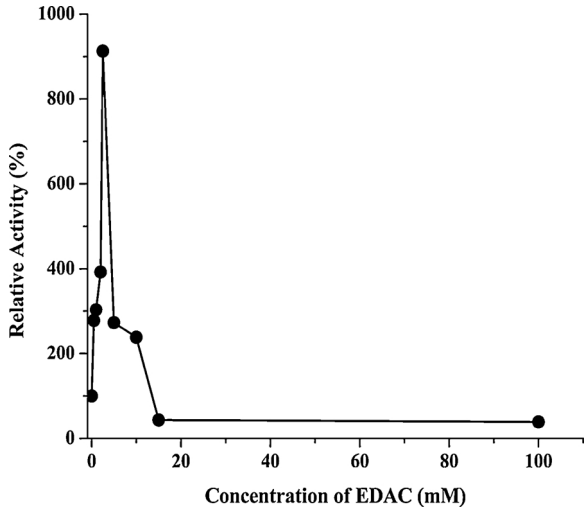

Fig. 2. Effect of varying concentrarion of EDAC on fibrinolytic activity. Bars represent standard deviation (n = 2). The error bars were smaller than the markers used.

comprises of a highly branched network with cross-linked γ-chains that facilitates interaction with bonded enzyme molecules and it is at the αC-domains in the fibrin structure where binding of plasminogen and tPA occurs to form a ternary complex. These domains play an important role in regulating fibrinolysis.

From all of the above modification reactions, only EDAC exhibited a significant enhancement in the fibrinolytic activity, while other modifiers such as DEPC, DTNB, PMSF and NBS led to reduced fibrinolytic enzyme activity (Figs. 1 and 2). In addition to these, experiments were conducted to investigate the specificity of the chemically modified enzyme (with 2.5 mM EDAC treatment) towards the substrates fibrin and fibrinogen as compared to the native enzyme (control) (Table 1). The ratio of fibrinolytic/ fibrinogenolytic activity was calculated for both modified enzyme as well as native enzyme. As seen in Table 1, fibrinolytic to fibrinogenolytic ratio did not change significantly.

3.6. Kinetic parameters

Kinetic parameters were determined under standard assay conditions using different concentrations of fibrin. As shown in Fig. 3A, chemically modified fibrinolytic enzyme obeyed Michaelis-Menten kinetics with fibrin as substrate. From the Lineweaver-Burk plot (Fig. 3B),

Table 1
Fibrinolytic and fibrinogenolytic activities of native and modified enzyme.

Specific activity* (U/mg)		Fibrinolytic/Fibrinogenolytic activity
Native enzyme		
Fibrinolytic	377.88 ± 0.87	1.69:1
Fibrinogenolytic	222.48 ± 8.69	
Modified enzyme with 2.5 mM EDAC		1.54:1
Fibrinolytic	7226.30 ± 0.00	
Fibrinogenolytic	4707.22 ± 19.68	

* Data represents mean ± SD (n = 2).

the chemically modified enzyme showed Michaelis constant (K_m) of 0.0013 mg/mL, with a V_{max} value of 62.62 U/mL, turnover number (K_{cat}) of 5.22 min^{-1} and catalytic efficiency (K_{cat}/K_m) of 4015.38 mL/(mg. min). In comparison to the results obtained in our previous study [5], as shown in Table 2, the native enzyme and chemically modified enzyme exhibited K_m value of 0.66 mg/mL and 0.0013 mg/mL respectively for fibrin substrate. This shows a significant enhancement of affinity towards fibrin substrate upon chemical modification. As a result of this, catalytic efficiency of the chemically modified enzyme increased to 4015.38 ml/(mg. min) as compared to that of the native enzyme, exhibiting an enhancement of 219 times (Table 2).

Most of the published literature on fibrinolytic enzymes reported kinetic values determined using casein, azocasein and synthetic substrates [33,34,27,35,36]. The K_m values reported by Simkhada et al. [33], Ju et al. [27], Bhargavi and Prakasham [35], Verma and Pulicherla [36] were 4.2, 0.96, 1.216, and 90–400 mg/mL respectively, using casein/azocasein as substrate. K_m obtained for the substrates casein/azocasein/synthetic substrate will not necessarily reflect a higher affinity for the substrate fibrin and thus, will not be of any significance. In such cases, the kinetics observed would differ from those of the natural enzyme substrates/target substrate (fibrin) and would fail to reflect the enzyme's kinetic properties in biological systems [37,38]. Reports on K_m values of the fibrinolytic enzyme using fibrin as the substrate is rather scarce. In view of aforesaid facts, K_m value of chemically modified enzyme studied in this case is extremely significant. Extremely low value (0.0013 mg/mL) indicates a very high affinity for fibrin. Furthermore, a very high value of catalytic efficiency (4015.38 ml/(mg. min)) indicates its very high capacity to reduce fibrin clots.

Yongjun et al. [34] reported improvement of fibrinolytic activity of nattokinase by following the method of DNA family shuffling

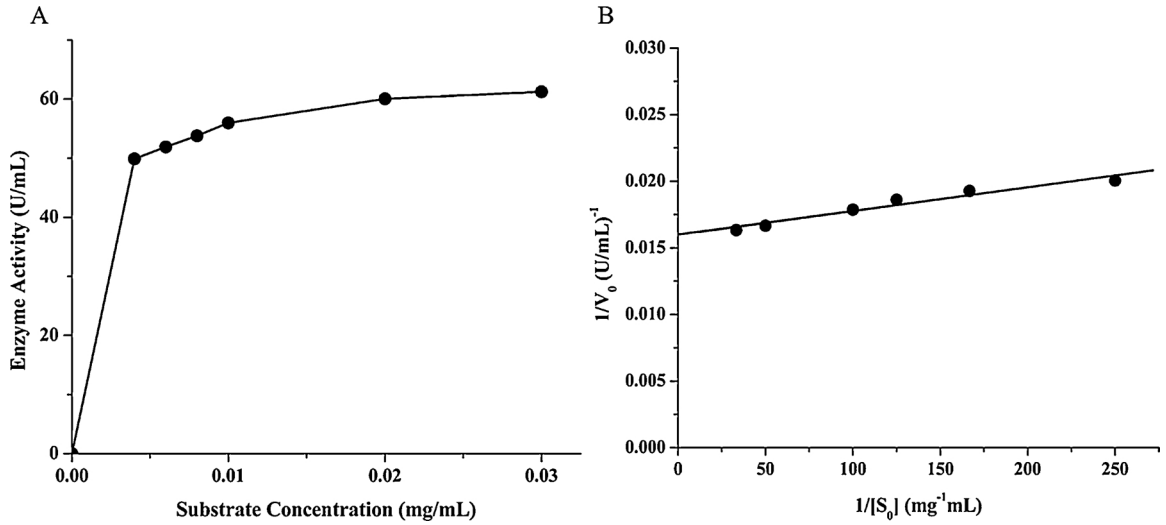

Fig. 3. (A) Michaelis-Menten plot for modified enzyme activity with fibrin. Bars represent standard deviation (n = 2). The error bars were smaller than the markers used. (B) Lineweaver-Burk plot for modified enzyme activity with fibrin as substrate.

Table 2
Comparison of kinetic parameters of native enzyme and chemically modified enzyme for fibrin substrate.

Kinetic Parameters*	Native Enzyme[a]	Chemically Modified Enzyme
K_m (mg/ml)	0.66	0.0013
V_{max} (U/ml)	158.73	62.62
K_{cat} (min^{-1})	12.21	5.22
Catalytic efficiency (mL/(mg min))	18.32	4015.38

* Data represents mean ± SD (n = 2).
[a] Data reported in our previous study [5].

Table 3
Comparison of effect of various metal ions on fibrinolytic activity of both chemically modified and unmodified enzyme.

Metal ion	Concentration (mM)	Residual activity* (%)	Residual activity* (%)
		Chemically modified enzyme	Native enzyme[a]
Control	–	100	100
Cu^{2+}	0.15	108.80 ± 0.74	65.96 ± 0.5
Mn^{2+}	0.15	107.78 ± 0.73	158.88 ± 4.71
Zn^{2+}	0.15	126.39 ± 0.73	103.33 ± 1.57
Li^{+}	0.15	72.57 ± 0.73	53.33 ± 0.49
Co^{2+}	0.15	89.13 ± 0.75	67 ± 2.87
Pb^{2+}	0.15	77.75 ± 0.71	67.76 ± 1.79
Ca^{2+}	1	104.66 ± 0.74	64.46 ± 2.86
Mg^{2+}	1	118.11 ± 0.75	107.77 ± 0
Fe^{3+}	5	96.38 ± 0.73	89.84 ± 1.43
Fe^{2+}	5	86.55 ± 1.46	68.78 ± 1.79
K^{+}	50	83.96 ± 0.73	68.78 ± 1.79
Na^{+}	150	93.27 ± 0.74	68.07 ± 3.47

* Data represents mean ± SD (n = 2).
[a] Data reported in our previous study [5].

technique. As compared to the wild-type nattokinase they had achieved an increase in the catalytic efficiency of the mutant nattokinase by 2.3 times and a decrease in K_m with the synthetic substrate suc-AAPF-pNA. Another report by Zhang et al. [39] quoted the enhancement of fibrinolytic activity upon genetic modification however, there is no supporting kinetic data to evaluate the enzyme catalysis. Increase in the catalytic efficiency was also observed in case of oxalate oxidase produced from *Ochrobactrum intermedium* CL6 upon chemical modification with DTNB [15]. In general, 4–10 fold increment in catalytic efficiency is common after strain improvement. However, in our study, 219-fold improvement in catalytic efficiency was witnessed, which is quite remarkable.

3.7. CD spectra analysis

The secondary structure of the modified and native enzyme was determined by CD spectrum in the UV region (Fig. 4A). The secondary structure of both the fibrinolytic enzymes (chemically modified and unmodified) was analyzed using K2D2 software. The percentages of secondary structure elements calculated using K2D2 software showed that there were slight differences in α- helix and β-sheet conformations between native and modified enzyme. Native enzyme had 2.11% α-helix, 51.77% β-sheet and 46.12% random coil contents, while modified enzyme had 1.78% α- helix, 52.93% β-sheet and 45.29% random coil contents (Fig. 4B and C).

3.8. Effect of metal ions on modified enzyme

As shown in Table 3, divalent metal ions such as Cu^{2+}, Mn^{2+}, Zn^{2+}, $Ca^{2+}$and Mg^{2+} enhanced the fibrinolytic activity of the chemically modified enzyme. The results obtained are comparable to that reported in the previous study [5], wherein the divalent metal ions such as Mn^{2+}, Mg^{2+} and Zn^{2+} increased the fibrinolytic activity of the native enzyme (Table 3).

4. Conclusions

Different amino acid specific modifiers such as DTNB, NBS, DEPC, EDAC and PMSF were used to chemically modify the fibrinolytic enzyme. Modification with 2.5 mM EDAC resulted in 19 times enhancement in specific activity of the enzyme signifying the role and presence of carboxylic acid residues in the catalytic site of the enzyme. On modification with DTNB, NBS, DEPC and PMSF, the fibrinolytic enzyme activity reduced, thus, denoting that cysteine, histidine, tryptophan and serine residues are essential for fibrinolytic activity and are present at the active site of the enzyme. The ratio of fibrinolytic to fibrinogenolytic activity for the modified enzyme was found to be 1.54:1 which was closely similar to that of the native enzyme. Affinity of the chemically modified enzyme for fibrin substrate increased notably resulting in an enhancement of catalytic efficiency by 219 times. CD

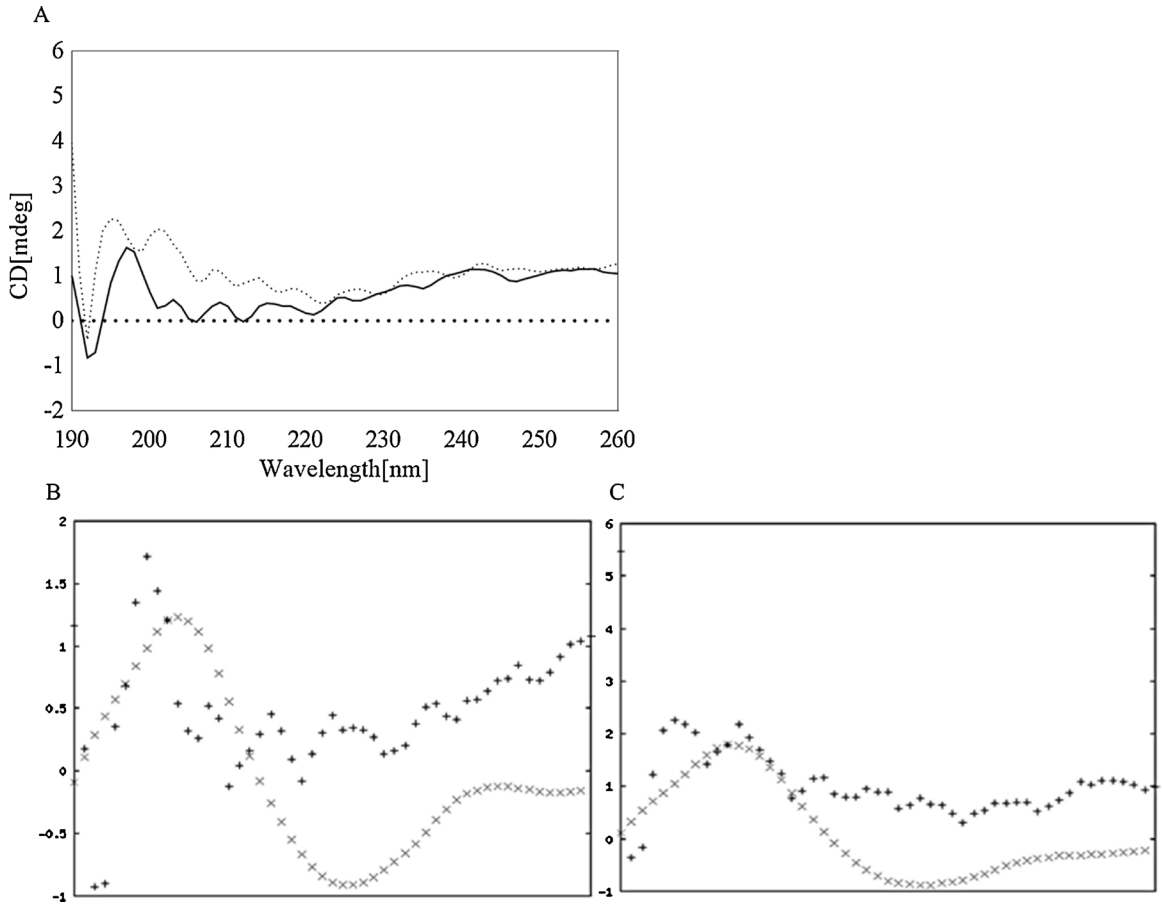

Fig. 4. (A) CD spectra of native (smooth line) and modified (dashed line) fibrinolytic enzyme. (B) K2D2 analysis: (+) input spectrum and (×): predicted spectrum of CD spectra of the native enzyme. (C) K2D2 analysis: (+) input spectrum and (×): predicted spectrum of CD spectra of modified enzyme.

spectrum analysis revealed changes in the percentages of secondary structure elements of the native and chemically modified enzyme. Furthermore, the modified enzyme was more responsive to the presence of all the tested metal ions in general. These findings suggest that the chemically modified fibrinolytic enzyme had certain improved characteristics as compared to the native enzyme and information on the amino acid composition of the modified enzyme would serve as a tool in further understanding its therapeutic role.

Conflict of interest

The authors declare that they have no conflicts of interest with the current work or its publication.

Acknowledgements

Anusha Krishnamurthy thanks the National Institute of Technology Karnataka (N.I.T.K.), India, for providing a fellowship to pursue doctoral research work.

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