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ScienceDirect
JOURNAL OF RARE EARTHS, Vol. 35, No. 5, May 2017, P. 510
JOURNAL OF RARE EARTHS
www.re-journal.com/en/

Effect of Tb(III) on activity and stability of nattokinase

MOU Xiaochao (牟小超)[1], YANG Renjia (杨仁佳)[2], ZHANG Wenlong (张文龙)[1], YANG Binsheng (杨斌盛)[1,*]

(1. Institute of Molecular Science, Key Laboratory of Chemical Biology of Molecular Engineering of Education Ministry, Shanxi University, Taiyuan 030006, China; 2. Blood Division, Shanxi Dayi Hospital, Taiyuan 030006, China)

Received 8 September 2016; revised 1 December 2016

Abstract: Nattokinase, is an effective fibrinolytic enzyme with the potential for fighting cardiovascular disease. The aim of study was to investigate the interaction of Tb(III) with nattokinase and the impact of Tb(III) on the enzyme activity and protein stability. The binding of Tb(III) with nattokinase was studied by fluorescence spectrum in 100 mmol/L Tris-HCl (pH 8.0). It could be seen that the protein bound one Tb(III) with low affinity, and the binding constants K were 2.90×10^4 L/mol at 288 K. Although the activity of nattokinase determined by tetra-peptide substrate method at proper pH and temperature was not influenced for the binding of Tb(III), the transformation rate of substrate was increased to 113%. To better assess the stability of protease in the absence and presence of Tb(III), nattokinase was unfolded through continuous concentrations urea. Based on the model of structural element, the results showed that Tb(III) could not change the average structural element free energy $<\Delta G^0_{element}(H_2O)>$ of nattokinase by the measurement of enzyme activity, but it could improve the stability of the global protein by the fluorescence spectral measurement.

Keywords: nattokinase; Tb(III); enzyme activity; protein stability; rare earths

Nattokinase (Subtilisin NAT or NK), one of the most considerable extracellular enzymes with strong fibrinolytic and thrombolytic activity, was first found in natto which is typical soybean food eaten in Japan[1]. Nattokinase has potential for fighting cardiovascular diseases including heart disease, high blood pressure and has also been used for curing other disease, such as Alzheimer's disease, pain, vitreoretinal disorder, chronic fatigue syndrome, uterine fibroids[2,3]. Thus, NK is currently considered as an efficient, secure, economic, and preventative drug.

As a number of subtilisin-like serine protease family, NK, encoded by *aprN*, was found code for a 29 residues signal peptide, a 77 residues propeptide and a 275 residues mature polypeptide, with a molecular mass of 27.7 kDa and an isoelectric point of 8.7[1,4]. NK not only could lyse fibrin directly or indirectly, but also showed amidolytic activity invested by several synthetic substrates, the most sensitive substrate for NK was the Suc-Ala-Ala-Pro-Phe-pNA for subtilisin[1].

So far, NK has been extensively studied not only for the insight into the mechanism of enzyme catalysis, but also for its significant applications in commercial field. The most important is to improve its activity and stability so as to broaden their utility in medical and commercial application. There are various methods to improve the activity and stability of NK at present. On the level of molecular biology, DNA family shuffling is one of the ways to improve the fibrinolytic activity of nattokinase[5]. Site-directed mutagenesis is another means to enhance activity and oxidative stability of NK[6,7]. During the NK purification process, the traditional protein expression and purification technology were changed and improved for better activity, but the results were less useful[8]. Beyond that, chemical modification has become an effective and popular approach to improve nattokinase activity and stability. NK was first well immobilized onto magnetic nanoparticles Fe_3O_4 in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC), and it turned out that it had much higher thrombolytic activity, even higher than the pure NK[9]. Immobilization of nattokinase upon polyhydroxybutyrate (PHB) nanoparticles resulted in a 20% increase in the enzyme activity[10]. The adsorption capacity of nanosilver (AgNPs) on nattokinase enhanced heat stability and anticoagulant effect of NK[11]. The effect of various metal ions, such as Ag(I), K(I), Na(I), Ba(II), Ca(II), Cd(II), Co(II), Cu(II), Hg(II), Mg(II), Mn(II), Ni(II), Zn(II), Fe(II), Fe(III) and Al(III), had been tested on enzyme activity with a final concentrations from 1 to 10 mmol/L[12–17]. It is not difficult to find that the metal ions and NK were added not at proper chemical stoichiometry in the experiment. So some new problems have arisen: whether and how these metal ions combined with NK, and how these metal ions impact the activity and stability of NK at the molecular level?

The first X-ray diffraction analysis of nattokinase revealed the protease crystal structure with two Ca(II),

Foundation item: Project supported by the National Natural Science Foundation of China (21571117) and the PhD Programs Foundation of the Ministry of Education of China (20131401110011)

* **Corresponding author:** YANG Binsheng (**E-mail:** yangbs@sxu.edu.cn; **Tel.:** +86-351-7016358)

DOI: 10.1016/S1002-0721(17)60941-4

which were important for activity and stability of serine proteinase[18–21]. While, deep studies about Ca(II) having impact on NK stability and activity, to date, had remained undetermined. Tb(III), as a fluorescence probe, due to the similarity to Ca(II) in ions radii, charge character, and coordination chemistry, was well known to replace Ca(II) and had been used to study protein structural characterization in many proteins[22–29]. Moreover, the application of rare earth element in medical science has hundred years of history at aboard, due to its broad pharmacological properties, low toxicity and good clinical curative effect, a shining example of this is the anticoagulant therapy[30–32].

In the present study, nattokinase was produced and purified successfully. Then, the interaction of nattokinase with terbium ion and the function of Tb(III) in the enzyme were analyzed in detail. Tb(III) fluorescence probe was first applied to characterize the binding of NK with Tb(III). Further, the effect of Tb(III) on activity and stability of NK was investigated for the first time.

1 Materials and methods

1.1 Materials

Synthetic substrate N-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide (Suc-AAPF-pNA), urea, HCl, Tris were obtained from Sigma-Aldrich, USA. Terbium oxide (Tb_4O_7) was 99.99%, purchased from Rare Earth Research Institute of Hunan, China. All the chemicals were analytical grade reagents.

The UV-Vis spectra were conducted by UV-Visible spectrophotometer (Varian Cary 50 BIO, Agilent). The fluorescence spectra were recorded on a fluorescence spectrophotometer (FluoroMax-4, HORIBA). All pH measurements were measured with a pH meter (Metter Toledo, Switzerland).

1.2 Methods

1.2.1 Production and purification of nattokinase

For the production of nattokinase, the stain of *Bacillus subtilis natto* YBNK-1 was previously isolated from health care products. In the experiment, nattokinase was produced by the liquid state fermentation according to the previous report[33]. After the purification procedures, the activity of purified enzyme was detected, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was applied to determine the enzyme's purity and the molecular mass.

1.2.2 Terbium stock solution

The terbium stock solution was prepared[25] by dissolving Tb_4O_7 with a small volume of concentrated hydrochloric acid (HCl) and then diluted with a specified volume of deionized water. The terbium solution was standardized by titration with standard ethylene diamine tetraacetic acid (EDTA) solution in hexamethylene tetramine buffer at pH 5.5. Titration endpoint was observed with a color change from pink to yellow by xylenol orange as an indicator.

1.2.3 Steady-state fluorescence studies

For the measurement of NK interacting with Tb(III), NK was titrated with Tb(III) solution in 100 mmol/L Tris-HCl (pH 8.0). Allowing for equilibrium time, spectra were recorded at 2-min intervals after the addition of Tb(III). The experiments with the same conditions except the temperature were done to obtain the thermodynamic data for the combination of NK with Tb(III). The excitation wavelength of the fluorescence spectra was set at 290 nm and the slit widths of excitation and emission were both 5 nm. To correct dilution errors after each titration, the fluorescence intensity at the maximum emission peak needs to deduct the dilution effect.

Using Tb^{3+} as fluorescence probe, we further explored the Ca(II)-NK binding properties from the competition between Ca(II) and Tb(III). In this study, Tb(III) and NK solution were mixed with the concentration rate of 1:1. Then the high concentration of Ca(II) was added to the NK-Tb to monitor the competition between Tb(III) and Ca(II). The reaction was monitored using aromatic residue sensitized Tb(III) fluorescence spectra, recorded between 500–560 nm with excitation at 290 nm, and the slit width of excitation and emission were both at 10 nm.

1.2.4 Enzymatic activity assays

The amidolytic activity of nattokinase was measured using the chromogenic substrate Suc-AAPF-pNA as previously reported[6]. Firstly, the protein sample was incubated in the 100 mmol/L Tris-HCl buffer (pH 8.0) at 303 K. Ten minutes later, the substrate Suc-AAPF-pNA was added into the protein samples. The absorbance of p-nitroaniline (pNA) produced by the hydrolysis of Suc-AAPF-pNA during the incubation was recorded on a UV-Vis spectrophotometer.

1.2.5 Effects of pH and temperature on enzyme activity

The effect of pH on enzyme activity was determined at pH 5.0–10.0 (pH 5.0–7.0 using 100 mmol/L citrate buffer, pH 7.4–10.0 using 100 mmol/L Tris-HCl buffer). The optimal temperature on enzyme activity was measured at different temperatures ranging from 25 to 70 ºC. The residual activity of NK was measured and the maximum activity was defined as 100%.

1.2.6 Enzyme-catalyzed kinetics

In order to introduce the effect of Tb(III) on nattokinase activity, Tb(III) solution was added into the protein sample (NK and Tb(III) in the stoichiometry of 1:1). The enzyme-catalyzed kinetic measurement was measured at the optimum pH and the optimum temperature as described above. Reactions were initiated by the addition of the Suc-AAPF-pNA. Dynamic data were acquired by measuring the change of absorbance at 390 nm on kinetic scan mode of UV spectrophotometer with a 30 s lag. To