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Roles of S3 Site Residues of Nattokinase on its Activity and Substrate Specificity

Shuming Wu[1,2], Chi Feng[2,3], Jin Zhong[1,*] and Liandong Huan[3,†]

[1]State Key Laboratory of Microbial Resource, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101; [2]Graduate School of the Chinese Academy of Sciences, Beijing 100039; and [3]Center for Metabolic Engineering of Microorganisms, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, P.R. China

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Nattokinase (Subtilisin NAT, NK) is a bacterial serine protease with high fibrinolytic activity. To probe their roles on protease activity and substrate specificity, three residues of S3 site (Gly[100], Ser[101] and Leu[126]) were mutated by site-directed mutagenesis. Kinetics parameters of 20 mutants were measured using tetrapeptides as substrates, and their fibrinolytic activities were determined by fibrin plate method. Results of mutation analysis showed that Gly[100] and Ser[101] had reverse steric and electrostatic effects. Residues with bulky or positively charged side chains at position 100 decreased the substrate binding and catalytic activity drastically, while residues with the same characters at position 101 could obviously enhance protease and fibrinolytic activity of NK. Mutation of Leu[126] might impair the structure of the active cleft and drastically decreased the activity of NK. Kinetics studies of the mutants showed that S3 residues were crucial to keep protease activity while they moderately affected substrate specificity of NK. The present study provided some original insight into the P3–S3 interaction in NK and other subtilisins, as well as showed successful protein engineering cases to improve NK as a potential therapeutic agent.

Key words: nattokinase, protease activity, S3 site, site-directed mutagenesis, substrate specificity.

Abbreviations: NK, nattokinase, subtilisin NAT; suc-, succinyl; k_{cat}, catalytic rate constant; t-PA, tissue-type plasminogen activator; DMF, *N*,*N*-dimethylformamide; ddH_2O, double-distilled H_2O; suc-AXPFpNA, succinyl-Ala-X-Pro-Phe-p-nitroanilide (where X represents the P3 amino acid); WT, wild-type nattokinase; The binding site notation is that of Schechter and Berger (*1*). Accordingly, Pn denotes a substrate position, and Sn denotes the corresponding enzyme binding subsite.

Nattokinase (Subtilisin NAT, NK, E.C. 3.4.21.62) is a highly active fibrinolytic enzyme secreted by *Bacillus subtilis* var. *natto* and was first found in the traditional Japanese soybean food natto (*2*). It can hydrolyse thrombi efficiently as well as increase the production of thrombi-dissolving agents such as t-PA *in vivo* (*3–6*). The fibrinolytic activity of NK can retain in blood for more than 3 h (*6*), making it feasible for clinical use. Compared with conventional clot-dissolving drugs, NK has several advantages including convenience of oral administration, confirmed efficacy, prolonged effects, low-cost and preventive effect (*7*). These characters made it a promising oral medicine for thrombolytic therapy.

NK is encoded by *aprN*, which encodes a 29-residue signal peptide, a 77-residue propeptide and a 275-residue mature polypeptide (*8*). It is highly homologous to many subtilisins in serine protease family. For example, the mature NK is 99.5% homologous to mature subtilisin E and 99.3% homologous to mature mesentericopeptidase (our alignment results). Despite of high homology among these enzymes, only NK shows high substrate specificity for fibrin (*9*).

Like many subtilisins, NK contains the conserved catalytic triad (Asp[32], His[64] and Ser[221]) and oxyanion hole (Asn[155]) (*10*). Because of their considerable commercial importance and being a good model to study structure–function relationship of proteins, subtilisins have been extensively studied. They are serine endopeptidases each of which has an extended binding cleft comprising of at least eight binding subsites, $S_5 - S_3$' (*11*). However, attempts to study substrate-binding regions have been concerned mainly with S1 and S4 sites (*11–19*). Although P3–S3 interaction is important in determining the protease activity and substrate specificity (*11*), the S3 site of subtilisins has received little attention.

Based on structure prediction and comparison, we chose Gly[100], Ser[101] and Leu[126] as the S3 site residues of NK. In order to analyse the roles of the S3 site residues on protease activity (includes fibrinolytic activity) and P3 substrate specificity of NK, we carried out mutagenesis analysis by substituting them with residues that had different steric or electric charge characters. Kinetics parameters of 20 mutants were determined using serial tetrapeptides that differed just at the P3 residue as substrates, and their fibrinolytic

*To whom correspondence should be addressed. Tel: +86-10-6480-7401, Fax: +86-10-6480-7401, E-mail: zhongj@im.ac.cn
†Correspondence may also be addressed. Tel: +86-10-6480-7439, Fax: +86-10-6480-7401, E-mail: huanld@sun.im.ac.cn

activities were determined by the standard fibrin plate method.

MATERIALS AND METHODS

Materials—Bacillus subtilis var. *natto* was kindly provided by Prof. John Spizizen (Scripps Clinic and Research Foundation, USA) and vector pUC18 was bought from Takara Co., Dalian, China. Vector pHY300PLK [a shuttle vector for *Escherichia coli* and *Bacillus subtilis* (*20*)] was a Takara (Japan) product and kindly provided by Dr Liqun Zhang (China Agricultural University). *Bacillus subtilis* DB104 was constructed by Kawamura (*21*) and kindly provided by Dr Juanmei Ye (National Taiwan Zhongxing University). The chromogenic peptide, suc-AAPFpNA, was from Sigma Chemical. Suc-AFPFpNA and suc-AEPFpNA were from Balchem Co., Sweden. Bovine fibrinogen, thrombin and urokinase were purchased from Chinese Medicine Testing Institute (Beijing, China). CM Sepharose CL-6B and Sephadex G-100 were from Pharmacia Biotech (Uppsala, Sweden). All other reagents were of the highest quality commercially available.

Molecular Simulations—The structure of nattokinase was modelled by SWISS-MODEL using default parameters (*22*), with mesentericopeptidase complexed with its inhibitor eglin C (PDB code: 1MEE), subtilisin E (PDB code: 1SCJ) and subtilisin BPN' (PDB code: 1SBH, 1YJB, 1YJA) as main templates. The energy computations and minimizations were done with the GROMOS96 (*23*) implementation of Swiss-PdbViewer (*22*). The quality of model was checked by PROCHECK (*24*). Structure comparison of the modeled nattokinase and mensentericopeptidase (PDB code: 1MEE) was done by DaliLite (*25*) as well as the former model was superimpose onto the later structure by DeepView v3.7 (*22*).

Construction, Expression and Purification of NK Variants—NK gene (*aprN*) including its promoter was previously amplified from *B. subtilis* var. *natto* and cloned into the *Eco*RI/*Sal*I sites of pUC18 vector to form pUC18-*aprN*. The protein sequence encoded by the cloned gene is identical to subtilisin NAT (*2*). The oligonucleotides (Table 1) for site-directed mutagenesis were synthesized by Invitrogene Co. (Beijing, China). With pUC18-*aprN* as template, site-directed mutagenesis was performed by using the same method as the QchickChang mutagenesis kit (Stratagene) according to manufacture's instructions. In each case, the entire encoding gene was sequenced to prove that only the expected mutation had occurred. The wild-type or mutant NK genes were inserted into the *Eco*RI/*Sal*I sites of pHY300PLK to form the expression vectors pHY300PLK-*aprN*.

Wild-type and mutant NKs were expressed in the protease-deficient host *Bacillus subtilis* DB104. The host cells were transformed with the expression vector pHY300PLK-*aprN* by electroporation. To express one protein, a single colony was inoculated into 5 ml LB broth supplemented with 30 μg/ml tetracycline and grew overnight at 37°C with shaking at 200 rpm. The overnight culture was diluted (3:100) into fresh media and grown at 37°C with shaking at 200 rpm for 72 h to express

Table 1. **Oligonucleotides used for site-directed mutagenesis.**

Mutant	Oligonucleotide[a]
G100A	5′-CTTGATTCAACAGCAAGCGGCCAATATAG-3′
G100V	5′-CTTGATTCAACAGTAAGCGGCCAATATAG-3′
G100T	5′-CTTGATTCAACAACAAGCGGCCAATATAG-3′
G100D	5′-CTTGATTCAACAGACAGCGGCCAATATAG-3′
G100H	5′-CTTGATTCAACACACAGCGGCCAATATAG-3′
G100K	5′-CTTGATTCAACAAAAAGCGGCCAATATAG-3′
S101A	5′-CTTGATTCAACAGGAGCAGGCCAATATAGCTG-3′
S101L	5′-CTTGATTCAACAGGACTGGGCCAATATAGCTG-3′
S101F	5′-CTTGATTCAACAGGATTCGGCCAATATAGCTG-3′
S101W	5′-CTTGATTCAACAGGATGGGGCCAATATAGCTG-3′
S101D	5′-CTTGATTCAACAGGAGACGGCCAATATAGCTG-3′
S101H	5′-CTTGATTCAACAGGACACGGCCAATATAGCTG-3′
S101K	5′-CTTGATTCAACAGGAAAAGGGCCAATATAGCTG-3′
S101R	5′-CTTGATTCAACAGGAAGGGGCCAATATAGCTG-3′
L126G	5′-GTTATCAACATGAGCGGTGGCGGACCTACTGGTT-3′
L126V	5′-GTTATCAACATGAGCGTTGGCGGACCTACTGGTT-3′
L126F	5′-GTTATCAACATGAGCTTTGGCGGACCTACTGGTT-3′
L126D	5′-GTTATCAACATGAGCGATGGCGGACCTACTGGTT-3′
L126E	5′-GTTATCAACATGAGCGAGGGCGGACCTACTGGTT-3′
L126K	5′-GTTATCAACATGAGCAAGGGCGGACCTACTGGTT-3′

[a]Only the forward oligonucleotides were showed. Base changes from the encode gene of the wild-type subtilisin NAT are underlined.

the enzyme. All the following purification steps were carried out at 0–4°C. Because NK was secreted into the culture, culture supernatant collected by centrifugation at 8500 rpm for 10 min was precipitated by 60% saturation of $(NH_4)_2SO_4$. The precipitate was collected by centrifugation (8500 rpm, 20 min) and dissolved in 20 mM Tris–Ac buffer, pH 7.0 (Buffer A) and dialysed against the same buffer. The insoluble materials were removed by centrifugation and the resultant supernatant was loaded onto a CM Sepharose CL-6B column ($1.6 \times 30\,cm^2$) pre-equilibrated with Buffer A. Unabsorbed material was washed from the column with the same buffer, and the enzyme was eluted at 0.2 ml/min with 0.2–0.8 M NaCl. The active fractions were pooled, dialysed against ddH_2O and loaded onto a Sephadex G-100 column ($0.8 \times 110\,cm^2$) pre-equilibrated with ddH_2O. The active fractions were pooled and lyophilized. The purity of all purified enzymes was >95% as determined by SDS–PAGE (Fig. 1). Concentrations were determined by the Bradford method (*26*) using bovine serum albumin as standard.

Determination of Kinetic Parameters—The enzymatic activity was assayed with the substrates suc-AXPFpNA (X represents Ala, Phe or Glu) (*27*) in 200 μl of Barbitol buffer (50 mM sodium barbital, 90 mM NaCl, 1.7 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.2 mM DMF, pH 7.75) at 25°C with the substrate concentrations varying between 0.01 and 2 mM. Reactions were initiated by the addition of 5 μl of the 5 μM corresponding enzyme (50 μM enzyme was also tested for L126D and L126E mutants) in a quartz cell on a spectrophotometer (DU800, Bechman) equipped with a thermostated cell compartment, then the release of *p*-nitroaniline was monitored at 410 nm ($\epsilon_{410} = 8480\,M^{-1}\,cm^{-1}$). The kinetic parameters k_{cat} and K_m were obtained from initial rate measurements of substrates hydrolysis monitored by following

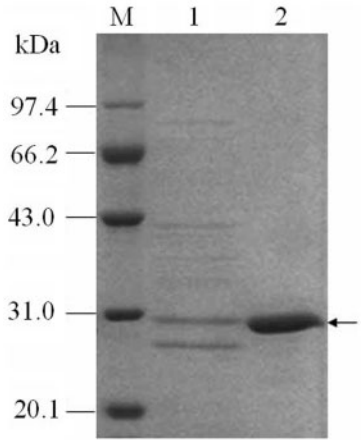

Fig. 1. **SDS–PAGE analysis of the purified nattokinase expressed in *B. subtilis* DB104.** Lane M, protein molecular weight marker; lane 1, proteins in the culture supernatant of *B. subtilis* DB101 harbouring pHY300PLK-*aprN*; lane 2, purified nattokinase. The arrow indicates the band of nattokinase.

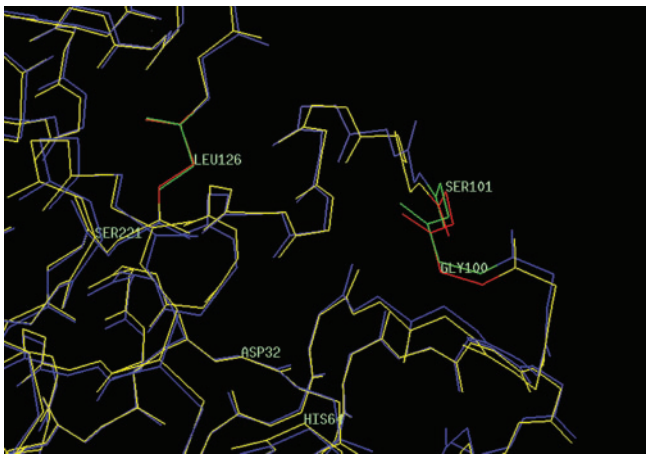

Fig. 2. **The backbone superimposition of the modelled nattokinase (white) and mesentericopeptidase (PDB code: 1mee. gray) showed the same structures of the catalytic triad (Asp^{32}, His^{64} and Ser^{221}) and the S3 site (Gly^{100}, Ser^{101} and Leu^{126}).** The structure of nattokinase was predicted by homology modelling on the SWISS-MODEL web server. The structure comparison was done by DaliLite (*25*) and the figure was made by DeepView v3.7. The catalytic triad and S3 site residues of nattokinase were labelled.

the absorption at 410 nm, and their values were calculated using an Eisenthal–Cornish–Bowden direct linear plot (*28*).

Assay of Fibrinolytic Activity—Quantitative analysis of fibrinolytic activity was conducted by the fibrin plate method (*29*) using urokinase as a standard. Briefly, 10 ml 1.5 mg/ml bovine fibrinogen solution [in Barbitol buffer (50 mM sodium barbitol, 90 mM NaCl, 1.7 mM $CaCl_2$, 0.7 mM $MgCl_2$, pH 7.75)] and 10 ml 1% agarose solution were brought to 45°C in a water bath, followed by the addition of 10 μl thrombin solution (0.1 BP/μl) into the agarose solution. The fibrinogen and agarose solutions were mixed in a 100 mm petri dish and kept at room temperature for 1 h to form fibrin clots. Enzymes were dissolved and diluted to a fitful concentration in Barbitol buffer. Holes were made on the fibrin plate and 10 μl of each enzyme solution was dropped in a hole. The plate was incubated at 37°C for 18 h. Two perpendicular diameters of the lyzed zone on the fibrin plate were measured and the fibrinolytic activity was determined according to the standard curve of urokinase. Specific activity of NKs was denoted by urokinase units of fibrinolytic activity in each milligram of enzyme.

RESULTS

Molecular Simulations—Mainly with mesentericopeptidase complexed with its inhibitor (PDB code: 1MEE), subtilisin E (PDB code: 1SCJ) and subtilisin BPN' (PDB code: 1SBH, 1YJB, 1YJA) as templates, we obtained the putative tertiary structure of NK and its S3 site (Fig. 2) by homology modelling. Nattokinase is highly homologous to mesentericopeptidase (99.3%, our alignment result) and differs by just three residues [183S versus A, 192V versus A and 259N versus S (*30*)]. Structure comparison of the modelled nattokinase (275 aa) and mesentericopeptidase (275 aa) was done by DaliLite (*25*). The comparison result was that 275 residues were aligned and the *Z*-score was 55.0, which indicated highly structural conservation between these two enzymes. The superimposed structures (Fig. 2) showed the root mean standard deviation (RMSD) was just 0.29 Å for all carbon alpha and 0.35 Å for all backbone atoms. X-ray study on mesentericopeptidase reported by Dauter *et al*. (*30*) clearly indicated that the S3 site of mesentericopeptidase consists of Gly^{100}, Ser^{101} and Leu^{126}(*30*), for the distance between each of these three residues and the side chain of the P3 substrate residue was within 0.4 nm. The structures of the catalytic triad and S3 site of nattokinase were the same as those of mesentericopeptidase (Fig. 2). Based on the above information, we chose Gly^{100}, Ser^{101} and Leu^{126} as S3 site residues of nattokinase. Furthermore, computational graphics analysis also showed S3 site of NK was adjacent to the surface of enzyme. Leu^{126} was close to the catalytic triad and buried in the active cleft, Gly^{100} was buried close to the surface of NK, while Ser^{101} lay on the surface with its side chain exposed to the solvent (Fig. 2).

Steady-State Kinetics Parameters and Fibrinolytic Activities—To probe the roles of S3 site residues on the protease activity and substrate specificity of NK, the steady-state kinetics parameters of the wild-type and 20 mutant enzymes were determined with a series of chromogenic tetrapeptides suc-AXPFpNA (X represents Ala, Phe or Glu). For it is an important parameter to evaluate the effect of a thrombolytic agent, fibrinolytic activity (specific activity) of the wild-type and mutant NKs was determined using the fibrin plate method (*29*). Both results of kinetics parameters (Table 2) and fibrinolytic activities (Table 3) are briefly described as follows.

Wild-Type nattokinase

The K_m value for the Ala substrate (0.79 mM) was the highest and a 2.1–2.6-fold decrease of the value was observed for the Phe and Glu substrates. The catalytic

Table 2. **Activities of wild-type and mutant subtilisin NATs with suc-AXPFpNA substrates[a].**

Mutant	P3 residue								
	Ala			Phe			Glu		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
Wild-type	16.28	0.79	20.55	5.97	0.30	19.65	2.76	0.37	7.38
G100A	3.99	0.56	7.07	7.65	0.62	12.30	1.44	0.57	2.54
G100V	2.07	0.82	2.51	3.82	0.57	6.72	2.09	1.66	1.25
G100T	0.75	1.02	0.74	0.93	0.66	1.40	0.21	0.85	0.24
G100D	34.20	1.94	17.64	41.62	1.94	21.42	2.09	1.66	1.25
G100H	9.07	0.75	12.11	6.75	0.39	17.50	4.50	1.16	3.89
G100K	1.35	0.35	3.87	2.92	0.40	7.33	2.04	0.83	2.44
S101A	5.47	0.34	16.02	13.30	0.52	25.55	3.39	0.47	7.20
S101L	17.20	0.58	29.86	22.38	0.55	40.34	12.95	1.05	12.31
S101F	10.83	0.29	36.91	60.57	0.39	154.63	7.51	0.25	29.59
S101W	20.17	0.51	39.29	29.49	0.55	53.54	22.44	1.19	18.83
S101D	0.98	0.10	9.75	4.20	0.87	4.82	0.51	0.43	1.18
S101H	35.59	0.98	36.47	28.26	0.76	37.38	45.62	2.06	22.20
S101K	13.55	0.47	28.58	54.23	1.84	29.40	15.48	0.62	24.97
S101R	23.32	0.67	34.71	20.10	0.31	65.39	12.11	0.61	19.88
L126G	6.66	1.98	3.35	3.32	0.56	5.92	0.67	0.35	1.91
L126V	5.08	7.14	0.71	1.82	1.05	1.74	0.13	0.31	0.42
L126F	2.16	0.95	2.26	6.11	1.70	3.61	2.10	1.32	1.58
L126D	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
L126E	0.0096	0.17	0.056	0.043	0.70	0.062	0.0032	0.54	0.060
L126K	4.36	1.45	3.01	2.36	0.39	5.98	0.59	0.41	1.45

[a]Kinetic parameters for the hydrolyses of suc-AXPFpNA, where X represents the P3 substrate residues Ala, Phe or Glu. Assays were done at 25°C, pH 7.75 (50 mM sodium barbital, 90 mM NaCl, 1.7 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.2 mM DMF). Units are as follows: k_{cat}, s^{-1}; K_m, mM and k_{cat}/K_m, $mM^{-1}\,s^{-1}$. SE were less than 15%.
[b]Parameters were not determined, because the catalytic efficiency was so low that the catalytic parameters could not be detected by the standard method of this paper.

efficiencies (k_{cat}/K_m) of the wild-type NK showed indistinguishable preference to the Ala or Phe substrates, while a 3-fold decreased substrate specificity was observed for a Glu at the P3 position (Table 2).

G100A, G100V, G100T, G100D, G100H and G100K Mutant NKs

Site-directed mutagenesis and kinetics study results (Table 2) showed that mutation of Gly^{100} changed the protease activity of NK obviously while modulated its substrate specificity moderately.

All six Gly^{100} mutants showed clear decrease in protease activities for the Ala substrate and Glu substrate. The catalytic efficiencies differed from 0.74 to 17.64 $mM^{-1}\,s^{-1}$ for the Ala substrate, and 0.24 to 3.89 $mM^{-1}\,s^{-1}$ for the Glu substrate. Except for G100D that showed higher protease activity, the other five mutants exhibited lower protease activities for the Phe substrate than the wild-type NK. Among mutants substituted with an uncharged residue, mutants with a bulky side chain were less active than those with a small side chain, with the order of WT>G100A>G100V>G100T. Among mutants substituted with a charged residue and for the Ala or Phe substrate, the protease activity of mutant with a negatively charged side chain was higher than those with positively charged side chains and the decreasing order was G100D>G100H>G100K (Table 2).

All Gly^{100} mutants exhibited increased substrate specificity for the Phe substrate. The G100K and G100V mutants increased, whereas the G100D mutant decreased substrate specificity for Glu substrate (Table 2).

Mutations of Gly^{100} interfered with the fibrinolytic activity of NK clearly (Table 3). The decrease of fibrinolytic activity of the six mutants is corresponding to the size and the charge of the side chain of the substituting residues, with the order of G100A>G100D>G100H=G100V>G100T=G100K. The longer the side chain, the lower the fibrinolytic activity. The mutants substituted with residues having a negatively charged side chain retained higher fibrinolytic activity than those with a positively charged side chain.

S101A, S101L, S101F, S101W, S101D, S101H, S101K and S101R Mutant NKs

Site-directed mutagenesis and kinetics study results (Table 2) showed that mutation of Ser^{101} altered the protease activity and substrate specificity of NK moderately.

Among mutants substituted with an uncharged residue and for the Ala substrate having a small side chain at the P3 position, the catalytic efficiencies of the mutants with bulky side chains were higher than those with small side chains and the increasing order was S101A<WT<S101L<S101F<S101W, the more bulky the

Table 3. **Fibrinolytic activities of wild-type and mutant subtilisin NATs[a].**

Mutant	Specific activity[b] (U/mg)	Rel. activity[c] (%)
Wild-type	3109 ± 257.4	100
G100A	2335 ± 231.9	75.1
G100V	872 ± 81.2	28
G100T	429 ± 22.5	13.8
G100D	1680 ± 210.3	54
G100H	897 ± 84.6	28.9
G100K	397 ± 37.7	12.8
S101A	2908 ± 249.5	93.5
S101L	5297 ± 532.2	170.4
S101F	2333 ± 315.4	75
S101W	6773 ± 702.0	217.9
S101D	1403 ± 207.5	45.1
S101H	2474 ± 309.6	79.6
S101K	5303 ± 456.1	170.6
S101R	4677 ± 244.2	150.4
L126G	579 ± 29.0	18.6
L126V	2172 ± 265.7	69.9
L126F	2302 ± 257.6	74.1
L126D	0.935 ± 0.065	0.03
L126E	90.32 ± 9.19	2.9
L126K	506 ± 17.9	16.3

[a]Fibrinolytic activities were detected by the fibrin plate method using urokinase as a standard, assays were done at 37°C for 18 h, pH 7.75 (50 mM sodium barbital, 90 mM NaCl, 1.7 mM $CaCl_2$, 0.7 mM $MgCl_2$).
[b]Values shown are the averages ± SE of three independent experiments, U is urokinase unit.
[c]Relative specificity calculated as the ratio of the fibrinolytic activities of each mutant relative to the wild-type subtilisin NAT.

side chain, the higher the protease activity. The S101W mutant showed 1.91-fold higher catalytic efficiency than the wild-type NK. For the large substrates (such as Phe and Glu substrates), the catalytic efficiency of the S101W mutant was decreased and the order changed to S101A<WT<S101L<S101W<S101F. The S101F mutant showed 7.87-fold higher catalytic efficiency than the wild-type NK. Among mutants substituted with a charged residue, the protease activities of mutants with a positively charged side chain were much higher than that of the S101D mutant which had a negatively charged side chain. For the Glu substrate, the S101K mutant showed 3.38-fold higher catalytic efficiency than the wild-type NK.

The S101H and S101K mutant NKs showed no clear preference to the Phe substrate or the Ala substrate. While the S101D mutant NK decreased the specificity, the S101A, S101L, S101F, S101W and S101R mutant NKs showed increased substrate specificity for the Phe substrate. Similar to G100D, the S101D mutant NK decreased substrate specificity for the Glu substrate mutant, whereas the other seven mutants showed increased catalytic efficiency for Glu as the P3 residue (Table 2).

Mutation of Ser^{101} moderately affected fibrinolytic activity of NK (Table 3). It is very interesting that some variants showed increased fibrinolytic activities relative to the wild-type enzyme with the order: S101W>S101L=S101K>S101R. The S101W and S101L mutant NKs, which introduce bulky residues into position 101, possessed 2.2-fold and 1.7-fold higher fibrinolytic activity compared with the wild-type NK, respectively. The S101K and S101R mutant NKs, which introduced negatively charged residues into position 101, possessed 1.7-fold and 1.5-fold higher fibrinolytic activity compared with the wild-type NK, respectively. The fibrinolytic activities of the other four mutant NKs was decreased and the following decreasing order was observed: S101A>S101H=S101F>S101D.

L126G, L126V, L126F, L126D, L126E and L126K Mutant NKs

Site-directed mutagenesis and kinetics study results (Table 2) showed that mutation of Leu^{126} drastically decreased the protease activity and moderately changed substrate specificity of NK.

The L126D and L126E NK variants, both with positively charged side chains at position 126, abolished the protease activity as well as the other four mutants (the L126G, L126V, L126F and L126K variants) decreased protease activities drastically. For all three tetrapeptide substrates, the catalytic efficiencies of L126E were less than 1% of the wild-type NK. The catalytic efficiencies of L126D were even lower than those of L126E and no protease activity could be detected in all of our experiment conditions (Table 2).

The L126V, L126K, L126G and L126F mutant NKs preferred Phe to Ala in the P3 position. The L126F, L126V, L126G and L126K mutant NKs showed clearly increased specificity for the Glu substrate.

Similar to the results of protease activity, mutation of Leu^{126} drastically interfered with fibrinolytic activity of NK (Table 3). The L126D and L126E mutant NKs abolished nearly all fibrinolytic activity. The fibrinolytic activity of the L126D and L126E variants decreased to just 0.03 and 2.9% of that of the wild-type NK, respectively. The fibrinolytic activities of the other four mutant NKs also decreased drastically and the following decreasing order was observed: L126F=L126V>L126G=L126K.

DISCUSSION

Our kinetics and fibrinolytic activity analyses showed that the S3 site of NK was crucial for its protease and fibrinolytic activity. Modification of the S3 site of NK might alter its protease and fibrinolytic activity obviously. These residues affected activity of the enzyme differently. Gly^{100} obviously affected, Ser^{101} moderately influenced, while Leu^{126} drastically affected the protease and fibrinolytic activities of NK. Different roles of these residues on the activity and substrate specificity of NK should result from their different structural positions (Fig. 2).

Gly^{100} and Ser^{101} reversely modulated the protease and fibrinolytic activity of NK. Substitution of Gly^{100} with hydrophobic residues (Ala or Val) led to obvious changes in protease and fibrinolytic activity. As the size of side chain increased in order of G100A, WT and G100V, the fibrinolytic activities and the k_{cat}/K_m values for the hydrolysis of the tetrapeptide substrates obviously decreased (Fig. 3). The longer the side chain,

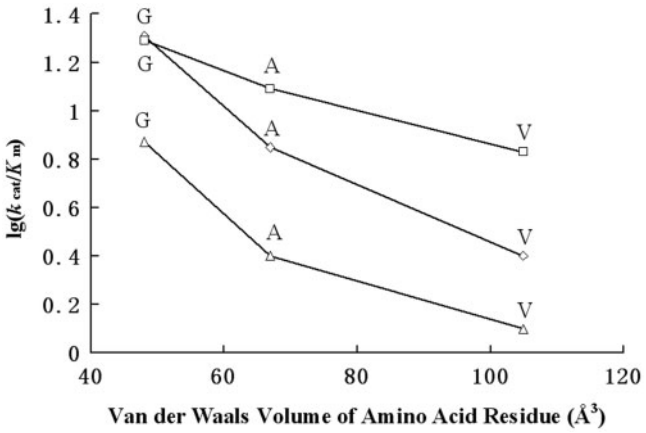

Fig. 3. **Effect of steric size (van der Waals volume) of residues at the position 100 of nattokinase for the hydrolysis of suc-AAPFpNA (open diamond), suc-AFPFpNA (open square) and suc-AEPFpNA (open triangle), by the wild-type and mutant nattokinases.** G, A and V represent the wild-type, G100A and G100V mutant NKs, respectively. The van der Waals volume values are taken from Richards (*31*). The k_{cat}/K_m values are from Table 2.

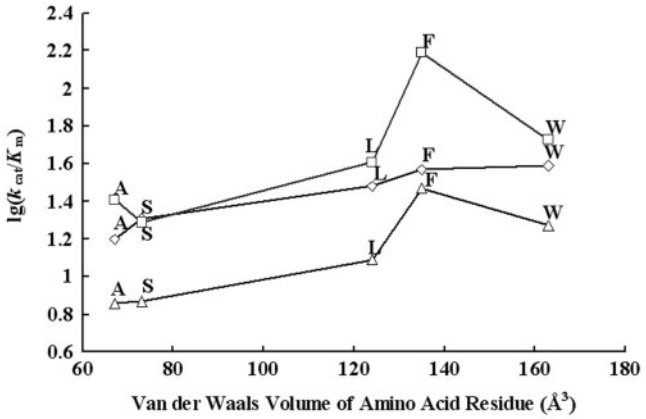

Fig. 4. **Effect of steric size (van der Waals volume) of residues at the position 101 of nattokinase for the hydrolysis of suc-AAPFpNA (open diamond), suc-AFPFpNA (open square) and suc-AEPFpNA (open triangle), by the wild-type and mutant NKs.** A, S, L, F and W represent the S101A, wild-type, S101L, S101F and S101W mutant nattokinases, respectively. The van der Waals volume values are taken from Richards (*31*). The k_{cat}/K_m values are from Table 2.

the lower the activity. For example, the G100V mutant showed just 28% of WT fibrinolytic activity (Table 3), and 12% of WT catalytic efficiency for suc-AAPFpNA substrate (Table 2). These results suggested the side chains of residues at this position that projected into the active site likely decreased substrate binding and catalytic activity of NK. Contrary to Gly^{100}, introduction of bulky side chains at position Ser^{101} resulted in increasing protease activities for the Ala substrate (Fig. 4), and the more bulky the side chain, the higher the activity. For suc-AAPFpNA substrate, the S101L, S101F and S101W mutants possessed a 1.45-fold, 1.80-fold and 1.91-fold higher catalytic efficiency, respectively compared with wild-type NK (Table 2). These results suggested the bulky side chains that built thicker hydrophobic walls at the outer edge of S3 site might improve the structure of NK, which could help to stabilizes both the transition state and the Michaelis complex formation during the enzyme reaction. The result that fibrinolytic activities increased in the order of S101A, S101L and S101W mutants might be due to the same reason. However, why fibrinolytic activity of the S101F mutant was relatively low was still unknown. For the Phe and Glu substrates, substitution of Ser^{101} with the bulky Trp resulted in decreased catalytic efficiency of the S101W mutant, suggesting Trp was too large to accept the substrates with bulky P3 residues. Introduction of residues with negatively charged side chains at position 100 did not significantly interfere with fibrinolytic activity and protease activity for the hydrophobic substrates (X = Ala or Phe), whereas introducing positively charged residues into this position severely interfered with the previous activities. In contrast, mutants substituted with a positively charged side chain at position 101 retained higher fibrinolytic activities and protease activities of NK for the hydrophobic substrates (X = Ala or Phe) than those with a negatively charged side chain. These data suggested that although conjoint at the surface of NK, Gly^{100} and Ser^{101} had reverse steric and electrostatic effects. These different effects might be due to the different positions of Gly^{100} and Ser^{101}.

Leu^{126} was an essential structure component of the active cleft of NK. Structure analysis showed this residue was buried in the active cleft of NK and was close to the catalytic triad (Fig. 2), suggesting it might be very important for the function of NK. Mutation analysis (Tables 2 and 3) substantiated this prediction and showed Leu^{126} was an essential structural component of the active cleft that could not be substituted without severely affecting catalytic activity of the enzyme. This observation was consistent with the fact that Leu^{126} is conserved among all subtilisins (*32*). All six mutants, even the mutant that just slightly changed the size of the side chain, drastically decreased catalytic efficiencies for all of the tetrapeptide substrates (Table 2). The fibrinolytic activities of these mutants were also drastically decreased (Table 3). These suggested even a conserved substitution of Leu^{126} would impair the enzyme conformation that normally stabilizes the transition state and the Michaelis complex formation during the enzymatic reaction. Introduction of positively charged residues at Leu^{126} might interrupt structure of the catalytic triad, thus the enzyme nearly abolished its protease activity. Relatively, mutants substituted with a hydrophobic side chain at this position retained higher fibrinolytic activity than those with a hydrophilic side chain (Table 3). This result suggested the hydrophobic environment within the active cleft was important to keep the fibrinolytic activity of NK.

However, S3 site was flexible to some degree in determining the substrate specificity of NK. Mutation on S3 residues led to only moderate (not more than 5-fold) alteration of P3 substrate specificity, suggesting that the S3 site of NK was flexible and the enzyme had a relatively broad P3 substrate specificity. The effect of the S3 site of NK on its substrate specificity was quite

different from those of S1 and S4 substrate-binding pockets of subtilisins. Mutations on S1 or S4 substrate-binding pockets drastically (much more than 10-fold) alter substrate specificity of substilins (*11–19*). Our data showed S3 site was quite more flexible than S1 and S4 pockets to determine substrate specificity of subtilisins. This result was also contrary to that of the S3 site of aqualysin (a bacterial subtilisin-related alkaline serine protease). Changing the S3 site residues (also locate at the enzyme surface) of aqualysin obviously affected its substrate specificity of the enzyme (*33*). The difference was not surprising for the primary sequence of aqualysin is just 28% homologous (our alignment) to that of nattokinse, suggesting their structures could be very different. Given the homology between nattokinse and most subtilisins was much higher than that of aqualysin, our result of S3 residues on the substrate specificity of NK might be more applicable to other subtilisins than that of aqualysin.

By this study we found some Ser mutants (S101F, S101K, *etc*.) exhibited obviously enhanced protease activities, and four Ser mutants (S101L, S101F, S101K and S101R) showed higher fibrinolytic activities relative to the wild-type NK. These results suggested that improving the protease activity and fibrinolytic activity of NK by introducing bulky or positively charged residues into Ser^{101} position at S3 site is effective. To our knowledge, this is the first report to improve NK as a potential therapeutic agent by protein engineering method.

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