RESEARCH LETTER

Directed evolution improves the fibrinolytic activity of nattokinase from *Bacillus natto*

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Abstract

Nattokinase (subtilisin NAT, NK) is a relatively effective microbial fibrinolytic enzyme that has been identified and characterized from *Bacillus natto*. In the current report, DNA family shuffling was used to improve the fibrinolytic activity of nattokinase. Three homologous genes from *B. natto* AS 1.107, *Bacillus amyloliquefaciens* CICC 20164 and *Bacillus licheniformis* CICC 10092 were shuffled to generate a mutant library. A plate-based method was used to screen the mutant libraries for improved activity. After three rounds of DNA shuffling, one desirable mutant with 16 amino acid substitutions was obtained. The mutant enzyme was purified and characterized. The kinetic measurements showed that the catalytic efficiency of the mutant NK was approximately 2.3 times higher than that of the wild-type nattokinase. In addition, the molecular modeling analysis suggested that the mutations affect the enzymatic function by changing the surface conformation of the substrate-binding pocket. The current study shows that the evolution of nattokinase with improved fibrinolytic activity by DNA family shuffling is feasible and provides useful references to facilitate the application of nattokinase in thrombolytic therapy.

Introduction

Thrombotic diseases, especially acute myocardial infarction, imperil the human lives and health in modern life. Compared with widely used thrombolytic agents, such as tissue plasminogen activator (t-PA) and urokinase (Mukhametova *et al.*, 2002), several cheaper and safer resources have been extensively investigated over the years (Nakanishi *et al.*, 1994; Moriyama & Takaoka, 2006). Among them, nattokinase (NK), which was extracted from a traditional Japanese fermented *natto*, has attracted interest. The molecular mass and isoelectric point of NK are about 28 kD and 8.6 respectively. NK has sufficient stability of pH and temperature to be stable in the gastrointestinal tract (Sumi *et al.*, 1987). NK directly cleaves cross-linked fibrin *in vitro*, catalyzes the conversion of plasminogen to plasmin or inactivates the fibrinolysis inhibitor (PAI-1) (Fujita *et al.*, 1993; Urano *et al.*, 2001). Until recently, most studies of NK have focused on its thrombolytic mechanism, effects, heterologous expression and purification.

In vitro molecular-directed evolution is a new strategy that has been used to change the characteristics of enzymes in recent years. The complete nucleotide sequence of the subtilisin NAT *aprN* has been obtained using shotgun cloning, and the amino acid sequence has been deduced from the DNA sequence (Nakamura *et al.*, 1992). We have previously introduced mutations into the *aprN* gene using site-directed mutagenesis to probe the importance of hydrogen bonds in the active site of the NK (Zheng *et al.*, 2006), increase the oxidative stability of NK (Weng *et al.*, 2009), and investigate the function of the propeptide of NK (Jia *et al.*, 2010). DNA family shuffling is a simple and efficient method for molecular-directed evolution by mimicking and accelerating the process of sexual recombination (Crameri *et al.*, 1998). This approach involves the recombination of homologous sequences, which are the same gene from related species or related genes from a single species to create a library of chimeras. A library of chimeric subtilisins has been created by DNA family shuffling and the mutants have improved properties compared to the parental enzymes (Ness *et al.*, 1999).

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NK belongs to the subtilisin family of serine protease, has the same conserved catalytic triad (D32, H64, S221) and substrate binding sites (S125, L126, G127) (Bryan, 2000). The homology of the encoding gene sequence between NK and subtilisin BPN′ (SB) from *Bacillus amyloliquefaciens* or NK and subtilisin Carlsberg (SC) from *Bacillus licheniformis* was 80% or 69%, respectively (Nakamura *et al.*, 1992). Therefore, we introduced random mutagenesis in the *aprN* gene using the DNA family shuffling method. The three encoding genes were recombined and shuffled to establish chimeric gene libraries. Combined with a high-throughput plate-based screening method, mutants that had the desired properties were selected, purified and characterized. In the current study, we reported for the first time the application of directed evolution to improve the fibrinolytic activity of subtilisin NAT from *Bacillus natto*.

Materials and methods

Bacterial strains, plasmids and materials

Bacillus subtilis var. *natto* strain AS 1.107 (Institute of Microbiology, Chinese Academy of Sciences, Beijing, China), *B. amyloliquefaciens* strain CICC 20164 and *B. licheniformis* strain CICC 10092 (China Center of Industrial Culture Collection, Beijing, China) were used to isolate the genomic DNA. *Escherichia coli* BL21(DE3) pLysS and the plasmid pET-26b$^+$ (Novagen) were used as the host-vector system for the cloning and expression of the gene encoding the enzymes. All of the enzymes for DNA manipulations were purchased from TaKaRa (Dalian). Thrombin and urokinase were purchased from the Chinese Medicine Testing Institute. Human fibrinogen and succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (suc-AAPF-pNA) were purchased from Sigma (St. Louis, MO).

Construction of recombinant plasmids pETSN, pETSB and pETSC

The oligonucleotide primers and plasmids used in the current study are listed in Table 1. The gene encoding the precursor NK was amplified by PCR from genomic DNA of *B. subtilis* var. *natto* using the primers PNB and PNX. Similarly, the gene encoding the precursor SB from *B. amyloliquefaciens* (Vasantha *et al.*, 1984) or Carlsberg from *B. licheniformis* (Jacobs *et al.*, 1985) were also obtained by PCR using the two sets of primer PBB and PBX or PCB and PCX, respectively. The PCR program was 5 min at 94 °C followed by 30 cycles of 60 s at 94 °C, 60 s at 56 °C, 90 s at 72 °C, and finally 10 min at 72 °C using *Pfu* DNA polymerase. The PCR products were digested with BamHI and XhoI and inserted between the same restriction sites of the plasmid pET-26b$^+$ to form pETSN, pETSB and pETSC, respectively. The three recombined plasmids were transformed into *E. coli.* BL21 (DE3) *plys* competent cells and plated onto Luria–Bertani (LB) plates with 30 μg mL^{-1} kanamycin. DNA sequences were sequenced by the Nanjing GenScript Biotechnology Co., Ltd.

Table 1. DNA sequence of oligonucleotide primers and plasmids in the current study

Primers/plasmid	DNA sequence (5′–3′)/relevant properties	Restriction sites/source
Primers		
PNB	agaggatccgatggccggaaaaagcagt	BamHI
PNX	agactcgagttgtgcagctgcttgtacgt	XhoI
PBB	gcgggatccgatggcagggaaatcaaacg	BamHI
PBX	tatctcgagctgagctgccgcctgtac	XhoI
PCB	agaggatccgatggctcaaccggcgaaaaat	BamHI
PCX	tatctcgagatgttattgagcggcagc	XhoI
Plasmids		
pETSN	pET-26b$^+$ with precursor sequence of NK	This study
pETSB	pET-26b$^+$ with precursor sequence of SB	This study
pETSC	pET-26b$^+$ with precursor sequence of SC	This study

DNA family shuffling and library construction

The plasmids pETSN, pETSB and pETSC which contain the correct gene sequence of the wild-type enzyme, served as the templates for DNA family shuffling. Initially, the target gene of the enzymes was amplified using PCR with the primers described above. The PCR products were purified and subjected to DNase I digestion to generate random fragments according to the method described by Suen *et al.* (2004). The procedures for DNA shuffling were performed based on the method described by Stemmer (1994) with minor modifications. The digested products were subjected to 2% agarose gel electrophoresis, and DNA fragments of 50–100 bp were recovered for primer-less DNA assembly. *Pfu* DNA polymerase was used in the PCR method to reduce new mutations that may be introduced into the parental gene sequences. The gradient PCR program of the primer-less PCR was 94 °C for 5 min, followed by 45 cycles of 94 °C for 30 s, 55 °C for 45 s, 50 °C for 45 s, 47 °C for 45 s, 44 °C for 45 s, and 72 °C for 2 min. The products of primer-less PCR were purified and diluted 10 times for PCR using the primers PNB and PNX (Table 1). After heating for 5 min at 94 °C, the reaction program was 94 °C for 1 min, 56.6 °C for 1 min, 72 °C for 2 min (30 cycles), with a final extension of 72 °C for 10 min. The mutated PCR products were

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purified, digested with BamHI and XhoI, and inserted into the pET-26b$^+$ vector, which was cut using the same enzymes, followed by the transformation into *E. coli* BL21 (DE3)pLysS competent cells to obtain the mutant library.

Screening for the hybrid enzymes with enhanced fibrinolytic activity

The mutant library was primarily screened on LB plates containing 30 μg mL^{-1} of kanamycin and 2% (w/v) skim milk (Tange *et al.*, 1994). After 24–48 h of cultivation at 37 °C, colonies that formed larger clear zones were isolated using sterile toothpicks and transferred to a 5-mL liquid LB culture containing 30 μg mL^{-1} kanamycin. The bacterial isolates were cultured at 37 °C for 12 h, induced for 4 h by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) and centrifuged. The pellets of bacteria were resuspended and diluted to $OD_{600\,nm}$ = 0.1 with 100 mM phosphate buffer (pH 8.0). The cells were lysed by sonication, and the crude enzyme fibrinolytic activity in the supernatant was assayed using the fibrin plate method (Astrup & Mullertz, 1952). Those colonies showing higher fibrinolytic activity compared to wild-type NK were selected as the parents for the next round of shuffling. Finally, the strains of mutants with the highest activity were retained for our experiments. Each plate contained the test strain with the plasmid pET26b$^+$ or with the plasmid pETSN as a control.

Expression, purification and characterization of wild-type and mutant enzymes

The selected transformants were cultured in LB medium containing 30 μg mL^{-1} kanamycin at 37 °C. IPTG was added to the medium at a final concentration of 0.7 mM to induce bacteria when the $OD_{600\,nm}$ reached approximately 0.8 (Liang *et al.*, 2007). After further induction overnight at 20 °C, cells were harvested by centrifugation, resuspended and then disrupted by sonication on ice. The supernatant of the whole-cell extracts was purified using the Ni-NTA column (Invitrogen) and DEAE Sepharose Fast Flow column (Amersham Biosciences) according to the manufacturers' instructions. The purification was performed at 0–4 °C. After the purification, the molecular mass of the purified enzymes was analyzed by SDS-PAGE using a 12.5% (w/v) polyacrylamide separating gel. The protein concentration was determined using the BCA protein assay reagent kit (Pierce). For immunoblotting, the proteins separated by SDS-PAGE were electrically transferred onto a polyvinylidene difluoride membrane. The membrane was blocked overnight at 4 °C in the blocking buffer (5% skim milk) and then incubated for 2 h at 37 °C with 1 : 3000 diluted mouse anti-His-tag monoclonal antibody, followed by incubation for 1 h at 37 °C with 1 : 6000 diluted HRP-Goat anti-mouse IgG (H + L) (Genscript, Nanjing, China). Finally, bands were visualized using enhanced chemiluminescence Western blotting detection reagents (Millipore).

Enzyme activity assay and kinetic analysis

Fibrinolytic activity was determined by measuring the areas of the lysed zone on the fibrin plate (Astrup & Mullertz, 1952; Liang *et al.*, 2007). In brief, the fibrin plate was made up of 0.4% fibrinogen, 0.6% agarose and 0.5 U mL^{-1} thrombin, which were dissolved in 50 mM barbitol buffer (pH 7.8) beforehand and mixed in a petri dish (9 cm in diameter). Purified enzymes were also diluted using the 50 mM barbitol buffer, and 20 μL of the samples were placed into holes which had been made previously on the fibrin plate. After measuring the dimension of the clear zone and incubating the plate at 37 °C for 18 h, the fibrinolytic activity was estimated using urokinase as a standard. The specific activity of the enzyme to hydrolyze fibrin was defined as urokinase units of fibrinolytic activity in each milligram of enzyme.

Enzymatic kinetics were determined by measuring the release of p-nitroaniline from the chromogenic substrate suc-AAPF-pNA in 100 mM phosphate buffer (pH 8.0) containing 4% (v/v) DMSO at (37 °C ± 0.2) (Sumi *et al.*, 1987). After incubation for 10 min at 37 ± 0.2 °C, the concentration of liberated p-nitroaniline was measured at an absorbance of 405 nm using an automatic microplate reader (Thermo Lab systems, Multiskan MK3). Kinetic parameters (V_{max} and K_m) were determined from initial rate measurements at different substrate concentrations ranging from 0.098 to 0.392 mM. The V_{max} values were converted to k_{cat} values from the relationship $k_{cat} = V_{max}/$(enzyme).

Protein modeling

To investigate why the observed mutations enhanced the fibrinolytic activity, the three-dimensional structures of the wild-type NK and the evolved mutant were performed using the AMBER9 software package (Pettersen *et al.*, 2004) based on the modeling template that was constructed by Zheng *et al.* (2005).

Results

Cloning and sequence analysis of *aprN* gene

The precursor encoding genes of NK, SB and SC were cloned into the plasmid pET-26b$^+$ to form the recombinant plasmids pETSN, pETSB and pETSC. After transformation,

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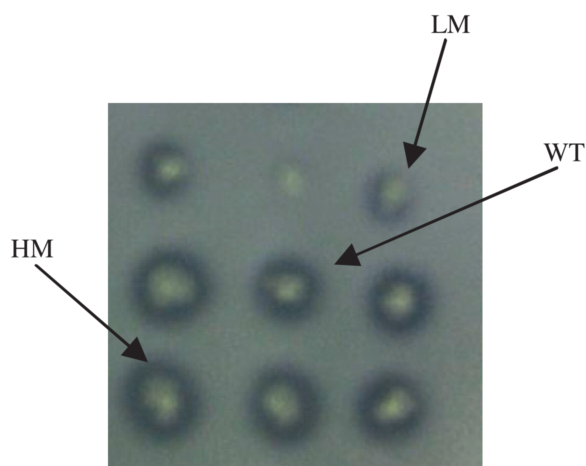

Fig. 1. Activity screening of colonies using skim milk plates. Indicators for arrows: WT, wild-type clone; LM, clone with lower enzyme activity; HM, clone with possible higher enzyme activity.

the positive transformants were selected and sequenced. The target gene sequences were analyzed with the NCBI database and revealed 100% homology with the reported NK gene (GenBank accession no. S51909), SB gene (GenBank accession no. K02496.1) and SC gene (GenBank accession no. X03341.1).

Directed evolution of *aprN* gene and screening of the mutant library

Random mutations were introduced into the nattokinase gene using the DNA family shuffling method as described in Materials and methods. After three rounds of DNA shuffling, more than 20 000 clones were screened for their possible increased fibrinolytic activity by the clear zone-forming method in the skim milk plates (Fig. 1). Subsequently, clones that showed a larger clear zone than the wild-type nattokinase were selected and screened by measuring the enzymatic activity of the cell-free extract using the fibrin plate method. A mutant showed an approximate 2.0-fold increase in fibrinolytic activity compared to the wild-type nattokinase was obtained. The DNA sequence of the evolved nattokinase gene showed 16 nucleotide substitutions resulting in amino acid substitutions in the translated enzyme sequence (Fig. 1a).

Purification of the enzymes from recombinant strain *E. coli* BL21 (DE3) transformant

To characterize the mutant NK with enhanced fibrinolytic activity, the wild-type nattokinase and the mutant enzyme were produced at a larger scale and purified. The plasmid pET-26b$^+$ carries an optional C-terminal His$_6$-tag sequence for protein purification using Ni^{2+} resins.

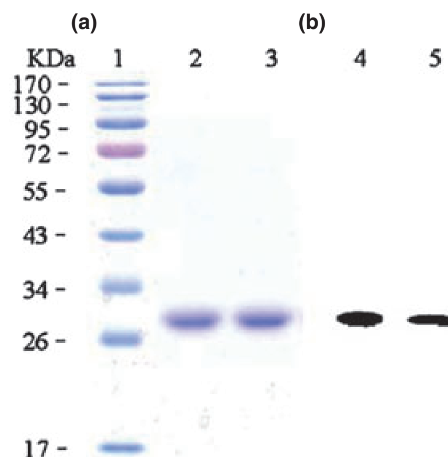

Fig. 2. SDS-PAGE and Western blotting analysis of purified wild-type NK and mutant NK that were expressed in *Escherichia coli* BL21 (DE3). (a) SDS-PAGE analysis of purified enzymes was visualized using Coomassie brilliant blue R-250. Lane 1, broad range prestained molecular weight markers; lane 2, purified wild-type NK; and lane 3, purified mutant enzyme. (b) Western blotting analysis of purified enzymes. Lane 4, purified wild-type NK; and lane 5, purified mutant enzyme.

Table 2. Specific activities of wild-type NK and mutant enzymes

Enzyme	Specific activity (U mg^{-1})	Relative activity (%)
Wild-type	601.12 (±132)	100
Mutant	1262.15 (±116)	210

Fibrinolytic activities were detected by the fibrin plate method using urokinase as a standard, assays were performed at 37 °C and pH 7.8 for 18 h. Relative specificity was calculated as the ratio of the fibrinolytic activities of the mutant NK relative to the wild-type NK. The data shown are expressed as means ± SD and are based on three or more independent experiments.

SDS-PAGE and Western blot analysis showed that the purified mutant enzyme has the same molecular weight as the wild-type nattokinase at 28 kDa (Fig. 2). The specific activities of the wild-type and mutant NK based on the protein concentration and the enzymatic activity analysis are summarized in Table 2. The results indicate that the specific activity of the purified mutant NK was approximately 1262 U mg^{-1} of protein, which is 2.1-fold higher than that of the wild-type nattokinase.

Kinetic analysis of the mutants

The kinetic parameters of the purified enzymes were determined based on the intercepts of the Lineweaver–Burk plots. As shown in Table 3, the mutant NK showed an apparent increase (approximately 1.4-fold) in the k_{cat} value and a visible decrease (approximately 30%) in the

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Table 3. Comparison of kinetics of wild-type NK and mutant NK for the hydrolysis of suc-AAPF-pNA

Enzyme	k_{cat} (s^{-1})	k_m (μM)	k_{cat}/k_m (M^{-1} s^{-1})
Wild-type	29.35 (±2.0%)	363.48 (±13.1)	0.807 × 10^5
Mutant	43.9 (±2.1%)	255.69 (±12.4)	1.717 × 10^5

Assays were performed in 0.1 M phosphate buffer, pH 8.0 containing 4% (v/v) DMSO with suc-AAPF-pNA as a substrate at 37 ± 0.2 °C. Each value is the mean of three measurements.

k_m value. Therefore, the catalytic efficiency (k_{cat}/k_m) of the mutant NK was 213% higher than that of wild-type NK. The catalytic parameters were also consistent with the fibrinolytic activity (specific activity) of the mutant NK and the wild-type NK (Table 2), which was determined using the fibrin plate method.

Protein modeling

A three-dimensional model of the evolved NK was constructed based on the three-dimensional structure of the parental NK. We can use the model structures to predict the roles that the mutations may play in NK function. As shown in Fig. 3b, many mutations were far away from the active pocket, and two mutations (V150 and T224) were close to the active site. The hydrophobic pocket of the active site was broadened as a result of all of the amino acid substitutions (Fig. 3c), which may lead to changes in the protein structure and the catalytic activity.

Discussion

In the current study, we investigated how to improve the fibrinolytic activity of NK using directed evolution to broaden its medical or commercial applications. *In vitro* molecular evolution strategies are the most efficient methods for creating proteins with improved or novel properties. We generated a library of NK variants by the shuffling of genes encoding subtilisin NAT (NK), BPN′ and Carlsberg. To screen large libraries, the NK variants were expressed in *E. coli*. BL21(DE3)pLysS using a prokaryotic signal peptide, PelB, for efficient secretion. NK variants were selected based on zone-forming activity on agar plates with skim milk or fibrin. A mutant NK showed a 2.3-fold increase in fibrinolytic activities compared to the wild-type NK from *Bacillus natto*. The further sequence and structural study of the mutant enzyme will offer some insight into the structure-function relationship of NK.

The amino acid sequence alignment of the three parents and the mutant enzyme revealed that the catalytic triad and the substrate-binding site were conserved. Nine amino acid substitutions were derived from SB, and the rest from SB or SC. No new mutations were introduced into the mutant enzyme sequence (Fig. 3a). To understand the functions of the amino acid substitutions, the identified mutations in the selected mutant was distributed throughout the model of the mutant structure based on the three-dimensional model of NK that was previously constructed by our lab (Zheng *et al.*, 2005). The

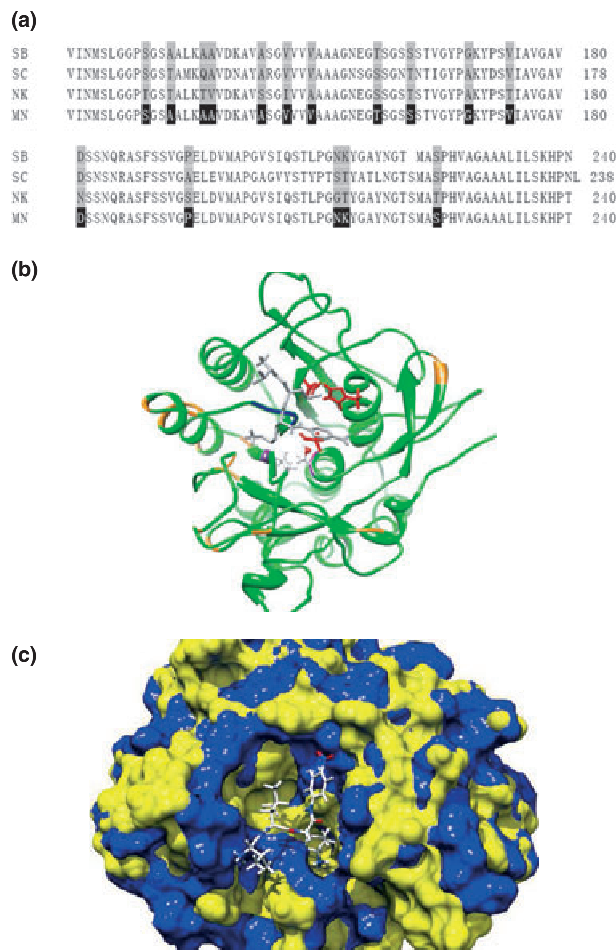

Fig. 3. (a) Amino acid sequence alignment of the three parents and the mutant enzyme.NK, nattokinase; MN, mutant NK. Letters in the gray boxes represent amino acid residues of the three parents. Letters in the black boxes represent amino acid residues of the mutant enzyme. For reasons of clarity, the sequence with no mutations is not shown. (b) The location of the amino acid mutations in the predicted structure of the mutant enzyme. Red, catalytic sites (D32, H64, S221); blue, substrate binding sites (S125, L126, G127); sliver, substrate; purple, two mutations (V150 and T224) close to the active site; and orange, mutations far away from the active site. Ribbon in green represents the enzyme structure. (c) Comparison of the hydrophobic pockets of wild-type NK (blue) and mutant enzyme (yellow) in the three-dimensional structure after molecular dynamic simulation.

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three-dimensional structure showed that the strictly conserved residues of the catalytic centre (D32, H64, S221) and the substrate-binding sites (S125, L126, G127) were positioned in the pocket, which comprised two α-helices and seven β-strands (Fig. 3b). However, in the current study, none of the mutations was located in those strictly conserved regions throughout the mutant.

Most of the mutations were located in the surface regions and far away from the pocket, with the exception of the substitutions A150V and T224S (Fig. 3b), which were very close to the Ser221 in the catalytic centre of the enzyme. This change may not be involved in hydrogen bonding with other residues. However, the combination of this change with other substitutions may result in the formation of a larger active-site pocket to improve the catalytic efficiency (Fig. 3c). These results indicate that the hydrophobic pocket plays an important role in the substrate association with the enzyme. The serine alkaline protease, SAPB, from *Bacillus pumilus* CBS is an effective additive in laundry detergent formulations (Jaouadi *et al.*, 2008). Twelve mutants of SAPB have constructed by site-directed mutagenesis and the results demonstrate that all the amino acids of the catalytic cluster and amino acids intimately related to the hydrophobic environments near the active site are important for engineering of kinetic performances of detergent-stable enzymes (Jaouadi *et al.*, 2010). Mutations outside of the catalytic centre or the binding sites resulted in increased catalytic activity of the enzyme, as has been observed in other studies (van der Veen *et al.*, 2004; Fan *et al.*, 2007). For the rational enzymatic design, the amino acid residues that are close to the active centre or the binding pocket are often modified. However, the amino acid residues that are located far from these two places may play an important role in enzymatic function. Random mutagenesis can be introduced into gene sequences when it is not necessary to know the identity of the structure–function relationship of the enzyme.

Currently, whether nattokinase may become a widely used thrombolytic agent mainly depends on the enhancement of its properties, e.g. prolonging the half-life with oral administration and improving the stability and catalytic efficiency.

In conclusion, the results of our work have demonstrated that it is feasible to generate a mutant library of nattokinase using the DNA family shuffling method to obtain a mutant with enhanced catalytic efficiency. With better catalytic efficiency, the mutant may become a desirable and economical source for use in thrombolytic therapy or other industrial applications. Further investigation of the selection of mutants with high catalytic efficiency using the DNA family shuffling and screening method is promising.

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