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Research article

Enzyme activity and thermostability of a non-specific nuclease from *Yersinia enterocolitica* subsp. *palearctica* by site-directed mutagenesis

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ABSTRACT

Background: To identify the critical amino acid residues that contribute to the high enzyme activity and good thermostability of *Yersinia enterocolitica* subsp. *palearctica* (Y. NSN), 15 mutants of Y. NSN were obtained by site-directed mutagenesis in this study. And their enzyme activity and thermostability were assayed. Effect of several factors on the enzyme activity and thermostability of Y. NSN, was also investigated.
Results: The results showed that the I203F and D264E mutants retained approximately 75% and 70% enzyme activity, respectively, compared to the wild-type enzyme. In addition to the I203F and D264E mutants, the mutant E202A had an obvious influence on the thermostability of Y. NSN. According to the analysis of enzyme activity and thermostability of Y. NSN, we found that Glu202, Ile203 and Asp264 might be the key residues for its high enzyme activity and good thermostability.
Conclusions: Among all factors affecting enzyme activity and thermostability of Y. NSN, they failed to explain the experimental results well. One reason might be that the enzyme activity and thermostability of Y. NSN were affected not only by a single factor but also by the entire environment.

1. Introduction

Non-specific nucleases are a group of enzymes that can degrade DNA and/or RNA molecules without sequence specificity [1]. They play an important role in genetic mechanisms, such as avoiding mutation, DNA repair, DNA replication and recombination, nucleoside deletion, phosphoric acid removal in growth and metabolism processes, host defenses against the invasion of exogenous nucleic acid molecules, and cell apoptosis [2,3,4,5,6]. In addition, non-specific nucleases have been widely used in molecular biology fields, such as the detection of nucleic acid structure, rapid detection of RNA, deletion of nucleic acids in the protein purification process, and application as antiviral agents [7,8]. To date, most of studies on non-specific nucleases have primarily focused on *Serratia marcescens* nucleases. The production, expression, extracellular secretion, catalytic mechanism, structure and application of *S. marcescens* nucleases have been studied by Michael and Benedik [7]. The studies on other nucleases, such as Staphylococcus nucleases, sugar non-specific nucleases, Vvn and ColE7, have also been reported [9,10].

Yersinia enterocolitica subsp. *palearctica* and *Yersinia enterocolitica* subsp. *enterocolitica* are two subspecies of *Yersinia enterocolitica* [11]. A non-specific nuclease from *Y. enterocolitica* subsp. *palearctica* (Y. NSN) was found in our group. In our previous work, a recombinant plasmid containing the Y. NSN gene and PET-24a(+) was expressed in BL21starTM(DE3)plysS. The results showed that Y. NSN had a high enzyme activity, good thermostability, and wide resistance to acid conditions, alkaline conditions, temperature and various chemical substances [3]. However, in our other research, we found that the nuclease from *Y. enterocolitica* subsp. enterocolitica 8081 had a lower enzyme activity and poorer thermostability compared to Y. NSN at the same conditions [12]. Thus, to identify the critical amino acid residues that contribute to the high enzyme activity and good thermostability of Y. NSN, Y. NSN mutants were obtained by site-directed mutagenesis in this paper. The enzyme activity and thermostability of the mutants and wild-type enzyme were compared. Moreover, the structure of Y. NSN was analyzed to interpret the effect of the mutations on the enzyme activity and thermostability of Y. NSN. The results of this work

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contribute to uncovering the factors involved in the high enzyme activity and thermostability of Y. NSN.

2. Materials and methods

2.1. Materials

StarPrep Plasmid Miniprep Kit, 2× Pfu PCR StarMix Loading Dye-free, and StarPrep Gel Extraction Kit were purchased from GenStar (Beijing, China). Calf thymus DNA was obtained from Sigma (Shanghai, China). Isopropyl-β-D-thiogalactopyranoside (IPTG), Dpn I, kanamycin sulfate and all other chemicals (analytical grade purity) were purchased from Sangon (Shanghai, China). The primer synthesis and mutant DNA sequencing were conducted by Sunny (Shanghai, China).

2.2. Mutation design

Our previous studies indicated that Y. NSN and the nuclease from *Y. enterocolitica* subsp. enterocolitica 8081 shared high homology, but the nucleases showed quite different enzyme characteristics [12]. The BLAST algorithm at NCBI was used to analyze the amino acid sequences obtained from GenBank. The accession numbers are YP_006005337 and YP_001007112, respectively.

2.3. Site-directed mutagenesis

A recombinant plasmid containing the Y. NSN gene and PET-24a(+) was used to over-express Y. NSN. Single mutations in the Y. NSN gene used in this study were generated using the quick-change site-directed method described by Fisher and Pei [13]. All primers are listed in Table 1. The PCR reaction mixture (50 μL) included 1.0 μL of plasmid DNA, 1.0 μL of each primer (10 μM), 25 μL of 2× Pfu PCR StarMix and 22 μL of ddH2O. The PCR program was performed as follows: 95°C for 30 s; then 15 cycles of 95°C for 30 s, 1 min at X°C for annealing, 6 min at 72°C for extension; 72°C for 5 min; and finally held at 4°C, (X represents the annealing temperature, which is determined by the Tm of the primers). To eliminate the effect of the PCR ingredients on the band, the PCR products were purified by a StarPrep Gel Extraction Kit, immediately digested at 37°C for 1.0 h by adding 1.0 μL of the restriction endonuclease Dpn I (10 U/μL), and finally transformed into DH5α *Escherichia coli* competent cells. Three transformants of each mutant were selected randomly, and then sequenced to ensure correct substitution mutations. Fifteen confirmed mutants by DNA sequencing were obtained for this study.

Table 1
Primers used for this study.

Mutants	Nucleotide sequences of primers
I29T	5′-CGCGCCCAAAACC**ACA**CTCTCGGCACCTAC-3′
	5′-GTAGGTGCCGAGAG**TGT**GGTTTTGGGCGCG-3′
L30P	5′-GCCCAAAACCATA**CCC**TCGGCACCTACTGT-3′
	5′-ACAGTAGGTGCCGA**GGG**TATGGTTTTGGGC-3′
V61I	5′-GTGGTAGTGATCAAACC**ATT**ATCCGTGATGTGTA-3′ 5′-TACACATCACGG
	AT**AAT**GGTTTGATCACTACCAC-3′
D105A	5′-ATCCCGACTTGCCA**GCC**TCAGATACATTGG-3′
	5′-CCAATGTATCTGA**GGC**TGGCAAGTCGGGAT-3′
E138N	5′-TTACTCGCTGGTAAC**AAC**GATTCGCAGGCACTT-3′ 5′-AAGTGCCTGCGA
	ATC**GTT**GTTACCAGCGAGTAA-3′
Q167K	5′-GTGAGGCTCGAAGAT**AAA**GAGCGCAATTTGG-3′ 5′-CCAAATTGCG
	CTC**TTT**ATCTTCGAGCCTCAC-3′
N170T	5′-AAGATCAAGAGCGC**ACT**TTGGCTAATCGCC-3′
	5′-GGCGATTAGCCAA**AGT**GCGCTCTTGATCTT-3′
P175Q	5′-ATTTGGCTAATCGC**CAA**GATGTTACGGCAGTC-3′ 5′-GACTGCCGTAAC
	ATC**TTG**GCGATTAGCCAAAT-3′
H191N	5′-CCCTTATTTGAACGC**AAC**ATTGCCACACTGC-3′
	5′-GCAGTGTGGCAAT**GTT**GCGTTCAAATAAGGG-3′
A193G	5′-TGAACGCCACATT**GGC**ACACTGCCAGCGAA-3′
	5′-TTCGCTGGCAGTGT**GCC**AATGTGGCGTTCA-3′
T200S	5′-TGCCAGCGAAACCG**TCT**GTCGAAATCCCCA-3′
	5′-TGGGGATTTCGAC**AGA**CGGTTTCGCTGGCA-3′
E202A	5′-GAAACCGACTGTC**GCA**ATCCCCAGCGGATA-3′
	5′-TATCCGCTGGGGAT**TGC**GACAGTCGGTTTC-3′
I203F	5′-AAACCGACTGTCGAA**TTC**CCCCAGCGGATATT-3′
	5′-AATATCCGCTGGG**GAA**TTCGACAGTCGGTTT-3′
N243T	5′-GCGATTATCAAGTC**ACC**GTTGATACCATCGA-3′
	5′-TCGATGGTATCAAC**GGT**GACTTGATAATCGC-3′
D264E	5′-CAAACTTGCCCGCT**GAA**GTTGCCCAAATAATT-3′ 5′-AATTATTTGGGC
	AAC**TTC**AGCGGGCAAGTTTG-3′

Bold and underlined nucleotides are the positions chosen for the mutations.

2.4. Enzyme recombinant expression

PET-24a(+) plasmids carrying Y. NSN mutant genes were transformed into BL21starTM(DE3)plysS competent cells. For each mutant, 10 colonies were selected to Luria–Bertani (LB) agar plates containing kanamycin sulfate (50 μg/mL). Ten milliliters of overnight pre-culture was seeded into 20 mL of fresh LB medium containing kanamycin sulfate (50 μg/mL). Afterwards, it was incubated at 37°C and 200 rpm until an OD600 of 0.8–1.0 was reached. After adding the inducer IPTG (final concentration, 1.0 mM) into the culture, the production of Y. NSN occurred under the same conditions for 22 h. And then, the cells were harvested by centrifugation at 8000 × g for 5 min at 4°C, re-suspended in 2.0 mL of distilled water supplemented with 20 mmol/L MgCl2. The supernatant of the cell lysate (3–5 cycles of freezing at −20°C and thawing at 50°C) was used for the study.

2.5. Enzyme activity assay

The enzyme activity assay was performed according to the method described previously by our group [12]. Briefly, approximately 2.0 μL of diluted enzyme solution was incubated in 36 μL of calf thymus DNA (100 ng/μL) as a substrate. After incubation at 55°C for 3 min, the reaction was stopped by adding 8.0 μL of 6× DNA loading buffer (10 mM of Tris–HCl, 60 mM of EDTA, 40% sucrose, 0.050% bromophenol blue, pH 7.6). The reaction products were then subjected to 1.0% agarose gel electrophoresis. The degree of nucleic acid degradation was visualized and calculated by staining with 0.25 μg/mL of ethidium bromide [12]. To analyze the enzyme activity of the mutants, the activity of the wild type enzyme was used as a control. The enzyme activity of the mutants was compared to that of the control.

2.6. Thermostability

To measure thermostability of the mutants, 30 μL of enzyme solution of the mutant and wild type enzyme was incubated at 80°C for 30 min, respectively. Afterwards, the samples were chilled using ice. The residual enzyme activities were determined as the method was described above.

2.7. Computational analysis

SWISS-MODEL was used to generate a 3D structure model of Y. NSN with the crystal structure of 4e3yB as a template. Swiss-Pdb Viewer was used to visualize and analyze the generated model structure. The hydrophobicity of amino acid residues was analyzed by Protscale. The hydrogen bond networks and salt bridges were analyzed by Discovery Studio 2.5 (Accelrys, San Diego, USA).

3. Results

3.1. Mutation selection in Y. NSN

The amino acid sequences of Y. NSN and *Y. enterocolitica* subsp. *enterocolitica* 8081 were analyzed using the BLAST algorithm at NCBI, respectively. The results are showed in Fig. 1. The results showed that

Range 1: 1 to 283 Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
526 bits(1356)	0.0	Compositional matrix adjust.	266/283(94%)	273/283(96%)	0/283(0%)

```
Query  1    MKFNLIKLLPVLLLTACTTTDHSSAPKTILSAPTVTEQNLPAAAIDNCLVGCPTGGSDQT  60
            MKFNLIKLLPVLLLTACTTTDHS+APKT  SAPTVTEQNLPAAAIDNCLVGCPTGGSDQT
Sbjct  1    MKFNLIKLLPVLLLTACTTTDHSTAPKTTPSAPTVTEQNLPAAAIDNCLVGCPTGGSDQT  60

Query  61   VIRDVYTLNNNSHTKFANWVAYKVTKSSQASNHPRKWAQDPDLPDSDTLAPADYTGANQK  120
            +IRDVYTLNNNSHTKFANWVAYKVTKSSQASNHPRKWAQDPDLP SDTLAPADYTGANQK
Sbjct  61   IIRDVYTLNNNSHTKFANWVAYKVTKSSQASNHPRKWAQDPDLPASDTLAPADYTGANQK  120

Query  121  LAVDRGHQAPLSLLAGNEDSQALNYLSNITPQKAALNQGAWVRLEDQERNLANRPDVTAV  180
            LAVDRGHQAPLSLLAGN DSQALNYLSNITPQKAALNQGAWVRLED+ER LANR DVTAV
Sbjct  121  LAVDRGHQAPLSLLAGNNDSQALNYLSNITPQKAALNQGAWVRLEDKERTLANRQDVTAV  180

Query  181  YSVTGPLFERHIATLPAKPTVEIPSGYWKIIFIGTSPDKGQYAAFLMDQNTAKSANFCDY  240
            YSVTGPLFER+I TLPAKP+V  PSGYWKIIFIGTSPDKGQYAAFLMDQNTAKSANFCDY
Sbjct  181  YSVTGPLFERNIGTLPAKPSVAFPSGYWKIIFIGTSPDKGQYAAFLMDQNTAKSANFCDY  240

Query  241  QVNVDTIEAKTNPQLTIWSNLPADVAQIIKSQKGTLAQTIGCD  283
            QV VDTIEAKTNPQLTIWSNLPA+VAQIIKSQKGTLAQTIGC+
Sbjct  241  QVTVDTIEAKTNPQLTIWSNLPAEVAQIIKSQKGTLAQTIGCN  283
```

Fig. 1. Amino acid sequence alignment of Y. NSN and the nuclease from *Yersinia enterocolitica* subsp. *enterocolitica* 8081. 15 mutant residues are showed in red. The underlined amino acids are primers used for PCR amplification when the Y. NSN gene was cloned into PET-24a(+). Abbreviations: Query, Y. NSN; Sbjct, the nuclease from *Yersinia enterocolitica* subsp. *enterocolitica* 8081.

only 17 amino acids were different in the sequences of the nucleases. Therefore, we hypothesized that these 17 amino acids played an important role in the enzyme activity and thermostability of the nucleases. The underlined amino acids were the primers used for PCR amplification when the Y. NSN gene was cloned into PET-24a(+). The remaining 15 amino acids in the recombinant plasmid were targeted for site-directed mutagenesis.

3.2. Enzyme activity of the mutant nucleases

Fig. 2 shows the residual enzyme activities of the 15 mutants as a percentage of the activity of the wild-type enzyme. I29T, V61I, E138N and N243T mutants maintained the activities close to the wild-type enzyme, while I203F and D264E mutants only retained about 75% and 70% activity of the wild-type enzyme, respectively.

3.3. Thermostability of the mutant nucleases

Fig. 3 shows the thermostability of 15 mutants. After treatment at 80°C for 30 min, the activities of the mutants I203F and D264E were completely inactive, and the mutant E202A only retained about 5% activity of the wild-type enzyme. Compared to that of the wild-type enzyme, enzyme activity of the mutants E202A, I203F, and D264E were decreased 90% (the mutant E202A retained about 95% wild-type enzyme activity without heating treatment, as showed in Fig. 2), 75%, and 70%, respectively. The rest mutants showed a slight decrease of enzyme activity.

3.4. Computational analysis of the mutant nucleases

The structural mode of the mutant is showed in Fig. 4. All mutations except V61I, Q167K, N170T, and D264E were located at the loop

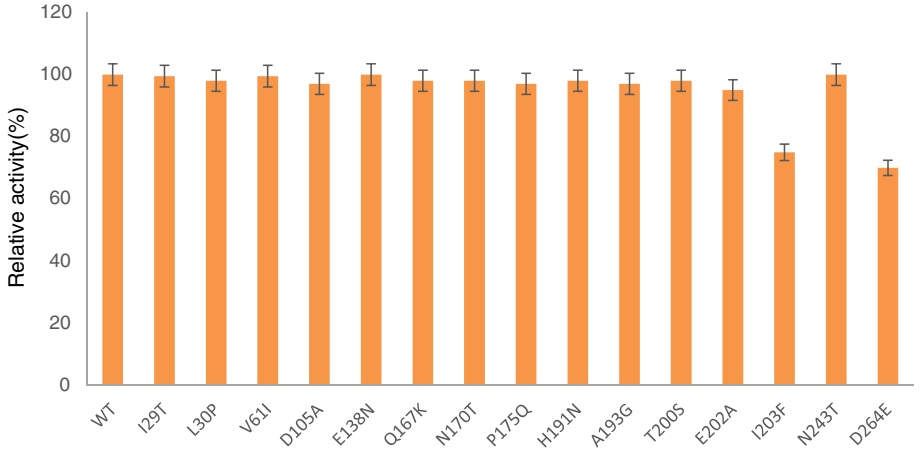

Fig. 2. Enzyme activity of the mutant nucleases.

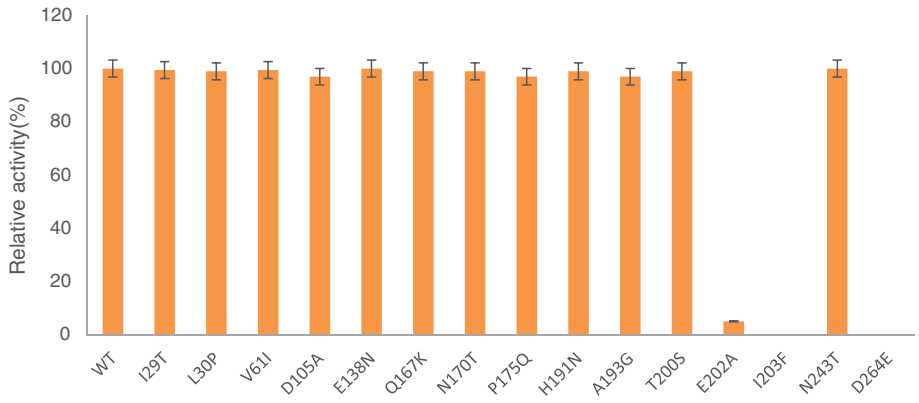

Fig. 3. Thermostability of the mutant nucleases.

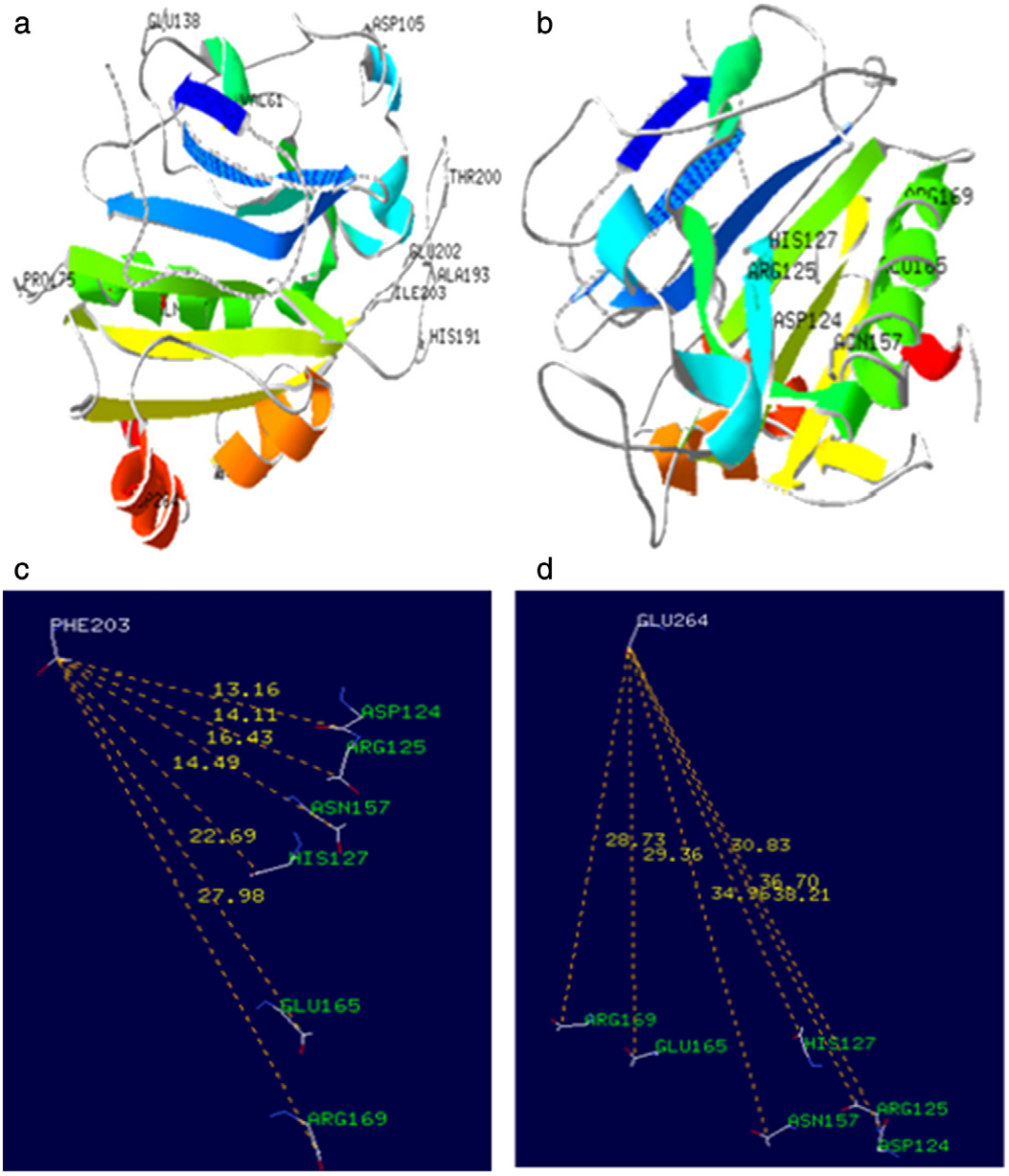

Fig. 4. The model structure of Y. NSN. (a) The positions of 13 mutants are showed in Ribbon diagram of the structure model of Y. NSN; (b) Asp124, Arg125, His127, Asn157, Glu165 and Arg169 constitute the catalytic center of Y. NSN; (c) the distance from Phe203 to respective catalytic residues in I203F; (d) The distance from Glu264 to respective catalytic residues in D264E; Catalytic residues are in green, and mutant residues are in white.

(Fig. 4a). The catalytic residues of Y. NSN, such as Asp124, Arg125, His127, Asn157, Glu165 and Arg169, are showed in Fig. 4b. His127 and Glu165 appeared to be the general base, whereas Asp124, Arg125 and Arg169 were substrate binding sites that formed a minor groove that was responsible for binding DNA or RNA. The distance from Phe203 to respective catalytic residues in I203F is showed in Fig. 4c. The distance from Phe203 to Asp124, Arg125, His127, Asn157, Glu165 and Arg169 was 13.16, 14.11, 14.49, 16.43, 22.69 and 27.98, respectively (Fig. 4c). The distance from Glu264 to respective catalytic residues in D264E is showed in Fig. 4d. The distance from Glu264 to Asp124, Arg125, His127, Asn157, Glu165 and Arg169 was 38.21, 36.70, 30.83, 34.96, 29.36 and 28.73, respectively (Fig. 4d).

4. Discussion

Site-directed mutagenesis is an effective tool to improve certain biochemical and catalytic properties of enzymes [14]. Generally, two key factors are involved in the implementation of site-directed mutagenesis. The first one is to select suitable mutation sites according to relevant structural information, which is the most critical step. The second step is the application of site-directed mutagenesis.

In recent years, many studies using site directed mutagenesis technology have been reported. Vladimir et al. [15] analyzed the 3D model of PvEGIII endo-1, 4-β-glucanase from *Penicillium verruculosum*, and selected Asp98 as the mutation site because the site was located at the place of hydrogen bond formation with Glu203 that played a role as a general acid in catalysis [15]. The pH optimum of the enzyme activity of the Asp98 mutant was shifted from pH 4.0 to 5.1.

Mahdieh et al. [16] explored the possibility of enhancing the thermostabilization of cABC I (chondroitinase ABC I) through site-directed mutagenesis. Gln140 with non-optimal φ and ψ values was selected as the mutation site. The results showed that the enzyme activity and thermostability of the Q140G and Q140A mutants were improved, whereas the enzyme activity of the Q140N mutant was reduced. Our previous work showed that the amino acid sequences of Y. NSN shared high similarity with those of the nuclease from *Y. enterocolitica* subsp. *enterocolitica* 8081. However, the characteristics of their enzyme activity and thermostability appeared quite different [12]. Compared to the nuclease from *Y. enterocolitica* subsp. *enterocolitica* 8081, Y. NSN had a higher enzyme activity and better thermostability. So, the purpose of this work was to investigate this interesting phenomenon.

As showed in Fig. 2, I203F and D264E mutants only retained about 75% and 70% activity of the wild-type enzyme, respectively. The activities of the other mutants were slightly decreased. These results indicated that these two amino acids residues, Ile203 and Asp264, might be key factors leading to the high enzyme activity of Y. NSN. As showed in Fig. 3, the activities of the mutants I203F and D264E were completely inactive after being treated at 80°C for 30 min. And the mutant E202A only retained about 5% activity of the wild-type enzyme after heat treatment at the same conditions. Therefore, the effect of the mutations on the thermostability of Y. NSN was ordered as E202A > I203F > D264E > remaining mutants. To analyze the effect of mutations on enzyme activity, a 3D model of Y. NSN was constructed by Swiss-Model with the crystal structure of 4e3yB as a template (Fig. 4). The differences between Y. NSN and the nuclease from *Y. enterocolitica* subsp. *enterocolitica* 8081 were primarily the amino acid residues located at the loop of the structural model (Fig. 4a). His127 and Glu165 appeared to be the general base, whereas Asp124, Arg125 and Arg169 were substrate binding sites that were responsible for binding DNA or RNA. Asn157 could bind the essential cofactor Mg2+ (Fig. 4b). Mutations near the catalytic residues most likely influence the catalytic mechanism [17,18]. The distance from the respective catalytic residues to the mutation sites, I203F and D264E (Fig. 4c, Fig. 4d), in which enzyme activity remarkably decreased. Unfortunately, by comparing with other mutations, no direct relationships between the distance from the mutation site to the catalytic residues and the effect of the mutations on enzyme activity were identified (results not shown).

Previously, Yu and Huang [19] reported that the thermostability of proteins was related to the simultaneous effect of several forces, such as hydrophobic interactions, disulfide bonds, salt bridges and hydrogen bonds. To improve the stability and catalytic efficiency of α-amylase from *Bacillus subtilis* under acidic conditions, Yang et al. [20] selected four basic histidine (His) residues (His222, His275, His293, and His310) in the catalytic domain as the mutation sites, and replaced the His residues with acidic aspartic acid (Asp). The results showed that the acid stability of the enzyme was significantly enhanced after mutation, and the catalytic efficiency of each active mutant was much higher than that of the wild type at low pH. Thus, these changes around the catalytic domain contributed to the significantly improved protein stability and catalytic efficiency at low pH.

Fei et al. [21] studied the relationship between *E. coli* AppA phytase's thermostability and salt bridges. A salt bridge subtraction mutant, E31Q, with a 13.96% decrease in thermostability and a salt bridge addition mutant, Q307D, with a 9.15% increase in thermostability, were observed. So, the salt bridge played a key role in *E. coli* AppA phytase's thermostability. To analyze the effect of the mutation on the thermostability of Y. NSN, only the forces of hydrophobic interactions and salt bridges were analyzed. One reason was disulfide bonds did not exist in the Y. NSN sequence. Another one was that the hydrogen bond networks did not change before or after mutation [12]. The hydrophobicity of Y. NSN amino acid residues in the wild-type and the mutants is shown in Table 2. The hydrophobicity of the E138N and D264E mutants did not change after mutation. Most other mutants displayed an increase in hydrophobicity. This result was inconsistent with the thermostability results in the order E202A > I203F > D264E > remaining mutants (Fig. 3). Six pairs of salt bridges in Y. NSN, Asp166-Arg169, Lys209-Glu168, Glu189-Lys250, Lys270-Glu248, Arg63-Asp100 and Asp228-Arg190, are shown in Table 3. None of the mutant sites were included in the amino acid resides that formed salt bridges. These results indicated that salt bridges might be not a primary factor for the thermostability of the mutants.

In this paper, we tried to analyze the effect of different factors on the enzyme activity and thermostability of Y. NSN. Unfortunately, none of these factors explained the experimental results well. One reason might be that the amino acid residues that affected the enzyme activity of Y. NSN were not in the catalytic domain, and that the amino acid residues that affected the thermostability of Y. NSN were not related to hydrophobic interactions, disulfide bonds, salt bridges and hydrogen bonds.

Table 2
Hydrophobicity analysis of *Y. NSN* and the mutants by Protscale.

Mutants	Former	Later
I29T	0.367	−0.811
L30P	0.089	−1.089
V61I	−0.4	−0.367
D105A	−0.844	−0.256
E138N	−0.867	−0.867
Q167K	−2.1	−1.833
N170 T	−2.322	−2.056
P175Q	−0.244	−0.456
H191N	0.533	0.256
A193G	−0.178	−0.456
T200S	−0.267	0.122
E202A	−0.422	−0.033
I203F	−0.133	0.256
N243T	−0.344	−0.033
D264E	0.444	0.444

The former and the later represent the hydrophobicity of corresponding amino acid residue in Y. NSN and the mutants, respectively.

Table 3
Salt bridges of *Y. NSN* analyzed by Accelrys Discovery Studio.v2.5 with all pairs of oppositely charged atoms less than 4 A apart in a molecule window.

Salt bridges	Salt bridges	Salt bridges distances C_α(Å)
Asp166-Arg169	1	2.769
Lys209-Glu168	1	2.974
Glu189-Lys250	2	3.275,3.765
Lys270-Glu248	2	2.757,3.828
Arg63-Asp100	4	2.859,2.869,3.507,3.753
Asp228-Arg190	4	2.779,2.971,3.575,3.734

5. Conclusion

The I203F and D264E mutants significantly affected the enzyme activity of Y. NSN. In addition, E202A, I203F and D264E, influenced the thermostability of Y. NSN. In other words, Glu202, Ile203 and Asp264 in Y. NSN may be the key residues for its high enzyme activity and thermostability. The enzyme activity and thermostability of Y. NSN might be affected not only by a single factor but also by the entire environment. While Glu202, Ile203 and Asp264 were key residues for the enzyme activity and thermostability of Y. NSN, to further investigate the high enzyme activity and thermostability of Y. NSN, double and triple mutants need to be investigated.

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