Development of a robust nitrilase by fragment swapping and semi-rational design for efficient biosynthesis of pregabalin precursor

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Abstract
Protein engineering is a powerful tool for improving the properties of enzymes. However, large changes in enzyme properties are still challenging for traditional evolution strategies because they usually require multiple amino acid substitutions. In this study, a feasible evolution approach by a combination of fragment swapping and semi-rational design was developed for the engineering of nitrilase. A chimera BaNIT harboring 12 amino acid substitutions was obtained using nitrilase from Arabis alpine (AaNIT) and Brassica rapa (BrNIT) as parent enzymes, which exhibited higher enantioselectivity and activity toward isobutylsuccinonitrile for the biosynthesis of pregabalin precursor. The semi-rational design was executed on BaNIT to further generate variant BaNIT/L223Q/H263D/Q279E with the concurrent improvement of activity, enantioselectivity, and solubility. The robust nitrilase displayed a 5.4-fold increase in whole-cell activity and the enantiomeric ratio (E) increased from 180 to higher than 300. Molecular dynamics simulation and molecular docking demonstrated that the substitution of residues on the A and C surface contributed to the conformation alteration of nitrilase, leading to the simultaneous enhancement of enzyme properties. The results obtained not only successfully engineered the nitrilase with great industrial potential for the production of pregabalin precursor, but also provided a new perspective for the development of novel industrially important enzymes.

KEYWORDS
nitrilase, fragment swapping, semi-rational design, enantioselectivity, pregabalin precursor

1 | INTRODUCTION

Enzyme, as a “green” catalyst conducting chemical reactions, is widely used in the fields of fine chemicals and pharmaceutical drugs (Cheng et al., 2018). To date, many kinds of enzymes have been developed as important industrial biocatalysts with higher efficiency and eco-friendliness in comparison to chemical catalysts (Li, Zheng, Ma, & Zheng, 2014). However, these enzymes are just the tip of the iceberg in the natural enzyme library. The potential of the natural enzymes in the practical application has not been fully explored. The original reason lies in their insufficient catalytic performances in the biotransformation of natural or nonnatural substrates, such as poor activity, incompetent chemo-, regio- or enantioselectivity, and so on (Chen et al., 2018; Zheng et al., 2017). Moreover, the vast differences between the cellular environment and industrial setting also impede their successful applications. Tremendous efforts have been undertaken to address these issues, including enzyme immobilization, chemical modification and protein engineering (Valikhani, Bolivar,
Among these efficient strategies, numerous successful examples have been reported in the past few decades using protein engineering. Desired mutants are usually generated by directed evolution, semi-rational or rational design approach (Gong et al., 2019; Luo, Ma, Chang, Yu, & Shen, 2016; Wang et al., 2015). However, directed evolution is often too time-consuming to be implemented in process development because it requires construction and screening of large libraries that mostly contain variants with reduced or even no activity. Semi-rational and rational design methods are the preferred options to tailor efficient biocatalysts, whereas their applications usually call for exact structure information and thorough mechanistic understanding (Cheng et al., 2018). In addition, large changes in enzyme properties are always challenging since multiple amino-acid substitutions are required. A problem thus arises as to whether we could efficiently improve multiple properties of enzymes simultaneously without relying on the precise structure data.

A positive solution to this problem is referred to structure-guided recombination, which utilizes structural and evolutionary information to design highly mutated and, yet still natively folded, chimeric proteins and protein libraries (Chang et al., 2016; Otey et al., 2006; Zheng et al., 2019). By taking this approach, the creation of chimeric proteins with higher biological activity or catalytic properties was achieved (Kang et al., 2014; Pardo, Vicente, Mate, Alcalde, & Camarero, 2012), indicating its effectiveness in protein engineering with unknown crystalline structures. Moreover, the difference between recombining sequences of expected chimeras and those of the parent enzymes also provides important clues for sequence-function clarification (Smith et al., 2012). Thus, a combination of structure-guided recombination and semi-rational design appears to be an efficient option for protein engineering.

Nitrilases (EC 3.5.5.1) are a class of industrially important hydrolyses that convert nitriles to carboxylic acids and ammonia in a single step (Thuku, Weber,Barsani, & Sewell, 2007). Given the prominent regio-, chemo-, and stereo-selectivity in biotransformation, nitrilase-mediated bioprocess has become a highly attractive method for manufacturing value-added carboxylic acids (Xue et al., 2015). (S)-3-cyano-5-methylhexanoic acid ((S)-CMHA) is a key chiral intermediate of the blockbuster drug pregabalin (Xie et al., 2006). Although the nitrilase-catalyzed chemoenzymatic route for pregabalin from isobutylsuccinonitrile (IBSN) featured higher atom economy and lower cost, its application was restricted by either low activity or inferior enantioselectivity of the reported nitrilases (Xie et al., 2006; Zhang, Wu, Hao et al., 2019; Zhang, Wu, Liu et al., 2019). In addition, semi-rational and rational design of nitrilases were severely plagued by a deficiency of precise crystal structure information (Schreiner, Steinkellner, Rozzell, Glieder, & Winkler, 2010).

In our previous work, two nitrilases derived from Arabis alpina (AaNIT) and Brassica rapa (BrNIT) were obtained. Although they shared high identity (82.6%), their catalytic performances differed significantly in terms of enantioselectivity and activity. Concretely, AaNIT displayed the highest enantioselectivity ($E > 300$) ever reported (Zhang, Wu, Hao et al., 2019), whereas BrNIT exhibited higher activity but lower enantioselectivity ($E = 180$) than AaNIT (Zhang, Wu, Liu et al., 2019). However, neither of them could serve as a robust biocatalyst for the synthesis of chiral pregabalin precursors.

In the present study, using AaNIT and BrNIT as the parent enzymes, a robust chimera with enhanced activity and enantioselectivity was designed and developed by a combination of fragment swapping and semi-rational design. Overviews of the evolution route were illustrated in Figure 1. First, the parent nitrilases were divided into five fragments, and five chimeric nitrilases were generated by swapping a single fragment from AaNIT to BrNIT, respectively. Second, chimer 4 (designated as BrNIT) with higher activity and enantioselectivity towards IBSN was obtained and the role of fragment 4 containing 12 amino acid substituents was investigated. Meanwhile, reverse mutagenesis of the 12 amino acid substituents was performed to locate the functional residues. Third, the semi-rational design was implemented based on the results of reverse mutagenesis, resulting in a series of variants with even higher activities than BrNIT. Finally, a desired robust nitrilase, BrNIT/L223Q/H263D/Q279E, was developed with enhanced solubility, activity, and enantioselectivity. On the basis of the results of structural modeling, molecular dynamic (MD) simulation, and docking studies, the mechanism responsible for enhanced properties of the nitrilase was elucidated. Furthermore, the protein engineering strategy described herein provides a new perspective for the development of novel industrially important enzymes.

## 2 | MATERIALS AND METHODS

### 2.1 | Reagents and materials

Isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin were purchased from Sigma (China), (Trimethylsilyl)diazomethane (TMSD) solution in hexane (2.0 M) was bought from Macklin Biochemical Co., Ltd. (Shanghai, China). Racemic IBSN was synthesized according to previously described methods with modifications (Burns, Weaver, & Wong, 2010). (S)-CMHA was purchased from Hui Chem Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade as commercially available.

### 2.2 | Strains, plasmids, and culture conditions

Nitrilase genes from BrNIT (GenBank: ABM55734.1; Ishikawa et al., 2007) and AaNIT (GenBank: KFK44999; Zhang, Wu, Hao et al., 2019) were synthesized and inserted into plasmid pET28b (+) providing a C-terminal 6×His tag. The recombinant plasmids were transformed into Escherichia coli BL21 (DE3). The recombinant E. coli cells were cultivated at 37°C in Luria–Bertani (LB) medium (10 g/L of peptone, 5 g/L of yeast extract, and 10 g/L of NaCl) supplemented with kanamycin (50 μg/mL). When the optical density at 600 nm reached 0.6–0.8, IPTG was added with a final concentration of 0.1 mM to induce the expression of proteins. After incubation at 28°C for 10 hr, cells were harvested by centrifugation at 9,000g for 20 min under 4°C.
2.3 Construction of chimeric nitrilases

On the basis of the structural information and multiple sequence alignment analysis (Figure S1), the amino acid sequences of AaNIT and BrNIT were divided into five fragments: fragment 1 (1–85), fragment 2 (86–175), fragment 3 (176–220), fragment 4 (221–285), and fragment 5 (286–350; Figure 2b). Five chimeric nitrilases (chimera 1–5) were constructed by swapping a single fragment from AaNIT to BrNIT. The construction of chimeras was performed by means of seamless cloning (Bolchi, Ottonello, & Petrucco, 2005; Dodev et al., 2014). The linearized vectors and corresponding fragments were amplified by polymerase chain reaction (PCR) using the corresponding primers (Table S1). The PCR reactions for amplifying linearized vectors were set up in a total volume of 50 µl containing 0.4 µM each of the primers, 25 µl 2 × Phanta Max Buffer, 5 ng of pET28b (+)-BrNIT plasmid template, 0.2 mM dNTP Mix, 1 U Phanta Max Super-Fidelity DNA Polymerase and distilled water. PCR program used
was 3 min at 95°C followed by 30 cycles of 15 s at 95°C, 15 s at 60°C and 6 min at 72°C, and a final extension step with 5 min at 72°C. The PCR products of linearized vectors were purified using a DNA purification kit and then digested for 3 hr at 37°C using DpnI to digest the parent plasmid. Reactions for amplifying the corresponding fragments using pET28b (+)-AaNIT as a template was performed with the 2× TSINGKE Master Mix (green). Subsequently, the linearized vectors and the corresponding amplified fragments were ligated using ClonExpress® II One Step Cloning Kit following the manufacturer’s instructions. The mixture samples were transformed into the competent E. coli BL21 cells by heat shock and then plated onto an LB agar plate with kanamycin (50 μg/ml) at 37°C. The clones harboring chimeric nitrilase genes were confirmed by sequencing.

2.4 Reverse and site-directed mutagenesis

Twelve amino acid substitutions (Leu223, Leu227, Arg241, Pro249, Asp257, Asp258, Asn259, Glu261, Asp262, His263, Gln279, and Val280) in the chimeric nitrilase BrNIT were subjected for reverse mutagenesis. Among these residues, Leu223, Asn263, Gln279, and Val280 were further subjected to site-directed saturation mutagenesis. The primers used for mutagenesis were listed in Table S2. The mutations were introduced into BrNIT using Phanta Max Super-Fidelity DNA Polymerase by PCR. The PCR products were digested with DpnI and transformed into competent E. coli cells by heat shock. Transformants were plated on an LB agar medium containing kanamycin (50 μg/ml) at 37°C. The clones harboring chimeric nitrilase genes were confirmed by sequencing for the next site-directed mutagenesis. The primers for site-directed mutagenesis were also listed in Table S2. The positive clones were chosen for enzyme activity assay and identified by sequencing.

2.5 Enzyme activity assay and kinetic analysis

The whole-cell activity of nitrilase was assayed in a reaction mixture (10 ml) containing Tris-HCl buffer (50 mM, pH 8.0), 30 g/L IBSN, and an appropriate amount of resting cells harboring nitrilase and amidase, respectively. The reactions were carried out at 30°C with shaking at 200 rpm for 10 min. The addition of amidase ensured that IBSN was completely converted to (S)-CMHA without byproduct amide accumulation (Zhang, Wu, Hao et al., 2019). Purification of nitrilases was carried out as described in the Material S2. The activity of purified nitrilase was evaluated at 30°C in 1 ml mixture system containing Tris-HCl buffer (50 mM, pH 8.0), 20 mM IBSN, an appropriate amount of purified nitrilase and amidase. All assays were performed in triplicate. One unit of the enzyme activity was defined as the amount of enzyme that produced 1 μmol of CMHA per minute.

The kinetic parameters were determined with varying concentrations of IBSN (1–40 mM) in Tris-HCl buffer (50 mM, pH 8.0) at 30°C. The values of $K_m$ and $V_{max}$ were obtained by performing a nonlinear regression analysis of the Michaelis–Menten equation using OriginPlot 8.0, and the turnover number ($k_{cat}$) was calculated by using the equation $k_{cat} = V_{max}/[E]$, where [E] is the molarity of the enzymes.

The enantiomeric excess (ee) of IBSN and CMHA were determined by chiral GC analysis as described previously (Zhang, Wu, Hao et al., 2019). The conversion ($c$) and enantiomeric ratio ($E$) were calculated based on ee$_{e}$ and ee$_{p}$ (Rakels, Straathof, & Heijnen, 1993). The
concentration of CMHA was determined using HPLC equipped with a Welch Chrome® C18 column (250 mm x 4.6 mm x 5 μm). The mobile phase was composed of 76% buffer (containing 0.58 g/L NH₄H₂PO₄ and 1.83 g/L NaClO₄, pH 1.8) and 24% acetonitrile with a flow rate of 1 ml/min. The column temperature was 40°C and the detection wavelength was set as 215 nm.

2.6 | Computational methods

The three-dimensional (3D) structures of the parent nitrilases and variants were constructed via the automated protein modeling server SWISS-MODEL (http://swissmodel.expasy.org/; Arnold, Bork, Dudi, Kopp, & Schwede, 2006) on the basis of homology modeling with reference to the crystal structure of Synechocystis sp. PCC6803 nitrilase (Nit6803, PDB ID: 3WUY; Zhang et al., 2014), which shared the highest sequence identity to the parent nitrilases.

The MD simulations for the parent nitrilases and variants were performed using GROMACS version 4.0 and OPLS-AA force field. Each nitrilase was placed in the center of a cubic box consisting of SPC216 water molecules, with periodic boundary conditions. After neutralization the redundant charges, the energy of the system was minimized with the steepest descents method to obtain a reasonable geometrical structure. To maintain the simulated systems at a constant temperature and pressure, 100 ps NVT and 100 ps NPT ensembles were used to obtain the stable environment parameters. After completion of equilibration, 10 ns long MD simulations were performed with a step time of 2 fs at a constant temperature of 300 K and a constant pressure of 1 bar pressure, respectively. The dynamics trajectories were analyzed by using VMD software, and Grace 5.1.25.

Equilibrated conformers of the parent nitrilases and variants were extracted from their dynamics trajectories using VMD software and docked to substrate IBSN. The docking studies were performed by Autodock 4.0, and the docking results were visualized using PyMOL ver 1.7.

2.7 | Biocatalytic hydrolysis of IBSN

The regio- and enantioselective hydrolysis of IBSN was mediated by recombinant E. coli cells. The biotransformation was carried out in 10 ml reaction system containing 50 mM Tris-HCl buffer (pH 8.0), 100 g/L IBSN, 15 g/L (wet cell weight [wcw]) whole cells harboring nitrilases, and 5 g/L wcw whole cells harboring amidase Pa-Ami (Zhang, Wu, Hao et al., 2019). Reactions were performed at 30°C with shaking at 200 rpm for 10 hr. Samples (200 μl) were taken every 1 hr to track the reaction and analyzed by GC analysis.

3 | RESULTS AND DISCUSSION

3.1 | Design and construction of chimeric nitrilases

Efficient protein engineering of nitrilases is practically challenging due to inadequate structure information of the enzyme. The location of functional fragments or residues could notably facilitate the evolution of nitrilase under such circumstances. As we have reported, BrNIT and AoNIT shared high sequence identity of 82.6% (Figure 2a), however, they exhibited significant differences in activity and enantioselectivity. On the other hand, high sequence identity not only enabled straightforward recombination of genes, but also suggested the high probability of yielding functional chimeras (Trudeau, Smith, & Arnold, 2013), which illuminated us to reduce the selection range of hotspot residues by design and construction of chimeric nitrilases.

The sequences of the parent nitrilases were divided according to the structural and functional information of the nitrilase superfamily, which was characterized by a conserved catalytic triad of Glu-Lys-Cys and a homo-dimeric building block with a αββαβα super sandwich fold (Dent, Weber, Benedik, & Sewell, 2009; Sewell, Thuku, Zhang, & Benedik, 2010). Generally, the quaternary structure of active nitrilases was a spiral or helical fiber formed by oligomerization of the dimers (Park, Mulelu, Sewell, & Benedik, 2016; Sewell, Berman, Meyers, Jandhyala & Benedik, 2003).

The active protein of BrNIT and AoNIT consisted of six dimers (Figure S2). The structure models of monomer and dimer of the parent nitrilases were built using the crystal structure of nitrilase Nite6803 (PDB ID: 3WUY) as a template. The 3D models indicated that the residues participating in the A and C surface were distributed in the region 221–285 (Figure S3). A surface is the dimerization surface that plays an important role in the polymerization of two monomers. C surface is asymmetric interaction between two dimers, which plays an essential role in the formation of the spiral quaternary structure of nitrilases. It was reported that substitution of residues in the A and C surface could greatly alter the substrate specificity, thermostability, and activity of nitrilase (Sewell et al., 2010; Woodward, Trompetter, Sewell, & Piotrowski, 2018; Xu et al., 2018). The catalytic triads of AoNIT and BrNIT were located at positions 65 and 66 (Glu), 152 and 153 (Lys), and 186 and 187 (Cys), respectively. It was proposed that amino acid residues within 10 positions near the catalytic triads had a close relationship with the catalytic property of nitrilase (Sosedov & Stolz, 2015; Yu et al., 2019). Sequence alignment also revealed that residues 77–85, 167–175, and 207–220 of BrNIT were identical to those of AoNIT (Figure 2a), which could be used as truncation points of the fragment in chimera construction. Taken these factors into consideration, sequences of the parent nitrilases were divided into five fragments: 1–85, 86–175, and 176–220, 221–285, and 286–350 (Figure 2b). Five chimeric nitrilases (chimera 1–5) were then constructed by swapping a single fragment from AoNIT to BrNIT, respectively.

3.2 | Characterization of the chimeric nitrilases

The activity and enantioselectivity of the chimeric nitrilases towards IBSN were investigated. As listed in Table 1, the whole-cell activity of chimera 1 and chimera 4 reached 100.1 and 150.8 U/g wcw, respectively, which was 1.6- and 2.4-fold higher than that of the wild-type BrNIT (61.8 U/g wcw). Conversely, the activities of chimera 3 and chimera 5 decreased to 57% and 49% of that of the wild-type...
The values of the specific activities and enantioselectivities represent the means ± standard deviation for three independent experiments. The reactions were conducted using whole cells as biocatalysts. Abbreviations: AaNIT, nitrilase genes from Arabis alpina; BrNIT, nitrilase genes from Brassica rapa; IBSN, isobutylsuccinonitrile; wcw, wet cell weight. BrNIT, respectively, and the activity of chimera 2 was completely abolished. More excitingly, chimera 4 displayed a notable improvement in enantioselectivity (E > 300), which indicated that the fragment 4 (221–285) had a dramatic effect on both activity and enantioselectivity. When the E value of nitrilase was increased from 180 to higher than 300, the optical purity of the product would be enhanced from 97.5% to 99.0% accordingly at a substrate conversion of 45.0%, which satisfied the requirements of pregabalin synthesis. Thus, chimera 4 (designated as BaNIT) with simultaneous improvement of activity and enantioselectivity was selected for further investigation.

3.3 Reverse mutagenesis of chimera BaNIT

Natural molecular evolution is caused by changes in protein primary sequence that then can fold to form higher-order structures with altered functions (Currin, Swainston, Day, & Kell, 2015; Kaushik, Mohan, & Banerjee, 2012). Vice versa, if a mutant with the enhanced property was obtained, we can trace the path of evolution and further identify the critical residues (Harms & Thornton, 2013; Kirsch & Joly, 1998). A close look at chimera BaNIT revealed that there were 12 substitutions in the fragment 221–285 compared with the wild-type BrNIT.

To further identify the roles of the key residues, reverse mutagenesis of the 12 substitutions was subsequently carried out. As shown in Table 2, a decreased enantioselectivity was only observed for mutant L227I with E value dropping from >300 to 180. To investigate the role of position 227 in enantioselectivity, the mutation of I227L and L226I were introduced into BrNIT and AaNIT, respectively. As expected, the E value of BrNIT/I227L increased from 180 to higher than 300 compared with the wild-type, while that of AaNIT/L226I dropped from >300 to 190. These results strongly indicated that residue 227 is instrumental in determining enantioselectivity. Mutants L223M, N259D, H263E, Q279K, and V280I showed 1.2–1.4-fold increase in their activity compared to BaNIT. By contrast, four mutations, L227I, P249A, D257Y, and D262Q, exerted an inhibitory effect on their activity. The above results clearly indicated that the enhanced activity of chimera BaNIT was a synergistic mutational effect of 12 substitutions, whereas the improvement of enantioselectivity was ascribed to the functional substituent L227. Therefore, we deduced that the residues situated on the A and C surface exerted remarkable effects on both activity and enantioselectivity of nitrilase. In addition, the results of reverse mutagenesis indicated that five residues (L223, N259, H263, Q279, and V280) were potential “hotspots” for a mutation to improve activity.

3.4 Site-directed saturation mutagenesis of chimera BaNIT

To further improve the activity of BaNIT, site-directed saturation mutagenesis was subsequently performed on the five “hotspots.” The mutant libraries were screened by a high-throughput fluorometric assay based on the reaction between liberated NH3 and o-phthalaldehyde-2-mercaptoethanol (Banerjee, Sharma, & Banerjee, 2003). Three variants L223Q, H263D, and Q279E exhibiting improved activity and excellent enantioselectivity were obtained. Compared with the wild-type BrNIT and chimera BaNIT, they showed 3.0–3.5-fold and 1.2–1.5-fold improvement in activity (Table 3).

A combination of beneficial mutations is often used to improve enzyme performance because of possible synergistic and cooperative effects of the mutations (Fuji, Nakagawa, Hiratake, Sogabe, & Sakata,

| Nitrilase | Specific activity (U/g wcw) | Relative activity (%) | Enantioselectivity (E) |
|-----------|-----------------------------|-----------------------|------------------------|
| BrNIT     | 61.8 ± 2.2                  | 100                   | 180                    |
| AaNIT     | 49.4 ± 1.1                  | 80                    | >300                   |
| Chimera 1 | 100.1 ± 2.6                 | 162                   | 180                    |
| Chimera 2 | 0                           | 0                     |                         |
| Chimera 3 | 35.2 ± 0.7                  | 57                    | 180                    |
| Chimera 4 | 150.8 ± 1.6                 | 244                   | >300                   |
| Chimera 5 | 30.3 ± 3.1                  | 49                    | 180                    |

Note: The values of the specific activities and enantioselectivities represent the means ± standard deviation for three independent experiments. Abbreviations: BrNIT, nitrilase genes from Brassica rapa; IBSN, isobutylsuccinonitrile.
TABLE 3  Specific activities and enantioselectivities of chimera BoNIT and its variants towards IBSN

| Nitrilase              | Specific activity (U/g wcw) | Relative activity (%) | Enantioselectivity (E) |
|------------------------|----------------------------|-----------------------|------------------------|
| BrNIT                  | 61.8 ± 2.2                 | 100                   | 180                    |
| BoNIT                  | 150.8 ± 1.6                | 244                   | >300                   |
| BoNIT/L223Q            | 197.8 ± 1.9                | 320                   | >300                   |
| BoNIT/H263D            | 187.9 ± 1.3                | 304                   | >300                   |
| BoNIT/Q279E            | 216.3 ± 2.5                | 350                   | >300                   |
| BoNIT/L223Q/H263D      | 260.8 ± 0.9                | 422                   | >300                   |
| BoNIT/L223Q/Q279E      | 270.7 ± 2.1                | 438                   | >300                   |
| BoNIT/H263D/Q279E      | 268.8 ± 1.7                | 435                   | >300                   |
| BoNIT/L223Q/H263D/Q279E| 335.6 ± 3.6                | 543                   | >300                   |

Note: The values of the specific activities and enantioselectivities represent the means ± standard deviation for three independent experiments. The reactions were conducted using whole cells as biocatalysts.

Abbreviations: BrNIT, nitrilase genes from Brassica rapa; IBSN, isobutylsuccinonitrile; wcw, wet cell weight.

2005). Therefore, we attempted to further construct double and triple mutants of the chimera by combining mutations. As shown in Table 3, the double and triple mutants L223Q/H263D, L223Q/Q279E, H263D/Q279E, and L223Q/H263D/Q279E showed 1.7-, 1.8-, 1.8-, and 2.2-fold improvement in whole-cell activity compared with the chimera BoNIT. Notably, the mutant BoNIT/L223Q/H263D/Q279E harboring 12 substitutions compared to the wild-type BrNIT, displayed a 5.4-fold increase in whole-cell activity. These results demonstrated that, despite inadequate structural information, successful engineering of nitrilases with simultaneous improvement of activity and enantioselectivity was achieved using fragment swapping and semi-rational design, suggesting the high efficiency of our evolution approach for protein engineering.

To evaluate the potential of the variants in the industrial synthesis of (S)-CMHA, biotransformations were investigated using whole cells harboring the wild-type BrNIT and its variants as biocatalysts. Enantio- and regio-selective hydrolysis of IBSN by BrNIT/L223Q/H263D/Q279E as biocatalyst resulted in 48.1% conversion and high enantioselectivity (E > 300) within 10 hr, which was much better than that using the wild-type BrNIT (35.1% conversion and E = 180) and the chimera BoNIT (40.4% conversion and E > 300; Figure 3). Compared to other reported nitrilases (Xie et al., 2006; Zhang, Wu, Hao et al., 2019; Zhang, Wu, Liu et al., 2019), BoNIT/L223Q/H263D/Q279E displayed the highest activity and enantioselectivity towards IBSN. These results highlighted the robustness of the mutant BoNIT/L223Q/H263D/Q279E for the efficient manufacturing of pregabalin.

3.5 | Kinetics analysis of nitrilases

To better illustrate the increased activity of nitrilases from a biochemical perspective, the parent nitrilases and their variants were purified and the kinetic parameters were measured using IBSN as the substrate. As shown in Table 4, the $K_m$ and $k_{cat}/K_m$ values of BrNIT were 11.34 mM and 0.45 s$^{-1}$·mM$^{-1}$, which were higher than that of AoNIT (9.34 mM and 0.30 s$^{-1}$·mM$^{-1}$). Compared with the wild-type BrNIT, the $K_m$ values of the chimera 1 and 4 (BoNIT) decreased by 13% and 26%, respectively, whereas the $k_{cat}/K_m$ of the chimera 1 and chimera 4 increased by 96% and 113%, respectively. These results indicated that the chimera 1 and 4 exhibited higher catalytic efficiency and substrate affinity than the parent nitrilases and other chimeras.

Among the mutants constructed on the basis of BoNIT, BoNIT/L223Q/H263D/Q279E showed the highest catalytic efficiency with $k_{cat}/K_m$ value of 1.24 s$^{-1}$·mM$^{-1}$, which was only 2.8 and 1.3 times higher than that of BrNIT and BoNIT. However, BoNIT/L223Q/H263D/Q279E displayed a 5.4- and 2.2-fold increase in the whole-cell activity compared with the wild-type BrNIT and chimera BoNIT (Table 3). These results suggested that the improvement in activity of purified BoNIT/L223Q/H263D/Q279E was much lower than that of the whole cells. Since the total activity of the whole-cell biocatalysts can be considered as the product of the specific activity of the enzyme and the expression level of soluble protein, we attempted to investigate the change of solubility of nitrilases in the process of protein engineering.
Aiming to investigate the effect of evolution on the solubility of nitrilases, the soluble and insoluble portions of nitrilases expressed from *E. coli* were examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. As shown in Figure 4, the wild-type *Br* NIT and chimera *Ba* NIT were highly prone to misfolding and aggregation, leading to the majority of overexpressed nitrilase found in the insoluble fractions of the cell lysate. The mutants L223Q, H263D and Q279E derived from *Ba* NIT displayed slightly enhanced solubility. When the three mutations were further combined (*Ba* NIT/L223Q/H263D/Q279E), a significant amount of soluble protein was obtained. The number of soluble target proteins was quantified using Image Lab software (Bio-Rad) and calculated based on calibration with varying concentrations of protein standards (0.25–2 μg; BCA protein assay kit). The results showed that the amount of soluble protein for *Ba* NIT/L223Q/H263D/Q279E was 8.50 mg/g wcw, which was 3.3-fold higher than that of the wild-type *Br* NIT and chimera *Ba* NIT (2.56 mg/g wcw). Taken together, the mutant *Ba* NIT/L223Q/H263D/Q279E displayed both improved catalytic efficiency and solubility, leading to a significant increase in the total activity of the whole-cell biocatalyst.

Till now, engineering of nitrilases have been mainly focused on activity, enantioselectivity and thermostability (DeSantis et al., 2003; Xu et al., 2018; Xue et al., 2015; Yu et al., 2019), but few examples of engineering of solubility have been reported. On the other hand, direct evolution for highly soluble variants of target proteins often led to the deleterious compromise of activity (Pedelacq et al., 2002). These results demonstrated that we succeeded in the concurrent improvement of nitrilase enantioselectivity, activity and solubility for the first time.

### 3.6 Solubility of nitrilases

Aiming to investigate the effect of evolution on the solubility of nitrilases, the soluble and insoluble portions of nitrilases expressed from *E. coli* were examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. As shown in Figure 4, the wild-type *Br* NIT and chimera *Ba* NIT were highly prone to misfolding and aggregation, leading to the majority of overexpressed nitrilase found in the insoluble fractions of the cell lysate. The mutants L223Q, H263D and Q279E derived from *Ba* NIT displayed slightly enhanced solubility. When the three mutations were further combined (*Ba* NIT/L223Q/H263D/Q279E), a significant amount of soluble protein was obtained. The number of soluble target proteins was quantified using Image Lab software (Bio-Rad) and calculated based on calibration with varying concentrations of protein standards (0.25–2 μg; BCA protein assay kit). The results showed that the amount of soluble protein for *Ba* NIT/L223Q/H263D/Q279E was 8.50 mg/g wcw, which was 3.3-fold higher than that of the wild-type *Br* NIT and chimera *Ba* NIT (2.56 mg/g wcw). Taken together, the mutant *Ba* NIT/L223Q/H263D/Q279E displayed both improved catalytic efficiency and solubility, leading to a significant increase in the total activity of the whole-cell biocatalyst. Till now, engineering of nitrilases have been mainly focused on activity, enantioselectivity and thermostability (DeSantis et al., 2003; Xu et al., 2018; Xue et al., 2015; Yu et al., 2019), but few examples of engineering of solubility have been reported. On the other hand, direct evolution for highly soluble variants of target proteins often led to the deleterious compromise of activity (Pedelacq et al., 2002). These results demonstrated that we succeeded in the concurrent improvement of nitrilase enantioselectivity, activity and solubility for the first time.

### 3.7 Mechanism of enhanced enzyme properties

To probe the mechanism of the remarkably enhanced enantioselectivity, activity, and solubility of the variants, the 3D structure models of the wild-type *Br* NIT, the chimera *Ba* NIT, and *Ba* NIT/L223Q/H263D/Q279E were constructed using homology modeling and optimized after a 10 ns MDs simulation (Figure S4). Subsequently, the equilibrated conformers of them were extracted for detailed structural analysis. A comparison between the modeled structures revealed that the significant differences were located in the flexible loops (C surface) and the spiral region (A surface). As illustrated in Figure 5a, the helix (residues 217–228) on the A surface of *Ba* NIT was shifted by about 3.7 Å compared with that of the

![TABLE 4 Kinetic parameters of the parent nitrilases and mutants towards IBSN](image)

| Nitrilase     | $K_m$ (mM) | $V_{max}$ (μmol·mg$^{-1}$·min$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$·mM$^{-1}$) |
|--------------|------------|-------------------------------------|---------------------|----------------------------------|
| *Br* NIT     | 11.34 ± 0.12 | 7.77 ± 0.24                         | 5.14 ± 0.16         | 0.45 ± 0.01                      |
| *Aa* NIT     | 9.34 ± 0.33  | 4.32 ± 0.52                         | 2.81 ± 0.34         | 0.30 ± 0.02                      |
| Chimera 1    | 9.86 ± 0.23  | 13.12 ± 0.35                        | 8.68 ± 0.23         | 0.88 ± 0.07                      |
| Chimera 2    | 0           | 0                                   | 0                   | 0                                |
| Chimera 3    | 18.21 ± 0.62 | 11.32 ± 0.23                        | 7.49 ± 0.15         | 0.41 ± 0.01                      |
| Chimera 4 (Ba NIT) | 8.36 ± 0.20 | 12.13 ± 0.62                        | 8.03 ± 0.41         | 0.96 ± 0.03                      |
| Chimera 5    | 21.72 ± 0.11 | 10.85 ± 0.14                        | 7.18 ± 0.09         | 0.33 ± 0.01                      |
| *Ba* NIT/L223Q | 8.32 ± 0.25 | 12.55 ± 0.62                        | 8.31 ± 0.41         | 1.00 ± 0.02                      |
| *Ba* NIT/H263D | 8.96 ± 0.17 | 14.05 ± 0.30                        | 9.30 ± 0.20         | 1.03 ± 0.08                      |
| *Ba* NIT/Q279E | 8.04 ± 0.13 | 12.95 ± 0.16                        | 8.57 ± 0.11         | 1.07 ± 0.03                      |
| *Ba* NIT/L223Q/H263D | 9.03 ± 0.08 | 13.94 ± 0.48                        | 9.23 ± 0.32         | 1.02 ± 0.03                      |
| *Ba* NIT/L223Q/Q279E | 8.13 ± 0.45 | 14.46 ± 0.50                        | 9.57 ± 0.33         | 1.18 ± 0.02                      |
| *Ba* NIT/H263D/Q279E | 9.91 ± 0.55 | 12.81 ± 0.84                        | 8.48 ± 0.56         | 0.86 ± 0.01                      |
| *Ba* NIT/L223Q/H263D/Q279E | 10.70 ± 0.75 | 20.03 ± 0.69                        | 13.26 ± 0.47        | 1.24 ± 0.04                      |

Note: The values of the reported parameters represent the means ± standard deviation for three independent experiments. The reactions were conducted using purified nitrilases as biocatalysts. Abbreviations: *Br* NIT, nitrilase genes from *Brassica rapa*; IBSN, isobutylsuccinonitrile.

![FIGURE 4 The solubility levels of nitrilase proteins. (a) SDS-PAGE of soluble fraction and (b) SDS-PAGE of the insoluble fraction. *Br* NIT, nitrilase genes from *Brassica rapa*; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis](image) [Color figure can be viewed at wileyonlinelibrary.com]
wild-type. The flexible loops on the C surface of the wild-type BrNIT were replaced by a helix (residues 242–256) of BaNIT (as illustrated in Figure 5c). As the A and C surface play critical roles in the formation of the spiral quaternary structure of nitrilases (Thuku, Brady, Benedik, & Sewell, 2009), these changes may affect the conformation of higher-order spiral structure. What is more, a helix (residues 188–191) located near the catalytic residue Cys187 of the wild-type BrNIT disappeared in the chimera BaNIT (Figure S5). We speculated that the structure model of the nitrilase was reshaped by fragment swapping. The most prominent change has taken place in the entrance to the active site (Figure S5), and the original cave shape in the wild-type BrNIT was replaced by a hydrophobic cleft one, which facilitated the interaction between substrate and the active site.

The substrate (S)-IBSN was docked into the active center pocket of BrNIT and BaNIT using Autodock 4.0 program. According to the elucidated catalytic mechanism of nitrilase (Brenner, 2002), Cys187 acted as the nucleophile and was responsible for attacking the carbon atom of substrate cyano group by the sulfur atom of itself, Lys153 stabilized the tetrahedral intermediate by providing positive charge, and Glu66 was a general base which could increase the nucleophilicity of substrate cyano group and the sulfur atom of catalytic Cys187 played a crucial role in determining the activity of nitrilase. As shown in Figure 6, D_{C-S} of chimera BaNIT was shortened from 4.1 to 3.4 Å compared with the wild-type and the shorter distance made it easier to initiate a nucleophilic attack. This was consistent with the experimental observation of enhanced activity towards IBSN (Table 4). In addition, we noted that there were significant differences in hydrogen bonds formed by the catalytic triads of BrNIT and BaNIT. In the wild-type BrNIT, Lys153 and Glu189 formed two pairs of hydrogen bonds, and Cys187 and Asn190 formed one pair of hydrogen bonds. However, only Cys187 formed a hydrogen bond with Ala185, Pro210, and Trp218 in the chimera BaNIT. We conjectured that hydrogen bonds between Lys153 and residue Glu189 destabilized tetrahedral intermediate, and further hindered the hydrolysis of (S)-IBSN. Meanwhile, the hydrogen bond between Cys187 and Asn190 of the wild-type might pull the residue Cys187 away from IBSN. Whereas in the chimera BaNIT, the hydrogen bonds kept Cys187 in a steady-state from three directions. Therefore, these multiple interactions together made the binding pocket in a highly reactive conformation and led to a highly enhanced activity of the chimera.

Reverse mutagenesis suggested that only the mutation L227I lowered the enantioselectivity of chimera BaNIT. As illustrated in Figure S6, Leu227 was located on the helix structure forming A surface of nitrilase and far from the catalytic triad (>18 Å). Obviously, the residue Leu227 itself did not directly participate in the catalytic reaction, but it could alter the structure of A surface that participated in the spiral structure formation of nitrilase. We speculated that the A surface of spiral structure might be the tunnel effect on the C surface of the wild-type BrNIT, the hydrogen bonds kept Cys187 in a steady-state from three directions. Therefore, these multiple interactions together made the binding pocket in a highly reactive conformation and led to a highly enhanced activity of the chimera.

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exhibited high enantioselectivity (E > 300). However, a drastic decrease of activity was observed when it was substituted by residues with bulky and complex side chains at position 227, such as I227P, I227R, I227K, I227E, I227Y, and I227W (data not shown). Our results revealed that the enantioselectivity of nitrilase could be effectively tuned by modifying the residues on the A surface.

The mutant BoNIT/L223Q/H263D/Q279E exhibited the highest activity and soluble expression level. We reasoned that the enhanced solubility was probably caused by the change of hydrophobic interaction and electronegativity of residues located on the interface. Substitution of Lys by Glu at position 223 weakened the hydrophobic interaction between the two monomers and affected the dimerization of nitrilase. As active nitrilases consisted of six dimer-based oligomers, the change of dimerization caused the large-scale alterations of protein folding, which were conducive to improving the solubility of protein (Kim et al., 2015).

It was reported that the increase of electronegativity of protein could enhance solubility at alkaline conditions (Shilpashree, Arora, Chawla, Vakkalagadda, & Sharma, 2015). Substituents D263 and E279 increased the electronegativity of protein and further improved the electrostatic repulsion between proteins, resulting in an increase of solubility.

Moreover, the enantiomeric ratio (E) increased from 180 to higher than 300. These results demonstrated the high efficiency of our built evolution approach for the engineering of nitrilase. The structural analysis revealed that residues situated on the A and C surface could be prominent targets to enhance the enantioselectivity, activity, solubility of nitrilase, as their substitution contributes to the conformation alteration of higher-order structures. In conclusion, the feasible evolution approach constructed in this study has successfully engineered nitrilase with good catalytic performances, providing a sound example for the engineering of enzymes without precise crystal structure information.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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