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Enhancement of thermo-stability and product tolerance of *Pseudomonas putida* nitrile hydratase by fusing with self-assembling peptide

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Self-assembling amphipathic peptides (SAPs) are the peptides that can spontaneously assemble into ordered nanostructures. It has been reported that the attachment of SAPs to the N- or C-terminus of an enzyme can benefit the thermo-stability of the enzyme. Here, we discovered that the thermo-stability and product tolerance of nitrile hydratase (NHase) were enhanced by fusing with two of the SAPs (EAK16 and ELK16). When the ELK16 was fused to the N-terminus of β-subunit, the resultant NHase (SAP-NHase-2) became an active inclusion body; EAK16 fused NHase in the N-terminus of β-subunit (SAP-NHase-1) and ELK16 fused NHase in the C-terminus of β-subunit (SAP-NHase-10) did not affect NHase solubility. Compared with the deactivation of the wild-type NHase after 30 min incubation at 50°C, SAP-NHase-1, SAP-NHase-2 and SAP-NHase-10 retained 45%, 30% and 50% activity; after treatment in the buffer containing 10% acrylamide, the wild-type retained 30% activity, while SAP-NHase-1, SAP-NHase-2 and SAP-NHase-10 retained 52%, 42% and 55% activity. These SAP-NHases with enhanced thermo-stability and product tolerance would be helpful for further industrial applications of the NHase.

[**Key words:** Active inclusion body; Nitrile hydratase; Product tolerance; Self-assembling peptide; Thermo-stability]

Nitrile hydratase (NHase, EC 4.2.1.84) is composed of α- and β-subunits. The enzyme contains either a non-heme iron (Fe-NHase) or non-corrin cobalt ion (Co-NHase) in the active center and catalyzes the hydration of a nitrile to the corresponding amide, which is followed by several consecutive reactions: amide → acid → acyl-CoA, as catalyzed by amidase and acyl-CoA synthetase, respectively (1). The iron and cobalt act as the active centers for the production of acrylamide and nicotinamide at the industrial level. Both Fe-NHases and Co-NHases require activators for functional expression. The activators for Fe-NHases have been shown to act as metallochaperones (2), the activators for Co-NHases from *Rhodococcus rhodochrous* J1 and *Pseudomonas putida* NRRL-18668 have been found to act as self-subunit swapping chaperones (3–7). Although NHase has been widely applied for the industrial production of acrylamide and nicotinamide, most of the NHases are thermolabile (8) and exhibit low product tolerance as well (9).

Self-assembling peptides (SAPs) are a category of peptides that have specific sequences with alternating hydrophobic and/or hydrophilic residues and can spontaneously assemble into ordered nanostructures (10). It has been reported that the attachment of SAPs to the N- or C-terminus of an enzyme can benefit the thermo-stability of the enzyme through the formation of hydrogelation (11–14). SAPs can induce protein to form inclusion body in some cases, and some of these inclusion proteins are still biologically active (11–14). Among those of the SAPs, three of them most likely induce the protein to active inclusion bodies. They are the terminally attached SAPs with self-complementary amphipathic peptides, EAK16 (AEAEAKAKAEAEAKAK) and ELK16 (LELELKLKLELELKLK) (13,15), the helix–turn–helix peptide DWLKAFYDKVAEKLKEA, which can form α-helical fibrils (16), and the small surfactant-like peptides EWLKAFYEKVLEKLKELF and LLLLLLDK, which can drive soluble proteins into active aggregates (17). The terminally attached SAP derived from the sequence of Zuotin protein (a putative Z-DNA binding protein in *Saccharomyces cerevisiae*) is able to spontaneously form β-sheet structure with a proposed pattern (Fig. 1A) (11,13).

In order to achieve a wide range of applications of the NHase, an NHase with high thermo-stability and product tolerance is expected. In this study, we fused SAPs to the terminus of NHase (SAP-NHase), the thermo-stability and product tolerance of the SAP-NHases were improved. These SAP-NHases would be helpful for further industrial applications of the NHase.

MATERIALS AND METHODS

Vectors and strains NHase from *P. putida* NRRL-18668 was used as wild-type NHase for SAP fusion. Plasmid pET-*BAP* containing NHase and its activator genes (*BAP*) was used for NHase expression (6) and used as a template for different plasmids pET-*SAP-BAP* construction. *Escherichia coli* JM109 (Promega, WI, USA) was used as the host for cloning. *E. coli* BL21(DE3) (Promega) was used as the host strain for gene expression. All the *SAP* genes are synthesized by Sangon Biotech Ltd. (China) (Table S1).

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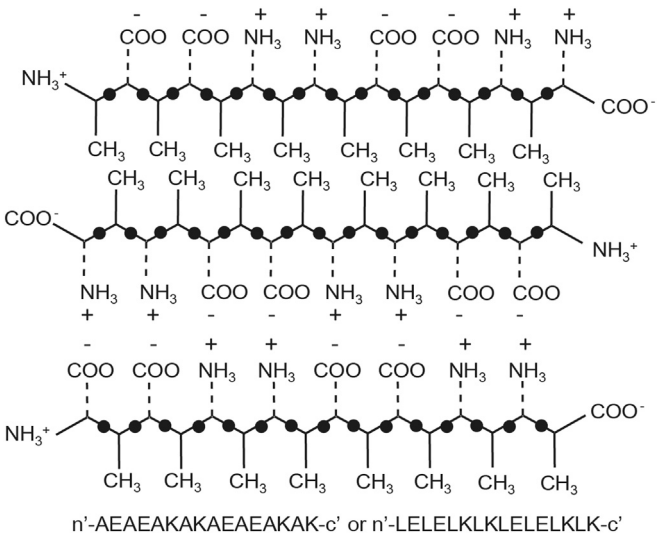

FIG. 1. Schematics for EAK16 or ELK16 aggregation. Antiparallel β-sheet is formed by hydrophobic bindings and salt bridges between self-complementary EAK16 or ELK16 peptides. Black circle represents the peptide bond. Dash line represents the methylenes of side chain of glutamate or lysine. The protons of residues are omitted.

Construction of plasmids The CloneEZ kit from GenScript (Piscataway, NJ, USA) was applied to rapidly construct the plasmids. This technique requires specific sequences to create 15 bp overhangs for recombination. Here, in order to create 15 bp overhangs, the template plasmid pET-*BAP* was abstracted from the pET-*BAP*-harbored *E. coli* cells and digested by the restriction enzyme *Nde* I. The overlapping 15 bp overhangs and an *Nde* I cleavage site sequence were designated in the end of primers for recombination (Table S2). For each plasmid pET-*SAP-BAP* construction, PCR was performed using the primer pairs (Table S2), the oligonucleotide sequence of peptide was used as the template (Table S1). The PCR product and digested linear plasmid pET-*BAP* were recombinated to generate the plasmid pET-*SAP-BAP* through the overlapping 15 bp oligonucleotide sequence by CloneEZ enzyme.

Expression and purification of enzymes *E. coli* BL21(DE3) transformants containing the recombinant plasmids were grown at 37°C in TB medium [1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4] containing $CoCl_26H_2O$ (0.05 g/l) and kanamycin (50 μg/ml) until the OD_{600} reached 0.8. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM, and then the cells were incubated at 24°C for 16 h.

All purification steps were performed at 4°C, and the procedures were conducted with an ÄKTA purifier (Pharmacia Biotech, Uppsala, Sweden). Potassium phosphate buffer (KPB) (10 mM, pH 7.5) containing 0.5 mM dithiothreitol (DTT) was used in the purification steps. NHase was purified with HisTrap HP column and Hiload 16/60 Superdex 200 pg column (Pharmacia Biotech) according to the protocols that described previously (7).

SAP-NHases supernatants and precipitations of cell lysates prepared from *E. coli* cells by sonication After incubation, the transformant cells were harvested and adjusted to OD_{600} of 6, and then lysated by sonication. All sonication steps were performed on ice. The cell lysates were sonicated until they became clear and transparent with 10 short burst of 10 s followed by intervals of 30 s for cooling. Supernatant and precipitation were separated by ultracentrifugation at 4°C for 10 min at 18,000 ×*g*.

Enzyme assay Activities of wild-type NHase and those fused NHases were assayed in a reaction mixture comprising 10 mM KPB (pH 7.5), 20 mM 3-cyanopyridine as a substrate and 0.1 μg enzyme in a total volume of 500 μl. The reaction mixture was incubated at 20°C for 20 min and terminated by addition of 500 μl of acetonitrile. The activity of NHase was determined by monitoring the formation of nicotinamide in the reaction mixture with high-pressure liquid chromatography (HPLC) (Hitachi, Tokyo, Japan) as previously described (3). One unit of NHase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of nicotinamide per min at 20/58°C.

RESULTS AND DISCUSSION

Effect of SAP fusion on NHase In order to improve the thermo-stability of NHase, nine SAPs were chosen to be fused to the N-terminus of β-subunit of NHase to construct fused gene of *SAP-BAP*. SAP and a linker are usually fused as a whole peptide to the target protein (11), here, peptide 1 was constructed by fusion of EAK16 and a PT linker (PTPPTTPTPPTTPTPTP) between the SAP and NHase; peptide 2 was constructed by fusing ELK16, a mutant of EAK16, to NHase and with the same PT linker between them (13) (Table 1). The sequences of other SAPs are shown in Table 1 (16,17). The transformant harboring each pET-*SAP-BAP* (Table 1) was used for SAP-NHase expression. The transformant harboring pET-*BAP* was used as control for wild-type NHase expression. After incubation, the transformant cells were lysated by sonication. The supernatant and precipitation of each *E. coli* cell lysates were obtained by centrifugation. Intriguingly, the precipitation of SAP-NHase-2-harbored cells had larger proportions than that of wild-type NHase (Fig. 2A). NHase activity of each supernatant was determined (Table 2). The activity in the supernatant of SAP-NHase-1 (109 U/mg) was comparable to that of wild-type (110 U/mg); although the activity in the supernatant of SAP-NHase-2 (13 U/mg) was much less than that of wild-type, SAP-NHase-2-harbored *E. coli* cells had larger proportions of precipitation. Therefore, the SAP-NHase-1 and SAP-NHase-2 were chosen for further study.

Fusion of ELK16 to N-terminus of NHase induced formation of active inclusion body The supernatants and precipitations from whole cell lysates of *E. coli* used for wild-type, SAP-NHase-1 and SAP-NHase-2 expression were analyzed by SDS-PAGE (the gene organization of SAP-NHase-1 and SAP-NHase-2 are shown in Fig. 2B, right panel). The molecular mass of each α- and β-subunits was corresponding to the calculation (α-subunit, 23.2 kDa; β-subunit, 24.1 kDa; EAK16-β-subunit, 27.3 kDa; ELK16-β-subunit, 27.7 kDa) (Fig. 2B). SDS-PAGE analysis showed that much more SAP-NHase-2 was in the precipitation, indicating that the fusion of ELK16 to N-terminus of the β-subunit of NHase resulted in formation of inclusion body. To further investigate whether fusion of ELK16 and EAK16 to the N-terminus of NHase affect the activity, the activities of SAP-NHase-2 in the precipitation and purified SAP-NHase-1 (Fig. 2C) were determined. As shown in Table 2, the activity of the purified SAP-NHase-1 was 426 U/mg, similar to that of wild-type; the activity of the SAP-NHase-2 in

TABLE 1. NHase fused SAPs in this study.

Peptide/corresponding NHase	Protein sequence
Peptide 1/SAP-NHase-1	AEAEAKAKAEAEAKAKPTPPTTPTPPTTPTPTP
Peptide 2/SAP-NHase-2	LELELKLKLELELKLKPTPPTTPTPPTTPTPTP
Peptide 3/SAP-NHase-3	DWLKAFYDKVAEKLKEAFKVEPLRADWLKAFYDKVAEKLKEAF
Peptide 4/SAP-NHase-4	DWLKAFYDKVAEKLKEAFGLLPVLEDWLKAFYDKVAEKLKEAF
Peptide 5/SAP-NHase-5	DWLKAFYDKVAEKLKEAFKVQPYLDDWLKAFYDKVAEKLKEAF
Peptide 6/SAP-NHase-6	DWLKAFYDKVAEKLKEAFNGGARLADWLKAFYDKVAEKLKEAF
Peptide 7/SAP-NHase-7	AEAEAKAKAEAEAKAKWISPTPPTTPTPPTTPTPTPAMD
Peptide 8/SAP-NHase-8	EWLKAFYEKVLEKLKELFPTPPTTPTPPTTPTPTP
Peptide 9/SAP-NHase-9	LLLLLLDKPTPPTTPTPPTTPTPTP
Peptide 10/SAP-NHase-10	PTPPTTPTPPTTPTPTPLELELKLKLELELKLK

The linker sequences are indicated by underlines.