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A novel molecular chaperone GroEL2 from *Rhodococcus ruber* and its fusion chimera with nitrile hydratase for co-enhanced activity and stability

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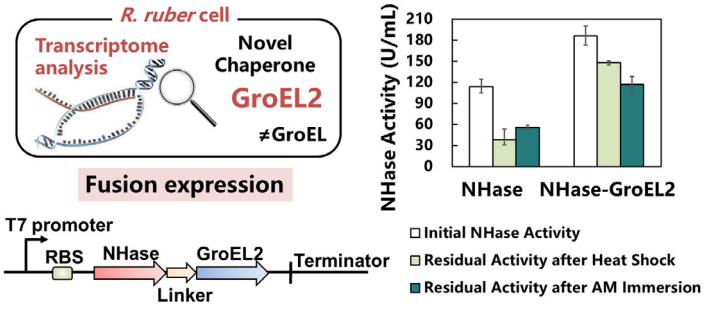
HIGHLIGHTS

- Novel *R. ruber* chaperones were identified by transcriptome analysis.
- *R. ruber* chaperone GroEL2 was distinguished from conventional GroEL.
- GroEL2 showed high self-thermal stability and protection effect on other proteins.
- NHase-GroEL2 fusion chimera enhanced the activity of NHase by 63.6%.
- It also enhanced thermal stability by 2.9× and acrylamide resistance by 1.1×.

GRAPHICAL ABSTRACT



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ABSTRACT

Rhodococcus ruber harboring intracellular nitrile hydratase (NHase) is widely used in large-scale acrylamide production. Transcriptome analyses of *R. ruber* under urea induction and heat shock revealed the novel chaperones GroEL2 and GroES. *M. jannaschii* chaperone rTHS, functional in organic solvent as reported in literature, was selected as control. In vitro experiments (70 °C/90 °C incubation) showed that GroEL2 from *R. ruber* was highly thermostable and can stabilize other proteins as well. GroEL2 was co-expressed with NHase in *E. coli* in three ways: (a) monocistronic expression with one T7 promoter, (b) bicistronic expression with double T7 promoters, and (c) fusion expression with one T7 promoter driving the NHase-GroEL2 chimera. Experimental results showed that the NHase-GroEL2 chimera was the most successful expression strategy. Maximal NHase activity was enhanced by 63.6% compared with the single NHase control. A stability assessment showed that the residual activity levels after heat shock and acrylamide (AM) immersion increased by 2.9× and 1.1×, respectively. For the fusion chimeras NHase-GroES and NHase-rTHS, their thermal stability also significantly enhanced by 1.6× and 64.0%, respectively; but their AM resistance both reduced (36.2% and 29.0%, respectively). In vivo heat inactivation curves further confirmed thermal stability enhancement of NHase by chaperone fusion, in which NHase-GroEL2 was the most stable one. Its inactivation constant k_d was only ~1/4 that of the single NHase. The K_{cat} of the intracellular NHase-GroEL2 was also remarkably higher than that of the NHase control.

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1. Introduction

Polyacrylamide (PAM) is synthesized from acrylamide monomer (AM). PAM is widely used in many fields such as enhanced oil recovery (EOR) (Samanta et al., 2013) and wastewater treatment (Walker and Kelley, 2003). Therefore, AM production is receiving increasing attention. Compared with traditional sulfuric acid hydration or Cu-catalyzed AM synthesis, the production of AM from the nitrile hydratase (NHase) biocatalysis of acrylonitrile is considered a promising alternative. It has significant advantages over other techniques, including high efficiency and the generation of highly pure product under relatively mild conditions (Martinez et al., 2014; Okamoto and Eltis, 2007; Prasad and Bhalla, 2010). NHase is an intracellular enzyme with α and β subunits. It is found in various bacterial species (Prasad and Bhalla, 2010). In the present study, we focused on *Rhodococcus ruber* because it has already been widely used as a stable industrial host with relatively high tolerance to salt (Yang et al., 2018) and organic solvents (Kosjel et al., 2003; Sun et al., 2016). Our previous study also confirmed its better acrylamide resistance compared with that of *E. coli* (Supplementary Fig. S1).

However, there are several serious problems with the NHase-bearing cellular catalyst. Previous studies have shown that NHase is inactivated by strong exothermic reactions and polar organic solvents, including the AM product. This inhibition effect is aggravated by increases in reaction temperature and/or AM product concentration (Gao et al., 2005). Therefore, it is imperative to improve the thermal stability and AM resistance of NHase. Moreover, it is preferable that NHase activity is co-enhanced. Researchers have attempted to realize these objectives by separating, domesticating, and mutating the catalytic bacterial strains used in AM synthesis (Liu et al., 2004; Yamada and Kobayashi, 1996). They also fused self-assembling peptides to the enzyme terminals (Liu et al., 2014) and used other approaches as well. In previous experiments, our group devised several ways to enhance the NHase-harboring cell catalyst. For instance, we mutated its σ^{70} global transcription factor (Ma and Yu, 2012), site-specifically mutated the NHase α-helix (Yu et al., 2006), and added a C-terminal salt bridge to the NHase (Chen et al., 2014; Chen et al., 2012). In most cases, however, increase in NHase stability was accompanied by a loss of NHase activity. Therefore, the research goal here is to co-enhance the activity and stability of NHase.

Chaperones are intracellular proteins which help nascent peptides fold properly, bind with unstable proteins to prevent aggregation, and help denatured proteins refold (Buchner, 1999; Bukau and Horwich, 1998; Cheetham and Caplan, 1998; Ellis, 1999; Georgopoulos and Welch, 1993; Hartl, 1996; Hartl et al., 2011). Chaperones are classified into several families according to their molecular weights (sHSP, HSP60, HSP90, and others). They have different functional focuses (Hartl, 1996). However, inter-family collaboration can occur (Hartl et al., 2011). To date, relatively few studies have tested chaperones on target NHases. Stevens *et al.* co-expressed the *E. coli* chaperones GroEL-GroES with NHase from *Comamonas testosteroni* in *E. coli* (Stevens et al., 2003). Song et al. attempted a similar strategy on NHase from *Rhodococcus erythropolis* in order to facilitate its active expression (Song et al., 2008). Tian et al. cloned GroEL-GroES into *Rhodococcus ruber* to improve cell catalyst performance (Tian et al., 2016). The chaperone rTHS from *Methanocaldococcus jannaschii* was reported to be highly stable (Bergeron et al., 2008) and could improve enzyme stability in organic co-solvents (Bergeron et al., 2009a, 2009b). However, few studies have focused on chaperones derived from *Rhodococcus ruber* or their application in enhancing NHase activity and stability.

Our aim was to identify novel chaperones from *R. ruber* to improve the performance of NHase. We analyzed transcriptome data for *R. ruber* under urea induction and heat shock conditions, and identified *R. ruber* chaperones GroES and GroEL2. We confirmed their thermal stability and enhancement in vitro, then selected chaperones to co-express with NHase to improve its activity and stability in *E. coli*.

2. Materials and methods

2.1. Genome and transcriptome analyses of R. ruber TH

R. ruber TH genome and transcriptome analyses (Ma et al., 2010) under urea-induction were performed as described previously (Sun et al., 2016). Briefly, *R. ruber* TH was fermented either with or without 10 g L^{-1} urea and 60 ppm Co^{2+}. Urea induced the overexpression of NHase and GroE chaperones (Komeda et al., 1996; Jiao et al., 2018) and Co^{2+} was the cofactor required for NHase activity (Komeda et al., 1996). The fermentation was performed at 28 °C (optimal NHase expression temperature) for 24 h. By that time, NHase expression had reached the midpoint. Cells were harvested for subsequent analyses.

The transcriptome of *R. ruber* TH under heat shock conditions was analyzed as follows: *R. ruber* TH cells were fermented with 10 g L^{-1} urea and 60 ppm Co^{2+} for 48 h until completion of the NHase fermentation. Cells were then harvested and cultured in flasks with shaking (200 rpm) at 50 °C for heat shock, 20 min. Then the cells were briefly centrifuged (12,000*g*, 5 min, 4 °C) for use in further testing.

The harvested bacterial sediment was frozen in liquid nitrogen for 10 min then sent out for transcriptome analysis. After discarding the redundancies at the 3′ and 5′ ends and the low-quality data (<20), the remaining transcriptome RNA data was compared with the reference genome by TopHat2 v. 2.0.11. The gene expression levels were calculated by Cufflinks v. 2.1.1 and expressed as fragments per kilobase per million mapped fragments (FPKM). The differentially expressed genes (DEGs) between two samples (28 °C/50 °C) were determined by Cuffdiff ($P < 0.001$). Gene sequencing and transcriptome analysis were performed by the Beijing Institute of Genomics at the Chinese Academy of Sciences, Beijing, China.

2.2. Plasmids, strains, and chemicals

All plasmids and strains used in this study are listed in Table 1. The genes of the *R. ruber* chaperones and NHase were cloned from laboratory samples of *R. ruber* TH. The chaperone rTHS gene was synthesized by Taihe (Beijing, China) according to the gene sequence reported in the literature (Bergeron et al., 2009a) and with the necessary codon optimization. The parent plasmid of the recombinants was pET28a (Merck KGaA, Darmstadt, Germany). The PCR primers used in this study were designed with Primer Premier v. 5.0 and synthesized by Taihe (Beijing, China), and are listed in Supplementary Table S1. *E. coli* TOP10 (Tiangen, Beijing, China) was used for cloning. *E. coli* BL21(DE3) (Tiangen, Beijing, China) served as a host to express various target proteins. Restriction endonucleases, T4 DNA ligase and isopropyl β-D-thiogalactopyranoside (IPTG) were obtained from TaKaRa Bio Inc., Kusatsu, Shiga, Japan. Lactose was purchased from BioDee Biotechnology Co. Ltd (Beijing, China).

2.3. Culture conditions of E. coli and the urea-induction in R. ruber TH

E. coli TOP10 and BL21(DE3) were grown in Luria-Bertani (LB) medium (tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; and NaCl, 10