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Soluble and functional expression of a recombinant enantioselective amidase from *Klebsiella oxytoca* KCTC 1686 in *Escherichia coli* and its biochemical characterization

Fa-Mou Guo, Jian-Ping Wu*, Li-Rong Yang*, Gang Xu

Institute of Biological Engineering, College of Chemical and Biological Engineering, Zhejiang University, 310027 Hangzhou, China

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ABSTRACT

A gene encoding an enantioselective amidase (KamH) was cloned from *Klebsiella oxytoca* KCTC 1686 and inserted into the *Eco*RI and *Hin*dIII sites of the vector pET-30a(+). When KamH with a peptide containing a His-tag and an enterokinase cleavage site was overexpressed in *Escherichia coli*, approximately half was found in the soluble fraction, but it lacked activity. After cleavage of the peptide by enterokinase, the enzyme activity was partly restored, reaching 420.2 ± 33.62 U/g dry cell weight (DCW). Another recombinant plasmid was constructed by inserting the KamH gene into the *Nde*I and *Eco*RI sites of pET-30a(+) to express KamH in its native form. The overexpressed amidase was found primarily in the soluble fraction and its maximum activity was 3613.4 ± 201.68 U/g DCW. This indicated that the peptide influenced not only soluble expression but also activity of KamH, perhaps by blocking the substrate-binding tunnel of KamH. Similar results were obtained with heterologously expressed amidases from *Rhodococcus erythropolis* MP50 and *Agrobacterium tumefaciens* d3. All of these amidases have an N-terminal α-helical domain. Therefore, amidases of this type may be functionally expressed in their native form. KamH hydrolyzed a range of aliphatic and aromatic amides and exhibited strict *S*-selectivity towards racemic amides.

1. Introduction

Amidases (EC 3.5.1.4), which catalyze the hydrolysis of amides to acid and ammonia, have been discovered in a variety of organisms, including bacteria, archaea, and fungi. A number of amidase-harboring microbes have been studied in detail, such as *Pseudomonas chlororaphis* [1], *Delftia tsuruhatensis* [2], *Agrobacterium tumefaciens* [3], *Klebsiella oxytoca* [4], *Brevibacterium epidermidis* [5], *Rhodococcus erythropolis* [6,7], *Sulfolobus tokodaii* [8], and *Pyrococcus yayanosii* CH1 [9]. Some amidases have very strict chemo-, regio-, and enantioselectivities, allowing them to be used to produce optically pure amides, carboxylic acids, and related derivatives. They have been used to produce (*S*)-2,2-dimethylcyclopropanecarboxamide [2,10–12], piperazine-2-carboxylic acid [13], phenylalanine [14], *S*-naproxen [15], and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide [16]. These chemicals and their derivatives have important applications in the fine chemical and pharmaceutical industries. Amidases have also been applied in the bioremediation of toxic chemicals [17,18].

Recombinant expression can increase the expression level of an exogenous gene, and *Escherichia coli* is the preferred recombinant expression host because of its high biomass-to-cost ratio. A number of amidase genes have been cloned and heterologously expressed. However, in most cases the expression efficiency has been low because of the formation of inactive or insoluble intracellular enzyme [2,15,19–23]. There have been numerous attempts to improve expression efficiency. First, different heterologous expression strategies have been investigated, such as using *Bacillus subtilis* as a heterologous expression host [23], expressing the amidase gene together with upstream and downstream flanking sequences [24], and using pET22b as the expression vector to add a six-histidine (6 × His)-tag to the C-terminal end of the protein [25]. Second, some studies have focused on the optimization of expression conditions, for example by using auto-induction media [25,26], adding ethanol to the culture medium [27], lowering the growth temperature to 29 °C, and cultivating cells without IPTG induction [28]. Although these strategies improved expression efficiency to a certain extent, they did not eliminate the problems of low expression level and formation of inclusion bodies. Suzuki and Ohta have reported the denaturation and refolding of an insoluble aggregated

* Corresponding authors at: Zhejiang University, Institute of Biological Engineering, College of Chemical and Biological Engineering, Hangzhou 310027, China. Tel.: +86 0571 87952363; fax: +86 0571 87952009.
E-mail addresses: wjp@zju.edu.cn (J.-P. Wu), lryang@zju.edu.cn (L.-R. Yang).

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6 × His-tagged fusion protein, but the steps were cumbersome and the yield was unsatisfactory [8].

In this study, we cloned an amidase gene from *K. oxytoca* KCTC 1686 [29] and studied how to achieve efficient expression in *E. coli*. First, the amidase (KamH) was expressed as a fusion protein with a 6 kDa peptide containing a 6 × His-tag and an enterokinase cleavage site at its N-terminus; approximately half of the recombinant protein was expressed in soluble form, but it had no amidase activity. Previous studies have indicated that extra amino acid residues at the terminus of overexpressed proteins can have a profound effect on the folding and solubility of these proteins [30–34]. Therefore, we used enterokinase to remove the extra amino acid residues from the fusion protein and found that the activity was partly restored. When KamH was expressed in its native form, almost no inclusion bodies were formed, and the recombinant protein had maximum activity. Similar results were achieved with heterologous expression of other amidases containing an N-terminus α-helical domain. Therefore, amidases of this type may be functionally expressed in their native form, and this strategy may be an alternative method for the efficient expression of amidases. Finally, characterization of KamH indicated that this enzyme has great application potential in the fine chemicals and pharmaceutical intermediates industries.

2. Materials and methods

2.1. Bacterial strains, plasmids, enzymes, and chemical reagents

The microbial strain *K. oxytoca* KCTC 1686 was procured from KCTC (Daejeon, Korea) and was routinely grown at 30 °C in lysogeny broth (LB) medium (1.0% [w/v] bactotryptone, 0.5% [w/v] yeast extract, 1.0% [w/v] NaCl, pH 7.2). *E. coli* strains DH5α and BL21(DE3) (Novagen, Madison, USA) were used as hosts for plasmid construction and recombinant amidase production, respectively. Plasmid pET-30a(+) (Novagen, Madison, USA) was used to construct recombinant vectors. The *E. coli* strains harboring recombinant vector were routinely cultured at 37 °C in LB liquid medium or on LB agar solid medium supplemented with kanamycin (50 μg/ml) as appropriate. Restriction enzymes (*Eco*RI, *Hin*dIII, *Nde*I) and T4 DNA ligase were purchased from Takara, Ltd. (Dalian, China). FastPfu DNA polymerase and protein markers were purchased from TransGen Biotech, Ltd. (Beijing, China). All other reagents were commercial products of analytical grade or higher and were purchased from standard suppliers.

2.2. Genomic DNA extraction and amidase gene subcloning

K. oxytoca KCTC 1686 was cultured in LB liquid medium at 30 °C and cells were harvested from the culture medium in the late log phase. Genomic DNA (GenBank accession No. CP003218.1) was extracted using a Genomic DNA Isolation Kit (Axygen, Hangzhou, China) according to the manufacturer's instructions.

Genomic DNA of *K. oxytoca* KCTC 1686 was used as template to amplify the amidase gene by polymerase chain reaction (PCR). Two sets of primers were designed to amplify the gene: primers A1 (forward) and A2 (reverse), with *Eco*RI and *Hin*dIII sites, respectively, and primers A3 (forward) and A4 (reverse), with *Nde*I and *Eco*RI sites, respectively. The primer sequences and restriction sites are listed in Table 1. The PCR program was carried out as follows: 1 cycle at 94 °C for 5 min; 30 cycles of 94 °C for 20 s, 57 °C for 20 s, and 72 °C for 90 s; and a final extension at 72 °C for 10 min. The PCR products were analyzed by 0.8% agarose gel electrophoresis. The products were named Ami1 and Ami2, respectively.

2.3. Plasmid construction

Ami1 was digested with *Eco*RI and *Hin*dIII, and Ami2 was digested with *Nde*I and *Eco*RI. Subsequently, the digested DNA fragments were inserted into the same restriction sites of the pET-30a(+) expression vector under the control of the T7 promoter. Ligations were performed according to the manufacturer's protocol (Novagen). The constructed recombinant plasmids were named pET-Ami1 and pET-Ami2.

2.4. Expression of KamH in E. coli

Transformation of *E. coli* BL21(DE3) with pET-Ami1 and pET-Ami2 was carried out essentially as recommended by Novagen. Transformed *E. coli* BL21(DE3) cells were cultivated in LB medium supplemented with 50 μg/ml kanamycin. Cells were incubated at 37 °C on a gyratory shaker until the OD_{600} reached 1.0. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cultures were incubated for 12 h at 18 °C. The cells were harvested by centrifugation (12,000 × *g* for 10 min), and cell suspensions in 50 mM Tris–HCl buffer (pH 8.0) were disrupted by sonication (Sonicator 400, Misonix, USA). The soluble and insoluble fractions were separated by centrifugation (12,000 × *g* for 20 min) at 4 °C and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12% polyacrylamide gels with a 4% stacking gel. Gels were stained with Coomassie Brilliant Blue.

2.5. Enterokinase cleavage of the extra peptide

The recombinant protein produced by *E. coli* BL21(DE3)/pET-Ami1 was purified by a combination of Ni-NTA affinity chromatography and Superose S10-300 gel filtration chromatography. The purified protein was dissolved in enterokinase cleavage buffer (50 mM Tris–HCl, pH 8.0, 2 mM $CaCl_2$, 0.1% Tween 80, 50 mM NaCl) to yield a protein solution with an apparent protein concentration of 6.0 mg/ml. A 2-ml aliquot of this enzyme solution was incubated with 3 U of enterokinase (Stratagene, Shanghai, China) at 35 °C for 20 h. Then, 100 μl of the enterokinase-digested protein was incubated with 60 μl of STI-agarose (Stratagene, Shanghai, China) and 1 ml of His-tag affinity resin at 4 °C for 1 h. The His-tag and enterokinase were removed by low-speed centrifugation (1000 × *g* for 10 min) and the supernatant was used to test enzyme activity.

2.6. Functional expression of other amidases

The microbial strains *R. erythropolis* MP50 (DSM 9675) and *A. tumefaciens* d3 (DSM 9674) were procured from DSMZ (Braunschweig, Germany) and were routinely grown in LB medium. Extraction of genomic DNA was performed as described for KamH. Amidase nucleotide sequence data from *R. erythropolis* MP50 (MamH) and *A. tumefaciens* d3 (DamH) appear in the GenBank nucleotide sequence database under the number AY026386 and AF315580. The primer sequences (A5–A12) used to amplify the amidase genes from these two strains and the restriction sites are listed in Table 1. The PCR, recombinant plasmid construction, transformation, and expression of recombinant amidases were carried out as described for KamH.

2.7. Amidase activity assays and determination of protein concentration

Amidase activity was determined in reaction mixtures (1.0 ml) consisting of Tris–HCl buffer (50 mM, pH 8.0), 10 mM benzamide, and an appropriate amount of enzyme. After a 5-min preincubation at 35 °C with shaking, the reaction was started by addition