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The study on D-aminoacylase gene synthesis via chemical and enzymatic combined method

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According to the amino acid sequence, the codons of D-aminoacylase gene from Alcaligenes A-6 were superseded by the most abundant codon in *E. coli*. The method is performed to avoid the rare codons and the lower abund+ance which can affect the expression of heterologous proteins. The total length of the synthesized gene is 1479 bp. 52 oligonucleotides were designed and synthesized by the phosphoramidite four-step chemical method. A three-stage assembling method was applied to assemble the whole DNA fragment. The reduction of the template complexity in the reaction system had a significant effect on reducing the number of byproducts to quickly obtain the synthetic gene. The synthesized DNA was connected to pET32a, and then put into *E.coli* BL21 (DE3) for expression. After induction by IPTG, the codon optimized D-aminoacylase gene can be expressed more efficiently in *E. coli* BL21 (DE3), accounting for 78% of total bacterial protein compared to 56% of codon unoptimized gene. The N-acetyl-D-valine amidohydrolase activity was assayed by measuring the D-valine formed using the fermentation bacterial cell to catalyze the N-acetyl-D,L-valine, the fermentation activity of strain *E. coli* BL21 (DE3) harboring pET-dan, which reaches 98 U/ml.

Keywords D-aminoacylase; Alcaligenes A-6; Gene synthesis; Expression.

INTRODUCTION

D-amino acids are involved in the synthesis of antibiotics, pesticides, and other products including food and agrochemical industries [1]. D-amino acids occur naturally in bacterial cell walls, some microbial metabolites including peptide toxins and antibiotics, plants, peptides from frogs and molluscs, several insects, earthworms and other animals [2]. But the D-enantiomers of amino acids are far less abundant in nature than the corresponding L-enantiomers, which are the predominant form occurring in biological molecules [3]. Since fermentation is not an efficient method of producing D-amino acids, the D-amino acids are produced in two ways, by direct organic asymmetric synthesis and by enzymatic resolution of racemates. In recent years, much attention has been paid to using N-acyl-D-amino acid amidohydrolase for the production of D-amino acids [4]. N-acyl-D-amino acid amidohydrolase (3.5.1.81) catalyzes the hydrolysis of N-acyl derivatives of various D-amino acids to D-amino acids and fatty acids, and is used for the optical resolution of DL-amino acids [5, 6]. N-acyl-D-amino acid amidohydrolase (D-aminoacylase) that acts on N-acyl derivatives of various neutral D-amino acids has been reported from *Alcaligenes*, *Pseudomonas*, *Streptomyces*, *Sebekia*, *Variovorax*. D-aminoacylase from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6 (*Alcaligenes* A-6) has been produced and sold by Amano Enzyme Co. as a commercial enzyme (D-aminoacylase "Amano") for the manufacture of neutral D-amino acids [6]. But the D-aminoacylase gene from *Alcaligenes* A-6 using to construct engineering bacteria *E. coli*, has some rare codons of host bacteria, which can affect the expression of heterologous proteins. Therefore, improving the expression of D-aminoacylase is a main goal which needs to be addressed.

The original purpose of this study was to synthesize an *Escherichia coli* codon optimized D-aminoacylase gene. After induction by IPTG, the codon optimized D-aminoacylase gene can be expressed more efficiently in *E. coli* BL21 (DE3), accounting for 78% of total bacterial protein and contrasted with 56% of codon unoptimized gene. The N-acetyl-D-valine amidohydrolase activity was assayed by measuring the D-valine formed

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using the fermentation bacterial cell to catalyze the N-acetyl-D,L-valine, the fermentation activity of strain *E. coli* BL21 (DE3) harboring pET-dan which can reach 98U/ml.

MATERIALS AND METHODS

Materials

E.coli BL21(DE3)、*E.coli* JM109、pUC18、pET-32a were obtained from ATCC. *Eco*R I, *Xba* I, *Bam*H I, *Bgl* II, *Xho* I, dNTP, T4 DNA ligase, *pfu* DNA polymerase, DNA marker were purchased from Fermentas, USA.

Gene and Oligonucleotide Design

The synthesis of D-aminoacylase gene was designed according to the literature [3,6] and retrieved database [7] information.

Chemical synthesis of oligonucleotides

The phosphoramidite four-step method was applied for chemical synthesis of oligonucleotides, which uses the reaction of acid-activated deoxynucleoside phosphoramidites with solid-phase tethered deoxynucleoside. The oligonucleotides were synthesized using the Biolytic Oligo Synthesizers Dr. Oligo-192 by BGI-Shenzhen at the 20 nmol scale, and PAGE method was used for purification.

Enzymatic assembly of oligonucleotides

In each DA-PCR reaction, a mixed solution of 5 μL was used in 50 μL of reaction solution; thus, the final concentration of each inner oligonucleotide in the PCR reaction mixture was 40 nmol/L, and the outer oligonucleotide was 0.2 μmol/L. DA-PCR reaction was carried out in 25 cycles of denaturing at 94℃ for 30 s, annealing at 53℃ for 30 s, and extension at 72℃ for 90 s. In each OE-PCR reaction, the final concentration of the template block or fragment in the PCR reaction mixture was 40 nmol/L The two outermost oligonucleotides as amplification primers, were used at a final concentration of 0.2 μmol/L. OE-PCR reaction was carried out in 25 cycles of denaturing at 94℃ for 1 min, annealing at 58℃ for 1 min, and extension at 72℃ for 3 min [7-9]. All PCR reactions were performed on a PTC-200. PCR products were purified using an agarose gel extraction kit.

Cloning and sequencing

The whole DNA fragment purified by agarose gel electrophoresis was connected into pUC18, constructed the pUC-*dan* recombinant plasmids and then transformed into *E.coli* JM109. After identification, the positive clones were sequenced by Sangon Biotech (Shanghai) Co., Ltd. Using other synthesized amplification primers with *Nde* I and *Bgl* II restriction sites, the D-aminoacylase gene was amplified from pUC-*dan*. Amplification fragments were purified and cloned into *Nde* I and *Bam*H I sites of pET32a. After identification, the plasmids pET-*dan* were put into *E.coli* BL21 (DE3) for enzyme expression.

Induced expression and activity assay

Strain of *E. coli* BL21 (DE3) harboring pET-*dan*, was inoculated into 4 ml LB medium containing 100 mg ampicillin/L (LB/Amp) and were incubated at 37℃ for 12 h. The inoculum (1%, v/v) was added to 100 ml LB/Amp and cultured at 37℃ to an OD_{600} of 0.6, after which protein production was induced by adding 0.1 mM IPTG and incubating at 37 ℃ for 3 h.

N-acyl-D-amino acid amidohydrolase activity was assayed by measuring D-amino acids formed from the hydrolysis of N-acetyl-D-amino acids as previously described [3-4]. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol D-amino acid per min.

RESULTS AND DISCUSSION

Gene and oligonucleotide design

D-aminoacylase gene (*dan*) from *Alcaligenes* A-6 has been reported by M. Moriguchi in 1993 [10]. The whole sequence can be found in GenBank using accession number D45918, containing 1455 nucleotides, which can be encoded as 484 amino acids.

To synthesize an *Escherichia coli* codon optimized D-aminoacylase gene, the codons were chosen to have either 1/3 or 2/3 GC content to ensure that the resulting GC content of the optimized DNA was between 40% and 60%. The codons encoding each amino acid were superseded by the most abundant codon in *Escherichia coli*[11] to avoid the rare codons which can affect the expression of heterologous proteins [12].

Adding the *Eco*R I and *Xba* I restriction endonuclease sites in the 5’ and 3’ end, the whole DNA fragment was 1479 bp in length. The design of the synthetically assembled oligonucleotides was that the length of each oligonucleotide was 50 bases and the overlapping region was 18 bases, therefore, 14 nt gaps between overlapping regions, 52 oligonucleotides were designed using Genedesign.

Oligonucleotide assembly

Chemically synthesized oligonucleotides were assembled by DNA polymerase based on polymerase chain reaction. *Taq* polymerase lacks 3′-5′ exonuclease activity and is known to be error-prone, and therefore, the *pfu* polymerase is used in this assembly process. Using a three-stage assembling method the 52 oligonucleotides were assembled into a full-length gene. The first stage used DA-PCR, and the second and the third stage used OE-PCR. The schematic diagram is shown in Fig.1.

In the first stage, six single-stranded oligonucleotides as a group were assembled into an intermediate fragment forming a block. The 1-48 oligonucleotides were assembled into block 1-8, the last four oligonucleotides 49-52 were assembled into block 9. In the second stage, three double-stranded block DNA as a group were assembled into a longer intermediate fragment forming a fragment. The 1-9 blocks were assembled into fragment 1-3. In the third stage, three double-stranded DNA fragments were assembled into the whole D-aminoacylase gene (Fig.2).

Cloning and sequencing

The whole DNA fragment purified by agarose gel electrophoresis was connected into pUC18, constructed the pUC-*dan* recombinant plasmids and then was transformed into *E.coli* JM109. After identification, five positive clones were sequenced by Sangon Biotech (Shanghai) Co., Ltd. The sequencing results showed that two of them were confirmed to be the correct products which have the accurate sequence with the design. Unfortunately, one of the five positive clones has the major bands error after the final amplication step appeared to be the correct size. There were total 5 base errors in the sequence of two clones which have no major bands error. It contained two base deletions, one single base insertion and two single point mutations. The average error rate determined by sequencing was approximately 0.68‰.

Induced expression

The strain of *E. coli* BL21 (DE3) harboring pET-*dan*, and the strain of *E. coli* BL21 (DE3) harboring pET-*dan*-N (D-aminoacylase gene amplified from *Alcaligenes* A-6, codons not optimized) were induced by 1 mM IPTG in LB/Amp medium and incubated at 37℃. The expressed codon optimized and unoptimized D-aminoacylase genes were detected by 10% SDS-PAGE (Fig.3).

The codon unoptimized D-aminoacylase gene efficiently expressed in *E. coli* BL21 (DE3), accounted for 56% of total bacterial protein. Correspondingly, the codon optimized D-aminoacylase gene synthesized in this paper, expressed more efficiently in *E. coli* BL21 (DE3), accounted for 78% of total bacterial protein (Tab. 1). Another better way is to directly express the D-aminoacylase gene in *E. coli* Rosetta 2 which harbors pRARE2 providing all the rare codon tRNAs for *E. coli*. The codon unoptimized D-aminoacylase gene can be efficiently expressed in *E. coli* Rosetta 2, accounting for 64% of total bacterial protein (Tab. 1). This shows that rare codon and codon abundance in the host affect the exogenous gene expression.

Activity assay

D-aminoacylase from *Alcaligenes* A-6 acts preferentially on N-acyl derivatives of d-Met, d-Phe, and d-Leu, and less effectively on those of d-Trp, d-Ala, and d-Val. We are using N-acetyl-D, L-valine as the substrate to assess the recombinant enzyme catalytic activity of the fermenting bacterial cell. The N-acetyl-D-valine amidohydrolase activity was assayed by measuring D-valine formed from the hydrolysis of N-acetyl-D-valine. The fermentation activity of the strain of *E. coli* BL21 (DE3) harboring pET-*dan* can reach 98 U/ml, higher than the wild type strain *Alcaligenes* A-6 (Tab. 2). The codon unoptimized D-aminoacylase gene can be expressed efficiently in *E. coli* Rosetta 2, accounting for 83 U/ml (Tab. 2).

Table 1. The expression of D-ANase synthetic D-aminoacylase gene in *E. coli* BL21 (DE3).

Number (% of)	1	2	3	4	5	Average value
Codon unoptimized	59	51	54	58	58	56
Codon optimized	63	68	65	61	63	64
Rosetta Express	78	84	72	76	80	78

Table 2. Fermentation enzyme activity of *D*-ANase.

Number (U/ml)	1	2	3	4	5	Average value
Codon unoptimized	68	59	66	74	78	70
Codon optimized	95	101	102	97	95	98
Rosetta Express	80	82	84	86	83	83

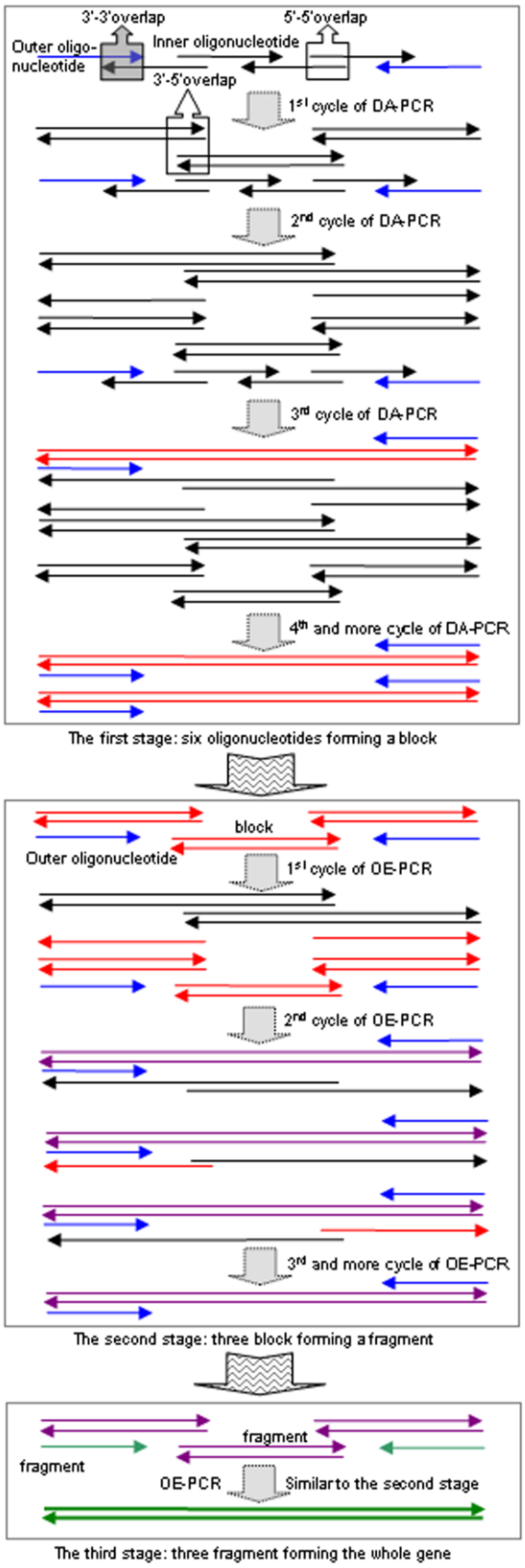

Fig. 1. Schematic diagram of the three-stage assembling method.

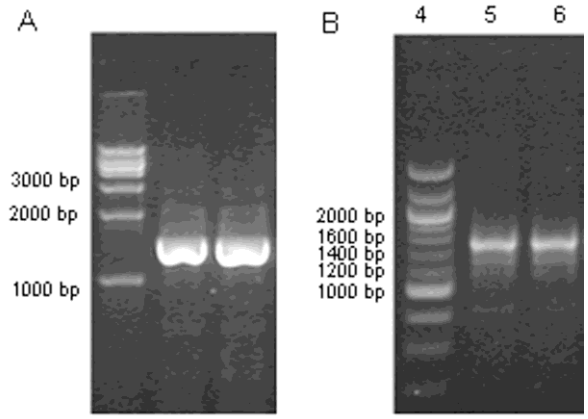

Fig. 2. Oligonucleotide assembly results. (A) three-stage assembling method. Lane 1: 100bp DNA marker, lane 2-3: synthesized D-aminoacylase gene; (B) two-stage assembling method. Lane 4:1000bp DNA marker, lane 5-6: synthesized D-aminoacylase gene.

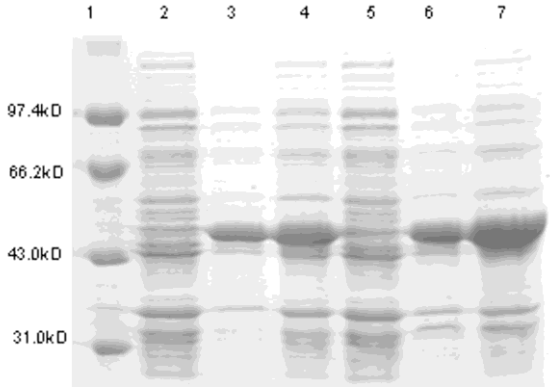

Fig. 3. SDS-PAGE analysis of the codon optimized and unoptimized D-aminoacylase gene expression. Lane 1: protein marker; lane 2-4: pET-dan-N induced by IPTG for 0, 1.5 and 3 h; lane 5-7: pET-dan induced by IPTG for 0, 1.5 and 3h.

D-aminoacylase chromatographic purification process

The 100 ml induced expression of *E.coli* BL21/pET-dan fermentation broth was centrifuged at 4000 rpm and 4°C for 10 min. After collecting the precipitated bacteria and weighing (wet weight), 20 mmol / L PBS buffer (pH 5.0) was added according to the proportion of 1g:10 mL. After mixing in an ice bath for ultrasound breaking (breaking conditions: output power 300W, breaking time 5 s, interval of 5 s times, 200 times), the supernatant was collected at 10000 rpm and 4°C for 10 min. After the supernatant was filtered through a 0.45 μm filter, the buffer solution was used to replace the buffer solution in the 8000 Da. Non fusion expression of D-ANase was purified by DEAE-FF anion exchange chromatography, CM-52 cation exchange chromatography and

Table 3. Separation and purification of non-fusion *D*-ANase.

Purification steps	Protein content (mg)	Total activity (U)	Specific activity (U/mg)	Recovery rate (%)	Total yield (%)	Purification rate
1. Before breaking	1564.3	6897.5	4.41			
2. Crude enzyme liquid	946.7	8254.3	8.72	120	120	
3.DEAE-FF	216.8	2137.8	9.86	26	31	1.13
4. CM-52	63.2	1769.1	27.99	83	26	2.84
6. Superdex-200	1.3	1489.6	1145.85	84	22	40.93

superdex200 AKTA-FPLC gel filtration chromatography. D-ANase was purified through multiple steps of chromatography, the final product recovery rate was 84% and the specific activity for 1145.85 (U/mg), purification rate was 40.93 times. The results of the chromatographic purification and detection of the various stages are shown in Table 3. The purity of D-ANase protein obtained by SDS-PAGE was higher, and the molecular weight of the protein was about 52 kDa (Fig.3).

CONCLUSIONS

The three-stage assembling method used in this paper includes a phase DA-PCR and two-stage OE-PCR [13]. Irrespectively of the stage, the heating step causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules. Then the reaction solution became a complex system of single-stranded DNA molecules, including the chemically synthesized oligonucleotides, the longer single-stranded fragments being assembled by DNA polymerase. The cooling step causes the primers or the overlapping segments containing sequences complementary to the target region by the stable DNA-DNA hydrogen bonds to form a local double-stranded DNA molecule. The annealing method yielded 3’-3’overlap, 3’-5’overlap and 5’-5’overlap. The DNA polymerase bound to the 3’-3’overlap and 3’-5’overlap double-stranded, synthesized a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The oligonucleotides alternate between sense and antisense directions, and the overlapping segments determine the order of the PCR fragments, thereby selectively producing the final long DNA product.

Considering the length of overlapping region of only 18 bases in average, the annealing of long single-stranded DNA molecules may easily produce a mismatch, so that assembling into full-length genes containing too many byproduct bands can be connected into a plasmid. Using the three-stage assembling method, the nine-block assembly by OE-PCR splits into two phases, reducing the complexity of the template in the reaction system, yielding single product bands, and reducing the workload of screening, by quickly producing the synthetic genes.

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СИНТЕЗА НА ГЕН ЗА D-АМИНОАЦИЛАЗА ПО ХИМИЧЕН И КОМБИНИРАН ЕНЗИМЕН МЕТОД

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(Резюме)

Според секвенцията на аминокиселините кодонът на гена за D-аминоацилаза в в щама *Alcaligenes* A-6 се замества от по-разпространения кодон в *E. coli*. Представеният метод позволява да се избегнат редки кодони и слабото разпространение, които могат да повлияят на експресията на хетероложни протеини. Общата дължина на синтезирания ген е 1479 bp. Петдесет и два олигонуклеотида са конструирани и синтезирани чрез четири-степенен химичен метод с фосфорамидит. Три-степенен метод е проложен за асемблирането на целия ДНК-фрагмент. Намаляването на сложността на темплейта в реакционната система има значителен ефект за намаляването броя на страничните продукти за бързото получаване на синтетичния ген.

Синтезираната ДНК е свързана с pET32a, след което е вкарана в *E.coli* BL21 (DE3) за експресия. След индукция с IPTG кодон-оптимизираният ген за D-аминоацилаза може да бъде експресиран по-ефективно в *E.coli* BL21 (DE3) с 78% общ бактериален протеин, при 56% при кодон-неоптимизиран ген. Определена е активността на N-ацетил-D-валин амидохидролазата чрез измерване на D-валина, образуван при хидролизата на N-acetyl-D,L-valine от щама *E. coli* BL21 (DE3) harboring pET-dan. Концентрацията на D-валина достига 98 U/ml.