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ORIGINAL PAPER

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Molecular chaperones facilitate the soluble expression of *N*-acyl-D-amino acid amidohydrolases in *Escherichia coli*

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Abstract The overproduction of D-aminoacylase (D-ANase, 233.8 U/mg), *N*-acyl-D-glutamate amidohydrolase (D-AGase, 38.1 U/mg) or *N*-acyl-D-aspartate amidohydrolase (D-AAase, 6.2 U/mg) in *Escherichia coli* is accompanied by aggregation of the overproduced protein. To facilitate the expression of active enzymes, the molecular chaperones GroEL-GroES (GroELS), DnaK-DnaJ-GrpE (DnaKJE), trigger factor (TF), GroELS and DnaKJE or GroELS and TF were coexpressed with the enzymes. D-ANase (313.3 U/mg) and D-AGase (95.8 U/mg) were overproduced in an active form at levels 1.3- and 1.8-fold higher, respectively, upon co-expression of GroELS and TF. An *E. coli* strain expressing the D-AAase gene simultaneously with the TF gene exhibited a 4.3-fold enhancement in D-AAase activity (32.0 U/mg) compared with control *E. coli* expressing the D-AAase gene alone.

Keywords *N*-Acyl-D-amino acid amidohydrolase · Chaperone · Inclusion body · Soluble expression · *Escherichia coli*

Introduction

N-Acyl-D-amino acid amidohydrolase 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 [31]. The activity is classified into three types based on its substrate specificity: D-ANase acts on *N*-acyl derivatives of various neutral D-amino acids, D-AGase is specific for *N*-acyl-D-glutamate, and D-AAase is specific for *N*-acyl-D-aspartate [15]. We previously reported the properties of these enzymes purified from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6 (*Alcaligenes* A-6) [27, 28, 29]. Genes encoding these *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6 were cloned and their nucleotide sequences were determined. D-ANase has 43.5 and 55.7% sequence homology with D-AGase and D-AAase, respectively. The high sequence homology among the three *N*-acyl-D-amino acid amidohydrolases could be indicative of their common structure. We previously constructed the plasmids pKNSD2 [30], pAGD5 [27] and pETAD1 [29], which express D-ANase, D-AGase, and D-AAase, respectively. With these plasmids, expression levels of D-AGase (1.0 U/mg) and D-AAase (8.3 U/mg) in *Escherichia coli* were lower than that of D-ANase (162.1 U/mg). Considering the specific activities of purified D-AGase (1,100 U/mg) [20], D-AAase (180 U/mg) [26], and D-ANase (2,023 U/mg) [30], the expression levels of these enzymes could be improved. In our efforts to enhance production of these enzymes, we found that D-ANase, D-AGase, and D-AAase were overexpressed in the insoluble fraction in *E. coli*.

The coexpression of molecular chaperones assists protein folding and enhances the production of active proteins in some cases [12, 14, 16, 17, 21, 22]. Several lines of evidence indicate that three major chaperone teams, DnaK-DnaJ-GrpE (DnaKJE), GroEL-GroES (GroELS), and trigger factor (TF) play distinct but cooperative roles in protein folding in vivo [2, 6, 23]. DnaKJE and TF bind to nascent proteins in unfolded states and possess similar substrate binding specificities [4, 19]. GroELS interacts with partially folded polypeptides and assists in additional folding, and plays roles different from those of DnaKJE and TF [5]. Nishihara et al. [16, 17] constructed plasmids by which the production of DnaKJE, GroELS, TF, TF together with GroELS, or DnaKJE together with GroELS, can be manipulated, and they examined the effects of

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coexpression of these chaperones on the production of several recombinant proteins.

DnaK is a member of the hsp70 family of heat shock proteins, and functions to cooperate with the co-chaperones DnaJ and GrpE. Deletion of the *dnaK* gene causes defects in growth at 42°C and the propagation of λ phage [32]. GroES and GroEL are essential for bacterial growth at all temperatures [2]. Upon binding of the co-chaperone GroES, the substrate is discharged into a microenvironment inside GroEL that promotes folding. TF is a non-essential ribosome-bound protein that displays chaperone and prolyl isomerase activities [24]. Combined deletion of the TF and DnaK genes is lethal under normal conditions [25].

In this paper, we describe improvement of the overexpression system for *N*-acyl-D-amino acid amidohydrolase genes in *E. coli* based on molecular chaperone function in vivo.

Material and methods

Construction of *N*-acyl-D-amino acid amidohydrolase expression plasmids

The plasmid pKNSD2 [30] was constructed by inserting a D-ANase gene into pKK223-3 (Amersham Pharmacia, Buckinghamshire, UK), and the plasmid pETAD1 [29] was constructed by inserting a D-AAase gene into pET-23d (Novagen, Darmstadt, Germany) as previously described (Fig. 1). The plasmid pKGSD2, which is composed of a DNA fragment containing the D-AGase gene and pKK223-3, was constructed as follows: the 2-kbp *Eco*RI–*Eco*RI fragment excised from pAGD1 [27] was inserted between the *Eco*RI sites of pUC118. The resultant pAG118 plasmid was introduced into *E. coli* CJ236 (*dut*− *ung*−) cells, and its single-stranded DNA, which contains uracil, was purified. Synthesis of mutant DNA was performed following the method of Kunkel [11]. Primer 1 (5′-TGCAACTTCC TCCTT CAT GGGGCCGG-3′), designed to contain mismatched bases (underlined) for site-directed mutagenesis, was used to introduce the Shine-Dalgarno (SD) sequence (GAAGGA) nine bases upstream of the ATG initiation codon of the D-AGase structural gene. The resultant plasmid was designated pAGSD1. Single-stranded DNA was prepared from pAGSD1 in the same way as for pAG118. Primer 2 (5′-ACAAGCGGCCGG GA ATTCCGCCGGCCCCAT-3′), containing mismatched bases (underlined), was used to introduce an *Eco*RI site 47 bases upstream of the ATG start codon. The resultant pAGSD1 plasmid was digested by *Eco*RI and the fragment encoding the D-AGase gene was ligated into pKK223-3. This new plasmid was designated pKGSD2 (Fig. 1).

Culture conditions

pKJE7, pTF16, pGro7, pGKJE8, and pG-Tf2 in the Chaperone Plasmid Set (Takara Shuzo, Kyoto, Japan) were used for chaperone expression. Strains harboring an *N*-acyl-D-amino acid amidohydrolase-expressing plasmid, alone or together with a chaperone expression plasmid, were cultured for 12 h at 30°C in 5 ml Luria-Bertani (LB) medium in the presence of 50 μg/ml ampicillin (plus 20 μg/ml chloramphenicol when necessary). The culture was transferred into 100 ml LB medium with antibiotics in 500 ml Erlenmeyer flasks, and grown to log phase ($OD_{610} = 1.0$) at 30°C on a reciprocal shaker at 100 rpm. The molecular chaperones were induced by first adding 2 mg/ml L-arabinose and/or 5 ng/ml tetracycline to the medium.

Protein extraction and analysis

Cell cultures were cooled rapidly in an ice-water bath. Cells were harvested by centrifugation for 10 min at 5,000 *g* at 4°C. Cell pellets were washed with saline and resuspended in ice-cold 20 mM KPB, Potassium phosphate buffer (pH 7.0) and disrupted by sonication (model UD-200 ultrasonicator; TOMY SEIKO, Tokyo, Japan) under preoptimized conditions at 4°C. Subsequent centrifugation at 9,000 *g* for 10 min separated the soluble and insoluble fractions. The supernatant was

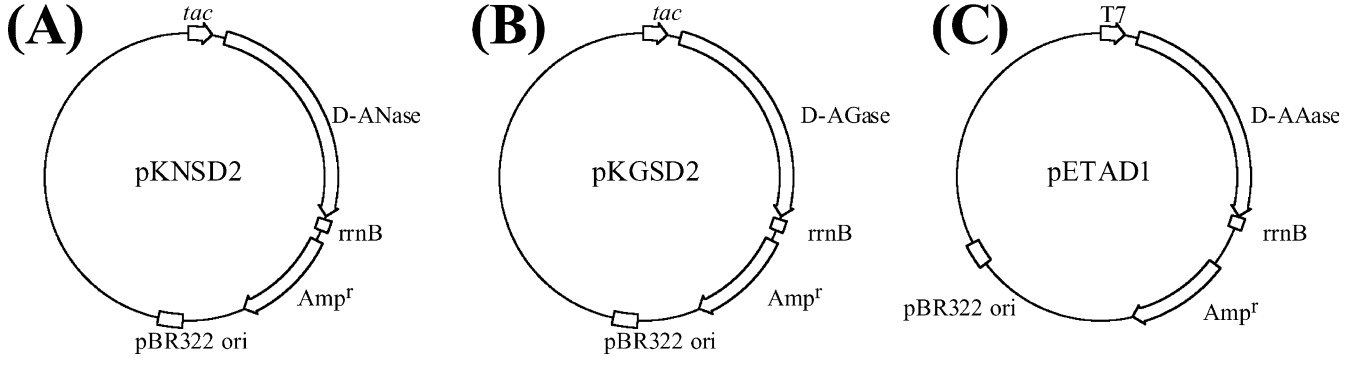

Fig. 1A–C Schematic diagram of the plasmid constructs. The β-lactamase gene (*Amp*^{r}), and the *tac* or T7 promoters are indicated by *arrows*. The pBR322 origin (*pBR322 ori*) and rrnB ribosomal terminator (*rrnB*) or T7 terminator (*T7 term*) are shown as *boxes*. **(A)** pKNSD2, **(B)** pKGSD2, **(C)** pETAD1

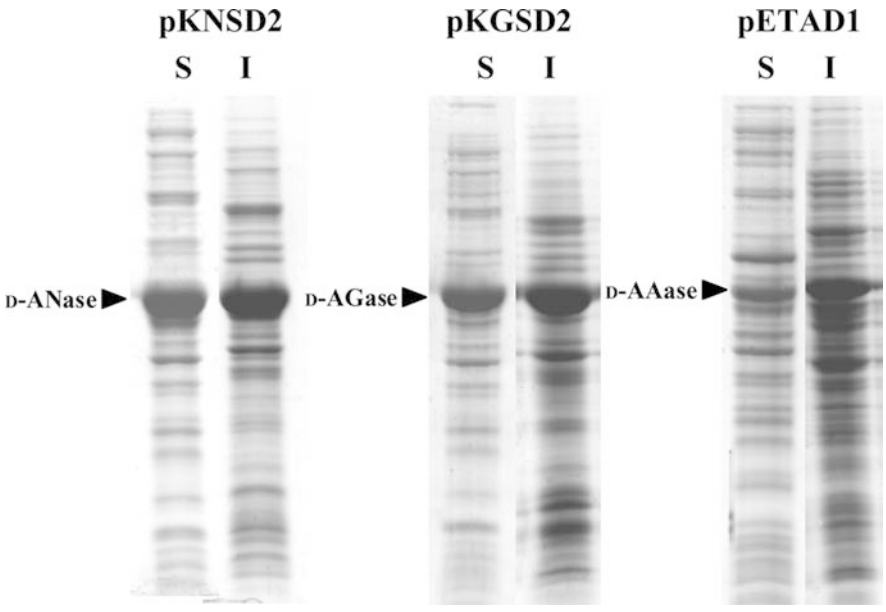

Fig. 2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of *N*-acyl-D-amino acid amidohydrolase expression patterns in *E.coli*. *E. coli* cells harboring pKNSD2, pKGSD2 or pETAD1 were cultured in the absence of IPTG at 30°C to produce of D-ANase, D-AGase or D-AAase, respectively. *S* Soluble fraction, *I* insoluble fraction

used as the soluble fraction. The pellet was washed with 50 mM KPB (pH 7.0) and resuspended in 50 mM KPB (pH 7.0) containing 8 M urea, and incubated for 1 h at 4°C. Cell debris was removed by centrifugation at 9,000 *g* for 10 min at 4°C, and the supernatant was used as the insoluble fraction.

Assay of enzyme activity

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 [27, 29, 30]. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol D-amino acid per minute. Specific activity was expressed as units per milligram of protein. Protein concentration was estimated using the Lowry method with crystalline egg albumin as the standard [13].

Results

Expression of the D-AGase gene in *E. coli*

For high-level expression of D-AGase, we constructed several expression plasmids in which the gene encoding D-AGase was placed under the control of the *lac* promoter or *tac* promoter, which are inducible using IPTG (isopropyl 1-thio-β-D-galactoside). Introduction of a canonical SD sequence and *tac* promoter were effective for the expression of the D-AGase gene in *E. coli*. pKGSD2 (38.1 U/mg) showed enzyme productivity 22-fold higher than that of pAGD1 (1.7 U/mg).

There was a significant reduction in the level of active enzyme (4.9 U/mg) when 0.1 mM IPTG was added to the culture. For the production of D-AGase, *E. coli* JM109 cells harboring pKGSD2 were cultured in the absence of IPTG for subsequent experiments.

Aggregation of *N*-acyl-D-amino acid amidohydrolases in *E. coli*

Our preliminary experiments showed that 1.0 mM IPTG in the culture reduced the production of active D-ANase or D-AAase, and that 0.1 mM IPTG was not effective for inducing D-ANase and D-AAase (data not shown). In subsequent experiments, *E. coli* cells harboring pKNSD2 or pETAD1 were cultured in the absence of IPTG. When cells carrying pKNSD2 or pKGSD2 were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the overexpressed protein was found in both the soluble and insoluble fractions (Fig. 2). SDS-PAGE revealed that most D-AAase was expressed as inclusion bodies, yielding only a small amount of active enzyme in cells carrying pETAD1 (Fig. 2). Table 1 shows the specific activities of transformants overexpressing the D-ANase, D-AGase, or D-AAase genes. The activity of each *N*-acyl-D-amino acid amidohydrolase was concomitant with the presence of the active enzyme in the soluble fraction of these cells, because *E. coli* host cells exhibit no *N*-acyl-D-amino acid amidohydrolase activity.

Roles of chaperones and chaperone teams in preventing aggregation of *N*-acyl-D-amino acid amidohydrolases

To determine whether chaperone teams affect the folding of recombinant *N*-acyl-D-amino acid amidohydrolases, pKNSD2 or pKGSD2 was transformed into JM109 cells, or pETAD1 was transformed into BL21 cells, carrying plasmids that permit the expression of several chaperone teams. As shown in Table 1,

Table 1 Effect of coexpression of molecular chaperones on the production of *N*-acyl-D-amino acid amidohydrolases. *Escherichia coli* strain JM109 or BL21 harboring the *N*-acyl-D-amino acid amidohydrolase expression plasmid, alone or together with pKJE7 (DnaKJE), pTF16 (TF), pGro7 (GroELS), pGKJE8 (DnaKJE + GroELS) or pG-Tf2 (TF + GroELS) was grown at 30°C in LB medium with (+) or without (−) inducer for expression of chaperones. Data are the average of more than two determinants

Inducer for chaperones	*N*-Acetyl-D-amino acid amidohydrolase activity (U/mg)					
	No chaperones	+pKJE7[a]	+pTF16[a]	+pGro7[a]	+pGKJE8[b]	+pG-Tf2[c]
pKNSD2/JM109						
+	104.8	186.0	53.0	57.1	46.6	313.3
−	233.8	289.7	139.9	148.6	152.8	20.8
pKGSD2/JM109						
+	7.8	3.6	36.0	72.4	16.2	66.9
−	38.1	26.2	39.5	49.5	88.8	95.8
pETAD1/BL21						
+	7.1	0.1	32.0	22.7	8.3	0
−	6.2	0	16.2	26.4	20.4	0

[a]Chaperones induced by 2 mg/ml L-arabinose
[b]Chaperones induced by 2 mg/ml L-arabinose 5 ng/ml tetracycline
[c]Chaperones induced by 5 ng/ml tetracycline

coexpression of GroELS and TF increased production of soluble and active D-ANase and D-AGase by 1.3- and 1.8-fold, respectively. By coexpressing GroELS, a 6.0-fold enhancement in soluble D-AAase expression was obtained. SDS-PAGE analysis revealed that a considerable amount of inclusion bodies formed in the cells even though the activity of *N*-acyl-D-amino acid amidohydrolase increased due to coexpression of the chaperones (Fig. 3).

Discussion

We constructed a series of multicopy plasmids that contain the D-ANase or D-AGase gene placed downstream of the *lac* or *tac* promoter or the D-AAase gene placed downstream of the *tac* promoter. The *lac* and *tac* promoters are inducible by IPTG. When the D-ANase, D-AGase or D-AAase gene was expressed in *E. coli* cells, *N*-acyl-D-amino acid amidohydrolase activities were higher in cells cultured in the absence of IPTG than in cells cultured in the presence of 0.1 mM IPTG. This suggests that the gene was not tightly controlled by the promoter, resulting in leaky expression, and that a high rate of protein synthesis led to the formation of inclusion bodies [8]. *N*-Acyl-D-amino acid amidohydrolases of *Alcaligenes* A-6 produced in *E. coli* aggregated into insoluble particles known as inclusion bodies. Factors considered to contribute to inclusion body formation are growth conditions of the host cells [10, 18], protein synthesis rate by a multicopy gene [8], and a lack of molecular chaperones required for the proper folding of overexpressed proteins [16]. To improve the production of active *N*-acyl-D-amino acid amidohydrolases, the coexpression of chaperones for folding is necessary. Therefore, a series of chaperones were coexpressed together with the *N*-acyl-D-amino acid amidohydrolases.

SDS-PAGE (data not shown) and specific activity analyses (Table 1) showed that the production of D-ANase or D-AGase was reduced by the addition of arabinose. Induction of the arabinose operon by the addition of arabinose might have decreased leaky expression of the D-ANase or D-AGase gene located downstream of the *tac* promoter, since it was not induced by IPTG.

Coexpression of GroELS together with DnaKJE or TF was more effective for production of active D-AGase, whereas coexpression of GroELS alone only slightly increased the amount of active enzyme. This suggests that DnaKJE and GroELS or TF and GroELS play synergistic roles in vivo. Coexpression of GroELS or TF individually was effective for production of active D-AAase; in fact active D-AAase was decreased by coexpression of GroELS together with TF. TF binds to GroEL and increases its affinity for proteins to promote their folding or degradation [7]. In this case, the coexpression of GroELS and TF might have led to the degradation of D-AAase. Our results show that the effects of DnaKJE, GroELS, and TF differ, even in cases where the target proteins have structural similarities.

We observed a considerable amount of inclusion bodies in cells that exhibited increased *N*-acyl-D-amino acid amidohydrolase activity due to the coexpression of the chaperones (Fig. 3). This suggests that it is possible to obtain a higher yield of *N*-acyl-D-amino acid amidohydrolases in soluble form. To obtain high yields of active enzyme, the level of expression of chaperones, ratio of chaperone teams, and cell culture conditions [18] all have to be considered.

Our results show that the effects of chaperone coexpression on the folding of D-ANase, D-AGase or D-AAase differs depending on the kind of chaperone. Previous studies have shown that DnaKJE, GroELS, and TF are not functionally interchangeable [2, 6, 23], and have common and divergent peptide binding specificities [1, 4, 19]. This substrate specificity may affect their ability to increase the amount of active enzyme in *E. coli*. DnaK, Bip, Hsc62, SecB, and endoplasmic reticulum hsp70 protein are not functionally interchangeable and have divergent peptide binding speci-

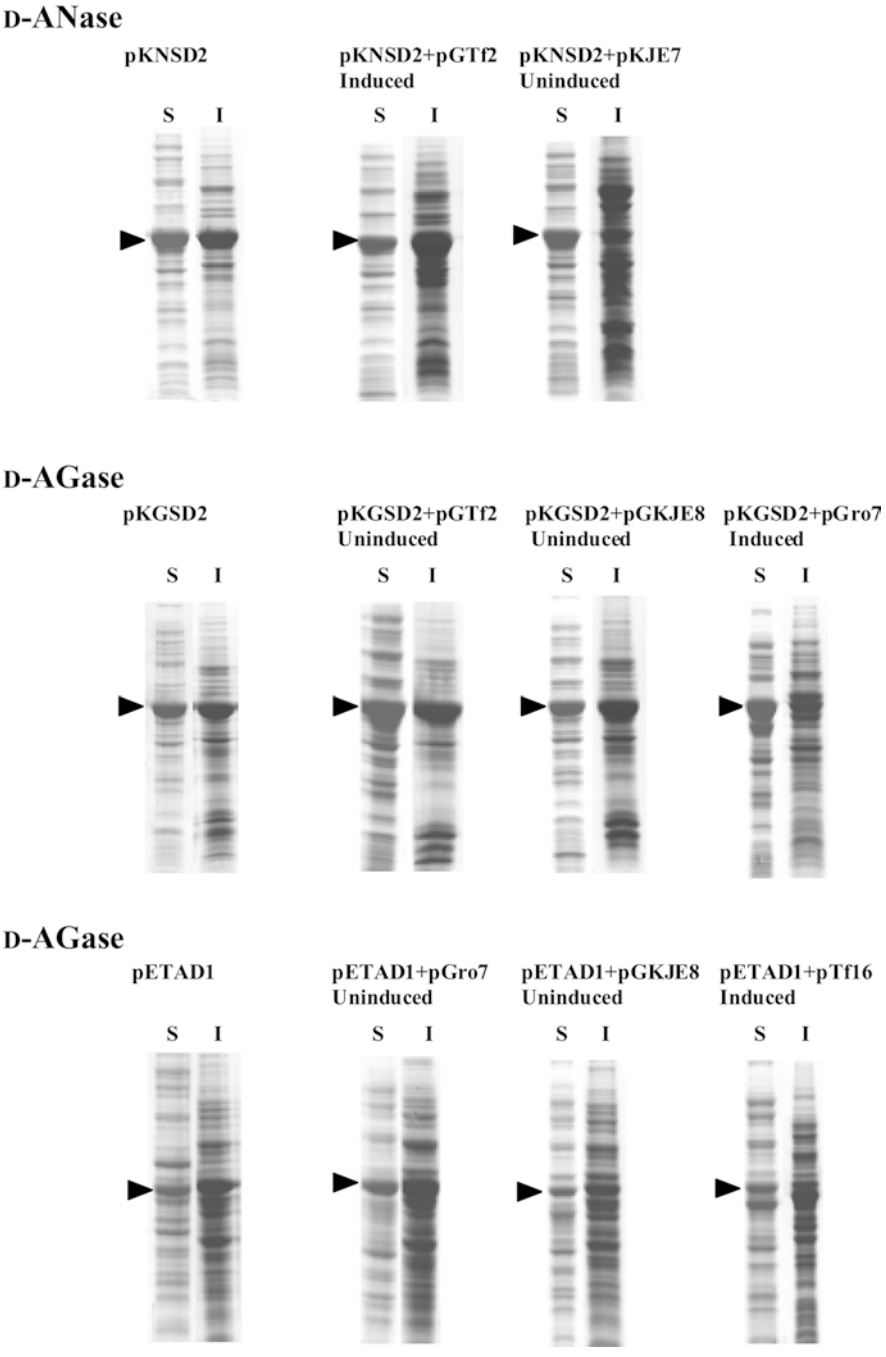

Fig. 3 Increased expression of D-ANase, D-AGase or D-AAase due to coexpression of a chaperone. *E. coli* strains harboring the D-ANase (pKNSD2), D-AGase (pKGSD2) or D-AAase (pETAD1) expression plasmids with or without the chaperone expression plasmid were grown as described in Table 1. pG-KJE8 possesses a *groELS* operon and a *dnaK-dnaJ-grpE* operon, pGro7 contains a *groELS* operon, pKJE7 contains a *dnaK-dnaJ-grpE* operon, pG-Tf2 possesses a *groELS* operon and a TF gene, and pTf16 contains a TF gene. *S* Soluble fraction, *I* insoluble fraction

ficities [3, 4, 9, 33, 34]. These chaperones are candidates for chaperones that might increase active *N*-acyl-D-amino acid amidohydrolases upon their coexpression.

Our results indicate that the coexpression of chaperones can be applied to increase production of *N*-acyl-D-amino acid amidohydrolases used for the industrial production of D-amino acids.

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