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Enhancement of Solubility, Purification, and Inclusion Body Refolding of Active Human Mitochondrial Aldehyde Dehydrogenase 2

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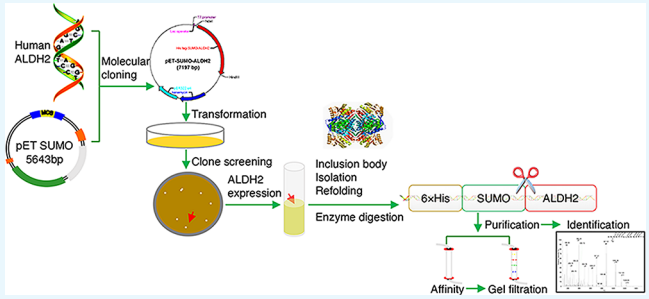
ABSTRACT: Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is predominantly linked with acetaldehyde detoxification in the second stage of alcohol metabolism. To intensively study ALDH2 function, a higher purity and uniform composition of the protein is required. An efficient *Escherichia coli* system for ALDH2 expression was developed by using His and a small ubiquitin-related modifier fusion tag. Most of the recombinant ALDH2s were expressed in the form of inclusion bodies. The ALDH2-enriched inclusion bodies were denatured with 6 M guanidine hydrochloride, and then ALDH2 was ultrafitrated. Finally, ALDH2 was successfully purified through affinity and gel filtration chromatography. The purified ALDH2 was finally preserved by the vacuum freeze-drying method, and its purity was determined to be higher than 95%, with a final media yield of 33.89 mg/L. The specific activity of ALDH2 was 6.1×10^4 U/mg. This work was the first to report pET-SUMO-ALDH2 recombinant plasmid expression in *Escherichia coli*, and the inclusion bodies were isolated and refolded. Finally, the purified ALDH2 had relatively higher purity, yield, and biological activity.



1. INTRODUCTION

The aldehyde dehydrogenase (ALDH) superfamily, which has an ancient origin, can be found in all living organisms. It is expressed in *Eukaryotic taxa*, *Eubacteria*, and *Archaea*.[1] ALDH plays a critical role in detoxification of organisms and exogenous aldehydes. Aldehydes are a kind of highly active compounds that can form adducts with proteins, lipids, and deoxyribonucleic acid (DNA), affecting the function of biomolecules and inducing cytotoxicity.[2] Among 19 kinds of human genome ALDH genes, ALDH2 has gained increased attention as a metabolite of aldehyde compounds in different directives, such as propionaldehyde, butyraldehyde, and 4-hydroxy-2-nonenal (4-HNE).[3,4] The human ALDH2 gene is located at chromosome 12q24. Its protein ALDH2 is a 517-amino acid polypeptide. ALDH2 is transported to the mitochondrial matrix process that is dependent on its NH_2 terminus 17-amino acid mitochondrial targeting sequence. The 17-amino acids are cleaved after the protein complete folding inside the mitochondria.[5] ALDH2 is a kind of tetrameric enzyme consisting of 56 kDa equivalent submits.[6] ALDH2 has three kinds of enzymatic activities. First, dehydrogenase activity converts aldehydes to carboxylic acids. Second, esterase activity converts carboxylic acid ester into free carboxylic acid and alcohol. Third, nitroglycerin reductase activity, which is a bioactivation enzyme for organic nitrates.[7]

Based on the important role studies of ALDH2 enzymatic activities in cell protection as well as how aldehydes participate in the pathology of human diseases, ALDH2 is a critical protein involved in a variety of disease phenotypes. ALDH2 has a critical function in cardiovascular diseases, including heart failure, myocardial infarct, and stroke.[8−10] ALDH2 is also associated with diabetes, cancer, neurodegenerative diseases, and osteoporosis.[11−13] Therefore, potential functional studies of ALDH2 and pharmaceutical development will impact the lifespan or quality of human life.[14]

Cellular protein functions usually involve coordinating interactions with one or more regulatory partners. Therefore, it is necessary to characterize proteins both in the separation process and in the context of complex chaperone interaction.[15]

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Therefore, research on ALDH2 function requires high purity and high concentration of homogeneous protein specimen. These requirements are usually the main obstacles to further protein research. Recently, commercial ALDH2 is mainly extracted from animal liver and pancreas, with limited resources and high price, making large-scale production difficult. Genetic engineering technology can produce ALDH2 on a large scale, but there is little research on ALDH2 in this area. The cost of genetic engineering technology is relatively high, and there is no significant result and optimal protein purification scheme to extract ALDH2. However, ALDH2 has been reported in the literature in almost all animals and yeast, and there is little research in prokaryotes. The prokaryotic system is the main host for heterologous expression. Bacteria are easily operated, durable, and grow rapidly and powerfully with high density and low cost.[16] Once 70% of the recombinant proteins are present in inclusion bodies (IBs), it will be difficult to study the heterologous expression necessary for the characterization of structural and functional proteins.[17] Heterologous expression of the human ALDH2 gene in *Escherichia coli* (*E. coli*), encoding the protein that exhibit complex structures, often induces accumulation of most of the expressed proteins in inactive and insoluble form. Therefore, exploring of optimal refolding conditions and biological activity maintenance of ALDH2 expressed in the form of IBs are the difficulties of purifying ALDH2.

The advantage of the His tag is that it can increase protein stability and solubility, prevent proteolysis, and still function under denaturing conditions.[18] A small ubiquitin-related modifier (SUMO) is a chaperone protein with about 100 amino acids. SUMO has functions that can form covalent attachments to target proteins.[19] SUMO can fuse into the N-terminus of the target protein and enhance protein folding via its molecular chaperone properties. Thus, SUMO can be an effective carrier in the expression of recombinant proteins. SUMO can improve target protein expression, folding, and solubility, reduce protein hydrolysis and degradation, facilitate protein purification, and detect target proteins.[20] SUMO protease 1 is also called Ubiquitin-like specific protease 1 (Ulp1), and it conveniently cuts the SUMO tag to obtain natural ALDH2 and can be implemented in difficult-to-express ALDH2 functional analysis. In this article, we also put forward a novel and effective solubilization and purification protocol for ALDH2 protein. Purified ALDH2 demonstrated higher biological activity, which will provide a basis for subsequent protein function research.

2. RESULTS AND DISCUSSION

2.1. Identification of the Recombinant ALDH2 Plasmids. We successfully constructed pET-28a(+)-ALDH2, pET-32a(+)-ALDH2, and pET-SUMO-ALDH2 recombinant plasmids using genetic engineering methods. Among the three recombinant plasmids, pET-SUMO-ALDH2 was our best choice according to total protein expression (Figure 1A). The ALDH2 cDNA fragments were ligated to the pET-SUMO expression vector, which was the most effective and widely used system for ALDH2 expression in *E. coli*. PCR detection and DNA sequencing confirmed that no changes occurred in the sequence of the target gene. We transformed the identified recombinant plasmids into BL21(DE3) competence and then selected three positive monoclonal colonies for amplification and culture. They were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot (Figure 1B,C).

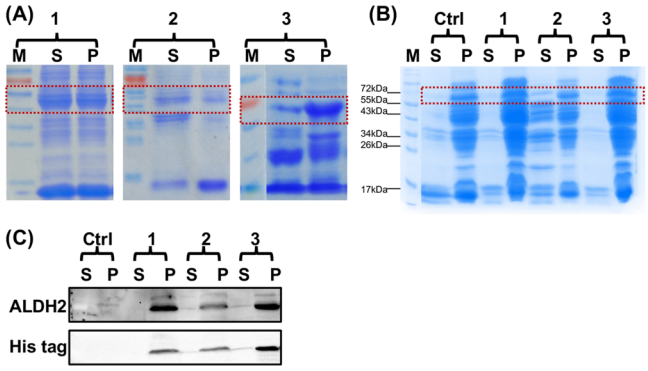

Figure 1. Identification of the recombinant ALDH2 plasmids expression in the bacterial expression system. Comparison of the recombinant ALDH2 plasmids by SDS-PAGE (A); lane 1, pET-28a(+)-ALDH2 recombinant plasmids; lane 2, pET-32a(+)-ALDH2 recombinant plasmids; line 3, pET-SUMO-ALDH2 recombinant plasmids. Identification of three positive clones by SDS-PAGE (B) and western blot (C). Ctrl, BL21(DE3); M, marker; S, supernatant; P, pellet.

2.2. Optimal Expression Conditions for Exploring ALDH2. The molecular weight of the His-SUMO-ALDH2 protein was predicted to be 70 kDa by web-based software (http://web.expasy.org/comedpute.pi). We used electrophoresis to analyze the effects of the four most commonly studied parameters (temperature, IPTG concentration, incubation time, and shaker rotation speed) to optimize the expression conditions of the ALDH2 recombinant protein in the bacterial expression system. SDS-PAGE and BandScan5 software were used to compare the band density on the gel, showing that maximum production of the recombinant protein occurring at an insoluble fraction of ALDH2 was obtained after induction by 0.3 mM IPTG at 37 °C and 100 rpm for 10 h. The maximal soluble fraction of ALDH2 was obtained after induction by 0.3 mM IPTG at 16 °C and 70 rpm for 12 h (Figure 2A−D).

2.3. Purification of the Recombinant ALDH2 from Soluble Fraction. ALDH2 with His tag had good binding affinity chromatography through nickel ions, and the non-specific binding part flowed out to the N1 penetration peak with a low concentration of imidazole. The high concentration of imidazole competed with the His tag for nickel ion groups, causing the target protein to flow out into the N2 elution peak (Figure 3A). The protein in the elution peak was subjected to gel filtration chromatography to remove imidazole and replace the buffer (Figure 3B).

The protein in the elution peak was cleaved by Ulp1 for the second affinity and gel filtration chromatography, and the results showed that the G UV absorption value was only 16 mAu (Figure 3C,D). In summary, after purification of ALDH2 expressed in soluble form, the final yield was very low, so we studied ALDH2 expressed in the form of IBs.

2.4. Refolding and Purification of ALDH2. ALDH2 has a highly hydrophobic N-terminal structural domain, and heterologous expression studies for protein characterization are laborious once the recombinant protein is present in IBs. Initially, this protocol consisted of washing the IBs with buffer [4 M urea, 10% glycerol, 50 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA, and 10 mM dithiothreitol (DTT)] followed by denaturing with buffer (8 M urea, 10% glycerol, 50 mM Tris

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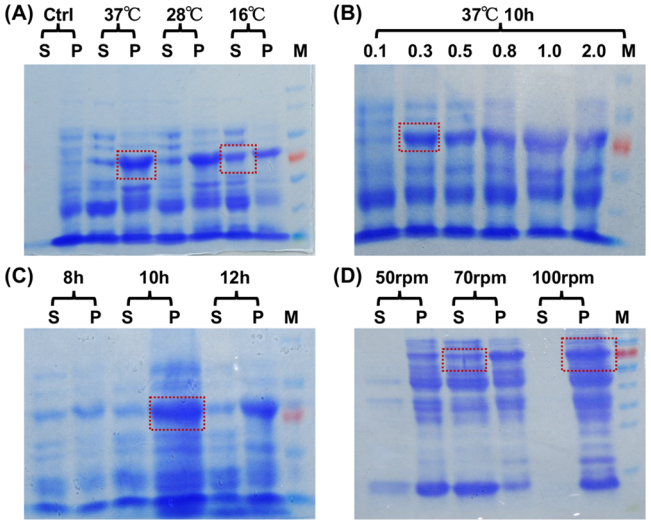

Figure 2. Exploring the optimal expression conditions of the recombinant ALDH2 protein. Effect of preincubation temperature (A), IPTG concentration (B), time (C), and rotation speed (D) on recombinant ALDH2 production (total protein analysis). Ctrl, the total proteins expression levels of *E. coli* BL21(DE3) competent cells; M, marker; S, supernatant; P, pellet.

pH 8.0, 100 mM NaCl, 10 mM EDTA, and 10 mM DTT). Two steps appear to be inappropriate for the high efficiency of ALDH2 refolding protocol development. After that, we observed protein denaturation by dropwise addition of 6 M guanidine hydrochloride (Gua-HCl) solution to the IBs in which constant dripping allowed better protein denaturation; this fact seems to be related to the kinetics of the protein.[21] A 6 M Gua-HCl solution had more than 95% dissolution capacity, and the dissolution was fast without causing covalent modification of the recombinant proteins. Another critical step was the protein renaturation procedure in which glycerol was essential for the stabilization of the hydrophobic regions of ALDH2. As shown in Figure 4A, the protein purity was higher after refolding. Refolding proteins (35 mg) were cleaved by Ulp1, as verified by SDS-PAGE (Figure 4B). The SDS-PAGE results indicated a 56 kDa band, corresponding to ALDH2, which was predicted by web-based software (http://web.expasy.org/compute.pi). The His-SUMO tag and ALDH2 were separated into two parts by enzyme digestion, and total proteins were purified by affinity and gel filtration chromatography (Figure 4C,D).

Purification and characterization of human ALDH2 have rarely been reported. They usually obtained ALDH2 from human[22] or grass carp[23,24] liver, but the yield and purity were low, and the activity was questionable. Zheng et al. reported that this protein has been expressed in the insect cell sf9 and an *E. coli* expression system with a specific activity of 1.10 U/mg. However, the yield was very low.[25] Zhao et al. reported that ALDH2 cDNA and 6×His tags were cloned into pPIC9K.[26] The recombinant protein was expressed in *P. pastoris* GS115 and purified by $Ni2^{+}$-Sepharose affinity chromatography. After purification of the fermentation supernatant, the enzyme had a specific activity of 1.2 U/mg. The yield was approximately 16 mg/L.[26] During the process of ALDH2 expression, the growth cycle of *P. pastoris* is longer, and the secretion of protein is serious.

The final ALDH2 yield was 33.89 mg/L (Table 1). In this work, we developed an effective strategy based on expression and purification to obtain ALDH2, which enabled us to perform an initial characterization of ALDH2 in the quantity and concentration range that can contribute to future intractable functional projects.

2.5. Identification of Purified ALDH2. The proteins in the penetration peak and the elution peak of affinity chromatography were separately collected and identified by SDS-PAGE and western blot. One band at a molecular weight of approximately 13 kDa was His-SUMO, and the other band at approximately 56.5 kDa was ALDH2 (Figure 5A,B). Freeze-dried ALDH2 was analyzed by SDS-PAGE. Preliminary analysis using BandScan software showed that the purity of ALDH2 was greater than 95%, and the purification efficiency was relatively high (Figure 5C).

At present, with the development of biological spectrometry technology sensitivity, specific degrees, and continuous high throughput, the application of spectrometry technology in proteomics is increasing. Currently, the most widely used technologies include electrospray ionization mass spectrometry and IC-MS-MS (Q-TOF).[27] Peptide mass fingerprinting (PMF) of ALDH2 is shown in Figure 5D. It was interesting to note that the amino acid sequences of 10 peptide fragments containing a total of 209 amino acid residues were 100% identical to human ALDH2 (gi: 48146098) (Figure 5E).

2.6. Detection of ALDH2 Activity by HPLC. When ALDH2 was not added, the absorbance of 2-hydroxy-3-nitrobenzaldehyde (2H3N-BA) at UV 280 nm was 632 mAU (Figure 6A); after 89 pmol/L, ALDH2 was added to the reaction system, and the absorbance was reduced to 344 mAU (Figure 6B), so it could be determined that the ALDH2 we purified was biologically active. To determine which storage method could better maintain ALDH2 activity, ALDH2 stored in three different ways was separately detected by HPLC. The results showed that the activity of freeze-dried protein was the highest (Figure 6C), so we freeze-dried ALDH2 in large quantities and stored it at −80 °C. When 893 pmol/L ALDH2 was added, the enzyme activity tended to plateau, and the calculated specific activity of ALDH2 was 6.1×10^{4} U/mg (Figure 6D).

3. CONCLUSIONS

The objective of this study was to produce biologically active human ALDH2 in a prokaryotic expression system. We obtained high purity refolding protein that was purified by two chromatography steps. Such studies have been facilitated by the improvement of existing techniques and the development of new techniques. This purification protocol is valuable to further research on the three-dimensional structure and function of ALDH2 and can contribute to a better understanding of the role of ALDH2 in human pathogenicity and relevant pharmaceutical development.

4. MATERIALS AND METHODS

4.1. Construction of Recombinant Plasmids. Coding sequences (CDSs) of the human ALDH2 gene (GenBank accession number: CR456991.1) were obtained from the database nucleotide sequence. The rare codons in *E. coli* were compared with the ALDH2 sequence; subsequently, the target genes were designed by web-based software such as GeneOptimizer (Thermo Fisher Scientific, USA),[28] Gene script (http://www.genescript.com), and *E. coli* rare codon analyzer2 (http://www.faculty.ucr.edu). The optimized se-

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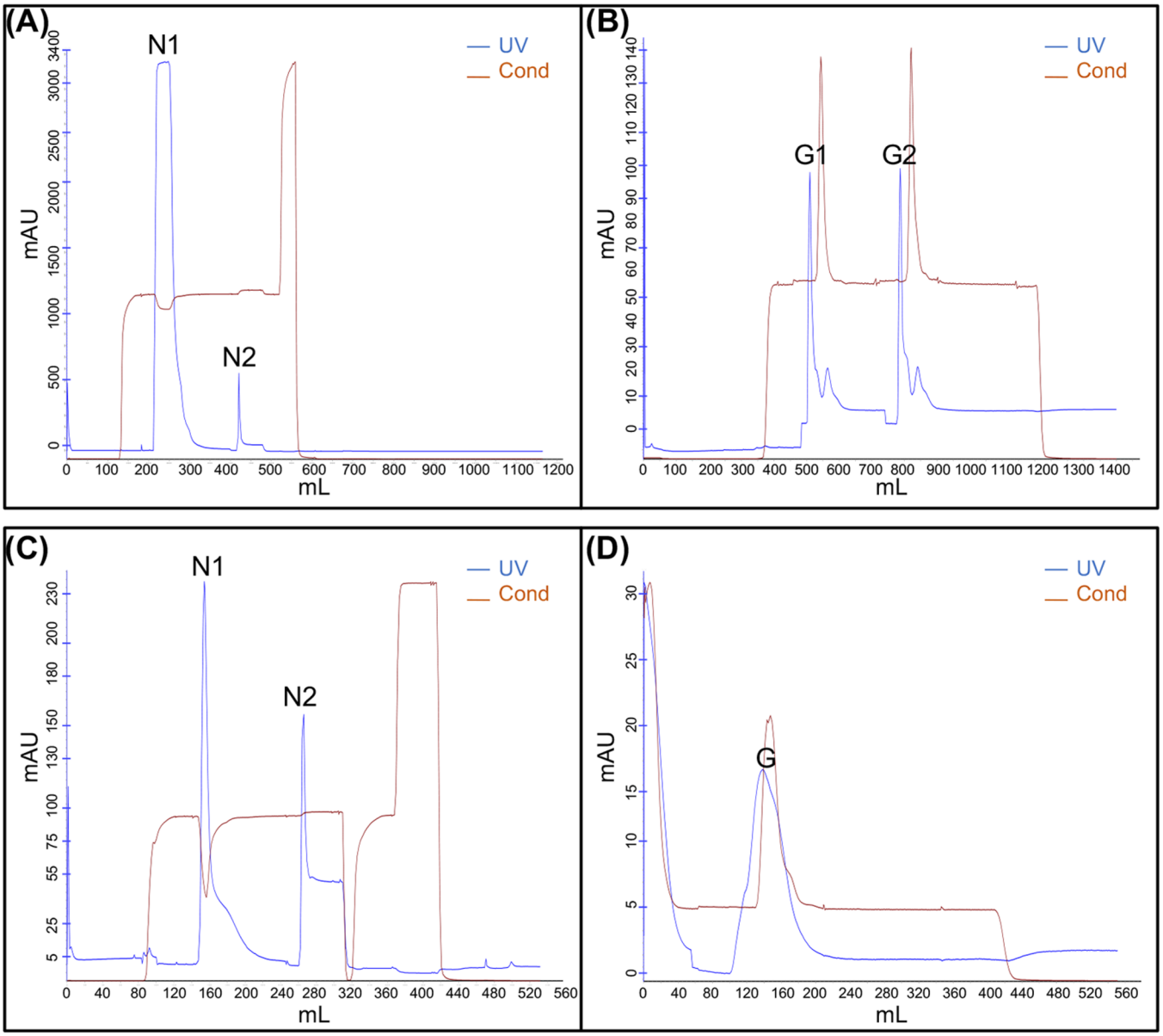

Figure 3. Purification of the recombinant ALDH2 protein from soluble fraction. Purification by affinity chromatography [N1, penetration peak-impure protein (3327 mAu); N2, elution peak-interest protein (622 mAu)] (A); gel filtration chromatography [G1 and G2, interest protein (97 mAu)] (B). Purification of G1 G2 peak fractions by affinity chromatography [N1, penetration peak-interest protein (238 mAu); N2, elution peak-impure protein (155 mAu)] (C); gel filtration chromatography [G, interest protein (16 mAu)] (D).

quences were synthesized by Generay Biotechnology Company (Shanghai, China), cloned into a pUC-SP vector, and delivered to our lab. A Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information (NCBI) was used for database searches and nucleotide sequence analysis. Multiple sequence alignments of nucleic acids and amino acids were performed by using ClustalW of the BioEdit program.

The ALDH2 bases were amplified by PCR using oligonucleotide sequence primers [Generay Biotechnology Company (Shanghai, China)] as follows: forward (5′ ATGCTGCGTGCTGCTCG 3′) and reverse (5′ TTAA-GAGTTTTTCTGCGGAA 3′). PCR was performed with one cycle at 94 °C for 5 min followed by 30 cycles of denaturation for 10 s at 98 °C, annealing for 30 s at 55 °C, and extension for 1.5 min at 72 °C and a final extension step for 9 min at 72 °C. The PCR product was separated by agarose gel electrophoresis and recycled from the gel using an agarose gel DNA extraction kit (Axygen, USA). The product was ligated to the pET-SUMO expression vector (Invitrogen, USA) with T4 DNA ligase (Figure 7), and then the ligation product was transformed into *E. coli* DH5α competent cells. The recombinant plasmid pET-SUMO-ALDH2 was confirmed by PCR and analyzed by DNA sequencing [Generay Biotechnology Company (Shanghai, China)] and finally transformed into BL21 (DE3) competent cells [CWBIO, Beijing, China].

4.2. Optimization of ALDH2 Expression Conditions in Bacterial Hosts. *E. coli* BL21 (DE3) host strains transformed with the pET-SUMO-ALDH2 recombinant plasmid were used to inoculate 20 mL of Luria-Bertani (LB) medium supplemented with 50 μg/mL kanamycin and grown overnight at 37 °C with shaking at 100 rpm on a vertical oscillator (Crystal, USA). Twenty microliters of this overnight preculture served to inoculate 20 mL of LB medium in 50 mL Erlenmeyer

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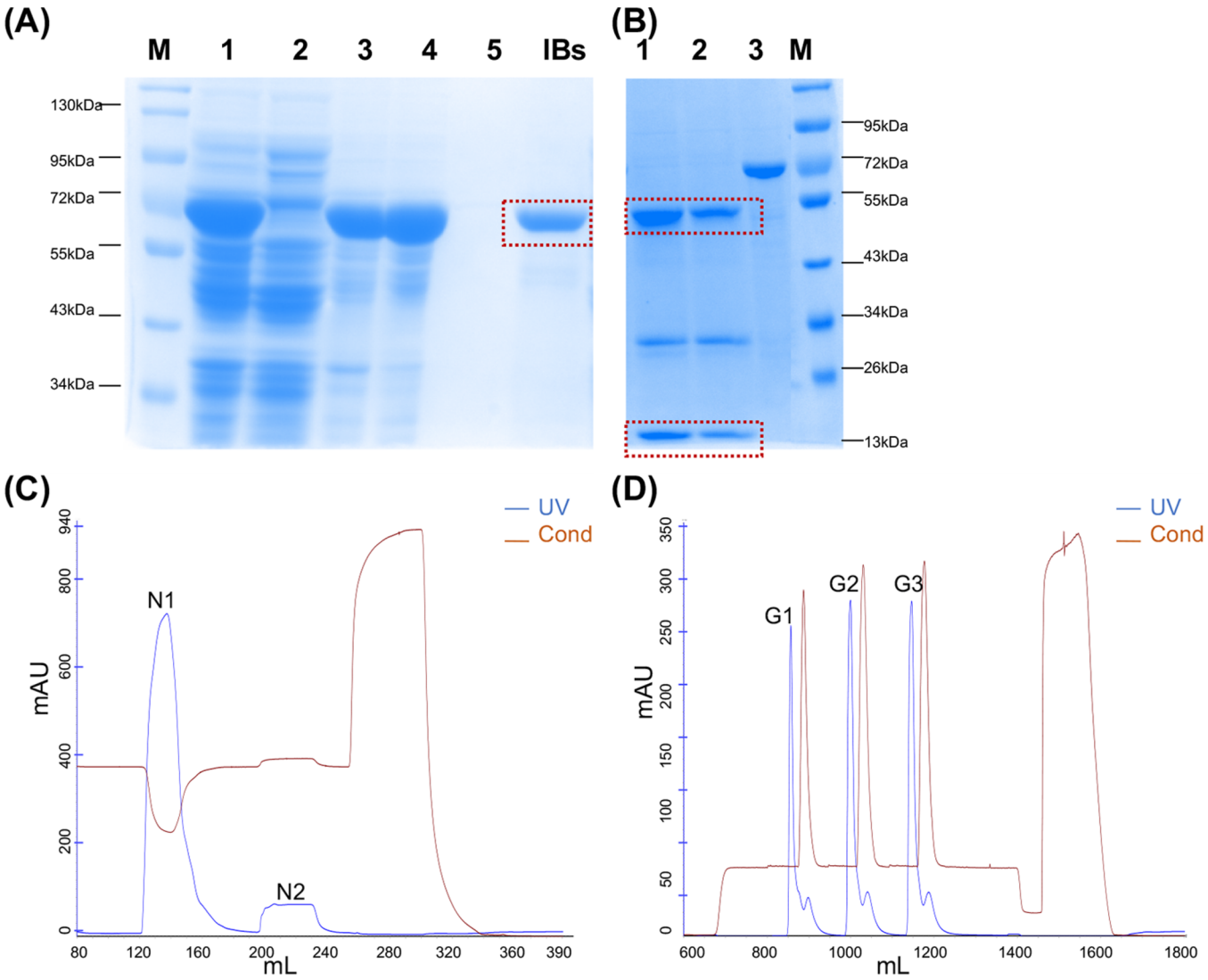

Figure 4. Refolding and purification of ALDH2. SDS-PAGE analysis of ALDH2 denaturation and renaturation (A); M, marker; lane 1, total proteins; lane 2, ultrasonic supernatant; lane 3, washing pellet; lane 4, resuspension pellet; lane 5, ultrafiltration liquid. Digestion of renatured ALDH2 by Ulp1 (B); lanes 1 and 2, Ulp1 digestion of total proteins; lane 3, total proteins. Purification of the renatured ALDH2 by affinity chromatography [N1, penetration peak-interest protein (736 mAu); N2, elution peak-impure protein (64 mAu)] (C) and gel filtration chromatography [G1, G2, G3, interest protein (260, 281, 283 mAu)] (D).

Table 1. Summary of the Total Protein Refolding and Purification from 1 L of *E. coli* BL21 (DE3)

steps	concentration (mg/mL)	volume (mL)	total protein (mg)
crude extract	12.4	32	396.8
refolding protein	2.67	68	181.6
nickel affinity column	0.97	49.4	47.9
Sephadex G-25	0.24	141.2	33.89

flasks. When the optical density at 600 nm (OD_{600}) of the culture reached approximately 0.4−0.6, the expression of recombinant ALDH2 was induced by adding various concentrations (0−2 mM) of isopropyl-β-D-thiogalactoside (IPTG, Sigma), and shaking was continued at 50 rpm, 70 rpm, and 100 rpm for 0−12 h at different temperatures (16, 28, and 37 °C). To analyze the expression levels and profiles, total proteins from the post-induction culture were further analyzed by SDS-PAGE and western blot.

4.3. Extraction of Intracellular Protein. Bacterial cells were centrifuged at 10,000*g* for 15 min, washed with PBS twice, and resuspended in 40 mL of lysis buffer (20 mM sodium phosphate, 0.5 M NaCl, and 10 mM imidazole, pH 7.4). Lysozyme (1 mg/mL) and deoxyribonuclease I (20 μg/mg) were added on ice for 30 min and homogenized using an ultrasonic sonicator continuously for 16 min with pulses of 5−7 s intervals at a frequency of 300 W in an ice bath. The lysate was centrifuged at 12,000*g* at 4 °C for 10 min to separate IBs from the soluble fraction. The supernatant presenting the soluble fraction was directly used for protein purification, and the pellet presenting the IB fraction was collected to perform ALDH2 refolding.

4.4. Protein Refolding from IBs. High-level expression of many recombinant proteins in *E. coli* leads to the formation of highly aggregated proteins commonly referred to as IBs.[29] The IBs were easily isolated by centrifugation from the cytoplasm and periplasmic space fraction and washed twice with buffer (0.5% Triton X-100, 50 mM Tris pH 8.0, 300 mM NaCl, 10

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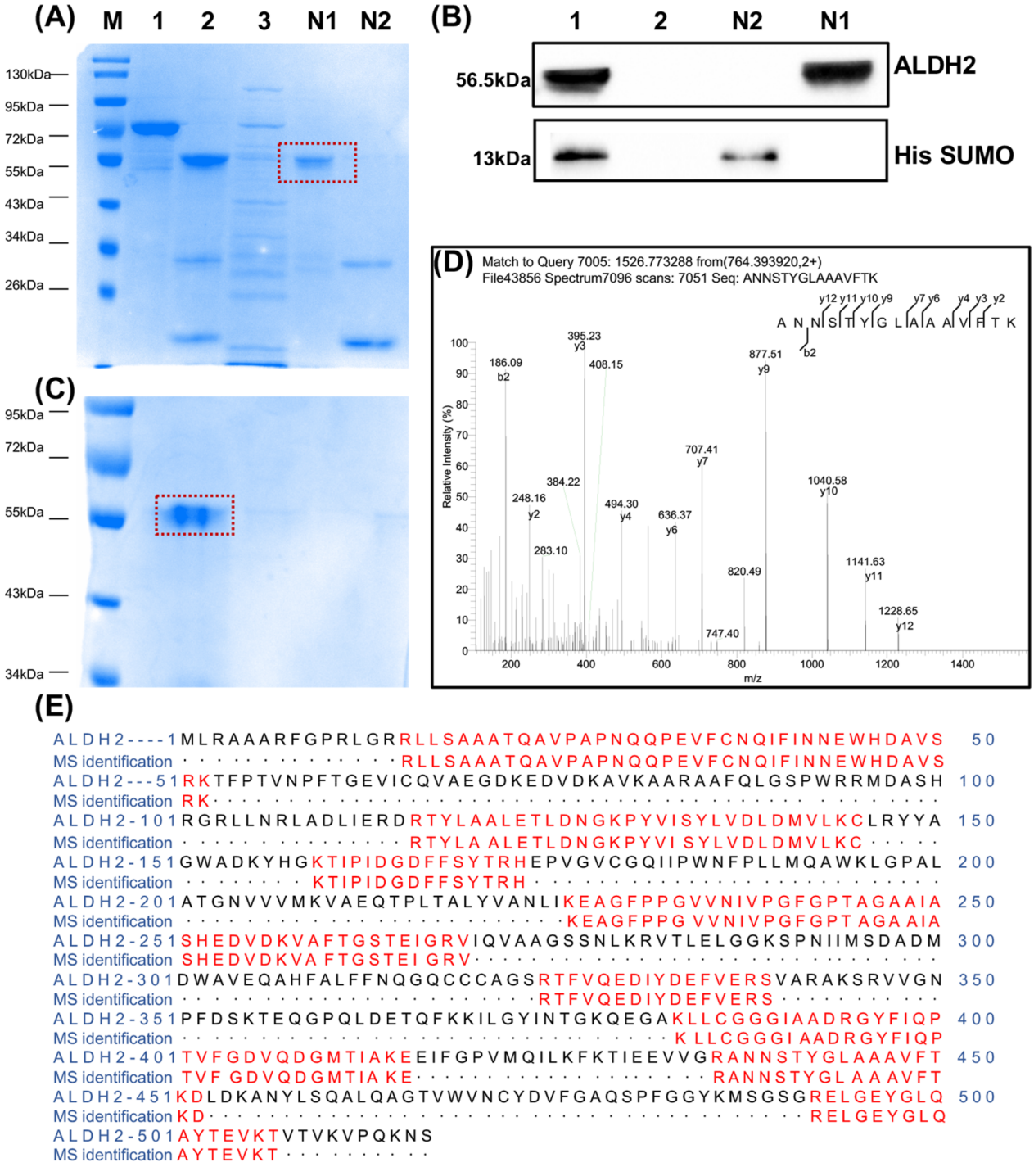

Figure 5. Identification of purified ALDH2. Identification of the chromatographic products by SDS-PAGE (A) and western blot (B); lane 1, total proteins; lane 2, Ulp1 digestion products; lane 3, Ulp1; N1, penetration peak; N2, elution peak. Analysis of freeze-drying ALDH2 by SDS-PAGE (C). Ions identified using MS (D). Protein sequence alignment of the MS identification of samples with human ALDH2. Identical amino acid residues are shown in red (E).

mM EDTA, and 10 mM DTT) to remove contaminant soluble proteins by centrifugation at 12,000*g* for 15 min at 4 °C. Triton X-100 can remove the *E. coli* membrane and cell wall material. Then, they were resuspended in 50 mL of buffer (50 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA, and 10 mM DTT) and homogenized using an ultrasonic sonicator continuously for 6 min. Each fraction of the resuspended IBs was solubilized in freshly prepared denaturing buffer [6 M Gua-HCl, 10% glycerol, 50 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA, and 10 mM DTT]. The final concentration reached 30 mg/mL, and the sample was subsequently incubated for 10 h at 4 °C with gentle stirring. Gua-HCl and DTT were used to solubilize the resuspended IBs. Denaturant buffer (100 mM Tris pH 8.0, 400 mM L-arginine, and 2 mM EDTA) was added dropwise to the IBs at 4 °C, and the renaturation time of each milliliter was 8 h.[30] After buffer-exchange steps via ultrafiltration and concentration, centrifugation at 10,000*g* for 30 min at 4 °C was performed using a

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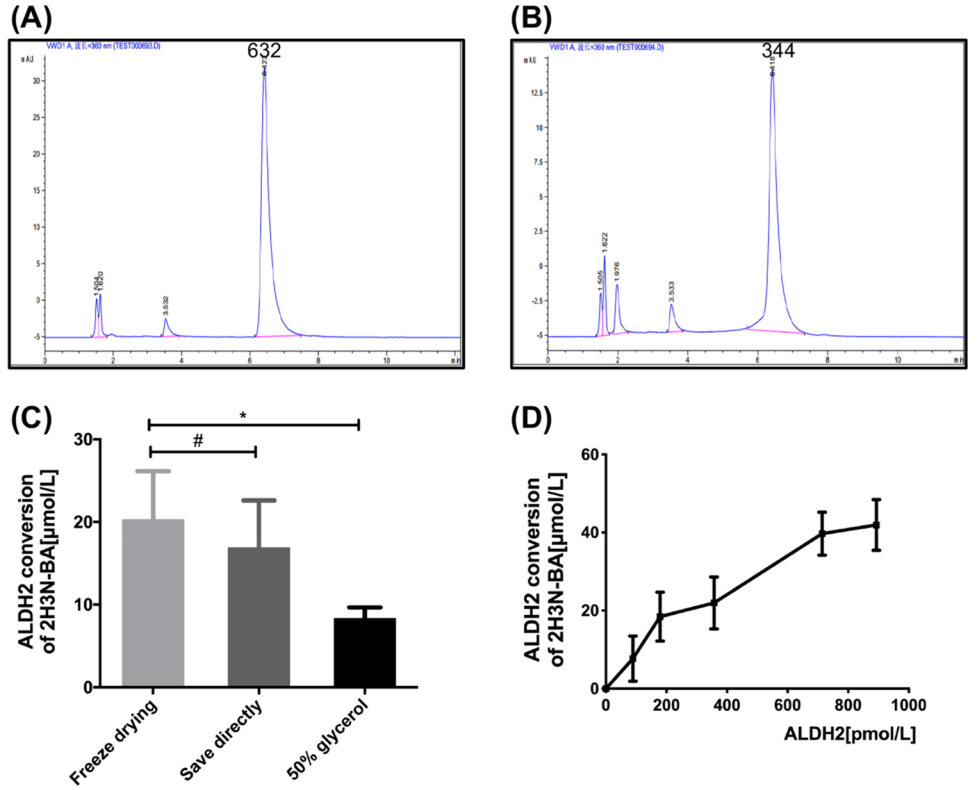

Figure 6. Detection of ALDH2 activity. Detection of 0 pmol/L (A) and 89 pmol/L ALDH2 catalytic substrate 2H3N-BA by HPLC (B). Detection of ALDH2 protein activity under different saving conditions (C). Different concentrations of the ALDH2 catalytic substrate 2H3N-BA by HPLC detection (D). Data are mean ± SD of five independent experiments. *, $P < 0.05$; #, $P > 0.05$.

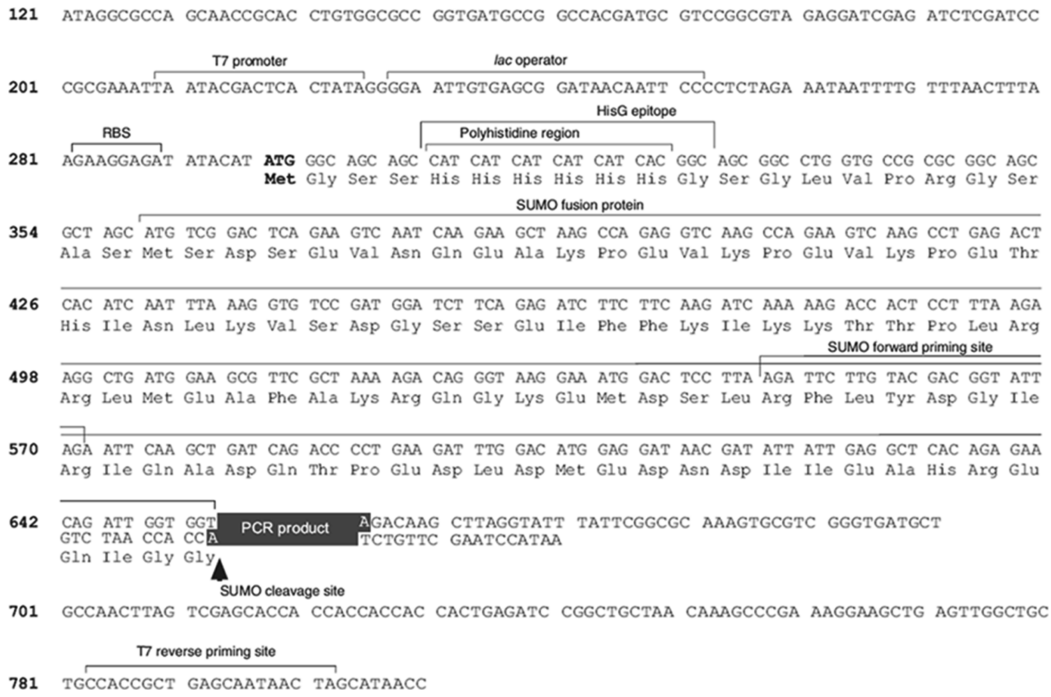

Figure 7. Multiple cloning site information of the pET-SUMO vector.

Centricon tube (Amicon Ultra15 Centrifugal Filter Devices, Millipore), and the denaturant agent was gradually removed.

4.5. Purification and Proteolytic Cleavage of Recombinant Protein. An AKTA Avant 150 (GE Healthcare Bioscience, USA) was used for protein purification. The supernatant containing the soluble fraction of His-SUMO-ALDH2 was loaded onto a 5 mL His-Trap affinity column chromatography machine (GE Healthcare Bioscience, USA) and equilibrated with wash buffer (20 mM sodium phosphate, 0.5 M NaCl, and 10 mM imidazole, pH 7.4). Then, an elution

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buffer (20 mM sodium phosphate, 0.5 M NaCl, and 250 mM imidazole, pH 7.4) was applied to elute the fusion protein. The elution fractions were collected and further loaded onto a Sephadex G-25 (16/40) column equilibrated with buffer (20 mM Tris-HCl and 50 mM NH_4COOCH_3). Ulp1 (Solarbio, China) was used to cut purified products with SUMO tags. Then, the cut products were subjected to second affinity and gel filtration chromatography, and finally, the target proteins were collected.

The other method was to purify the protein expressed in the form of IBs. Ulp1 was added to the refolding total protein according to the protein qualities and then incubated at 16 °C to release ALDH2 following the manufacturer's instructions (Invitrogen, USA).[31] The purification method of the proteolytic cleavage product of IBs was only one-step affinity and gel filtration chromatography. The purified protein was preserved in the following three groups: the first group was treated with 50% glycerol, the second group was treated by freeze-drying, and the third group was not treated and stored at −80 °C for 2 weeks until subsequent biophysical activity experiments.[32]

4.6. Mass Spectrometry Identification and Activity Analysis of ALDH2. IC-MS-MS (Q-TOF) (BGI, Beijing) was used to determine ALDH2. HPLC was used to analyze the activity of ALDH2 treated in different ways. ALDH2 (89 pmol/L) was preincubated with PBS containing 1 mM NAD^+, and activity was measured upon the addition of 100 μM 2H3N-BA and incubation for 30 min at 37 °C by its conversion to 2-hydroxy-3-nitrobenzoic acid using HPLC analysis.[33] In the same way, HPLC was used for analysis of freeze-dried ALDH2 enzyme catalysis kinetics at different concentrations (89, 179, 357, 714, and 893 pmol/L). The conversion product was subjected to an HPLC-based analysis on its absorbance at 340 nm as described previously.[34]

4.7. SDS-PAGE and Western Blot Analysis of Recombinant Protein. Protein concentration was determined by using a Micro bicinchoninic acid protein assay reagent kit (Beyotime, China) with standard bovine albumin. The protein loading quality was 10−20 μg. Each sample was identified by molecular weight using electrophoresis in SDS-polyacrylamide gel and analyzed after SDS-PAGE or western blot.[6] The 10% running gel separation proteins were washed with deionized water and stained with 0.1% Coomassie Brilliant Blue R-250 on a shaker for 2.5 h. The decolorizing solution was composed of 454 mL of methanol, 75 mL of glacial acetic acid, and 471 mL of deionized water. The liquid was replaced every 20 min until the decolorization was complete and then replaced with deionized water. The bands were analyzed by taking photos.

The 10% running gel separation proteins were subsequently transferred to a PVDF membrane (BioRad, USA). The membrane was incubated for 2 h in blocking solution containing 5% skimmed milk powder dissolved in pH 7.4 PBS contained with 0.05% Tween 20 (PBST). The membrane was washed three times before incubation with the primary His tag antibody and ALDH2 antibody (Abcam) overnight at 4 °C. The next day, the membrane was washed three times before incubation for 80 min at room temperature with a goat anti-mouse IgG/goat anti-rabbit IgG secondary antibody diluted (1:5000). After washing with PBST, the immunoreactive bands were detected using a Super ECL reagent (HaiGene, China).

4.8. Statistical Analysis. Data from independent experiments are shown as the mean ± standard deviation (SD). Statistical analysis between two groups was performed by two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant (*). All statistical analyses were completed using GraphPad Prism 8.0 software.

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Notes

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