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Article

Recombinant preparation and characterization of ADH1C and ALDH2 in alcohol metabolism

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SUMMARY

The observation that some individuals experience facial redness after consuming alcoholic beverages prompted this investigation. Alcohol metabolism, which involves the catalytic action of two key enzymes—alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)—with NAD^+/NADH as coenzymes, produces acetaldehyde (MeCHO), a toxic intermediate responsible for facial redness, nausea, and other health issues. With the increasing consumption of alcohol worldwide, alcohol-related health concerns have been drawing more attention and need to be addressed in more effective ways. We hypothesized that ADH and ALDH can be recombinantly purified and that they can detoxify alcohol *in vitro*. In this study, we cloned and expressed human ADH1C and ALDH2 in *Escherichia coli* cells and purified them using sequential chromatography. Enzymatic activity tests were performed using a microplate spectrophotometer that detected changes in NADH concentration. The results showed that both enzymes were active individually. We co-incubated ADH1C and ALDH2 with ethanol in the presence of NAD^+. Gas Chromatography-Mass Spectrometry (GC-MS) detected acetic acid, and no MeCHO accumulation was observed. This confirmed that both ADH1C and ALDH2 are essential for the detoxification of alcohol *in vitro*. The potential applications of recombinant ADH1C and ALDH2 to detoxify alcohol await further research on an effective encapsulation strategy for delivering enzymes through the complex environments of the gastrointestinal (GI) tract.

INTRODUCTION

Harmful alcohol consumption is a global concern. In 2016, the World Health Organization (WHO) reported that there were about 3 million alcohol-attributable deaths, including deaths from injuries, diseases, cancer, which adds up to 5.3% of all deaths globally (1). Upon ingestion, alcohol is absorbed in the gastrointestinal (GI) tract and then carried to the liver by the bloodstream for metabolism. Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) play major roles in ethanol metabolism in humans (**Figure 1**) (2). First, ADH functions to oxidize ethanol to acetaldehyde (MeCHO) in the presence of nicotinamide adenine dinucleotide (NAD^+) (2). Next, ALDH oxidizes MeCHO to acetic acid while NAD^+ is present (2). Finally, acetic acid decomposes to produce carbon dioxide and water via the tricarboxylic acid (TCA) cycle (3). MeCHO is highly reactive and can be harmful in the human body because it readily forms covalent bonds with proteins, DNA, and other cellular components, leading to cellular and tissue damage. This reactive feature makes MeCHO one of the most harmful byproducts, causing short-term phenomena including facial redness, nausea, and headaches, as well as long-term accumulation-induced chronic disease, such as liver damage or cancer (4-6).

Extensive research has been carried out to study both critical enzymes in alcohol metabolism. ADH and ALDH have many variants; since the different genotypes determine the diverse phenotypes, the response to alcohol consumption in different populations varies (7). Seven ADH genes are located on human chromosome 4, including *ADH1A, ADH1B, ADH1C,* and *ADH4-7*. The encoded ADH proteins may have more subclasses due to single nucleotide polymorphisms or key amino acid mutagenesis (7,8). A total of 19 ALDHs have been characterized in humans, among which ALDH1A1, ALDH2, and ALDH1B1 are most relevant to metabolizing MeCHO in the liver. *ALDH* is also known to have natural polymorphisms that may affect enzyme function (7,9,10). The collective enzymatic activities of ADH and ALDH are responsible for the overall response to alcohol consumption in different populations. Recent innovations explored a variety of strategies that manipulate the enzymatic activity of both enzymes to prevent the accumulation of MeCHO. Examples of these strategies include introducing a compound to increase the activities of both enzymes, intravenously administering both enzymes encapsulated by liposomes or erythrocytes, or oral administration of recombinantly engineered probiotics containing both enzymes (11-13).

We hypothesized that human ADH and ALDH enzymes can be recombinantly purified and that they can detoxify alcohol *in vitro*. Among the ADH and ALDH isozymes, ADH1B and ADH1C are most prominently linked to alcohol metabolism in the liver, with ADH1C being particularly well-expressed and frequently studied in liver tissue. ALDH2, likewise, is essential for liver function in alcohol metabolism and is a primary focus in research on alcohol-related metabolic pathways and toxicity (7, 14). Thus, in this study, we chose to focus on ADH1C and ALDH2 to further investigate the molecular mechanisms underlying alcohol metabolism and to explore potential treatments for facial redness. We first cloned, expressed, and purified both enzymes as Maltose Binding Protein (MBP)-fusion proteins in the recombinant expression system of *Escherichia coli*. *In vitro* activity assays showed that both enzymes are individually active. Next, we co-incubated ADH1C and ALDH2 with ethanol in the presence of NAD^+; using Gas Chromatography-Mass Spectrometry (GC-MS), we could detect that acetic acid concentration increased over time without the accumulation of MeCHO. Together,

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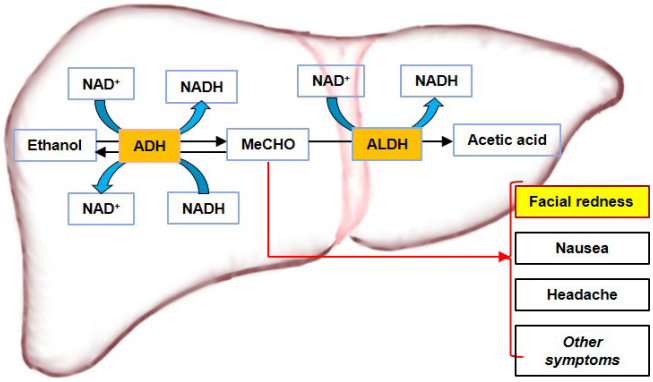

Figure 1: Two key enzymes in alcohol metabolism in the human liver. Ethanol is first converted to MeCHO in a reversible reaction catalyzed by ADH in the presence of NAD^+/NADH (left); MeCHO is converted to acetic acid by ALDH in the presence of NAD^+ (center). MeCHO, one toxic byproduct in the process, causes facial redness, nausea, headache, and other symptoms (right).

these results support our hypothesis that recombinant ADH and ALDH can detoxify alcohol *in vitro*. This paves the way for the potential application of these enzymes to alleviate the alcohol-induced toxic effects.

RESULTS

Cloning, expression, and purification of ADH1C and ALDH2

We first sought to purify ADH1C and ALDH2. *ADH1C* was subcloned into a 9C expression vector with an N-terminal His6 - MBP - Tobacco Etch Virus protease (TEVp) cleavage site tag (simplified as MBP hereafter) (**Figure 2A**). After confirmation via Sanger sequencing, the recombinant plasmid ADH1C-9C was transformed into *E. coli* strain Rosetta (DE3) cells. Expression was evaluated at 37°C or 16°C by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The amplified recombinant bacteria at 16°C were harvested and lysed using a high-pressure homogenizer. The supernatant was applied to the affinity column filled with amylose resin for initial purification (**Figure 2B**). Next, the eluted proteins were purified using sequential chromatography, including anion exchange and size exclusion chromatography (**Figure 2C**). Pure MBP-ADH1C fusion enzyme was concentrated to 1 mg/mL, flash frozen in liquid nitrogen (LN), and stored at -80°C (**Figure 2D**). Meanwhile, cloning, expression, and purification of ALDH2 were carried out using the same strategy as ADH1C (**Figure 3**). The result of SDS-PAGE showed that MBP-ALDH2 could also be expressed and purified in soluble form using the affinity column and following sequential chromatography (**Figure 3B**). The gel filtration elution profile of MBP-ALDH2 indicated a retention volume of 11 ml, corresponding to an approximate molecular weight of 400 kDa based on the standard elution profile provided by the column manufacturer (**Figure 3C**). Given that the molecular weight of MBP-ALDH2 is 101 kDa, we concluded that it exists as a tetramer. Pure MBP-ALDH2 fusion protein was concentrated to 1.1 mg/mL for later use (**Figure 3D**).

Initially, we attempted to remove the MBP tag from both fusion proteins using TEV protease digestion, but this was unsuccessful as cleaved ADH1C became unstable and

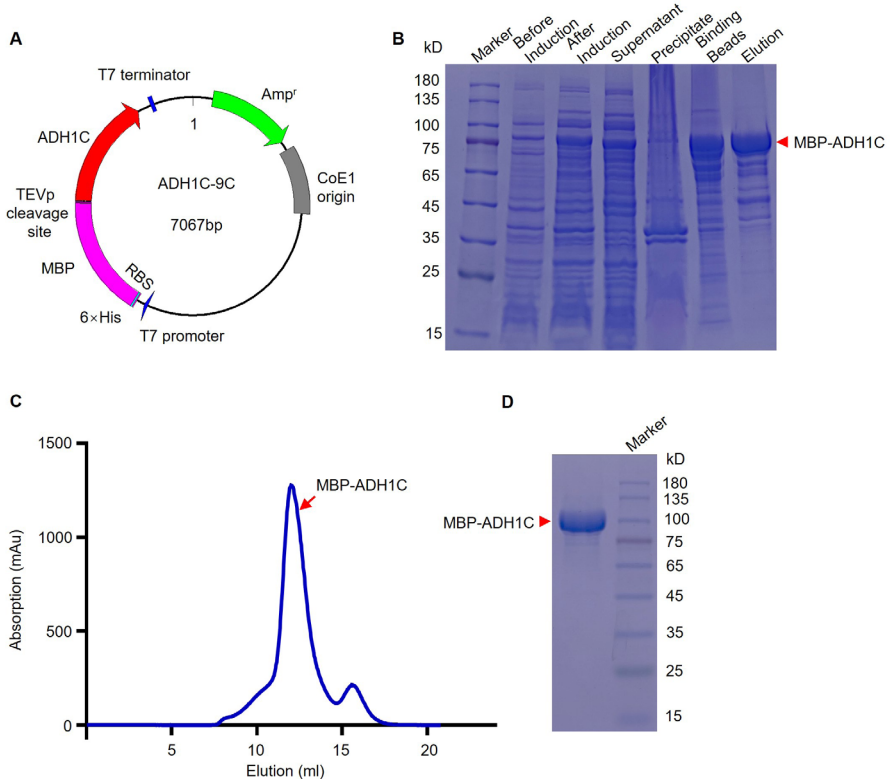

Figure 2: Cloning, expression, and purification of ADH1C. A) Construction of the recombinant expression plasmid, ADH1C-9C (Ampr, Ampicillin resistant; RBS, Ribosome binding site). B) SDS-PAGE to analyze the expression of ADH1C-9C. C) Gel filtration chromatography of MBP-ADH1C. D) SDS-PAGE of the final pure MBP-ADH1C fusion protein.

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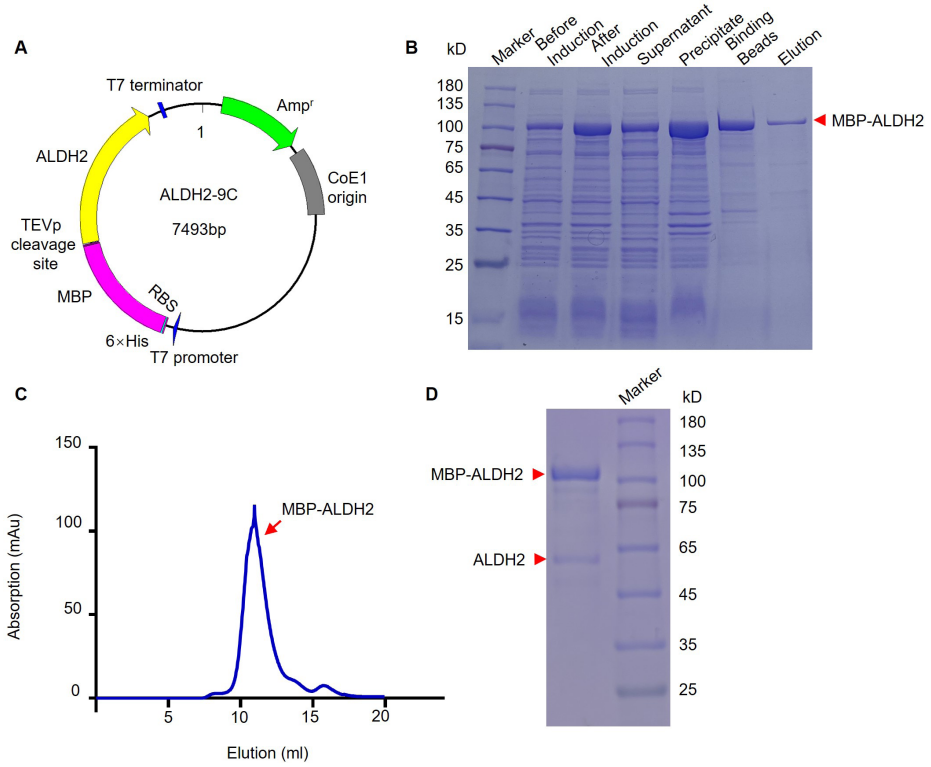

Figure 3: Cloning, expression, and purification of ALDH2. A) Construction of the recombinant expression plasmid, ALDH2-9C. B) SDS-PAGE to analyze the expression of ALDH2-9C. C) Gel filtration chromatography of MBP- ALDH2. D) SDS-PAGE of the final pure MBP-ALDH2 fusion protein.

ALDH2 tended to aggregate. This demonstrated that the MBP fusion strategy not only enhances stability but also improves yield.

ADH1C and ALDH2 are active individually

The enzymatic activity of ADH1C was assayed using a commercially available kit to detect changes in NADH concentration. The conversion between ethanol and MeCHO catalyzed by ADH1C is reversible (**Figure 1**). We measured the decrease of NADH in the reverse reaction when MeCHO was used as substrate in the presence of ALDH2 and NADH. According to the manufacturer's instructions, ADH1C enzymatic activity can be determined by calculating the decline in absorbance at a wavelength of 340 nm (A340), which indicated a reduction in NADH amount. One enzyme active unit (U) is the oxidation of 1 µmol of NADH per minute per milligram of protein. We observed that A340 gradually decreases over time under test conditions, which is consistent with our expectations (**Figure 4A**). According to the formula provided by the manufacturer, the final enzymatic activity of ADH1C was 0.024 U. Additionally, the reaction with purified MBP tag had no detectable change in the A340 values, which indicates that the MBP tag has no dehydrogenase activity (**Figure 4A**).

The enzymatic activity of ALDH2 was assayed using another commercially available kit via measurement of NADH levels. When ALDH2 converts MeCHO to acetic acid, NAD^+ is reduced to NADH. We observed that the A340 gradually

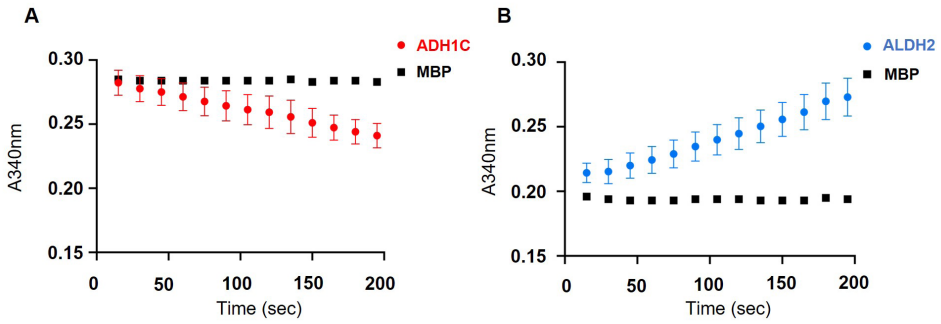

Figure 4: Enzymatic activity of individual ADH1C or ALDH2. The enzymatic activity of ADH1C and ALDH2 was assessed by monitoring the change in NADH concentration through spectrometry at 340 nm (A340). A) Graphic representation of the A340 decrease for ADH1C. B) Graphic representation of the A340 increase for ALDH2. MBP served as a negative control. Error bars represent the standard deviation from three independent experiments.

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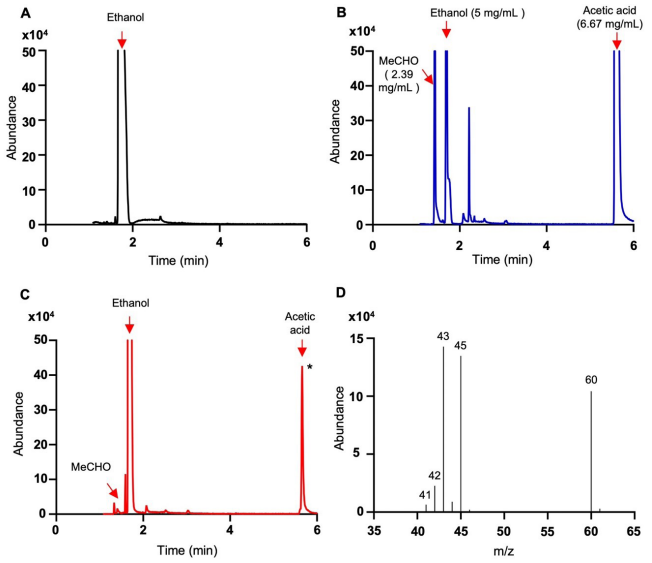

Figure 5: GC-MS analysis of the cooperation of ADH1C and ALDH2 in metabolizing ethanol. A) Gas chromatography of standard ethanol sample. B) Gas chromatography of standard ethanol (5 mg/mL), MeCHO (2.39 mg/mL), and acetic acid (6.67 mg/mL) mixture. C) Gas chromatography of the reaction of ADH1C and ALDH2 in metabolizing ethanol. The asterisk indicates the peak that was used for further analysis. D)The acetic acid peak labeled with an asterisk in panel C was analyzed by mass spectrometry.

increased over time, indicating that NADH amounts were increasing and that ALDH2 was active (**Figure 4B**). The amount of enzyme for catalytic reduction of 1nmol NAD^+ per milligram of protein per minute is one enzyme active unit. According to the formula provided by the manufacturer, the final enzymatic activity of ALDH2 was 21.49 U. In addition, the reaction with purified MBP tag had no detectable change on the A340, indicating that the MBP tag has no dehydrogenase activity (**Figure 4B**).

Co-incubation of ADH1C and ALDH2 could convert ethanol to acetic acid

In the human body, ethanol is metabolized by the stepwise cooperation of ADH and ALDH and converted to nontoxic acetic acid. Next, we measured the *in vitro* cooperation of purified ADH1C and ALDH2 in metabolizing alcohol. Briefly, we co-incubated ADH1C and ALDH2 at a molar ratio of ~1:1 in the presence of NAD^+ (1 mM) and ethanol substrate (15% v/v) for 4.5 min at room temperature. The reaction mixture was then subjected to detection using GC-MS. Standard ethanol, MeCHO, and acetic acid samples could be detected in GC-MS with specific peaks and were used as references (**Figure 5A-B**). It was shown that ethanol could be converted to acetic acid by purified MBP-ADH1C and MBP-ALDH2 under test conditions (**Figure 5C-D**, **Table 1**). Notably, MeCHO did not accumulate obviously in this *in vitro* reaction system (**Figure 5C**, **Table 1**), suggesting that these purified enzymes could potentially be used to detoxify alcohol.

DISCUSSION

The N-terminal MBP tag aids the recombinant preparation of ADH1C and ALDH2 without affecting their enzymatic activity. Each enzyme was fused with a His-MBP tag to enhance solubility and aid purification via affinity chromatography (**Figure 2A**, **3A**). While a TEVp cleavage site was included to remove the tags, cleaved ADH1C became unstable, and ALDH2 tended to aggregate. These observations are in line with prior reports that ALDH is primarily expressed as inclusion bodies in the *E. coli* system (15,16). We tested various tags, including the His, Glutathione-S-transferase (GST), and MBP tags. We found that the MBP tag significantly increased the soluble yield in *E. coli*, consistent with its known role in improving protein solubility (17). Enzymatic assays confirmed that the His-MBP tag had no dehydrogenase activity or impact on the enzymes. Thus, both fusion proteins were purified for this study, and this strategy may enhance future applications by boosting recombinant yield.

The combinations of ADH and ALDH lead to various responses to alcohol ingestion. It is generally accepted that different ADH or ALDH variants have diverse enzymatic activities (7). Therefore, combinations of variants of both enzymes are responsible for the response to alcohol intake. The imbalance of ADH and ALDH's activities may lead to the accumulation of ethanol when the relative activity of ADH is significantly lower than that of ALDH. In this case, ethanol may be metabolized very slowly or removed by other remediation pathways (18). On the other hand, when the relative activity of ADH is significantly higher than ALDH, it may lead to the accumulation of toxic MeCHO. This is commonly observed in the Asian population with deficient ALDH2 variants (19). Those people generally have more severe facial redness after alcohol consumption and may have potential liver damage and cancer risk in the long run (5,6). In this study, we tested whether the use of purified ADH1C and ALDH2 could have potential for combatting facial redness following alcohol consumption. Comprehensive enzymatic activity assays for all ADH and ALDH variants are currently unavailable, making it challenging to determine which enzymes exhibit the highest activity. Among the ADH variants, ADH1B and ADH1C are most closely associated with alcohol metabolism in the liver, with ADH1C being particularly well-expressed and extensively studied in liver tissue (7, 14). Similarly, ALDH2 plays a critical role in alcohol metabolism and is a central focus in research on alcohol-related metabolic pathways and toxicity (7, 14). Therefore, this study concentrated on ADH1C and ALDH2. *In vitro* experiments confirmed that when ADH1C and ALDH2 proteins were present at a molar ratio of approximately 1:1,

Name (RT, min)	Std Sample Con (mg/mL)	Std Sample Peak Area	Sample Peak Area	Sample Con (mg/mL)
MeCHO (1.43)	2.39	725500	21957	0.072
Ethanol (1.7)	5	634584	2896504	22.822
Acetic acid (5.57)	6.67	3702792	258041	0.465

Table 1: GC-MS result of the co-incubation of ADH1C and ALDH2 sample. In gas chromatography, the concentration of each component corresponds to its peak area. We started by mixing three standard samples with known concentrations, which we then analyzed using gas chromatography and measured their peak areas. Next, we examined the co-incubated sample of ethanol with both enzymes using the same method to measure the peak area of each component. By comparing these peak areas to those of the standards, we could calculate the concentrations of the components in our sample. RT, retention time of gas chromatography; Std, standard; Con, concentration.

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they effectively converted ethanol to acetic acid with minimal MeCHO accumulation. This indicates that the combination of both enzymes could offer a potential therapy to reduce facial redness and other alcohol-induced effects in individuals with low-activity ADH/ALDH variants.

ALDH functions as a tetramer, increasing the potential for regulating its enzymatic activity (20-21). We observed that purified ALDH2 forms a homo-tetramer (**Figure 3C**). It is known that incorporating a single ALDH2*2 subunit (ALDH2 E504K), an inactive variant common in Asian populations, into the tetrameric ALDH2 structure nearly eliminates the enzyme's activity (19, 22). This suggests that ALDH2's enzymatic function relies on the cooperative interaction of all four subunits. This interdependence adds complexity to future research on high-activity ALDH2 variants, which could potentially enhance the metabolism of MeCHO more effectively.

The reversible reaction catalyzed by ADH may be a potential self-protective mechanism in the human body. Previous studies have shown that ADH can catalyze the interconversion between ethanol and acetaldehyde (MeCHO) (23). In our study, we also tested the enzymatic activity of ADH1C by detecting the reverse reaction. This suggests that as MeCHO accumulates, excess MeCHO can be converted back to ethanol by ADH. Although this may prolong the metabolism and clearance of ethanol, it prevents the excessive accumulation of MeCHO, which could serve as a potential protective mechanism.

This study has a few limitations. First, we only investigated ADH1C and ALDH2, and did not investigate other isozymes in detail. Second, while the results show that the recombinant enzymes can effectively metabolize alcohol *in vitro*, additional *in vivo* experimental validation is needed.

To further advance this research, we plan the following studies. First, we will broaden our investigation to include other isozymes in the ADH and ALDH families, using the methods developed in this study to identify candidates with high stability and activity. Second, we will conduct activity tests under simulated gastric conditions, exploring different encapsulation or coating strategies to extend the release and survival of the recombinant enzymes in conditions resembling that of the GI tract. Finally, we will perform mouse experiments to assess the effectiveness of these recombinant enzymes in alcohol metabolism, laying the groundwork for future clinical trials.

In summary, this study showed that human ADH1C and ALDH2 could be recombinantly prepared using the MBP-fusion *E. coli* expression system, and the purified MBP-fused ADH1C and ALDH2 could cooperate to convert ethanol to acetic acid efficiently *in vitro*. Considering the complicated environments of the GI tract, especially the extensive distribution of proteases, the potential applications of recombinant ADH1C and ALDH2 to detoxify alcohol in GI tract await further research on an effective encapsulation strategy for delivering enzymes (24).

MATERIALS AND METHODS

Recombinant plasmid construction

The plasmids containing the coding sequences of human *ADH1C* (1128 bp, NM_000669, Youbio, Cat# G120224) and *ALDH2* (1554 bp, NM_000690, Youbio, Cat# G113419) were used as templates. The primers were designed using Oligo 6 and synthesized by Sangon. ADH1C was sub-cloned with these primers (Forward: 5' TACTTCCAATCC AATGCAATGAGCACAGCAGGAAAAGTAATC 3' and reverse: 5' TTATCCACTTCCAATGTTATTATCAAAACGTCA GGACGGTACGG 3'), while ALDH2 was sub-cloned with these primers (Forward: 5' TACTTCCAA TCCAATGCAATGTTGCGCGCTGCCGCCC 3' and reverse: 5' TTATCCACTTCCAATGTTATTATTATGAGTTCTTCTGAGGC ACTTTGAC 3'). The amplified PCR fragments of the target genes were inserted into a 9C expression vector (Addgene, Cat# 48286) using a ligation-independent cloning strategy (25). The amplified fragments and linearized 9C vector were mixed at a molar ratio of 3:1, supplemented with T4 DNA polymerase (NEB, Cat# M0203), and incubated for 30 min at room temperature. Next, 4 µL of the ligation mixture was transformed into 100 µL *E. coli* strain DH5α competent cells pre-thawed on ice (Weidi Bio, Cat# DL1005) using the heat shock method. Briefly, the cell-DNA mixture was placed in an ice bath for 30 min, then transferred to a water bath at 42°C for 90 s, and then returned to an ice bath for 2 min. Next, the cells were supplemented with 900 µL of antibiotic-free LB medium containing 10 g/L tryptone (OXOID, Cat# LP0042B), 5 g/L yeast extract (OXOID, Cat# LP0021B), and 10 g/L NaCl (Sinopharm, Cat# 10019360) and grown in a shaker at 37°C, 220 rpm for 40 min. The cell sample was centrifuged at 18000 x g for 1 min at 4°C, and 800 µL supernatant was removed. The remaining cell sample was resuspended, spread on an LB agar plate prepared with LB medium containing 15 g/L agar (Acmec, Cat# A48392) and supplemented with 100 µg/mL ampicillin (Sangon, Cat# A610028), then incubated at 37°C for about 12 hours.

Single colonies of the transformed strain were inoculated into 2 mL of LB medium with ampicillin, and incubated overnight, before sequencing was carried out using the Applied BiosystemsTM 3730XL DNA analyzer by Tsingke. After sequence confirmation, the recombinant strain was amplified as detailed above, and the plasmid was extracted using the Mini Plasmid Kit (TIANGEN, Cat# Y1324) according to the manufacturer's manual.

Cell growth

Using the heat shock method, 1 µL of the recombinant plasmid was transformed into 100 µL *E. coli* BL21 Rosetta2 competent cells pre-thawed on ice (Weidi Bio, Cat# EC1014). Single colonies of the transformed strain were obtained and inoculated individually into 10 mL of LB medium. The cells were grown overnight in a shaker at 37°C, 220 rpm. The *E. coli* strain was inoculated in 1L LB culture at a 1:100 dilution ratio and incubated at 37°C, 220 rpm, until the OD600 reached 0.6–0.8. The cell culture was supplemented with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Yeasen, Cat# 10902ES60) and incubated for 16-20 hours at 16°C before collection.

Protein purification

Cells were harvested by centrifugation at 4000 g for 20 min at 4°C. The cell pellet was re-suspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% v/v glycerol) (40 mL buffer/L cell culture) and lysed using a high-pressure homogenizer (Yonglian, UH-06). After lysis, samples were centrifuged at 42000 x g for 30 min at 4°C to remove insoluble debris.

The supernatant was applied to gravity columns filled with 5 mL amylose resin (NEB, Cat# E8021L) at 4°C. After a thorough wash, the target fusion proteins were eluted by

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lysis buffer supplemented with 10 mM maltose (Sigma, Cat# 63418). Next, the eluted proteins were purified using sequential chromatography, including anion exchange (5 mL HiTrap Q column, Cytiva, Cat# 17115401) and size exclusion (Superdex 200 10/300 GL column, Cytiva, Cat# 28990944) chromatography. The final pure proteins were concentrated, aliquoted, flash-frozen in liquid nitrogen, and stored at -80 °C for later use.

Enzymatic assays

The enzymatic activity of ADH1C was assayed using a commercially available kit (Qiyuan Bio, Cat# 231029). In short, 20 µL of ADH1C (1 mg/mL) or MBP tag (lab purified, 1 mg/mL) was added to the reaction system containing 180 µL buffers supplemented with MeCHO and NADH, provided by the manufacturer. Then, the A340 values of the reaction mixtures were measured every 15 seconds using a spectrophotometer (Biotek, SynergyH1). The enzymatic activity of ADH1C was calculated according to the manufacturer's manual.

The enzymatic activity of ALDH2 was assayed using another commercially available kit (Qiyuan Bio, Cat# 231032). Forty µL of ALDH2 (1.1mg/mL) or MBP tag (lab purified, 1 mg/mL) was mixed with 160 µL buffers containing MeCHO and NAD^+, provided by the manufacturer. Then, the A340 values of the reaction mixtures were measured every 15 seconds using a spectrophotometer. The enzymatic activity of ALDH2 was calculated according to the manufacturer's manual.

Gas Chromatography-Mass Spectrometry

To measure the cooperation of ADH1C and ALDH2 in metabolizing alcohol *in vitro*, 20 µL ADH1C (1 mg/mL) and 20 µL ALDH2 (1.1 mg/mL) were added into a 200 µL reaction system containing 5 mM Tris-HCl PH 8.0, 75 mM NaCl, 1 mM NAD^+ (Psaitong, Cat# 53849), and ethanol substrate (15% v/v). After incubation for 4.5 min at room temperature, the reaction mixture was analyzed using GC-MS (Agilent Technologies 6890N Network GC System and Agilent Technologies 5975B inert XL EI/CI MSD). A standard sample of 15 mg/mL ethanol (Analytical Reagent (AR), Sinopharm, Cat# H1Y0424) and a mixture of 5 mg/mL ethanol, 2.39 mg/mL MeCHO (AR, Macklin, Cat# A823262), and 6.67 mg/mL acetic acid (AR, Sinopharm, Cat# 100002610) were also analyzed using GC-MS as references.

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