Hemiacetal Dehydrogenation Activity of Alcohol Dehydrogenases in Saccharomyces cerevisiae

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Some methylotrophic yeasts produce methyl formate from methanol and formaldehyde via hemiacetal formation. We investigated Saccharomyces cerevisiae to find whether this yeast has a carboxylate ester producing pathway that proceeds via hemiacetal dehydrogenation. We confirmed that the purified alcohol dehydrogenase (Adh) protein from S. cerevisiae can catalyze the production of esters. High specific activities were observed toward the hemiacetals corresponding to the primary alcohols when ether groups were substituted for methylene groups, resulting in the formation of formate esters. Both ADH and methyl formate synthesizing activities were sharply reduced in the Adh1 Adh2 mutant. The ADH1 and ADH2 genes encode the major Adh proteins in S. cerevisiae. Thus, it was concluded that the S. cerevisiae Adh protein catalyzes activities for the production of certain carboxylate esters.

Key words: alcohol dehydrogenase; Saccharomyces cerevisiae; carboxylate ester synthesis; hemiacetal dehydrogenation

The production of aliphatic carboxylate esters is a phenomenon widely observed in various yeasts, but the functions of these compounds are unknown. The esters, 3-methyl-1-butyl acetate (isoamyl acetate), ethyl acetate, ethyl hexanoate (ethyl caproate), and 2-phenylethyl acetate are typical of the carboxylate esters produced by yeasts of the genus Saccharomyces, which are widely used in brewing. Two kinds of ester synthetic pathway have been proposed for yeast. One involves the transfer of an acyl group from acyl-CoA to alcohol, catalyzed by alcohol acyltransferase2 or alcohol acetyltransferase (AATase).3 To date, the AATases involved in the synthesis of 3-methyl-1-butyl acetate and ethyl acetate have been purified from Saccharomyces cerevisiae,4,5 and the genes encoding these enzymes have been cloned as ATFl6 and ATF2.7 The other pathway comprises the reverse reaction of esterases, in which esters are made directly from alcohols and carboxylates. Certain esters, especially the ethyl esters, have been reported to be synthesized by this pathway.7

We have observed that methylotrophic yeasts produce a large quantity of methyl formate. This activity was considered to result from an alternative formaldehyde oxidation pathway. In this reaction, methanol replaces glutathione to form a hemiacetal with formaldehyde in solution, then methyl formate is produced from the hemiacetal by dehydrogenation.8 This methyl formate synthesizing activity was isolated from the mitochondrial fraction of Pichia methanolica and Candida boidinii, and was named "methyl formate synthase (MFS)". By purification and characterization of this enzyme from these yeast strains, it was shown that this enzyme is an alcohol dehydrogenase that is specific for medium to higher chain length alcohols. Also the N-terminus amino acid sequence of this enzyme had high similarity to that of the S. cerevisiae alcohol dehydrogenase (Adh1) protein.9

The synthetic pathway of methyl formate via a hemiacetal is somewhat similar to the alcohol dehydrogenase reaction mechanism by which alcohols are converted into aldehydes (Fig. 1). Thus, this reaction and the activity catalyzing it is referred to as hemiacetal dehydrogenation (HADH) and HADH activity. In this study we investigated whether S. cerevisiae has a synthetic pathway for carboxylate esters involving HADH activity in addition to the known pathways.

Fig. 1. Reactions Catalyzed by Alcohol Dehydrogenase (ADH), and Its Hemiacetal Dehydrogenase (HADH) Activity.

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Abbreviations: HADH, Hemiacetal dehydrogenation; MFS, Methyl formate synthase or Methyl formate synthesis; EFS, Ethyl formate synthesis; MAS, Methyl acetate synthesis; EAS, Ethyl acetate synthesis; ADH, Alcohol dehydrogenase
Materials and Methods

Chemicals. NAD⁺, NADH, NADP⁺, and NADPH were purchased from Oriental Yeast (Osaka, Japan). Alcohol dehydrogenase from baker’s yeast was purchased from Sigma (Product number: A7011). Other reagents were reagent grade or higher and were purchased from local commercial sources unless otherwise stated in the text.

Gas chromatography (GC). The head-space gas chromatography units HSS-4A and GC-17A (Shimadzu, Kyoto, Japan) were used. Data was analysed using a Chromatopac CR-7A (Shimadzu). The columns and the conditions used are described elsewhere in this report.

Yeast strains. Saccharomyces cerevisiae TD4 (MATα leu2-3, 112 ura3 his4 trp1 can1) was used as the parent strain.

Media.YPD medium contained 1% yeast extract, 2% peptone, and 2% d-glucose. Solid media were made by adding 2% agar before autoclaving. The media were solidified in sterilized laboratory dishes. Yeast minimal medium contained 0.67% yeast nitrogen base w/o amino acids (Difco), 2% d-glucose and an amino acid mix without leucine, uracil, or leucine and uracil for marker selection (LEU2, URA3, or LEU2 and URA3, respectively). Solid media contained 3% agar.

Yeast cultivation. All strains were first incubated at 30°C in 5 ml of YPD for 16 to 24 h. This seed culture was added to 100 ml YPD in a 500-ml shaking flask to a density equivalent to an OD600 of 0.05 to inoculate the main cultures.

Measurement of volatile components in the culture media. A sample of culture was collected and centrifuged at 18,000 × g for 5 min. A 4-ml sample of the supernatant was used in GC analysis. The conditions were: column: DB-WAX (J&W Scientific, 30 m × 0.53 mm); syringe temperature: 140°C; vial temperature: 40°C; vial warming time: 15 min; injector temperature: 200°C; detector temperature: 200°C; column temperature program: 0 min, 40°C; 5 min, 40°C; 15 min, 140°C; 18 min, 140°C. Acetaldehyde, ethanol, ethyl acetate, 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butyl acetate, and 3-methyl-1-butanol were measured by the absolute standard method.

Preparation of cell-free extract. All procedures were done at 4°C or on ice. A sample of culture medium equivalent to an OD600 of 100 was collected (if the OD600 was less than 10, then 10 ml was collected). Cells were collected by centrifugation at 12,000 × g for 5 min. The cells were then washed with 5 ml of distilled water once and twice with 5 ml of buffer A (10 mM potassium phosphate, 0.1 M potassium chloride, 10%(v/v) glycerol, and 1 M dithiothreitol, pH 7.5). Then 0.5 ml of buffer A and 1.5 g of acid-washed glass beads (425–600 mesh) were added to the cells. The cells were disrupted by vigorous shaking for 10 min using a Taiyo Mix-Tower A-14 (Tai Tec, Japan). After the supernatant was transferred to another tube, the residues were washed twice with 0.5 ml of buffer A, and the washing solutions were added to the supernatant in the new tube. This solution was centrifuged at 18,000 × g for 20 min, and the supernatant was collected as the cell-free extract.

ADH assay. The reaction mixtures (1 ml) contained cell-free extract, alcohol as a substrate (200 μmol of methanol, ethanol, 1-propanol, or 1-butanol; 100 μmol of 2-butanol; 50 μmol of 2-pentanol) and 100 mM Tris·HCl, pH 8.5. The reaction was started by the addition of 40 μl of 125 mM NAD(P)⁺ (5 μmol). After 5–10 min of reaction at 25°C, the amount of NAD(P)H produced was calculated by measuring the change in absorbance at 340-nm absorbances. The control was treated by the same procedure and contained a reaction mixture without alcohol.

HADH Assay. The reaction mixtures (1 ml) contained 500 μmol alcohol (methanol or ethanol), 50 μmol aldehyde (formaldehyde or acetaldehyde), and 100 mM potassium phosphate, pH 6.8. These mixtures were put into GC vials (for head space GC) and sealed. The reaction was started by the addition of 40 μl of 375 mM NAD(P)⁺ (15 μmol). After 2 h (except MFS which was incubated for 15 min) incubation at 25°C, the reaction was stopped by the addition of 500 μl of 0.5%(v/v) H2SO4 containing 60 ppm 2-propanol (in methyl formate synthesis: MFS), 2-butanol (in methyl acetate synthesis: MAS) or 1-butanol (in ethyl formate synthesis: EFS and ethyl acetate synthesis: EAS) as an internal standard. The esters formed were measured by GC with internal standards. The GC conditions were as follows: (a) for MFS, MAS, and EAS; column: Gaskuro pack 54 60/80 (GL Science, 2 m × 33 mm packed column); syringe temperature: 60°C; vial temperature: 55°C; vial warming time: 15 min; injector temperature: 200°C; detector temperature: 200°C; column temperature program: 0 min, 40°C; 5 min, 40°C; 15 min, 140°C; 18 min, 140°C. Acetaldehyde, ethanol, ethyl acetate, 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butyl acetate, and 3-methyl-1-butanol were measured by the absolute standard method.

Construction of ADH1 and/or ADH2 gene disrupted strains. The ADH1 or ADH2 gene was disrupted by replacement of its open reading frame by the URA3 or LEU2 gene, respectively. Fragments for the disruptions were constructed by PCR, and introduced into the parental strain, TD4. Gene disruptions were confirmed by genomic Southern blot analysis of both the TD4 and adh strains, using the ADH1 or ADH2 open reading frame sequences as probes.

Protein Assay. Protein was measured by the method of Bradford, using bovine serum albumin as a standard.

Results

Estimation of HADH activity of the S. cerevisiae Adh protein
Table 1. Confirmation of the HADH Activities of a Commercial Adh Protein. Compounds in parentheses indicate either the substrate (for ADH) or the products (for HADH).

<table>
<thead>
<tr>
<th>Coenzymes</th>
<th>Activities</th>
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<tbody>
<tr>
<td>ADH (ethanol)</td>
<td>NAD⁺</td>
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<tr>
<td></td>
<td>NADP⁺</td>
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<tr>
<td>HADH (methyl formate)</td>
<td>NAD⁺</td>
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<td></td>
<td>NADP⁺</td>
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<tr>
<td></td>
<td>none</td>
</tr>
<tr>
<td>HADH (ethyl acetate)</td>
<td>NAD⁺</td>
</tr>
<tr>
<td></td>
<td>NADP⁺</td>
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<td></td>
<td>none</td>
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The ADH activity toward each alcohol was correlated well with the reported data. Straight chain primary alcohols were better substrates for ADH than the straight chain secondary alcohols. Methanol was not recognized as a substrate.

Investigation of HADH activity showed that the hemiacetals in which the methylene group had been substituted with the ether group and which corresponded to the primary alcohols (methyl formate, ethyl formate, etc. as products) were much better substrates than the hemiacetals corresponding to the secondary alcohols (methyl acetate, ethyl acetate, etc.). These results suggested that the HADH activity of the S. cerevisiae Adh proteins only acted on hemiacetals with formaldehyde as the aldehyde moiety.

**HADH activities of the ADH1 and/ or ADH2 gene disruptant strains**

To investigate the HADH activity in vivo, a mutant lacking the genes encoding the Adh proteins was constructed. S. cerevisiae has five ADH genes. The ADH1 gene encodes the protein which is active during fermentative conditions, while the ADH2 gene encodes the glucose-repressed protein. ADH3 encodes a mitochondrial protein, and ADH5 encodes a protein characterized by its similarity to the Adh proteins described above as a result of DNA sequencing by the Saccharomyces genome project (its function is still unknown). These four Adh proteins have high similarity to each other at the amino acid sequence level. The ADH4 gene encodes a rather different protein, similarity to ADH2 protein of Zymomonas mobilis, its function also remains unknown. Based on this information, the ADH1 and/or ADH2 genes, which encode the major proteins of the five independent genes, were disrupted and their HADH activities were estimated. Three kinds of disruptants were constructed, these were Δadh1, Δadh2, and Δadh1 Δadh2. The gene disruptions were confirmed by

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Since the Mfs protein of methylotrophic yeast was reported to an Adh protein, the ADH, MFS, and EAS activities of a commercial preparation of baker’s yeast (S. cerevisiae) were measured to find whether the Adh protein has HADH activity. The results are shown in Table 1. Both methyl formate and ethyl acetate were synthesized by the Adh protein, suggesting that this protein has HADH activity. Since the HADH activity was greater than ADH activity when NADP⁺ was used in place of the NAD⁺ coenzyme, the coenzyme specificity of HADH was thought to be lower than that of ADH.

**Substrate specificity of HADH activity**

It is thought that HADH activity is derived from the Adh proteins’ recognition of the hemiacetals formed from alcohols and aldehydes as a kind of alcohol. Therefore, it was considered that the relative substrate specificity of HADH activity would be measured using alcohols that had the methylene group replaced by the ether group of the hemiacetals. The ADH and HADH specificities of the Adh protein were then examined using various alcohols and hemiacetals as substrates (Fig. 2).

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**Fig. 2. Substrate Specificities of ADH and HADH Activities.**

The substrates and the products of hemiacetals dehydrogenation are shown on the left, and the alcohol formulas are shown on the right. The alcohols with outlined ethylene groups shown on the right correspond to the hemiacetals on the left. The ethylene group is replaced by an ether group in the hemiacetal. Numbers show the degree of ADH activity compared to ethanol (which has a value of 100).
Southern hybridization analysis (data not shown). These three disruptants and their parent strains were grown in YPD liquid medium with shaking at 30°C. The course of cell growth (measured as the OD600 of the culture), the major volatile flavor components in the medium (Fig. 3), and the ADH and MFS (as HADH) activities (Fig. 4) were examined. Basically, the profiles of the courses of the ADH and MFS activities resembled each other. Both ADH and MFS activities of the Adh1 strain were repressed during the early growth stage, but these activities increased to a level greater than the parent strain at the latter stage of cultivation. On the other hand, the activities of the Adh2 strains decreased equally, irrespective of cultivation time, compared with the parent. Both activities of the Adh1 Adh2 strain decreased sharply. Thus, it was concluded that the Adh proteins not only have ADH activity, but also have HADH activity in S. cerevisiae.

Discussion

It was shown for the first time that the Adh proteins in S. cerevisiae can catalyze the production of carboxylate esters from alcohols and aldehydes via hemiacetal formation, in addition to their function in the oxidation of alcohols or the reduction of aldehydes.

Although the coenzyme NAD⁺ is essential for alcohol (ethanol) oxidation by ADH, NADP⁺ was able to fill the role of NAD⁺ to some degree in MFS/EAS (HADH) activity. The reason for this phenomenon remained unclear, but we think that the electronic environment around the catalytic center of the enzyme was changed by substitution of an ether group for the methylene group adjacent to the dehydrogenated carbon. As a result NAD⁺, which is not recognized as a coenzyme in ADH, was able to play the NAD⁺ role in HADH activity (No NAD⁺ was detected from the commercial NAD⁺ using an anion exchange HPLC analysis, data not shown). Although we have hypothesized that the HADH activities belong to the Adh proteins, we only observed the effects of Adh1 and/or Adh2 gene disruption HADH activities described in this study. This is because both the ADH and MFS (taken as HADH) activities of the double gene disruptant strain were reduced to about 10% and 3%, respectively, compared with the parent strain. Thus, most of the ADH and MFS activities were accounted for by the Adh1 and Adh2 transcripts. To demonstrate that the HADH activities were due only to the Adh proteins, it must be shown that ADH and HADH activities are completely lost when not only Adh1 and Adh2, but also the Adh3 and Adh5 (and Adh4, if needed) genes are simultaneously disrupted.

Research on the formaldehyde tolerance mechanism in S. cerevisiae showed that SFAI (Sensitive to FormAl-
dehydroy (glutathione-dependent formaldehyde dehydrogenase), is a resistance gene. 17) The tolerance mechanism was demonstrated by radioisotope tracer experiments as follows; formaldehyde is oxidized by the Sfa1 protein to formate, which is further oxidized to carbon dioxide by the formate dehydrogenase-like protein, then formaldehyde is detoxified. 18) A strain overexpressing ADH1 appeared to be hyper-resistant to formaldehyde, suggesting that formaldehyde is detoxified by the Adh1 protein by reduction to methanol. The Sfa1 protein was characterized as a long chain alcohol specific ADH (glutathione dependent formaldehyde dehydrogenase), the reaction mechanism involves the dehydrogenation of the thiohemiacetal of formaldehyde and glutathione. However, little homology is observed between Sfa1 and ADH at the amino acid level. It is possible that the Sfa1 protein is involved in HADH activity, but this is unlikely as most HADH activity was abolished when the ADH1 and ADH2 genes were both disrupted. Also, the glutathione dependent formaldehyde dehydrogenase in the methylotrophic yeasts generally does not show HADH activity. 3)

Compared with the parent or ADH2 strain, ethyl acetate formation by ADH1 and ADH1 ADH2 were reduced, but their growth rates were delayed and reduction of 2-methyl-1-propanol and 3-methyl-1-butanol (branched alcohols) production were much higher than that of ethyl acetate. Diminution of branched alcohol formation might be owing to the decrease of carbon metabolism caused by poorer growth. So it was thought that ADH gene disruption might not directly reduce in the ability to produce ethyl acetate by the EAS reaction, but might have an indirect effect due to poor growth. Thus, the EAS activity of Adh proteins may not contribute greatly to the production of ethyl acetate in vivo in S. cerevisiae.

It will be of interest to investigate whether the Adh proteins in other kinds of yeasts generally show HADH activities. It will be of particular interest to find whether yeasts that produce high levels of esters show these activities, as this will help to discover the roles of esters in yeast cells.

References


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