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Use of novel assays to indicate that O-esters and S-esters are produced by the same enzyme in brewing yeast

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Abstract

Ethanol- and methanethiol-dependent removal of acetyl-CoA by crude extracts of ale yeast has been monitored using a decrease in OD_{232}. Activity has also been detected in these extracts after fractionation on polyacrylamide gels, in this case using a novel assay in which the coenzyme A produced in the reaction is linked via DCPIP reduction to color formation from nitroblue tetrazolium. Ethanol- and methanethiol-dependent activities migrate identically on such gels, and only one band of color formation was observed. Furthermore they displayed closely similar sensitivity to heating at 40°C and 60°C and pH optima, with activity maximal at pH 7.5. It is likely that a single enzyme is responsible for the formation of O-esters and S-esters in yeast. Initial kinetic studies indicate that methanethiol has higher affinity for the enzyme than has ethanol and a higher maximum velocity. However, the enzyme has a much lower K_m for acetyl-CoA, suggesting that the alcohol or thiol substrate is the more likely substrate to be limiting.

Keywords: Ethanol; Methanethiol; Ethyl acetate; Methyl thioacetate; Acetyl-CoA; Alcohol acetyl transferase

1. Introduction

Various esters can make a sizeable contribution to the flavor of beer [1]. These include O-esters, such as ethyl acetate and *iso*-amyl acetate [2] but also the S-esters (thioesters), notable amongst which is methyl thioacetate [3]. Both types of ester also make major contributions to the aroma of other foodstuffs, for example O-esters to that of fruits [4] and S-ester to that of cheeses [5]. It has been firmly established that the production of the O-esters is catalyzed by the enzyme alcohol acetyl transferase (AAT; EC 2.3.1.84) [6,7]. This is a membrane-linked activity that catalyzes the nucleophilic addition of alcohols to acyl-CoA molecules, notably acetyl-CoA. There are three or more distinct AAT gene products in yeast but as yet no clear understanding of the true physiological role for this type of enzyme [7]. It is believed that AAT may function in fatty acid homeostasis and/or detoxification mechanisms [7]. Methyl thioacetate seems to be produced in analogous fashion to the O-esters, with methanethiol replacing the alcohol [8,9]. An unanswered question is whether the enzyme(s) responsible for O-ester and S-ester production are distinct or identical.

It has been the custom to assess the production of esters (including in enzyme assays) by use of gas chromatography. However, it would be convenient if an assay was available which would allow the O-ester- and S-ester-producing enzymes to be measured in a simpler manner. Accordingly we have explored the possibility of using the alcohol- or thiol-dependent consumption of acetyl-CoA, as monitored by a decrease in OD_{232}, as the basis of an assay for these enzymes. Furthermore, we have developed a new procedure for staining polyacrylamide gels for the specific detection therein of these enzymes. Using these techniques we present evidence which suggests that a single enzyme may be involved in the production of both O-esters and S-esters.

2. Materials and methods

2.1. Organism and chemicals

The yeast used was an American Ale Yeast 1056 (*Saccharomyces cerevisiae*) from Wyeast, Odell, OR, USA.

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Yeast from stock was pre-cultured at 20°C in 200 ml of an ale wort (10° Plato; Brewers Gold wort concentrate, Briess Malting Company, Chilton, WI, USA) before transferring 1% of the contents to 6 l of the same medium for growth under static conditions for 72 h at 20°C.

Methanethiol and coenzyme A were purchased from Sigma-Aldrich. Acetyl-CoA was produced as follows: to 10 μmol coenzyme A in 1 ml of ice cold deionized water was added 0.2 ml 1 M potassium bicarbonate and the solution adjusted to pH 7.5. Ice cold 0.1 M acetic anhydride was added (0.15 ml) with mixing and the solution allowed to stand at 0°C for 5 min. The pH was then adjusted to 6.0 with 1.5 N HCl and the volume made up to 2 ml to yield a 5 mM solution, as determined from the absorbance at 232 nm (molar extinction coefficient = 4.5×10^3 1 mol^{-1} cm^{-1}.)

2.2. Extraction

Yeast was harvested by centrifuging at $3100 \times g$ for 10 min and washed with 50 mM potassium phosphate buffer pH 7.5 containing 0.9 M sodium chloride. The yeast pellet (40 g) was frozen overnight and resuspended in 80 ml 25 mM imidazole-HCl pH 7.5 containing 20% glycerol, 1 mM dithiothreitol and 0.1% Triton X-100 at 4°C. The cells were ground with glass beads (40 g) in a chilled mortar and pestle. Breakage was assessed microscopically, and was estimated to exceed 60%. Unbroken cells and debris was removed by centrifuging at $15\,000 \times g$ for 20 min at 4°C. The resultant supernatant is termed the crude extract. Typically it has a protein concentration of ca. 25 mg ml^{-1}, as measured by the method of Bradford [10].

2.3. Enzyme assay

Silica glass cuvettes contained, in a total volume of 1 ml: 50 mM potassium phosphate pH 7.5; 0.1–1.0 mM acetyl-CoA; either 0.05–1 M ethanol or 0.5–10 mM methanethiol and crude extract of yeast. Reaction was started by the addition of enzyme solution and monitored over 30 min by the decrease in OD_{232}.

2.4. Polyacrylamide gel electrophoresis

Crude extracts were fractionated by native polyacrylamide gel electrophoresis at pH 8.9 [11]. Gels were stained by a novel approach based on the acetyl-CoA hydrolase method described by Manchenko [12]. The principle is illustrated in Fig. 1. Gels were steeped for 10 min at 37°C in 100 ml of 50 mM potassium phosphate buffer pH 7.5 containing 0.1 mM acetyl-CoA, 0.2 mg 2,6-dichlorophenolindophenol and either 1 M ethanol or 10 mM methanethiol. Then 10 ml of a solution containing 100 mg nitroblue tetrazolium was added and incubation continued at 37°C until a reddish-purple color developed.

3. Results

3.1. The spectrophotometric assay

We have been able to demonstrate the ethanol- and methanethiol-dependent consumption of acetyl-CoA, as monitored by a decrease in absorbance at 232 nm. Reaction was linear over a time period of 30 min. Using 1 mM methanethiol and 0.1 mM acetyl-CoA as substrates, a ΔOD_{232} of 0.041 was caused by 0.025 ml of crude extract in 30 min. When the methanethiol was replaced by 1 M ethanol the ΔOD_{232} was 0.053.

3.2. Is a single enzyme responsible for the reaction of acetyl-CoA with ethanol and methanethiol?

The rate of thermal inactivation of ethanol- and methanethiol-dependent acetyl-CoA consumption is very similar, displaying half-lives of 67 ± 3 min at 40°C and 7 ± 0.5 min at 60°C. This is consistent with the same enzyme(s) being responsible for both activities.

On polyacrylamide gel electrophoresis at pH 8.9, both the methanethiol- and ethanol-dependent conversion of tetrazolium in the assay described in Fig. 1 were located at an identical R_F, namely 0.64 relative to the tracking dye (bromophenol blue). No other such bands were observed, although a number of other clear bands of unknown provenance were observed on all gels. Again it appears that only a single activity is present that is capable of effecting the formation of coenzyme A from acetyl-CoA in an alcohol- or thiol-dependent process.

3.3. Some basic properties of the enzyme fraction

Lineweaver–Burk plots revealed the K_m for ethanol to be 170 mM (at an acetyl-CoA concentration of 0.1 mM). We have been unable to find published information on the K_m value for ethanol for the yeast enzyme; however, the

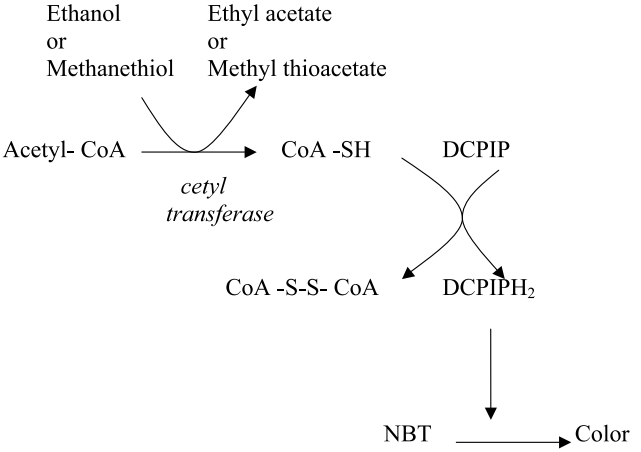

Fig. 1. The principle of the method for detecting ethanol- or methanethiol-dependent production of coenzyme A on polyacrylamide gels.

enzyme from strawberry displays a value of 45 mM [13]. The V_{max} for the ethanol-dependent reaction was 63 nmol acetyl-CoA consumed h^{-1} mg $protein^{-1}$. The K_m for methanethiol at the same acetyl-CoA concentration was 20 mM, demonstrating that the enzyme has a much greater affinity for the thiol substrate. The V_{max} was also much higher, at 3.7 μmol acetyl-CoA consumed h^{-1} mg $protein^{-1}$.

The K_m for acetyl-CoA (as determined at 1 M ethanol) was 2.5 mM, with the V_{max} being 77 nmol acetyl-CoA consumed h^{-1} mg $protein^{-1}$. This K_m value is substantially higher than that reported by Malcorps and Dufour [14], yet is virtually identical with that reported for the strawberry-derived AAT [13]. When assayed at a fixed methanethiol concentration of 1 mM, the K_m for acetyl-CoA was calculated to be 0.8 mM whilst V_{max} was 1.7 μmol acetyl-CoA consumed h^{-1} mg $protein^{-1}$, confirming that the enzyme has a greater catalytic capability with the thiol.

The optimum pH was 7.5, with 28% of the activity observed at pH 7 and 8, and zero activity at pH 6 and 8.5. This was observed irrespective of whether the substrate was ethanol or methanethiol.

4. Discussion

The evidence presented in this paper is consistent with a single enzyme being responsible for the production of O-esters and S-esters in brewing yeast. Indeed a comparison of ethanol and methanethiol as substrates suggests that the enzyme has a greater affinity for the latter. As shown by others previously for AAT, the enzyme has a much higher affinity for acetyl-CoA than for the nucleophilic substrate, suggesting that it is either the alcohol or thiol that will be limiting for the production of esters [15]. Walker and Simpson [16] showed that lager strains of *S. cerevisiae* produce substantially more methyl thioacetate than do ale strains, but that this is due to the much lesser production of methanethiol in the latter type of yeast. If ale yeast is supplied with exogenous methanethiol it readily produces methylthioacetate. There is no a priori reason why, because the same enzyme is responsible for producing both O- and S-esters, the levels of these esters should rise or fall in tandem in brewery fermentations. The regulating factor will be the level of either alcohol (notably ethanol or *iso*-amyl alcohol) or thiol (notably methanethiol) which is available. It may also be the case that the enzyme is limited in its ability to act by an adverse pH: the intracellular pH of high viability yeast is between 6 and 6.5 [17], at which pH AAT displays much lower levels of activity than at pH 7.5.

The spectrophotometric assay described in this paper should prove valuable in allowing more detailed study of ester- and thioester-producing systems in yeast and other organisms. It demands less intricate analytical chemistry and is more economical in respect of resources.

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