Purification and Properties of an Esterase from the Yeast *Saccharomyces cerevisiae* and Identification of the Encoding Gene

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We purified an intracellular esterase that can function as an \( S \)-formylglutathione hydrolase from the yeast *Saccharomyces cerevisiae*. Its molecular mass was 40 kDa, as determined by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point was 5.0 by isoelectric focusing. The enzyme activity was optimal at 50°C and pH 7.0. The corresponding gene, *YJL068C*, was identified by its N-terminal amino acid sequence and is not essential for cell viability. Null mutants have reduced esterase activities and grow slowly in the presence of formaldehyde. This enzyme may be involved in the detoxification of formaldehyde, which can be metabolized to \( S \)-formylglutathione by *S. cerevisiae*.

Esterases catalyze the hydrolysis of aliphatic and aromatic esters and have been widely studied because of their metabolic functions (29), their utility in flavor development (11), and their role in the breakdown of insecticides (10).

Esterases from the yeast *Saccharomyces cerevisiae* have been partially characterized (18, 20) and localized (13). Although intracellular esterase activity is the basis of a fluorescent staining method of yeast (1), the genetic and physiological roles of esterases in *S. cerevisiae* are not well understood. Our objectives in this study were (i) to purify and characterize a new esterase from the yeast *S. cerevisiae* and (ii) to identify the correspondent gene and to study its possible function.

Nonmethylotrophic yeasts like *S. cerevisiae* are unable to metabolize methanol to formaldehyde. However, the aldehyde can apparently be formed in some other metabolic reaction(s), because the oxidation of formaldehyde is effectively catalyzed in *S. cerevisiae* (4). In *S. cerevisiae* the SFA gene encodes a glutathione-dependent formaldehyde dehydrogenase that can oxidize formaldehyde in the presence of glutathione (4, 28).

The novel esterase purified in this study can catalyze the hydrolysis of the \( S \)-formylglutathione that is produced by the glutathione-dependent formaldehyde dehydrogenase.

**MATERIALS AND METHODS**

Protein purification. A crude extract was prepared from *S. cerevisiae* S283C grown aerobically in yeast extract-peptone-dextrose medium (Difco Laboratory, Detroit, Mich.) at 30°C for 3 days on a rotary shaker (200 rpm) and harvested by centrifugation (4,000 × g, 10 min). Twenty grams of yeast cells washed with 50 mM Tris-HCl (pH 7.5) was resuspended into 20 ml of 50 mM Tris-HCl (pH 7.5)–1 mM dithiothreitol and mixed with 100 g of chilled glass beads (diameter, 0.45 mm). The cells were broken in an MSK cell homogenizer (B. Braun AG, Melsungen, Germany) in a single 1-min cycle. Cell walls were removed by centrifugation (12,000 × g, 15 min). The supernatant was fractionated by ammonium sulfate precipitation into five fractions. Esterase activity was assayed with 1 mM \( p \)-naphthyl acetate as the substrate for the detection of acetyl esterase (15). Active fractions, precipitating between 40 and 80% of saturation, were pooled, and the corresponding pellet was resuspended in 100 mM sodium phosphate (pH 7.0)–1 M ammonium sulfate, filtered through a 0.45-μm-pore-size membrane, and loaded onto a phenyl Sepharose HP 16/10 column (Pharmacia Biotech, Uppsala, Sweden).

Further purification (Table 1) was according to the procedure of Degrassi et al. (2). An additional chromato-focusing step was performed; after gel filtration chromatography, the active fractions were collected, dialyzed, and concentrated to 1 ml by ultrafiltration with a YM30 membrane (Amicon Inc., Beverly, Mass.). The sample was loaded onto a Polybuffer Exchanger 94 column (30 ml, column model, CI1040; Pharmacia) previously equilibrated with 80 ml of 25 mM imidazole-HCl (pH 7.4) and then eluted with 400 ml of diluted Polybuffer PB74 (1:18) titrated to pH 4.0 with 5 M HCl. Active fractions were pooled, dialyzed against 20 volumes of 50 mM Tris-Cl (pH 7.5), and concentrated.

Enzyme assays. We determined substrate specificity by measuring activities for \( \alpha \)-naphthyl acetate, butyrate, caprylate, laurate, and oleate at 0.25 mM concentrations. Activities for \( \alpha \)-nitrophenyl acetate and 4-methylumbelliferyl acetate were determined by monitoring the release of \( p \)-nitrophenol photometrically at 420 nm (7) and of 4-methyl umbelliferone at 354 nm (19), respectively.

The enzymatic hydrolysis of carboxyfluorescein diacetate was monitored by the appearance of carboxyfluorescein at 470 nm after the addition of 5% (vol/vol) of a 5 mM stock solution in methanol to the reaction mixture. These assays were carried out in 20 mM sodium phosphate (pH 7.0) at 37°C.

We also determined if the purified esterase could catalyze the hydrolysis of \( S \)-formylglutathione in 50 mM potassium phosphate buffer (pH 7.0) at 30°C (25). \( S \)-Formylglutathione and \( S \)-acetylglutathione were prepared by mixing formaldehyde and glutathione in 5 mM stock solutions in methanol to the reaction mixture. We checked the progress of the reactions by monitoring the enzymatic hydrolysis of \( S \)-formylglutathione (molar absorbance, 3,300 cm\(^{-1}\)) and \( S \)-acetylglutathione (molar absorbance, 2,980 cm\(^{-1}\)) at 240 nm (24) and of 4-methylumbelliferyl acetate at 340 nm (9). \( V_{\text{max}} \) and \( K_m \) values for \( S \)-formylglutathione were determined with the substrate concentration range of 0.05 to 0.45 mM. One unit of enzyme is defined as the amount of enzyme that hydrolyzes 1 μmol of the substrate per min. Protein concentration was estimated with a Bio-Rad Protein Assay Kit I with bovine serum albumin as the standard.

**Amino acid sequence.** The purified protein was subjected to N-terminal amino acid sequence determination by automated Edman degradation on a pulsed liquid-phase protein sequencer (model 470A; Applied Biosystem, Foster City, Calif.) equipped with an on-line phenylthiohydantoin amino acid analyzer (model 120A, Applied Biosystem). The amino acid sequence was used to identify the open reading frame (ORF) coding for the purified protein, through FASTA analysis (14) of sequences in the SwissProt database.
Construction and phenotypic analysis of null mutant. A null mutant of ORF YJLO68c was obtained by replacing 100% of the gene with a dominant resistance module, kanMX4, as previously described (27), and the correct gene deletion and integration of the kanMX4 cassette was verified by the PCR method described by Huxley et al. (6).

We quantified total esterase activities on the crude extracts from both the deletion mutant and the wild-type strain on α-naphthyl acetate, p-nitrophenyl acetate, and carboxyfluorescein diacetate.

RESULTS

Enzyme purification and characterization. After chromatofocusing, only one band of 40 kDa was obtained on a sodium dodecyl sulfate–12% polyacrylamide gel. The molecular mass of the purified protein estimated by Sephacryl HR200 gel filtration was also 40 kDa. These data suggest that the purified yeast esterase is a monomer. The pI of the esterase was estimated by isoelectric focusing to be 5.0, while the pI predicted from the deduced sequence was 6.7. The pH and temperature optima of the enzyme were determined, showing that the enzyme can be routinely assayed at pH 7.0 and 50°C.

The purified esterase could use α-naphthyl acetate, 4-methylumbelliferyl acetate, p-nitrophenyl acetate, carboxyfluorescein diacetate, and S-formylglutathione as substrates (Table 2). The specific activities for α-naphthyl butyrate (9 U/mg) and α-naphthyl caprylate (0.4 U/mg) were lower than for the above-named substrates, and the enzyme was unable to hydrolyze α-naphthyl laurate and α-naphthyl oleate. The $K_m$ and $V_{max}$ values for carboxyfluorescein diacetate, which we observed with the purified esterase, differed from those previously reported for the crude extract (12 nmol per min per mg of protein and 0.29 mM, respectively [1]). These differences may be attributable to the presence of other esterases active on this substrate in the crude supernatant, as was previously reported by Parkkinen (13). We found two peaks with esterase activity after hydrophobic interaction chromatography. The peak with the highest specific activity on α-naphthyl acetate that was purified in this study accounted for approximately 80% of the esterase activity found after ammonium sulfate fractionation.

Identification of the YJLO68c gene. The NH$_2$-terminal haptenptide of the purified protein was MKVKEF. The only ORF in the yeast genome with this N-terminal sequence is YJLO68c. Based on amino acid sequence, among proteins showing similarity with the esterase identified here was the S-formylglutathione hydrolase (FghA) protein of Paracoccus denitrificans, which has 40.8% identity with our esterase and is involved in the detoxification of formaldehyde (5). No S-formylglutathione hydrolase has been reported for S. cerevisiae; however, a gene, SFA, for resistance to formaldehyde which is a putative glutathione-dependent formaldehyde dehydrogenase has been described previously (28). An ORF, HRE299, with 52% sequence similarity to the gene encoding S-formylglutathione hydrolase in P. denitrificans has been identified on chromosome X (26). HR299 encodes the esterase purified in this study and is equivalent to ORF YJLO68c.

The specific activities obtained in assays with S-formylglutathione, S-acetylglutathione, and 4-methylumbelliferyl acetate were much less effective. $K_m$ and $V_{max}$ values for S-formylglutathione were 0.88 mM and 220 U/mg, respectively (Table 2). Therefore, S-formylglutathione, although it was measured at a lower temperature than the other compounds, was clearly the most rapidly reacting substrate of the enzyme but had a relatively high $K_m$ value. When the catalytic efficiencies of the different substrates were compared by calculating their catalytic constant ($K_m/V_{max}$ ratios (Table 2), it was found that S-formylglutathione and carboxyfluorescein diacetate were the best substrates, closely followed by 4-methylumbelliferyl acetate. p-Nitrophenyl acetate and α-naphthyl acetate were much less effective.

Total esterase activities of the null mutant on α-naphthyl acetate, p-nitrophenyl acetate, and carboxyfluorescein diacetate were reduced by 30, 25, and 60% of the activity of the wild type, respectively. Growth of the mutant appeared to be similar to that of the wild type on a rich, undefined medium such as yeast extract-peptone-dextrose. When grown in the presence of 1 mM formaldehyde, the null mutant grew significantly more slowly than the wild type (data not shown).

DISCUSSION

The enzyme purified in this study preferentially hydrolyzes aliphatic and aromatic short-chain fatty acid esters and S-formylglutathione. To our knowledge this is the first report of the purification and characterization of a yeast (S. cerevisiae) esterase, followed by the identification and inactivation of the corresponding gene. The enzyme we have purified differs from the esterase purified by Toshimitsu et al. (20) because the former is a monomer while the latter is a homodimer with subunits of 40 kDa. Moreover, we observed a different optimum pH (7.0 instead of 8.0) and a higher specific activity (12

### Table 2. Kinetic constants of yeast esterase on different compounds functioning as substrates for the enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$/mg of protein (U/mg)</th>
<th>$k_{cat}/K_m$ (mol$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthyl acetate</td>
<td>0.86</td>
<td>19</td>
<td>1.5 x 10$^4$</td>
</tr>
<tr>
<td>4-Methylumbelliferyl acetate</td>
<td>0.076</td>
<td>15</td>
<td>1.3 x 10$^2$</td>
</tr>
<tr>
<td>p-Nitrophenyl acetate</td>
<td>0.35</td>
<td>12</td>
<td>2.3 x 10$^4$</td>
</tr>
<tr>
<td>Carboxyfluorescein diacetate</td>
<td>0.056</td>
<td>15</td>
<td>1.8 x 10$^3$</td>
</tr>
<tr>
<td>S-Formylglutathione</td>
<td>0.88</td>
<td>220</td>
<td>1.7 x 10$^5$</td>
</tr>
</tbody>
</table>

*a $K_m$ and $V_{max}$ values for the substrates were determined with the most purified enzyme preparation. The $k_{cat}$ is $V_{max}$/[E]$_o$, where [E]$_o$ is the (total) concentration of the enzyme. Activities for S-formylglutathione were measured in 50 mM potassium phosphate (pH 7.0) at 30°C.*
instead of 3.8 U/mg) using p-nitrophenyl acetate as the substrate.

The pI value of our purified yeast esterase by isoelectric focusing is close to the pI values reported for S-formylglutathione hydrolases from human liver (25) and from human erythrocytes (23) by similar techniques. The same pI value has been reported for esterase D (12), which is probably identical with S-formylglutathione hydrolase (3). The pI values of two genetic variants of human esterase D were found to be 5.08 and 5.10 by isoelectric focusing (16). However, Tschida et al. (21) predicted the pI of the two human esterase D variants from the cDNA sequences to be 6.58 and 6.38, respectively. Therefore, the rather large difference that we found for the yeast esterase in the experimentally determined and calculated pI values (5.0 and 6.7, respectively) is similar to that described for the human S-formylglutathione and esterase D. We speculate that this esterase might have a native conformation containing some shielded basic residues.

The phenotypic behavior of the null mutant suggests a possible role in formaldehyde detoxification. This conclusion is supported by the direct demonstration of the ability of our purified enzyme to catalyze the hydrolysis of S-formylglutathione. The yeast esterase had a specific activity on S-formylglutathione lower than those of similar enzymes from mammalian sources (8, 24, 25), and S-acetylglutathione was a poor substrate for the yeast enzyme. Moreover, a higher relative rate than with the mammalian enzymes was obtained with the yeast esterase for 4-methylumbelliferyl acetate, which gave a rate of almost 8% of that for S-formylglutathione.

In S. cerevisiae, a nonmethylotrophic yeast, S-formylglutathione is the product of the oxidation of formaldehyde by the glutathione-dependent formaldehyde dehydrogenase (28). Although this enzyme was able to catalyze the in vitro oxidation of some long-chain alcohols in addition to formaldehyde, investigations on the kinetic properties of the enzyme from S. cerevisiae strongly suggested that the enzyme functions in vivo in the detoxification of formaldehyde (4). Although the metabolic reaction(s) which produces formaldehyde in S. cerevisiae is not well established, it appears that the present enzyme, which catalyzes well the hydrolysis of S-formylglutathione, may be involved in the detoxification pathway of formaldehyde.

REFERENCES