Purification and Characterization of Glycerol Dehydratase from
Lactobacillus reuteri†

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A coenzyme B12-dependent glycerol dehydratase from Lactobacillus reuteri has been purified and characterized. The dehydratase has a molecular weight of approximately 200,000, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis yielded a single major band with a molecular weight of 52,000. Km values for substrates and coenzyme B12 were in the millimolar and the submicromolar range, respectively.

In previous studies, it was shown that when Lactobacillus reuteri strains are exposed to glycerol, they synthesize and secrete reuterin, a potent, broad-spectrum antimicrobial substance (1, 3, 5). This substance was subsequently purified and determined to be an equilibrium mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA) (14, 15). In this report, we describe the purification and characterization of the enzyme responsible for synthesis of reuterin, a coenzyme B12-dependent glycerol dehydratase first described by Smiley and Sobolov (12, 13).

Dehydratase activity was monitored by conversion of the substrate, 1,2-propanediol (1,2-PD) or glycerol, to propionaldehyde or 3-HPA, respectively. When 1,2-PD was used as the substrate, the propionaldehyde product was treated with 2,4-dinitrophenylhydrazine and assayed spectrophotometrically at A440 as previously described (8). The assay was carried out in a total volume of 1 ml, 100 μl of which was the reaction mixture consisting of 200 mM 1,2-PD, 40 mM KCl, 1 mM coenzyme B12 (Sigma Chemical Co.), and 0.05 U of enzyme, all dissolved in 75 mM potassium phosphate buffer (pH 7.2) (KPB). The reaction was allowed to proceed for 10 min at 37°C. Glycerol was converted to 3-HPA by the dehydratase and assayed by the method of Circle et al. (4).

The assay was carried out in a total volume of 1 ml and contained 100 μl of a mixture consisting of 50 mM glycerol, 1 mM coenzyme B12, and 0.2 U of enzyme, all dissolved in 75 mM KPB. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of product min⁻¹.

L. reuteri was cultured in 5 liters of Lactobacillus-carrying medium (6) at 37°C for 14 h, harvested, washed twice with sterile deionized water, and suspended in 20 ml of 0.05 KPB containing 0.15 M potassium chloride (KCl) and 2% 1,2-PD. Cells were disrupted by passage twice through a French pressure cell at 500 kg cm⁻². Deoxyribonuclease (2 mg) and acid-washed activated charcoal (0.2 g) (Sigma) were added to this disrupted solution, and the cell debris was removed by centrifugation at 20,000 × g for 20 min. The cell-free supernatant was decanted and centrifuged again at 40,000 × g for 20 min.

Purification of the dehydratase was accomplished from cleared cell extracts by using three successive chromatographic steps, each carried out at 4°C. The cleared extract was applied to a column (2.4 by 50 cm) of DE53 DEAE-cellulose (Whatman) equilibrated with 0.05 M KPB containing 0.15 M KCl and 2% 1,2-PD. This enzyme was eluted with equilibration buffer, and the column was cleared of other proteins by passage of 0.05 M KPB buffer containing 0.5 M KCl and 2% 1,2-PD.

Two distinct dehydratase activity peaks were eluted with equilibration buffer; the first peak fractions were pooled and concentrated to 2 ml by using a stirred Amicon ultrafiltration cell with an Omega series OM-0100 membrane (Pharmacia). The protein corresponding to the second dehydratase activity peak separated by DEAE-cellulose chromatography was not further purified in this study.

Klebsiella pneumoniae has also been reported to possess two immunochromically distinct dehydratases, glycerol dehydratase and diol dehydratase (16).

The concentrated enzyme fraction from DEAE-cellulose chromatography was applied to a column (100 by 1.6 cm) of Sepharose CL-6B (Pharmacia) equilibrated in 0.05 M KPB containing 0.5 M KCl and 2% 1,2-PD and eluted with the same buffer. Fractions containing dehydratase activity were pooled and concentrated to 3 ml, diluted with an equal volume of 2 M KPB containing 2% 1,2-PD, and applied in 2-ml aliquots to a column (10 by 1.6 cm) of propyl agarose (Sigma) equilibrated in 1.5 M KPB containing 2% 1,2-PD. Dehydratase activity was eluted by a gradient from 1.5 M KPB (2% 1,2-PD) to 0.75 M KPB (2% 1,2-PD). The fractions displaying dehydratase activity were concentrated and stored at −20°C.

The preservation of dehydratase activity in L. reuteri required the presence of K⁺ and 1,2-PD in moderately high concentrations. Deletion of either component during DEAE-cellulose chromatography resulted in complete loss of activity, which could not be recovered by recombination of all protein-containing fractions. The protective effect of 1,2-PD has also been reported for purification of glycerol and diol dehydratases from K. pneumoniae (9). The enzyme purification protocol is shown in Table 1. The low specific activity of the purified dehydratase is believed to be due to the instability of the enzyme complex and a large loss of activity during purification. Poznanska et al. (9) reported that the presence of glycerol was required along with 1,2-PD during purification of diol dehydratase from K. pneumoniae to retain maximal activity.

Polyacrylamide gel electrophoretic patterns of the active fractions were determined by the method of Laemmli (7) without the inclusion of sodium dodecyl sulfate. The polyacrylamide gel electrophoretic pattern in each column step is shown in Fig. 1. Protein concentrations were determined by...

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TABLE 1. Purification protocol for glycerol dehydratase
from L. reuteri

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Amt (mg) of protein</th>
<th>Activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>225</td>
<td>187</td>
<td>0.83</td>
<td>100</td>
</tr>
<tr>
<td>2. DEAE-cellulose</td>
<td>75</td>
<td>73</td>
<td>0.97</td>
<td>39</td>
</tr>
<tr>
<td>3. Sepharose CL-6B</td>
<td>15</td>
<td>32</td>
<td>2.2</td>
<td>17</td>
</tr>
<tr>
<td>4. Propyl agarose</td>
<td>1.8</td>
<td>7.2</td>
<td>4.0</td>
<td>4</td>
</tr>
</tbody>
</table>

the method of Bradford (2), using bovine serum albumin as
the standard (Bio-Rad Laboratories).

The pH optimum for the conversion of 1,2-PD to propi-
onaldehyde by the dehydratase was measured in 500 mM
KPB adjusted with hydrochloric acid or potassium hydrox-
ide, and activity was assayed, using 1,2-PD as the substrate.
Dehydratase activity was optimal between pH 6.5 and 8 and
declined sharply outside that range. The highest activity
occurred at pH 7.2, approximately one pH unit higher than
glycerol dehydratase examined in Lactobacillus 208A by
Smiley and Sobolov (12).

The molecular weight of the native enzyme was estimated
by gel filtration on a Sepharose CL-6B column (1.6 by 100
cm) by comparison to standards of known molecular weight
(stdards listed in the legend to Fig. 2). The purified
enzyme was eluted from the Sepharose CL-6B column with
0.05 M KPB, 0.5 M KCl, and 2% 1,2-PD at a flow rate of 12
ml h⁻¹. Elution of the dehydratase corresponded to a
molecular weight of approximately 200,000. Sodium dodecyl
sulfate-polyacrylamide gel electrophoresis carried out on
this protein by the method of Laemmli (7) yielded a major
protein band of molecular weight 52,000 (Fig. 2). Two other
protein bands (molecular weights, 70,000 and 40,000)
accompanied the major band, and thus one cannot eliminate
the possibility that the dehydratase is composed of some
combination of two nonidentical subunits. However, it ap-
pears more likely that the dehydratase is composed of four
identical subunits of molecular weight 52,000. This remains
to be confirmed until genetic studies on L. reuteri can be
used to complement existing biochemical analyses.

Propionaldehyde production from 1,2-PD was found to be
linear with time up to 30 min. However, when glycerol was
the substrate, production of 3-HPA was linear for only 10
min; thereafter, the reaction fell close to zero, indicating that
an inactivation of the enzyme had occurred. The inactivation
of the dehydratase could not be attributed to substrate
deficiency, since the concentration of glycerol in such sys-
tems was substantially larger than the amount of product
formed. This inactivation appeared not to be caused by free
product, 3-HPA, because inactivation was not observed
when 3-HPA was included in the reaction mixture containing
1,2-PD as the substrate. The glycerol dehydratase from K.
pneumoniae was also shown to become inactivated after
reaction with glycerol, but the product of the reaction,
3-HPA, when added to purified enzyme, was shown not to
be the inactivating agent (10).

The apparent Kₘ values for glycerol and 1,2-PD were
calculated from standard Lineweaver-Burk plots and deter-
mined to be 3.3 and 7 mM, respectively. The Kₘ values
were determined in the presence of 0.5 mM coenzyme B₁₂.

The affinity of the dehydratase for coenzyme B₁₂ was
considerably higher than that of either substrate. The appar-
ent Kₘ of the purified enzyme for coenzyme B₁₂ was 0.3 μM,
determined in the presence of 200 mM 1,2-PD. The Kₘ of
the purified dehydratase (0.3 μM) was shown to be lower than

FIG. 1. Native polyacrylamide gel electrophoretic separation of
proteins from L. reuteri after each column step utilized in purifica-
tion. Lane 1, Propyl agarose column; lane 2, Sepharose CL-6B
column; lane 3, DEAE-cellulose column; lane 4, crude extract.

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electropho-
retic separation of protein purified from L. reuteri after elution from
propyl agarose. Lane 1. Protein from L. reuteri; lane 2, molecular
weight standards (phosphorylase b [97,400], bovine serum albumin
[66,200], ovalbumin [42,699]), carbonic anhydrase [31,000], soybean
trypsin inhibitor [two bands] [21,300], and lysozyme [14,400].
the $K_m$ of the second dehydratase (approximately 50 $\mu$M). This was deduced by a comparison of the activity of crude extracts and purified dehydratase in the presence of varied concentrations of coenzyme B$_12$ (Fig. 3). The purified dehydratase activity in this system coincided with the initial reaction seen in Fig. 3, while the second dehydratase eluted from the DEAE-cellulose column corresponded to the second activity phase seen in this figure. Addition of vitamin B$_12$ (cyanocobalamin) inhibited the dehydratase reaction, consistent with previous reports (12).

Smiley and Sobolov (12, 13) first reported a coenzyme B$_{12}$-dependent glycerol dehydratase which was responsible for the production of 3-HPA, a transient intermediate subsequently reduced by NADH to the end product, 1,3-propanediol. This work involved a Lactobacillus species isolated from grain mash (13). Schutz and Radler (11) later showed that Lactobacillus brevis and Lactobacillus buchneri possess coenzyme B$_{12}$-dependent glycerol dehydratases. In the present study, L. reuteri was shown to possess two coenzyme B$_{12}$-dependent dehydratases, each with different affinities for coenzyme B$_{12}$. The high-affinity dehydratase ($K_m$, 0.3 $\mu$M) was purified and shown to have a pH optimum of 7.2, one unit higher than the dehydratase described by Smiley and Sobolov (12). Although the presence of dehydratase activity and the ability to reduce the dehydratation product, 3-HPA, has been observed in the lactobacilli previously mentioned, L. reuteri appears to be unique in its ability to accumulate and excrete 3-HPA (14, 15). Preliminary studies are under way to purify the 3-HPA oxidoreductase and to determine the role glycerol plays in L. reuteri metabolism.

**LITERATURE CITED**


**FIG. 3.** Enzyme activity of dehydratase from purified (○) and crude (●) extracts of L. reuteri as a function of coenzyme B$_{12}$ concentration. Inset is the Lineweaver-Burk plot used for determination of the $K_m$ value of the purified dehydratase for coenzyme B$_{12}$. The units of the Lineweaver-Burk plot are as follows: micromolar for s and micromoles per minute for v.