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Propanediol-1,2-dehydratase and metabolism of glycerol of *Lactobacillus brevis**

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Abstract. While most strains of heterofermentative lactobacilli and strains of *Leuconostoc* species contained only traces of a dehydratase reacting with glycerol or propanediol-1,2, three strains of *Lactobacillus brevis* and one strain of *L. buchneri* that metabolized glycerol readily in the presence of glucose, contained propanediol-1,2 dehydratase (EC 4.2.1.28). This cobamide requiring enzyme from *L. brevis* B 18 was partially purified. It reacts with the substrates propanediol-1,2, glycerol and ethanediol-1,2 with the relative activities of about 3:2:1. This ratio remained unchanged throughout the purification procedure. The substrate affinities were measured: propanediol-1,2 K_m = 0.6 mM, glycerol K_m = 4 mM, ethanediol-1,2 K_m = 5.3 mM coenzyme B_{12} (substrate glycerol) K_m = 0.007 mM. The activity of the dehydratase was promoted by potassium or ammonium ions and inhibited by sodium, lithium, magnesium or specially manganese. The apparent molecular weight of propanediol-1,2 dehydratase was determined as M_r = 180,000.

Key words: Lactic acid bacteria – *Lactobacillus brevis* – Propanediol-1,2-dehydratase – Propanediol-1,2 – Glycerol – Ethanediol-1,2

Diol dehydratase (EC 4.2.1.28) was first observed by Lee and Abeles (1963) in *Klebsiella pneumoniae* grown anaerobically on propanediol-1,2. This enzyme catalyzes the dehydratation of propanediol-1,2, ethanediol-1,2, and glycerol to propanal, acetaldehyde, and 3-hydroxypropanal respectively. The glycerol dehydratase (EC 4.2.1.30) of *K. pneumoniae* reacts with the same substrates but immunochemical tests (Toraya and Fukui 1977), polyacrylamide gel electrophoresis, and ion exchange chromatography (Forage and Foster 1979) have shown that the two enzymes are different. Both enzymes were also found in *Citrobacter intermedium, C. freundii* and *Propionibacterium freudenreichii* (Toraya et al. 1980). Smiley and Sobolov (1962) have found a glycerol dehydratase in a heterofermentative species of *Lactobacillus*. An investigation of the anaerobic metabolism of glycerol by heterofermentative lactobacilli has shown that most strains formed some propanediol-1,3 from glycerol and contained traces of a dehydratase but only 3 strains of *Lactobacillus brevis* and one strain of *Lactobacillus buchneri* metabolized glycerol and contained significant amounts of a dehydratase (Schütz and Radler 1984). This paper describes the partial purification of the glycerol metabolizing enzyme of *L. brevis* B 18 and its characterization as a coenzyme B_{12} dependent diol dehydratase (propanediol-1,2 hydro-lyase EC 4.2.1.28).

* Dedicated to Prof. Dr. H. G. Schlegel on behalf of his 60th birthday

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Materials and methods

Cultures and preparation of extracts. The lactic acid bacteria and the culture conditions were described recently (Schütz and Radler 1984). For the purification of diol dehydratase the cells of *Lactobacillus brevis* B 18 were grown in 4 l glycerol medium in two 2-l Erlenmeyer flasks for 18 h at 30° C (1% inoculum). After centrifuging and washing twice in buffer (10 mM potassium phosphate, pH 7.2, 1 mM dithiothreitol) the cells (about 4.5 g wet weight) were suspended in 15 ml of this buffer and were homogenized in a CO_2-refrigerated ball mill Braun MSK for 2.5 min after addition of 30 g glass beads (0.10–0.11 mm diameter). Cells and debris were removed by centrifuging at 15,000 × *g*. All procedures were performed at 4° C.

Purification of propanediol-1,2-dehydratase. Step 1. Streptomycin sulphate solution (5% w/v) was added drop by drop (71 mg streptomycin per 100 mg protein according to Linn and Lehmann 1965) to the extract, that was stirred for 10 min, then centrifuged (15,000 × *g*, 15 min) to remove the precipitate that was discarded. – *Step 2.* Dialysis overnight against 200 volumes of buffer (as above). By these two steps the content of nucleic acids was lowered to about 5%. – *Step 3.* Saturated ammonium sulphate solution was slowly added up to a concentration of 10% (w/v). The precipitate was centrifuged and discarded. While stirring vigorously further ammonium sulphate solution was slowly added up to a final concentration of 30% (w/v). The pink-red sediment containing the dehydratase was recovered by centrifuging (15,000 × *g*, 20 min) and dissolved in 0.5 ml 0.05 M phosphate buffer, pH 7.2 containing 5 mM dithiothreitol. – *Step 4.* The preparation obtained after step 3 was applied to a column of Sephacryl S-300 (diameter 9 mm, 600 mm). The column was equilibrated and run with the phosphate buffer used in step 3, to which 10 µM coenzyme B_{12} and 0.02% sodium azide had been added to avoid the inactivation of the dehydratase. To the fractions of 5 ml containing the dehydratase activity dithiothreitol was added to a final concentration of 100 mM to stabilize and activate the enzyme.

Table 1. Partial purification of propanediol-1,2 dehydratase of *Lactobacillus brevis* B 18

Fraction	Protein mg	Specific activity			Total activity[a] U	Yield[a] %	Purification factor[a]
		Substrate propanediol-1,2 U/mg	Substrate ethanediol-1,2 U/mg	Substrate glycerol U/mg			
Cell extract[b]	256	0.19	0.06	0.12	31	100	1
Streptomycin sulphate (step 1)	195	0.38	0.13	0.26	51	165	2.2
Dialysis (step 2)	139	0.53	0.18	0.33	46	148	2.8
Ammonium sulphate (step 3)	3.2	15.5	5.4	9.9	32	103	82
Sephacryl S-300 (step 4)	0.6	82.2	28.8	54.0	32	103	450

[a] For substrate glycerol

[b] Due to the spontaneous inactivation and activation by dithiothreitol, the measurement of dehydratase activity gives varying figures in extracts

Table 2. Influence of cations on the activity of dehydratase with the substrates propanediol-1,2 or glycerol. (1.9 µg protein of enzyme preparation after step 4 was used for the assay without ammonium sulphate, that was replaced by the salts indicated)

Salt added (50 µmol)	Activity of propanediol-1,2 dehydratase		Activity of glycerol dehydratase	
	U/mg	%	U/mg	%
None	48.8	59	21.1	39
NH_4Cl	82.2	100	53.5	99
KCl	64.0	78	31.6	58
NaCl	29.5	36	14.1	26
LiCl	21.8	27	10.2	19
$MgCl_2$	13.8	17	5.8	11
$MnCl_2$	0	0	0	0
$(NH_4)_2SO_4$[a]	81.1	99	54.2	100

[a] Usual assay

Enzyme assays. Propanediol-1,2-dehydratase. The method of Toraya et al. (1977) was used to determine the activity of dehydratase with the substrates propanediol-1,2, glycerol and ethanediol-1,2. Reaction mixture: 0.2 ml 0.01 M potassium phosphate buffer, pH 7.2; ammonium sulphate (50 µmol), coenzyme B_{12} (20 µmol), substrate (100 µmol), enzyme solution in phosphate buffer (0.03–0.3 U). Total volume 1 ml. After 7 min at 35°C the reaction was stopped with 1 ml sodium citrate buffer (0.1 M, pH 3.6). Immediately 0.5 ml 3-methylbenzo-2-thiazolone solution (0.1% in water) and after 15 min at 35°C 1 ml water were added and the absorbance was read at 350 nm. – One unit (U) of enzyme activity is the amount that catalyzes the formation of 1 µmol aldehyde per minute. – *Lactate dehydrogenase.* The activity of this enzyme was determined by measuring the oxidation of NADH at 340 nm with pyruvate as substrate.

Analytical determinations. Glycerol was determined according to the enzymatic method of Eggstein and Kuhlmann (1974), propanediol-1,3 by gas chromatography (Schütz and Radler 1984) and protein either with the biuret method or spectrophotometrically according to Warburg and Christian (1941) in the preparations after dialysis.

Chemicals. Enzymes, coenzymes and dithiothreitol were obtained from Boehringer, Mannheim, FRG. Coenzyme B_{12} was purchased from Fluka, Neu-Ulm, FRG. L-Cysteine · HCl, D,L-α-aminobutyric acid and hydroxyapatite were supplied by Serva, Heidelberg, FRG. Sephacryl S-300 superfine was obtained from Pharmacia, Freiburg, FRG. Materials for gas chromatography were purchased from Varian, Darmstadt, FRG and all other chemicals from Merck, Darmstadt, FRG.

Results

Glycerol fermentation and dehydratase activity

When the anaerobic metabolism of glycerol was studied during growth of 49 strains of heterofermentative lactobacilli (*Lactobacillus brevis, L. buchneri, L. fermentum, L. fructivorans, L. fructosus* and *L. cellobiosus*), *Leuconostoc mesenteroides* and *Lc. oenos* most strains fermented less than 0.8 g glycerol per l, formed less than 90 mg propanediol-1,3 per l and the cell extracts showed a specific activity of dehydratase of less than 0.1 U/mg with glycerol as substrate (Schütz and Radler 1984). In cell extracts the specific activity of lactate dehydrogenase was high (2–46 U/mg) thus showing that this enzyme activity had not been inactivated by the preparation of the extracts. Three strains of *L. brevis* and one of *L. buchneri* fermented a large part of the glycerol and contained the dehydratase with a specific activity of up to 0.6 U/mg.

Partial purification of diol dehydratase of L. brevis B 18

Lactobacillus brevis B 18 was one of the strains that showed good glycerol fermentation when grown in a medium containing glycerol plus glucose (Schütz and Radler 1984). For the partial purification of the dehydratase cell extracts were prepared from *L. brevis* before they reached the stationary growth phase. The enzyme was purified by treatment with streptomycin sulphate, dialysis, precipitation with ammonium sulphate (10–30%) and gel chromatography on Sephacryl S-300. Table 1 shows a typical result. During the purification procedure an increase in the total activity of the dehydratase was observed. The reason is not known, but this influences the values "yield" and "purification factor".

Throughout the purification procedure outlined in Table 1 the dehydratase activities for the substrates propanediol-

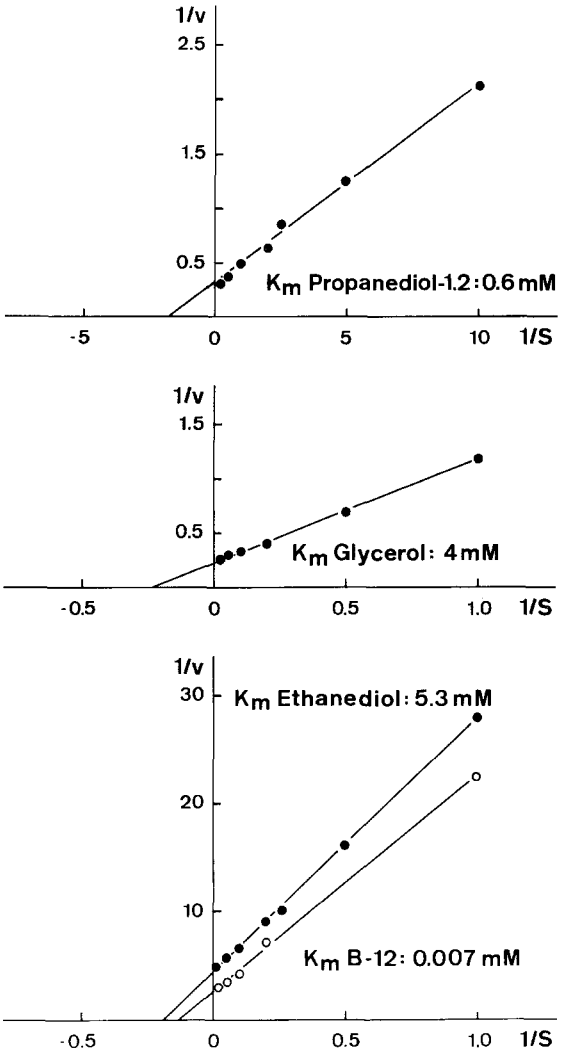

Fig. 1. Lineweaver-Burk plots with the dehydratase of *Lactobacillus brevis* for the substrates propanediol-1,2, glycerol, and (●) ethanediol-1,2, and for (○) coenzyme B_{12} with 100 mM glycerol as substrate

1,2, glycerol and ethanediol-1,2 showed a ratio of about 3:2:1. The highest specific activities for these substrates were 82.2 U/mg, 54.0 U/mg and 28.8 U/mg, respectively. This relative ratio of the dehydratase activity was also observed when the cells were grown on propanediol-1,2 plus glucose, on fructose, or on fructose plus glycerol instead of glucose plus glycerol.

When meso-butanediol-2,3 was used as substrate no reaction was observed with the test described. However, extending the reaction time from 7 to 30 min revealed a weak activity with meso-butanediol-2,3. This activity was only about 1% of the dehydratase activity measured with propanediol-1,2. The substrate affinity of the diol dehydratase for meso-butanediol-2,3 is low (K_m = 0.04 mol/l).

Characterization of the propanediol dehydratase of L. brevis B 18

Dehydratases are known to be influenced in their activity by the presence of monovalent or divalent cations. Therefore the dehydratase activities with the substrates glycerol and propanediol-1,2 were determined in the presence of various cations. As shown in Table 2 the enzyme activity is increased by ammonium and to a lesser extent by potassium. Sodium and lithium were slightly inhibitory whereas magnesium and in particular manganese caused a pronounced inhibition.

The affinity for the different substrates of the dehydratase of *L. brevis* grown on glycerol plus glucose was determined. Figure 1 shows the Lineweaver-Burk plots for the substrates glycerol, propanediol-1,2, ethanediol and the coenzyme B_{12}. In the latter case an enzyme preparation was used, that had been treated with hydroxyapatite to remove the coenzyme from the apoenzyme. The following K_m-values were determined: K_m = 0.6 mM (propanediol-1,2), K_m = 4 mM (glycerol), K_m = 5.3 mM (ethanediol-1,2) and K_m = 7 µM (coenzyme B_{12}).

Several experiments were performed to characterize the diol dehydratase of *L. brevis* B 18. In 0.1 M phosphate buffer the maximum activity was observed in the range of pH 7–7.5. When kept for 24 h at 4° C the best activity was observed at pH 7 in 0.1 M phosphate buffer. The optimum temperature was in the range of 30–40° C. At 70° C, 60° C and 50° C the enzyme lost 50% of the activity after about 10, 30 and 50 min respectively. By chromatography on Sephacryl S-300 in the presence of standard proteins (M_r = 12,500–450,000) and dextrane blue the apparent molecular mass of the diol dehydratase of *L. brevis* B 18 was determined as M_r = 180,000. The Table 3 compiles these results.

Polyacrylamide electrophoresis with gradient gels type PAA 4/30 (as described by Caspritz and Radler 1983) of a preparation of diol dehydratase after step 4 revealed one main band ($M_r = 2.3 \times 10^5$). By visual comparison less than about 10% of the protein was detected as three very faint bands. This indicates that the enzyme preparation obtained after step 4 contains about 10% impurities.

Dehydratase activity of Lactobacillus brevis B 41

Lactobacillus brevis B 41 is one of the many lactic acid bacteria that reduced only small amounts of glycerol, formed only traces of propanediol-1,3 and showed a weak activity of dehydratase. After growth in MRS-medium with glycerol cell extracts were found to contain some dehydratase activity and the specific activities were 0.019, 0.020, 0.012 and 0.008 U/mg protein for the substrates glycerol, propanediol-1,2, ethanediol-1,2 and butanediol-2,3, respectively. This enzyme activity was completely lost after precipitation with streptomycin sulphate or dialysis, and no purification was achieved by gel filtration on Sephacryl S-300. We found that contrary to the propanediol dehydratase of *L. brevis* B 18 the dehydratase activity of *L. brevis* B 41 was very strongly promoted by sodium ions. Potassium, lithium and ammonium ions increased the enzyme activity in cell extracts, whereas magnesium and manganese caused a complete inhibition.

Discussion

Most strains of heterofermentative lactobacilli and *Leuconostoc* had been found to contain a low activity of a dehydratase (substrate glycerol), but only 3 strains of *Lactobacillus brevis* and one strain of *L. buchneri* that reduced glycerol to propanediol-1,3 contained a propanediol

Table 3. Comparison of dehydratases of *Lactobacillus brevis* B 18, *Lactobacillus sp.* and *Klebsiella pneumoniae* (n.d. = not determined)

Characteristics	*L. brevis* B 18	*L. sp.*[a]	*K. pneumoniae*
Substrates	Propanediol-1,2 Glycerol Ethanediol-1,2 (meso-Butanediol-1,2)	Glycerol	Glycerol Propanediol-1,2 Ethanediol-1,2
K_m: Propanediol-1,2	0.6 mM	n.d.	0.02 mM[b]
K_m: Glycerol	4.0 mM	n.d.	1.6 mM[b]
K_m: Ethanediol-1,2	5.3 mM	n.d.	0.66 mM[b]
K_m: Coenzyme B_{12} Substrate glycerol	0.007 mM	n.d.	n.d.
Effect of cations Activity increase by Activity decrease by	NH_4, K Na, Li, Mg, Mn	NH_4, Rb, K Na	K, Mg[c]
pH-Optimum[d] pH-Stability[d]	7.0–7.5 7.2	5.8–6.0 n.d.	n.d. 6.0–8.8[c]
Temperature optimum[d] Temperature stability[d]	30–40°C 5 min at 70°C	n.d. n.d.	n.d. 4 min at 75°C[c]
M_r Inhibitors[e]	1.8×10^5 p-CMB	n.d. p-CMB, PMA vitamine B_{12}	1.88×10^5[c] EDTA[c]

[a] According to Smiley and Sobolov (1962, 1964)
[b] According to Bachovchin et al. (1977)
[c] According to Schneider et al. (1970)
[d] Substrate glycerol
[e] p-CMB = p-Chloromercuribenzoate, PMA = Phenyl mercuric acetate, EDTA = Ethylenediamine tetra-acetate

dehydratase, and in addition a propanediol-1,3 dehydrogenase. The propanediol dehydratase is inducible, but it is probably not necessarily induced by glycerol, for cells grown on fructose contain this enzyme in high activity. On the other hand, cells grown on ribose plus glycerol, neither metabolize glycerol nor contain diol dehydratase (Schütz and Radler 1984).

The function of propanediol dehydratase in *L. brevis* is obviously only the first step in the reduction of diols and glycerol to the corresponding alcohols. *L. brevis* is unable to use glycerol as sole substrate anaerobically, but its reduction enables this bacterium to ferment glucose more efficiently. *Klebsiella pneumoniae* and *Citrobacter freundii* ferment glycerol, by partially reducing it to propanediol-1,3 and partially oxidizing it via dihydroxiacetone and pyruvate to acetate and CO_2 (Forage and Lin 1982). Inspite of the different metabolism the enzyme of *L. brevis* shows many similarities with the propanediol dehydratase of *K. pneumoniae*, see Table 3. The $M_r = 180{,}000$ is similar to the values of $M_r =$ 188,000 and 190,000 for the dehydratase of *K. pneumoniae* determined by Schneider et al. (1970) and Forage and Foster (1979). Similar to the dehydratase of *Lactobacillus spec.* (Smiley and Sobolov 1962) and *K. pneumoniae* (Schneider et al. 1970) the enzyme of *L. brevis* is activated by monovalent cations, although some differences exist.

Our observations indicate that the dehydratase of *L. brevis* B 18 is a propanediol-1,2 dehydratase (propanediol-1,2 hydro-lyase EC 4.2.1.28). The enzyme reacts with the substrates propanediol-1,2, glycerol, ethanediol-1,2, and very weakly with meso-butanediol-2,3. The relative ratio of the activities for propanediol-1,2, glycerol, and ethanediol-1,2 is about 3:2:1 and did not change during the purification, this indicates that only one enzyme is involved in these reactions. In accordance with this assumption the dehydration of propanediol-1,2 and glycerol is similarly effected by cations, whereas the two dehydratases of *Klebsiella pneumoniae* show differences (Toraya and Fukui 1977). Furthermore the same ratio of activities for the substrates is observed with the dehydratase of *L. brevis* B 18 independent of the presence of glycerol or propanediol-1,2 in the growth medium. Of the diols tested, the dehydratase of *L. brevis* has the highest affinity for propanediol-1,2. Therefore, the enzyme involved in the metabolism of glycerol is a propanediol-1,2 hydro-lyase (EC 4.2.1.28).

So far it cannot be decided, if the weak activity of glycerol dehydratase found in many strains of lactic acid bacteria is caused by the same cobamide requiring propanediol-1,2 dehydratase purified by us. Distinguishing characteristics are the different ratio of activities with the various diols and the activation by sodium ions. It may be that a further dehydratase is present or that this activity is due to a side reaction of some other cobamide dependent lyase. Attempts to purify this dehydratase from *L. brevis* B 41 have failed. The purification of the propanediol-1,2 dehydratase was only possible when *L. brevis* B 18 was grown in glycerol-medium. This medium does not support good growth of most lactic acid bacteria. The use of MRS medium for growth of *L. brevis* B 18 even prevented the purification of the dehydratase from these cells.

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