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Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*

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Summary. Reliable assay systems were developed for the detection and quantitation of butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. Butanol dehydrogenase was NADPH-dependent. The enzyme could be sparated by ultracentrifugation from a NADH-specific enzyme which probably represents the ethanol dehydrogenase but which also reacted with butyraldehyde to form butanol. Butyraldehyde dehydrogenase proved to be NADH-specific. All enzymes were induced shortly before butanol formation began. Specific activities decreased at the end of the fermentation process. An explanation for contradictory data in the literature is proposed.

Introduction

Only a few strictly anaerobic bacteria are able to synthesize 1-butanol as a major fermentation product. These are *Clostridium acetobutylicum, C. aurantibutyricum, C. beijerinckii,* and *C. tetanomorphum* (George et al. 1983; Gottwald et al. 1986). Among them, *Clostridium acetobutylicum* received most scientific attention. Due to the renewed interest in butanol production from biomass a lot of information concerning the biochemistry of this organism and the fermentation process has accumulated in the last few years (for a recent review see Rogers 1986). However, little is known about the regulation of butanol formation at the molecular level and the respective enzymes, i. e. butanol dehydrogenase and butyraldehyde dehydrogenase. Data published are even contradictory with respect to their coenzyme specificity, optimal assay conditions, and reliable detection in cell extracts (Andersch et al. 1983; Gerckens 1980; Petitdemange et al. 1969; Rogers 1986).

This study was undertaken in order to develop reliable methods for the detection of the two enzymes and to determine unequivocally their coenzyme specificity. Such assay systems are prerequisites for all regulatory studies, the isolation of mutants, and the cloning of the respective genes.

Materials and methods

C. acetobutylicum DSM 1732 was used throughout this study and obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, FRG. The organism was grown in the so-called "clostridial basal medium" (CBM) described by O'Brien and Morris (1971) which was modified by omission of $MnSO_4$. Addition of clarified corn steep liquor ensured butanol production in batch culture (Bowles and Ellefson 1985; Gottwald 1986; Lemmel 1985). All media were prepared anaerobically (Costilow 1981).

For the preparation of cell extracts, 500-ml cultures were grown until the onset of butanol synthesis as revealed by gas chromatography. Conditions for analysis were described earlier (Bahl et al. 1982). During growth experiments aliquots of 500–1000 ml were removed under sterile conditions. Cell harvest was performed under strictly anaerobic conditions, employing tightly sealing centrifuge bottles and an anaerobic cabinet (Mecaplex, Switzerland). The cell pellet was resuspended in 3–5 ml Tris(hydroxymethyl)aminomethane hydrochloride buffer (0.1 M; pH: 7.5), supplemented with 1 mM dithioerythritol. Lysis was either achieved by one passage through a French pressure cell at 77 MPa or by ultrasonication (30 kHz, 6 intervals of 30 seconds and 10 seconds pausing). The resulting suspension was either centrifuged at 10000 × g for 10 min or at 95000 × g for 60 min and the respective supernatant or the pellet of the high-speed centrifugation were then used for enzymatic investigations. Protein concentration of cell extracts was determined according to Lowry et al. (1951).

The following assay systems were used for butanol dehy-

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drogenase: (A) 11 mM butyraldehyde; 0.23 mM NADPH; 77 mM Tris-HCl, pH 7.8; final volume 0.86 ml (this study); (B) 19.6 mM butanol; 0.39 mM NAD^+; 73.5 mM semicarbazide hydrochloride; 68.8 mM Tris-HCl, pH 7.8; final volume 0.83 ml (Andersch et al. 1983); (C) 363 mM butanol; 1.8 mM $NADP^+$; 6.2 mM semicarbazide hydrochloride; 1 mM reduced glutathione; 19.1 mM glycine; 85.5 mM sodium pyrophosphate; final volume 0.62 ml (George and Chen 1983); (D) 4 mM butyraldehyde; 0.34 mM NADH; 85 mM Tris-HCl, pH 6.0; final volume 0.85 ml (Gerckens 1980); (E) 1.7 mM butyraldehyde; 0.13 mM NADPH; 50 mM potassium phosphate buffer, pH 7.2; final volume 0.85 ml (Petitdemange et al. 1969); (F) 66.7 mM butanol; 0.1 mM $NADP^+$; 50 mM potassium phosphate buffer, pH 7.2; final volume 0.84 ml (Petitdemange et al. 1969).

Butyraldehyde dehydrogenase activity was measured using the following test systems: (G) 0.2 mM butyryl-CoA; 1 mM dithioerythritol; 0.27 mM NADH; 72 mM semicarbazide hydrochloride; 67 mM Tris-HCl, pH 6.0; final volume 0.87 ml (this study); (H) 0.2 mM butyryl-CoA; 0.4 mM NADH; 72 mM semicarbazide hydrochloride; 80 mM Tris-HCl, pH 6.0; final volume 0.89 ml (Andersch et al. 1983); (I) 0.2 mM butyryl-CoA; 106 mM KCl; 0.3 mM NADH; 3.4 U of alcohol dehydrogenase; 74 mM Tris-HCl, pH 6.0; final volume 0.97 ml (Gerckens 1980); (K) 50 mM butyraldehyde; 0.2 mM CoASH; 0.2 mM NAD^+; 5 mM reduced glutathione; 100 mM Tris-HCl, pH 7.8; final volume 0.90 ml (Petitdemange et al. 1977); (L) 45 mM butyraldehyde; 0.1 mM CoASH; 80 mM dithioerythritol; 0.5 mM NAD^+; 50 mM glycylglycine, pH 9.0; final volume 1.01 ml (Rogers 1986).

50 µl of the various extracts were added. The light path was 1 cm. The absorption change at 365 nm was followed in all cases. Determination of activity of the oxygen-labile hydrogenase served as an internal control of strict anaerobiosis. The assay was performed as described earlier (Andersch et al. 1983).

Results

Using the different assays for butanol dehydrogenase described in Materials and methods, confusing results were obtained with respect to coenzyme specificity and specific activity. These contradictions could not be explained until we found that the centrifugation speed during extract preparation had an effect on enzymatic activity. The data summarized in Table 1 show that a NADPH-dependent butanol dehydrogenase could be detected in the low-speed extract as well as in the high-speed extract (that consequently was missing or only present in small amounts in the high-speed sediment). The enzyme also used acetaldehyde as a substrate, however, the activity with butyraldehyde on an average was 2.4fold higher than with the C-2 compound. Assay systems with a high pH value were required for the measurement of maximum activities and, indeed, a broad pH optimum between 7.8 and 8.5 was determined (not shown). The enzyme was hardly affected by oxygen and lost about 60% of its activity in one week after storage at 4° C.

A second butyraldehyde-reducing activity could be detected in low-speed extracts that, however, was NADH-dependent. This enzyme was sedimented by ultracentrifugation. Again, C-4 and C-2 aldehydes could serve as a substrate, but the activity with butyraldehyde was on an average only 1.7fold higher than that with acetaldehyde. In contrast to the NADPH-dependent enzyme the activities determined at pH 7.8 and at 6.0 were about the same. This enzyme lost about 50% of its activity in one week when stored at 4° C.

Butyraldehyde dehydrogenase activity was NADH-dependent. The enzyme could be measured best in the low-speed extract. Ultracentrifugation resulted in the precipitation of approximately 70% of the activity. The enzyme was oxygen-labile and lost about 75% of its specific activity within 7 days when stored at 4° C.

Table 1. Butanol dehydrogenase activity in extracts prepared at different centrifugation conditions. Cells were grown in a 500-ml culture and harvested shortly after the onset of butanol production (butanol concentration was 8.6 mM). Test system A was used

Coenzyme	Centrifugation condition[a]	Fraction	Specific activity	
			at pH 7.8 [U/mg]	at pH 6.0 [U/mg]
NADPH	10000 × g	S[b]	0.104 (0.047)[d]	0.048 (0.021)
NADH	10000 × g	S	0.18 (0.18)	0.10 (0.062)
NADPH	95000 × g	S	0.14 (0.053)	0.058 (0.025)
NADH	95000 × g	S	0.038 (0.022)	0.044 (0.018)
NADPH	95000 × g	P[c]	0.035 (n.d.)[e]	n.d. (n.d.)
NADH	95000 × g	P	0.75 (0.60)	0.81 (0.59)

[a] centrifugation at 10000 × g for 10 min and at 95000 × g for 60 min
[b] S, supernatant
[c] P, pellet
[d] values in parentheses were determined with acetaldehyde as substrate
[e] n.d., not detectable

Using the high-speed and low-speed extracts we tried to detect enzymatic activities using all assay systems described so far for butanol dehydrogenase and butyraldehyde dehydrogenase (A—L, see Materials and methods, data not shown). In our hands some test systems failed to show any activity, although we followed precisely the described procedures. In our newly developed test systems (A and G, see Materials and methods) maximal specific activities were obtained. In general, much better results were observed using the physiological substrate, i.e. butyraldehyde (instead of butanol) for butanol dehydrogenase and butyryl-CoA (instead of butyraldehyde) for butyraldehyde dehydrogenase. Extracts for all experiments mentioned so far had been obtained by French pressure treatment. Preparation by ultrasonication resulted in a sharp decrease or total loss of activity.

A growth experiment was conducted in order

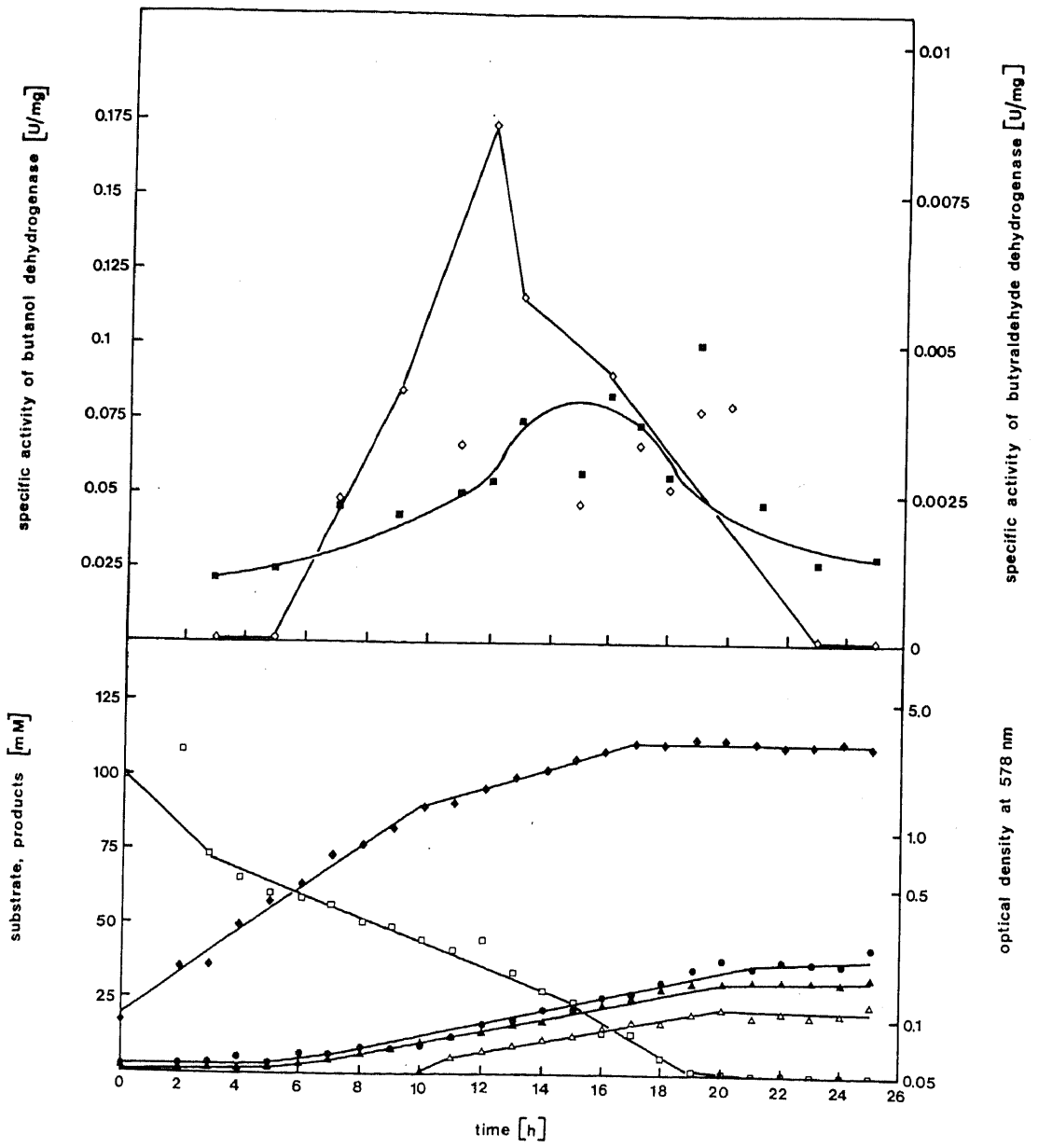

Fig. 1. Growth of *C. acetobutylicum* in batch culture and levels of butanol-forming enzymes. The lower part shows growth parameter, substrate consumed, and products formed. Optical density, ◆; glucose, □; acetate, ●; butyrate, ▲; butanol, △. The upper part shows the level of butanol-forming enzymes during growth. NADPH-dependent butanol dehydrogenase, ■; butyraldehyde dehydrogenase, ◇

to follow the NADPH-specific butanol dehydrogenase activity and the NADH-specific butyraldehyde dehydrogenase activity during the fermentation process (Fig. 1). Test systems A and G were used. For technical reasons only a high-speed extract was used, thus, values determined for butyraldehyde dehydrogenase were not maximal (compare Table 2). The latter enzyme proved to be inducible with an onset of activity 3 h before butanol production. The specific activity reached a maximum shortly after butanol synthesis had started, then it decreased sharply and was not detectable anymore at the end of the fermentation. The NADPH-dependent butanol dehydrogenase was also an inducible enzyme, however, a low level of activity was expressed constitutively. Specific activity started to increase 1 h before butanol production, reached a maximum 7 h later, and then decreased to the basal level. The formation of 1-butanol started after approximately 10 h of growth. Acetone and ethanol were formed in concentrations of only up to 2 mM and 5.5 mM, respectively. For clarity these data are not included in Fig. 1. Whereas the acetone concentration was practically constant throughout the whole fermentation, the increase of ethanol concentration from the basal level of 2.7 mM to a final value of 5.5 mM paralleled the butanol production. Furthermore, the NADH-specific butyraldehyde- and acetaldehyde-reducing activity was induced at the same time (data not shown). The basal level of ethanol found was due to the corn steep liquor added to the medium.

Discussion

From the data presented it becomes clear that *Clostridium acetobutylicum* contains two enzymes that are able to reduce butyraldehyde and acetaldehyde, respectively. One uses NADH as a coenzyme, is partly sedimented by ultracentrifugation, and reacts 1.7fold faster with butyraldehyde than with acetaldehyde. It is induced shortly before butanol and ethanol formation starts. The other enzyme is NADPH-specific, is not sedimented as easily as the above enzyme, and reacts 2.4fold faster with butyraldehyde than with acetaldehyde. This enzyme, too, is induced shortly before the onset of production of the alcohols. At the present stage of our knowledge on the properties of the two enzymes, the function of these dehydrogenases in the fermentation cannot be specified. The NADH-specific enzyme could be involved in ethanol formation (Fogarty and Ward 1970) and the NADPH-specific one in butanol formation, but it cannot be excluded that the two enzymes are involved in both, butanol and ethanol production. Further investigations of the role of the enzymes must await their purification and the isolation of mutants defective in one of the dehydrogenases. So far, only a note about a partial purification of a NADPH-linked butanol dehydrogenase from *C. beijerinckii* has been published (Hiu and Chen 1986).

The results reported here may explain the contradictory data about butanol dehydrogenase published earlier. If one compares specific activi-

Table 2. Comparison of specific activities and characteristics of butanol dehydrogenase and butyraldehyde dehydrogenase in cell-free preparations from *C. acetobutylicum* as reported in the literature

Butanol dehydrogenase			Butyraldehyde dehydrogenase			Cell lysis procedure	Centrifugation conditions	Reference
Specific activity [U/mg][a]	Substrate	Coenzyme	Specific activity [U/mg][a]	Substrate	Coenzyme			
0.0096	butanol	NAD^+	0.018	butyraldehyde	NAD^+	ultrasonication	20000 × g, 20 min	Petitdemange et al. 1977
0.014	butanol	$NADP^+$	0.0017	butyraldehyde	$NADP^+$			
0.02	butyraldehyde	NADH	0.006	butyryl-CoA	NADH	French press	8000 × g, 25 min	Gerckens 1980
0.06	butanol	NAD^+	0.04	butyryl-CoA	NADH	ultrasonication	10000 × g, 30 min	Andersch et al. 1983
0.007	butanol	$NADP^+$	0.0048	butyryl-CoA	NADPH			
—[b]	—	$NADP^+$	0.11	butyraldehyde	NAD^+	—	—	Rogers 1986
0.14	butyraldehyde	NADPH	0.038	butyryl-CoA	NADH	French press	95000 × g, 60 min	this study test systems A, G
0.012[c]	butanol	$NADP^+$	n.d.[d]	n.d.	n.d.	lysozyme	37000 × g, 30 min	George and Chen 1983

[a] maximal values reported [b] conditions not specified [c] data published for *C. beijerinckii* [d] n.d., not determined

ty, coenzyme specificity, and mode of extract preparation reported by different laboratories (Table 2), it is obvious that extracts prepared by a high-speed centrifugation step always contained a NADPH-dependent butanol dehydrogenase, whereas in extracts from low-speed centrifugation a NADH-dependent activity was found. For reliable detection and quantitation of butanol dehydrogenase activity in cell extracts usage of the physiological substrate butyraldehyde seemed advantageous. Measurement of the reverse reaction was possible, but yielded much lower specific activities. The test employed in this study yielded the highest values reported so far.

Butyraldehyde dehydrogenase proved to be NADH-specific which is in agreement with the data from the literature (Table 2). Most test systems allowed detection of activity in low-speed extracts, however, with high-speed extracts only the assay used here and the very similar one (H) of Andersch et al. (1983) worked. The enzyme could mostly be sedimented by ultracentrifugation. The test system used here (G) gave maximal activities; a significant higher specific activity (2.9fold) for butyraldehyde dehydrogenase has only been reported by Rogers (1986). Butyraldehyde dehydrogenase clearly is inducible and also decays rapidly as reported earlier (Rogers 1986).

Finally, it should be mentioned that cells of *C. acetobutylicum* producing solvents in large concentrations are not very suitable for enzyme studies. Usually they are very difficult to disrupt. A medium containing low concentrations of glucose but corn steep liquor to assure a reproducible shift yields solvents in relatively small concentrations. Cells from such cultures have been found to be advantageous for the study of enzymes involved in solvent formation. A second important point is to harvest the cells exactly at the maximum of enzyme activity, since all three activities decay rapidly. Similar results have been obtained using enzymatic tests (Rogers 1986) and rates of conversion of ^{14}C-butyrate to ^{14}C-butanol (Terracciano and Kashket 1986). These findings will allow future work on the regulation and genetics of the butanol-forming enzymes.

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