FEBS Letters 351 (1994) 95–99

FEBS 14433

Purification and properties of the α-acetolactate decarboxylase from *Lactococcus lactis* subsp. *lactis* NCDO 2118

Vincent Phalip[a], Christophe Monnet[a], Philippe Schmitt[a,*], Pierre Renault[b], Jean-Jacques Godon[b], Charles Diviès[a]

[a]*Laboratoire de Microbiologie, ENS.BANA, 1 esplanade Erasme, 21000 Dijon, France*
[b]*Laboratoire de Génétique Microbienne, Institut National de la Recherche Agronomique, 78352 Jouy en Josas, France*

Received 24 June 1994

Abstract

α-Acetolactate decarboxylase from *Lactococcus lactis* subsp. *lactis* NCDO 2118 was expressed at low levels in cell extracts and was also unstable. The purification was carried out from *E. coli* in which the enzyme was expressed 36-fold higher. The specific activity was 24-fold enhanced after purification. The main characteristics of α-acetolactate decarboxylase were: (i) activation by the three branched chain amino acids leucine, valine and isoleucine; (ii) allosteric properties displayed in absence and Michaelis kinetics in the presence of leucine. The enzyme is composed of six identical subunits of 26,500 Da.

Key words: *Lactococcus*; α-Acetolactate decarboxylase; Branched-chain amino acids

1. Introduction

Many lactic acid bacteria, including *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, are able to ferment citrate. This results in the generation of C_4 compounds such as diacetyl, acetoin and 2,3-butanediol via the key intermediate, pyruvate. The main enzymes involved in pyruvate metabolism in *Lactococcus* are L-lactate dehydrogenase (LDH; EC 1.2.1.22), leading to L-lactate and α-acetolactate synthase (ALS; EC 4.1.3.18) leading to α-acetolactate. α-Acetolactate is an unstable compound which can be chemically transformed to acetoin (decarboxylation) or diacetyl (oxidative decarboxylation). α-Acetolactate can also be decarboxylated in acetoin by an α-acetolactate decarboxylase (ALDC; EC 4.1.1.5). In *L. lactis*, the gene encoding ALDC is located downstream of the last *ilv* gene [1]. It can be transcribed from three promoters. P1 and P2, located upstream of the *leu* and *ilv* genes, respectively, are negatively controlled by isoleucine [2]. Another promoter, P3, is specific for the transcription of the ALDC gene. Recently, we have shown that ALDC is involved both in the citrate catabolism and in the control of the biosynthesis of branched-chain amino acids (BCAA) in *L. lactis*. The expression of this gene is thus subject to multiple control [1]. In addition to the genetic control, we have shown that ALDC from *L. lactis* subsp. *lactis* biovar *diacetylactis* has allosteric properties and is strongly activated by BCAA [3].

In this paper, we report the purification of the α-acetolactate decarboxylase from *L. lactis* subsp. *lactis* NCDO 2118 overexpressed in *E. coli* to achieve its biochemical characterization. The properties of this enzyme are consistant with its dual role in *L. lactis*.

2. Materials and methods

2.1. Bacterial strains

NCDO 2118 is a prototrophic strain of *Lactococcus lactis* subsp. *lactis* isolated from frozen peas. JIM 4460 is an α-acetolactate decarboxylase negative (*ald*B$^-$) derivative of NCDO 2118 where the *tet*M gene from Tn916 interrups the coding phase of the gene. TG1 [4] was used as *Escherichia coli* strain host for overexpression of ALDC from pJIM 540. pJIM 540 is pBluescript(*Amp*R) which contains the *ald*B gene from *L. lactis* subsp. *lactis* NCDO 2118 under control of its own promotor. *E. coli* strains and *Lactococcus* strains were grown in LB broth [5] supplemented with 50 mg/l ampicilline, and M17 broth [6], respectively.

2.2. Assay of α-acetolactate decarboxylase activity

The ester of α-acetolactate (α-methyl-α-acetoxyethyl acetoacetate) was obtained from Oxford Chemical Ltd. (Brackley, Northands, UK). It was transformed to α-acetolactate, ethanol and acetate by adding two equivalents of NaOH. α-Acetolactate solutions were prepared immediately before use, at the concentration of 500 mM. The saponification reaction was carried out at 20°C for 30 min. The efficiency of the reaction was controlled by the assay of α-acetolactate produced by the method of Westerfeld [7], as modified by Veringa et al. [8]. We verified that ethanol and acetate formed during the saponification had no effect upon ALDC activity. The α-acetolactate obtained from saponification is a racemic mixture of D- and L-α-acetolactate. *Klebsiella pneumoniae* [9] and *Lactobacillus casei* [10] ALDCs use the D-isomer. By comparison, we proved that ALDC from *L. lactis* subsp. *lactis* NCDO 2118 also uses the D-isomer.

ALDC activity was assayed at 30°C; the reaction mixture contained 200 mM potassium phosphate (pH 6.0) and enzyme solution. The

*Corresponding author.
Fax: (33) 8039-6611; E-mail: Schmitt2@satie.u.bourgogne.fr

Abbreviations: ALDC, α-acetolactate decarboxylase; LDH, lactate dehydrogenase; BCAA, branched-chain amino acids; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

SSDI 0014-5793(94)00820-5

reaction was started by addition of α-acetolactate at varying concentrations as indicated in the text. Acetoin concentration was assayed just after addition of α-acetolactate in the reaction mixture and again 15 min later by the method of Westerfeld. Since α-acetolactate undergoes a slow non-enzymatic decarboxylation (approx. 3% per hour at pH 6.0 and 30°C) into diacetyl or acetoin, which gives a similar colour reaction, a control reagent was run without enzyme and acetoin production was corrected for the non-enzymatic decarboxylation. One unit of ALDC activity represents the formation of 1 μmol acetoin per min.

During the purification procedures, ALDC activity was measured in the presence of 10 mM leucine, which increases the sensibility of the determination.

Protein was determined by the method of Lowry et al. [11] with BSA as a standard or by monitoring the optical density at 280 nm during the purification procedure.

2.3. Purification of ALDC

The purification was carried out from strain TG1(pJIM 540). Unless otherwise stated, all steps were performed at 4°C.

Step 1: Preparation of the crude extract. *E. coli* TG1(pJIM 540) cells from a 6-liter overnight culture (under strong agitation at 37°C) were harvested by centrifugation, washed in 50 mM phosphate buffer pH 6.5 (Buffer A) and resuspended in 40 ml of the same buffer. The cells were broken by passage through a French pressure cell and the debris removed by centrifugation at 25,000 × *g*, 4°C for 20 min.

Step 2: Ammonium sulfate fractionation. The supernatant from Step 1 was subjected to fractionation with ammonium sulfate. The enzyme was shown to precipitate between 30 and 55% saturation at 0°C. The pellet was dissolved in 5.5 ml of Buffer A. The solution was concentrated by ultrafiltration using a Macrosep unit (PM 30, Filtron, Northborough, MA) to a volume of 3.5 ml. It was then diluted to a final volume of 20 ml with Buffer A. This concentration/dilution resulted in desalting of the solution allowing it to be submitted to ion-exchange chromatography.

Step 3: Ion exchange chromatography. The desalted solution was applied to a 100 ml Macro-Prep 50 Q column (Bio-Rad, Richmond, CA), previously equilibrated with Buffer A. After loading the sample, the column was washed with one volume of Buffer A containing 0.05 M potassium chloride. No ALDC activity was detected in these fractions. A linear gradient from 0.05 M to 0.4 M KCl was then applied. The enzyme was eluted at approximately 0.3 M KCl. The column was then washed with buffer A containing 1 M KCl. The active fractions were pooled and concentrated again with a 30 kDa Macrosep unit.

Step 4: Hydroxylapatite chromatography. The solution from Step 3 was loaded in a 1 ml Econo Pac HTP cartridge (Bio-Rad) previously equilibrated with Buffer A. After the sample was loaded, the column was washed with four volumes of Buffer A. Then, a linear gradient from 0.05 to 0.5 mM phosphate buffer, pH 6.5, was applied. The enzyme was eluted between 0.11 and 0.14 mM phosphate. The active fractions were pooled, stored at −30°C, and alternatively used for the enzyme characterization.

2.4. Electrophoresis

SDS-PAGE was performed according to Laemmli [12] with 12.5% (w/v) polyacrylamide gels. The proteins were stained with Coomassie blue. The molecular weight of the enzyme was determined from a calibration curve constructed by using the following markers: phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500) and lysozyme (14,000). The isoelectric point of the purified enzyme was determined as described by O'Farrell [13].

2.5. Molecular weight of the native ALDC

The molecular weight of the native enzyme was determined by gel filtration with a Sephacryl S-200-HR column (Pharmacia, Uppsala, Sweden). The following proteins were used as M_r markers: alcohol dehydrogenase (150,000), bovine serum albumin (66,200), ovalbumin (45,000) and pepsin (34,700).

3. Results

3.1. Overexpression of ALDC in E. coli

ALDC activity in *L. lactis* is relatively weak and the enzyme is unstable which renders its purification difficult. We thus decided to overexpress the gene in *E. coli* which is devoid of ALDC. To achieve this, we subcloned a *L. lactis* NCDO 2118 DNA fragment containing its own promotor P3 and the *ald*B gene on a high copy number plasmid pBluescript. Indeed, we observed that P3 is a strong promoter in *E. coli* which leads to a lethal expression of lacZ fusion (data not shown).

Crude extracts from *L. lactis* subsp. *lactis* NCDO 2118 and *E. coli* TG1(pJIM 540) were compared to ensure that the enzyme extracted from the two strains had the same properties. Data listed in Table 1 shows that the ALDC from *L. lactis* subsp. *lactis* NCDO 2118 and the enzyme expressed in *E. coli* TG1(pJIM 540) were both activated about 12-fold, 8-fold and 3-fold by leucine, valine and isoleucine, respectively. In addition, the molecular weights of the native enzymes determined by gel filtration were proved to be identical. Thus, the enzyme expressed in *E. coli* TG1(pJIM 540) has the same characteristics as the ALDC from *L. lactis* subsp. *lactis* NCDO 2118. The *E. coli* TG1 and *L. lactis* subsp. *lactis* NCDO 2118 *ald*B$^-$ strains displayed no ALDC activity.

3.2. Purification of the α-acetolactate decarboxylase

The purification was carried out from *E. coli* TG1(pJIM 540) which displayed a 36-fold higher specific activity than *L. lactis* subsp. *lactis* NCDO 2118 (Table 1). The four-step procedure described in section 2 is sum-

Table 1
Comparison of some characteristics of the ALDC in crude extracts of different bacterial strains

	Specific activity[a] (U/mg)				Molecular weight (dal)
	None	Leucine	Valine	Isoleucine	
L. lactis subsp. *lactis* NCDO 2118	0.005	0.065	0.048	0.017	150,000
L. lactis subsp. *lactis* NCDO 2118 *ald* B$^-$	< 0.0005	< 0.0005	< 0.0005	< 0.0005	NA[b]
E. coli TG1(pJIM 540)	0.180	1.970	1.308	0.568	150,000
E. coli TG1	< 0.0005	< 0.0005	< 0.0005	< 0.0005	NA

[a] Measured in crude extracts with 3.6 mM D-α-acetolactate, pH 6.0 and with addition of 10 mM of each amino acid.
[b] Not applicable.

marized in Table 2. It resulted in a 24-fold purification with a 3% yield. The relatively low yield is due to the instability of the enzyme. The enzyme was purified to apparent homogeneity on SDS-PAGE through the over-Call procedure as shown in Fig. 1. Its molecular weight was estimated at 26,500. Gel filtration, performed with crude extracts of NCDO 2118, TG1(pJIM 540) (Table 1) as well as with the purified enzyme indicated that the native molecular weight was 150,000. Taken together, these data suggest that the enzyme is constituted of six identical subunits. The pI was estimated by isoelectric focusing at about 4.5.

3.3. Characterization of the purified enzyme

In order to ascertain the stimulatory effect of BCAA on ALDC activity, these amino acids were tested on the purified enzyme. Specific activity was measured with increasing concentrations of each amino acid and a mixture of the three BCAA was also tested (Fig. 2). ALDC (measured with 3.6 mM D-α-acetolactate) was greatly stimulated by addition of each amino acid separately. The specific activity enhancement near saturation, which was observed with about 40 mM of each amino acid, was 40-fold, 23-fold and 14-fold with leucine, valine and isoleucine, respectively. In presence of the three BCAA, the specific activity was also increased. The enzyme was then saturated with 30 mM of BCAA (10 mM each). The enhancement of specific activity was however always lower with the mixture. The maximal stimulation observed was indeed lower than 10-fold.

Saturation of the enzyme with α-acetolactate in absence or in presence of 40 mM leucine is shown in Fig. 3. In absence of leucine, the enzyme displayed sigmoidal kinetics. The K_m for D-α-acetolactate was 75 mM and the Hill coefficient 2.62 suggesting a strong positive cooperativity in substrate binding. The enzyme was saturated with 120 mM of D-α-acetolactate. When leucine was added, the saturation curve obeys Michaelis-Menten kinetics and the enzyme was saturated with 50 mM D-α-acetolactate. The K_m (10 mM) and Hill coefficient (1.18) were lowered. This indicates that the cooperativity is suppressed and the enzyme works at relatively low α-acetolactate concentrations in presence of leucine. Furthermore, in the presence of leucine, the maximum specific activity was increased. The 17 others amino acids tested at the concentration of 10 mM, have no effect upon the ALDC activity (measured with 3.6 mM D-α-acetolactate) except cysteine which inhibited the enzyme (approx. 50%).

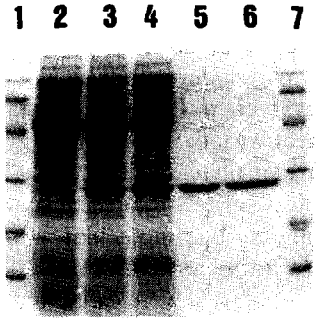

Fig. 1. SDS-PAGE of ALDC at various stages of purification. Lanes: (1) molecular weight markers; (2) crude extract from *E. coli* TG1; (3) crude extract from *E. coli* TG1(pJIM 540); (4) after ammonium sulphate fractionation; (5) after elution through ion-exchange column; (6) after elution through the hydroxylapatite column; and (7) molecular weight markers.

The purified enzyme was very unstable, especially when diluted: at 5 μg/ml the activity was totally lost within 24 h at 4°C. Addition of bovine serum albumin (5 mg/ml) allowed the preservation of ALDC activity for at least two weeks at 4°C.

When the purified enzyme was incubated with 0.5 mM EDTA during 20 min at 37°C, a 3-fold decrease in ALDC activity was observed. Further incubation for 10 min at 37°C after addition of 2 mM Mg^{2+} did not restore the initial activity, in contrary to Mn^{2+} and Zn^{2+} with which 82 and 69% of the initial activity was recovered, respectively.

ALDC activity was unaffected by addition of avidin suggesting that the enzyme did not require biotin as cofactor. The effect of pH upon ALDC activity, measured with 40 mM leucine and 50 mM α-acetolactate, was investigated. Maximal activity was found at pH 6.0 and 75% of the maximal activity was conserved within a pH range of 5.4 to 6.9.

4. Discussion

ALDC from *L. lactis* was purified from *E. coli* strain TG1(pJIM 540) which overexpressed the enzyme 36-fold as compared to *Lactococcus lactis* subsp. *lactis* NCDO 2118. ALDC from *L. lactis* is very unstable. Instability of ALDC was also described by Rasmussen et al. [10]

Table 2
Summary of ALDC purification

	Protein (mg)	Activity (U)	Spec. act. (U/mg)	Yield (%)	Purification-fold
Crude extract	816	15571	19.1	100	1
Ammonium sulfate	110.7	4020	36.3	25.8	1.9
Ion exchange	8.07	990	122.7	6.4	6.4
Hydroxylapatite	1.05	477	454.3	3.1	23.8

which reported a half-life of 15 min at 37°C and pH 5 for the purified enzyme from *Lactobacillus casei*. A protective effect of bovine serum albumin was also observed.

The molecular weight (26,500) estimated on SDS-PAGE is consistent with the molecular weight calculated from the sequence of the *ald*B gene which is 26,200 [14]. Gel filtration results suggest that the enzyme is a six-mer of identical subunits. The molecular weights of denatured ALDC from *Lactobacillus casei* [10] and *Brevibacterium acetylicum* [15] were estimated at 27,500 and 31,000, respectively, which is close to that determinated in this study, but these enzymes are dimeric. The isoelectric point deduced from the gene sequence, 4.54 [14] was very close to the experimental pI (4.5) determinated in this study and also close to the pI of the ALDC from *Lactobacillus casei* (4.7) [10] and from *Brevibacterium acetylicum* (4.4) [15]. Addition of the chelating agent EDTA inhibited α-acetolactate decarboxylase activity, suggesting that one or several divalent ions may be part of the enzyme. The stimulatory effect of Mn^{2+} and Zn^{2+} ions on EDTA treated enzyme and the absence of effect of Mg^{2+} was also reported by Rasmussen et al. [10]. On the contrary, the *Klebsiella pneumoniae* enzyme was not inhibited by EDTA and some metal ions were inhibitors [16].

A very strong activation of ALDC was observed by leucine, valine and isoleucine. The property is unique among the ALDC studied up to date. It is interesting that in *L. lactis*, the gene encoding this enzyme is located downstream of the genes involved in the biosynthesis of BCAA. In contrast, in *Bacilli* and *Enterobacteriaceae* it is linked to genes involved in the catabolism of pyruvate [17–19]. The particular location of *ald*B in *Lactococci* suggests that ALDC is involved in the regulation of leucine and valine biosynthesis [1]. Indeed, it is transcribed with the *leu* and *ilv* genes. It has been proposed that

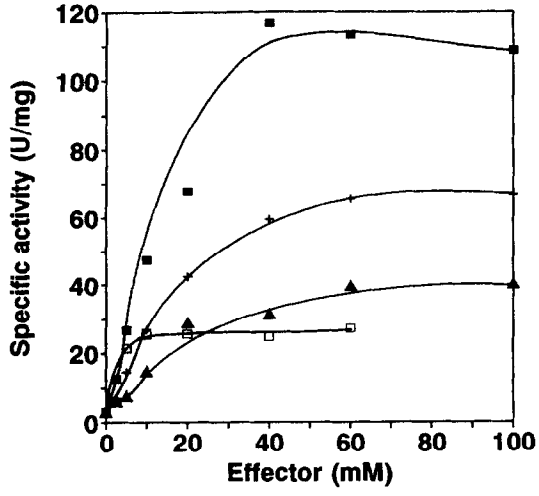

Fig. 2. Effect of increasing BCAA concentrations upon ALDC activity measured with 3.6 mM D-α-acetolactate: leucine (■), valine (+), isoleucine (▲), mixture of the three BCAA at the indicated concentrations (□).

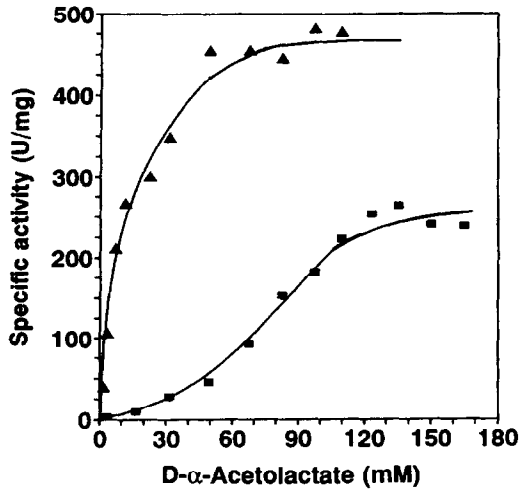

Fig. 3. α-Acetolactate saturation curve of ALDC from *Lactococcus lactis* subsp. *lactis* without leucine (■) and with 40 mM leucine (▲).

translation of *ald*B was controlled by secondary structures able to sequester the ribosome binding site. However, leak in this control would result in the expression of significant amount of ALDC which would interfere with the synthesis of leucine and valine. The low activity of ALDC in the absence of BCAA is a second control to avoid the escape of α-acetolactate to acetoin when cells are starved for these amino acids. It is particularly interesting that leucine and valine, which production depends on the availability of α-acetolactate, activate ALDC much more than isoleucine does. The same phenomenon was already described with crude extracts from *L. lactis* subsp. *lactis* biovar *diacetylactis* [3]. Most *Lactococci* isolated from dairy products are auxotrophic for BCAA and it has been proposed that they became auxotrophs by adaptation to milk while their ancestors were prototrophs [2]. Despite, the characteristics of the ALDC, and in particular its activation by BCAA, are conserved.

Acknowledgements: We thank Yoplait (Ivry sur Seine, France) for its financial support. Grant 91G0558 Agrobio 2002.

References

[1] Renault, P., Godon, J.J., Goupil, N., Delorme, C. and Ehrlich, S.D. (1994) Dev. Biol. Standard. In press.
[2] Godon, J.J., Delorme, C., Bardowski, J., Chopin, M.C., Ehrlich, S.D. and Renault, P. (1993) J. Bacteriol. 175, 4383–4390.
[3] Monnet, C., Phalip, V., Schmitt, P. and Divies, C. (1994) Biotechnol. Lett. 16, 257–262.
[4] Gilson, T.J. (1984) Ph. D. thesis, University of Cambridge, Cambridge, UK.
[5] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[6] Terzaghi, B.E. and Sandine, W.E. (1975) Appl. Microbiol. 29, 807–813.

[7] Westerfeld, W.W. (1945) J. Biol. Chem. 161, 495–502.
[8] Veringa, H.A., Verburg, E.H. and Stadhouders, J. (1984) Netherlands Milk Dairy J. 38, 251–263.
[9] Juni, E. (1952) J. Biol. Chem. 195, 715–726.
[10] Rasmussen, A.M., Gibson, R.M., Godtfredsen, S.E. and Ottesen, M. (1985) Carlsberg Res. Commun. 50, 73–82.
[11] Lowry, O.H., Roseborough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] O'Farrell, P.H. (1974) J. Biol. Chem. 250, 4007–4021.
[14] Godon, J.J. (1992) Thèse de doctorat. Université de Paris XI, Paris.
[15] Ohshiro, T., Aisaka, K. and Uwajima, T. (1989) Agric. Biol. Chem. 53, 1913–1918.
[16] Loken, J.P. and Stormer, F.C. (1970) Eur. J. Biochem. 14, 133–137.
[17] Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B.R. and Sjoholm, C. (1990) J. Bacteriol. 172, 4315–4321.
[18] Blomqvist, K., Nikkola, M., Lehtovaara, P., Suihko, M.L., Airaksinen, U., Straby, K.B., Knowles, J.K.C. and Penttila, M.E. (1993) J. Bacteriol. 175, 1392–1404.
[19] Renna, M.C., Najimudin, N., Winik, L.R. and Zahler, S.A. (1993) J. Bacteriol. 175, 3863–3875.