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PURIFICATION OF α-ACETOLACTATE DECARBOXYLASE FROM LACTOBACILLUS CASEI DSM 2547

by

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α-Acetolactate decarboxylase has been purified to homogeneity, by fast protein liquid chromatography and high performance elution chromatography, from a partially purified α-acetolactate decarboxylase preparation from Lactobacillus casei DSM 2547. The pure enzyme exhibited a specific activity of 375 kU · mg^{-1} and exerted its optimal activity at pH 4.5 to 5.0 and at a temperature of 40 °C. Its isoelectric point was estimated to pH 4.7 and its molecular weight was found to be 48,000. The enzyme was inhibited by o-phenanthroline and could be partially reactivated by zinc ions. An HPLC method for the determination of α-acetolactate is described.

1. INTRODUCTION

Enzymes with the ability to decarboxylate α-acetolactate are widely found amoung bacterial strains but not in other groups of microorganisms (7, 8, 10). The only α-acetolactate decarboxylase (EC 4.1.1.5.) which has been isolated in homogeneous form is the enzyme from Aerobacter aerogenes isolated in 1970 by LØKEN and STØRMER (12). This enzyme has more recently been demonstrated to be effective for removal of α-acetolactate from freshly fermented beer and thus for acceleration of the beer maturation process (6). However, the practical use of this procedure is complicated by a limited stability of the Aerobacter α-acetolactate decarboxylase at the pH of beer (7). A search for an enzyme with higher stability in the acid pH region led, among others (7, 8, 10), to an α-acetolactate decarboxylase in a strain of Lactobacillus casei which met this criteria (8, 9). This enzyme removed as expected all α-acetolactate from freshly fermented beer within 48 hrs. However, attemps to use the enzyme during the main fermentation of beer were unsuccessful due to removal of zinc atoms from the enzyme by the rapidly metabolizing yeast cells. Since this was a

Abbreviations: ALDC = α-acetolactate decarboxylase; AUFS = absorbance units full scale; BSA = bovine serum albumin; DSM = Deutsche Sammlung von Mikroorganismen; EDTA = ethylene diaminetetraacetic acid; HPEC = high performance exclusion chromatography; HPLC = high performance liquid chromatography.

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clear demonstration that the α-acetolactate decarboxylase from Lactobacillus differed from the enzyme from Aerobacter (6, 12) attempts were made to purify the enzyme.

The present paper describes the purification and characterization of the α-acetolactate decarboxylase from Lactobacillus casei DSM 2547.

2. MATERIALS AND METHODS

2.1. Materials

O-acetyl-acetolactate ethyl ester (the precursor for the substrate (D,L)-α-acetolactate), was synthesized and its purity checked by gas chromatography as described in (6).

Thiamine pyrophosphate and n-hexylamine were obtained from Sigma Chemical Co., USA. Gel filtration and ion-exchange resins were from Pharmacia, Uppsala, Sweden. Ampholines were supplied by LKB, Sweden, and creatine monohydrate was obtained from Fluka AG, Switzerland. Other reagents were obtained from Merck, Darmstadt, FDR and were of analytical grade.

2.2. Methods

2.2.1. Determination of α-acetolactate decarboxylase activity

α-Acetolactate solutions were prepared immediately before use by mixing O-acetyl-acetolactate ethyl ester with 0.1 M-sodium hydroxide in a ratio of 1:100 by volume. When the starting material had dissolved, the solution was incubated for 5 min at 37 °C to complete the reaction. 200 µl aliquots of the resulting 0.05 M-acetolactate solution were mixed with 200 µl of acetate buffer (1.0 M, pH 5.0) and 600 µl of enzyme solution. The enzymatic decarboxylation was allowed to proceed for 30 min at 37 °C before the reaction was terminated by addition of 1 ml 2.5 M-sodium hydroxide. 100 µl aliquots of the reaction mixtures were diluted with 1.1 ml water containing 1.0 mg creatine and the colorimetric acetoin determination was started by addition of 200 µl of α-naphthol solution (50 mg per ml 2.5 M-NaOH). After incubation for 1 hour at room temperature the absorbance was measured at 520 nm. Since, in solutions at pH 5, α-acetolactate undergoes a slow non-enzymatic oxidative decarboxylation into diacetyl which gives a similar colour reaction with creatine plus α-naphthol (4, 21) a reagent blank was run without the enzyme. In addition, a calibration curve was made for each assay by mixing 100 µl aliquots of the reagent blank with 2, 4, and 6 µg of acetoin, adding creatine and α-naphthol and measuring the colour after 1 hour as described above. One unit of enzyme activity is defined as the amount of enzyme that forms 1 µmole of acetoin per hour at 37 °C (12). Since the colour formation was linearly related to the amount of acetoin only within a range from approx. 2 µg to 5 µg per ml assay solution, it was necessary to adjust the enzyme samples to contain between 1.0 and 2.5 units of activity per assay.

As a supplement to the colorimetric determination of acetoin a high performance liquid chromatography (HPLC) method was developed which directly determines the residual amount of (D,L)-α-acetolactate. A Waters liquid chromatography system was used with a C-18 reverse phase radial compression column (10 cm × 0.5 cm, 10 µm Nova-Pak). Elution was performed isocratically with 0.01 M-di-ammonium hydrogen phosphate containing 2% acetonitrile and 0.2% n-hexylamine adjusted to pH 7.3 with ortho-phosphoric acid. The flow rate was 60 ml · h^{-1} and peaks were detected by measurement of absorbance at 210 nm. α-Acetolactate was eluted as an assymetrical peak with a retention time of approx. 4.5 min. Acetoin and diacetyl were not eluted under these conditions. It was found that use of a new column required conditioning with a small amount of protein. In this study 4 mg of oxidized ribonuclease was used to achieve adequate peak separation (3).

2.2.2. Isolation procedures

Partially purified α-acetolactate decarboxylase was prepared as described from a 250 litre culture of Lactobacillus casei fermented by Chr. Hansens Laboratory (10). The resulting solution, containing 3350 kU in 1.9 l, was concentrated to 600 ml by ultrafiltration and then divided into six aliquots each of which was dialysed against distilled water (5 l, 20 hrs, 5 °C). After centrifugation at 10.000 g for 15 min the supernatant from each portion was adjusted to pH 8.0 with 0.1 M-NaOH and applied to a

column (d = 30 mm, h = 112 mm) of Fractogel DE 650 S anion exchanger previously equilibrated with 25 mM-Tris buffer, pH 8.0 at room temperature. Elution was performed at room temperature with a linear sodium sulphate gradient (350 ml 25 mM-Tris buffer, pH 8.0 and 350 ml 0.215 M-Na_2SO_4 in 25 mM-Tris buffer, pH 8.0, flow rate 220 ml · h^{-1}) and fractions containing α-acetolactate decarboxylase activity were pooled and dialysed at 2 °C against distilled water (10 volumes). Enzyme containing fractions from two columns were pooled and rechromatographed on the same Fractogel column.

The column was regenerated in situ with 110 ml 0.2 M-HCl, distilled water until the eluent pH was 4, 110 ml of 0.2 M-NaOH, distilled water until the eluent pH was 8 and finally equilibrated with the Tris buffer.

Each enzyme pool obtained by rechromatography on Fractogel was dialyzed as before and concentrated by ultrafiltration to 1 ml in an Amicon stirred cell, Model 8010, equipped with an Amicon YM 10 filter. The resulting enzyme solution was applied to a Pharmacia FPLC Mono Q column type HR 5/5 previously equilibrated with 10 mM-Tris, pH 8.0 at room temperature. This column was similarly eluted with a linear gradient of sodium sulphate (30 ml of 10 mM-Tris at pH 8.0 and 30 ml 0.215 M-Na_2SO_4 in 10 mM-Tris buffer, pH 8.0, flow rate 60 ml · h^{-1}). Fractions containing α-acetolactate decarboxylase activity were pooled, dialysed at 2 °C against water (10 volumes), and again concentrated by ultrafiltration to 1 ml. The concentrated enzyme solutions were rechromatographed twice as above on the Mono Q column whereupon enzyme containing fractions were pooled and concentrated to 1 ml. This solution was divided into aliquots of 100 µl and further purified by high performance gel filtration on a TSK 3000 column (flow rate 33 ml · h^{-1}, eluent: 0.1 M-Na_2SO_4 in 50 mM-phosphate buffer at pH 6.7). The fractions containing α-acetolactate decarboxylase were pooled and stored at -20 °C.

2.2.3. Molecular weight

Molecular weight was estimated by means of high performance gel filtration using a TSK 3000 column as described in section *2.2.2* after calibration with ferritin, catalase, bovine serum albumin, ovalbumin, chymotrypsinogen A and

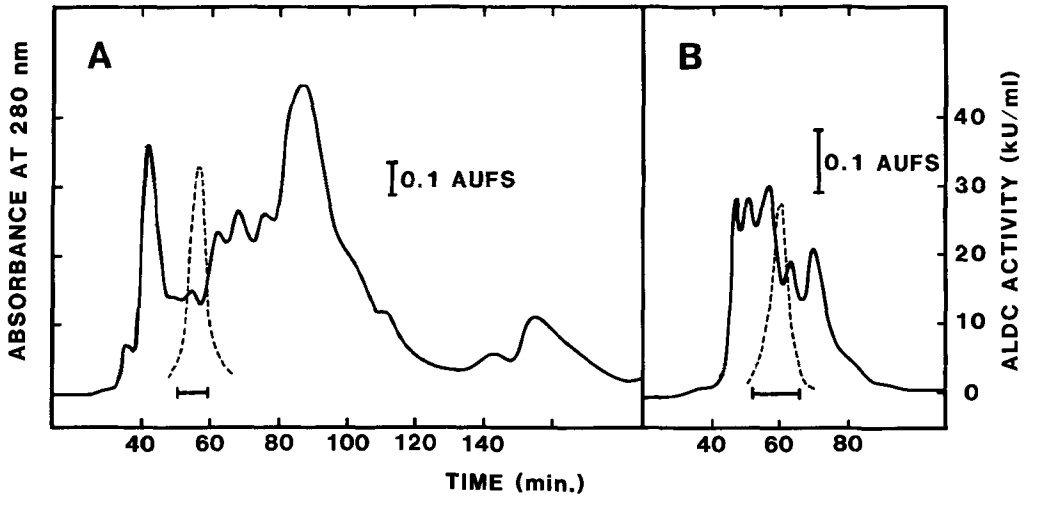

Figure 1. A: Chromatography of 670 mg of α-acetolactate decarboxylase, purified as in (10), on a Fractogel DE 650 S column at room temperature with a linear gradient of 350 ml 25 mM-Tris, pH 8.0 and 350 ml of 0.215 M-Na_2SO_4 in 25 mM-Tris, pH 8.0, flow 220 ml · h^{-1}. (– –) indicates ALDC activity and (⊢—⊣) the fractions pooled. B: Rechromatography of fractions from A, 109 mg protein. The resulting pool (⊢—⊣) was applied to a Mono Q column as shown in Figure 2.

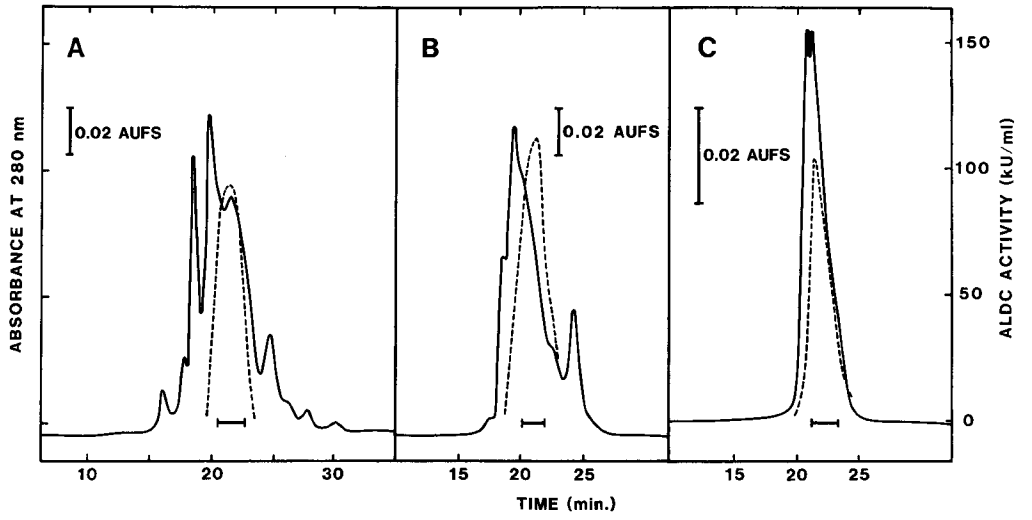

Figure 2. A: Chromatography of 3.5 mg of the resulting pool from the second Fractogel DE chromatography (Figure 1 B) on a Mono Q HR 5/5 column at room temperature with a linear gradient of 30 ml of 10 mM-Tris, pH 8.0 and 30 ml of 0.215 M-Na_2SO_4 in 10 mM-Tris, pH 8.0, flow 60 ml $\cdot$ h^{-1}. B: Rechromatography of the pool from A (⊢—⊣), 2.4 mg. C: Rechromatography of the pool from B, 1.4 mg yielding 0.4 mg of protein. (– –) indicates ALDC activity.

cytochrome c.

Molecular weight under denaturing conditions was determined by SDS polyacrylamide gel electrophoresis using homogeneous slabs (1 mm × 140 mm × 80 mm, 7.5% polyacrylamide gel) with the Weber and Osborn SDS-phosphate continuous buffer system in the Pharmacia Apparatus GE-2/4 as described in the Pharmacia Fine Chemicals laboratory Manual (14). β-Mercaptoethanol was omitted from the sample buffer and the Pharmacia LMW kit was used for molecular weight calibration.

2.2.4. Isoelectric focusing

Isoelectric focusing was carried out with 4% ampholines at pH 4-6 in a sucrose gradient using the LKB 110 column as described by the manufacturer.

2.2.5. Amino acid analysis

Amino acid analysis were performed with a Durrum Model D-500 automatic analyser after hydrolysis of the samples at 110 °C with 6 M-HCl in evacuated tubes for 24 hrs.

2.2.6. Optical rotation

Optical rotation was measured using a Perkin Elmer model 141 Polarimeter fitted with a 1 ml capacity cell of 1 dm pathlength. Rotations were measured at 589 nm and 20 °C.

3. RESULTS

3.1. Isolation of α-acetolactate decarboxylase

The starting material for the isolation of α-acetolactate decarboxylase from Lactobacillus casei DSM 2547 had already undergone chromatography steps on DE and CM cellulose as described in (10). Further purification on the relatively slow DE-cellulose and CM-cellulose columns resulted in great loss of activity while it was found that better results were obtained with resins and columns which could be operated at higher flow rates: approx. 670 mg of the partially purified enzyme could be chromatographed on Fractogel DE 650 S, as described in section *2.2.2*, within 3 hrs at room temperature with a five fold purification as seen from Figure 1 A and Table I. When α-acetolactate decarboxylase from two runs on the Fractogel column were combined and rechromatographed on the same

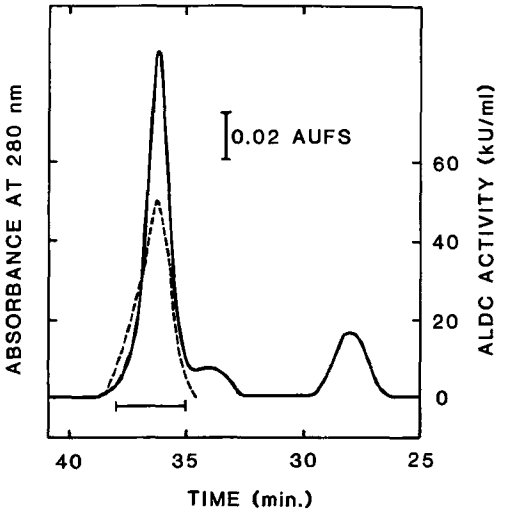

Figure 3. HPEC chromatography of 0.30 mg of the resulting α-acetolactate decarboxylase preparation from the Mono Q column on a TSK 3000 column with a flow rate of 33 ml · h^{-1}, eluent 0.1 M-Na_2SO_4 in 50 mM-phosphate, pH 6.7. The fractions with ALDC activity (– –) were pooled (⊢—⊣) and contained 0.09 mg of pure enzyme.

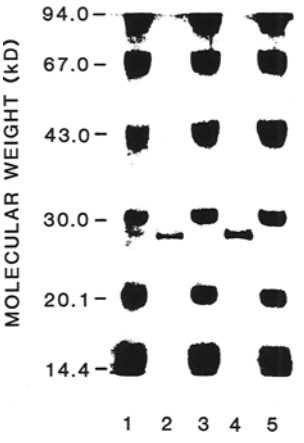

Figure 4. SDS gel electrophoresis of the pure α-acetolactate decarboxylase obtained by a final HPEC chromatography. Lanes 2 and 4 contain approximately 3.2 μg of the enzyme in non-reduced and reduced form, respectively. Lanes 1, 3 and 5 contain LMW standards.

column an additional seven fold purification was achieved (Figure 1 B) while further rechromatography on this column did not result in additional purification.

However, when a Mono Q column was used as described in section *2.2.2*, the preparation from the Fractogel column separated in many protein components, as shown in Figure 2 A. Repeated rechromatography on the Mono Q column eliminated most impurities (Figure 2 B and C) but a high performance gel filtration step was required to obtain homogeneous α-acetolactate decarboxylase (Figure 3), as judged by SDS gel electrophoresis (Figure 4). The purification scheme in Table I shows that the final product

Table I.
Purification scheme of α-acetolactate decarboxylase from a 250 l fermentation of Lactobacillus casei DSM 2547.

Fraction and step	Protein mg	Total ALDC kU	Specific activity kU · mg^{-1}	Enzyme Yield %	Purification -fold
Supernatant of homogenized L.casei cells	1,500,000	18,700	0.012	100	1
ALDC purified as in (10)	18,000	3,350	0.21	18	18
After ultrafiltration and dialysis	5,480	5,070	1	27	83
Fractogel, run 1	670	3,270	5	18	416
Fractogel, run 2	90	2,890	33	16	2,750
Mono Q, run 1	8	990	125	5	10,300
Mono Q, run 2	4	990	250	5	20,600
Mono Q, run 3	3	815	270	4	23,000
HPEC	1	375	375	2	31,000

Table II.
The amino acid composition of a 24 hrs hydrolyzate of α-acetolactate decarboxylase from L.casei. Trp and Cys were not determined.

Amino acid	Mole %	Amino acid	Mole %
Asp	9.1	Ile	3.5
Thr	7.6	Leu	12.3
Ser	3.2	Tyr	2.2
Glu	11.3	Phe	6.4
Pro	4.6	His	4.6
Gly	11.0	Lys	5.4
Ala	9.4	Arg	2.6
Val	6.8		

with a specific activity of 375 kU · mg^{-1} was obtained by 31,000 fold purification and that only 1 mg of α-acetolactate decarboxylase was obtained from the original 250 litres of fermentation liquid.

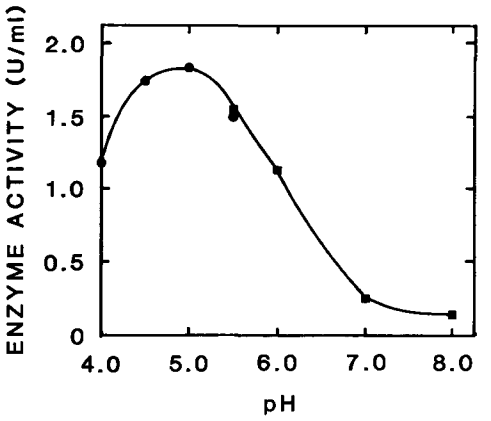

Figure 5. pH optimum curve for α-acetolactate decarboxylase. The assay was performed in acetate buffer (—●—) or phosphate buffer (—■—) at the indicated pH values for 30 min at 37 °C, after which the acetoin formed was estimated colorimetrically.

3.2. Physico-chemical characterization

The molecular weight of native α-acetolactate decarboxylase was estimated to be 48,000 by means of high performance gel filtration, while the molecular weight under denaturing conditions using SDS gel electrophoresis was estimated to be 27,500. This suggests that the enzyme is composed of two subunits.

The α-acetolactate decarboxylase had an isoelectric point at pH 4.7 and its approximate amino acid composition is listed in Table II. The content of glutamic acid and leucine was relatively high while no methionine was found. Due to lack of material the content of cysteine and tryptophan were not determined. The purified enzyme was relatively unstable with a half-life of approx. 15 min at 37 °C and pH 5. A study of possible stabilizing agents showed bovine serum albumin (BSA) to have the best effect: 1 mg · ml^{-1} of BSA increased the half-life at 37 °C and pH 5 to $4^1/_2$ hrs and in further experiments this concentration of BSA was added to all α-acetolactate decarboxylase solutions. The enzyme was unstable below pH 4 and above pH 9. It had the same temperature optimum as the partially purified enzyme (9) and the pH profile (Figure 5) showed a broad optimum around pH 4.5-5.0.

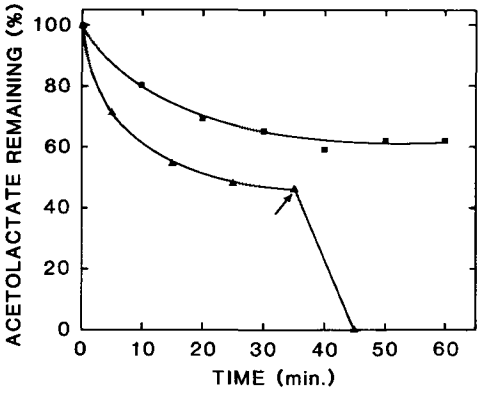

Figure 6. The degradation of α-acetolactate by α-acetolactate decarboxylase as judged by HPLC analysis. Solutions containing 25 ng (■) and 75 ng (▲) of purified enzyme preparation are incubated, at 37 °C, with BSA (1 mg · ml^{-1}), α-acetolactate (10 μmoles · ml^{-1}) and sodium phosphate buffer, pH 5.0 (200 μmoles · ml^{-1}). Aliquots of these solutions were removed at various times and subjected to HPLC analysis as described. A blank value was subtracted from all measurements: this was calculated from the rate of non-enzymatic decarboxylation of α-acetolactate as measured by HPLC analysis (approx. 0.2% · min^{-1}).

At the point indicated by the arrow 20 μl of concentrated H_2SO_4 was added to the assay which brought about total decarboxylation of the remaining α-acetolactate.

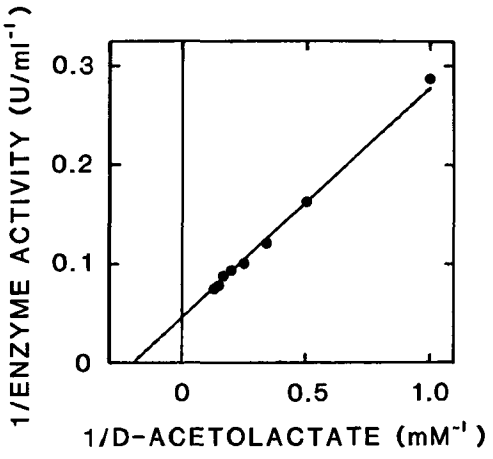

Figure 7. Double reciprocal plot of D-α-acetolactate concentration versus α-acetolactate decarboxylase concentration. Enzyme (0.051 μg) in a solution of 200 mM-acetate buffer, pH 5.0 and BSA (5 mg · ml^{-1}) was added to solutions containing various amounts of D,L-α-acetolactate (2-16 μmoles), 200 μmoles acetate buffer, pH 5.0 and 5 mg BSA to a final assay volume of 1 ml. The reaction was stopped after 5 minutes at 37 °C and the amount of acetoin estimated.

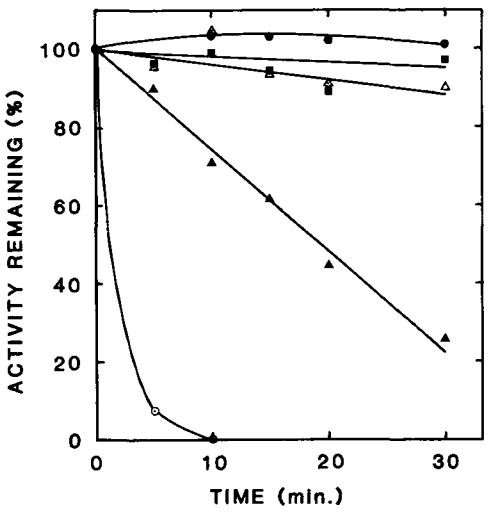

Figure 8. Inhibition of α-acetolactate decarboxylase by various metal ion chelating agents. 0.127 μg aliquots of α-acetolactate decarboxylase were incubated at 37 °C in solutions containing metal ion chelator (0.25 mM), 200 mM-acetate buffer, pH 5.0, BSA (5 mg · ml^{-1}) in a volume of 100 μl. At the time intervals indicated 10 μl aliquots of each solution were removed and assayed for ALDC activity. The metal ion chelators employed were: none (●); EDTA (▲); 1,10-phenanthroline (○); α, α'-dipyridyl (△) and 8-hydroxyquinoline (■).

3.3. Enzymatic properties

After incubation of a solution of (D,L)-α-acetolactate (10 μmoles · ml^{-1}) at 37 °C for 10 minutes with 1 kU α-acetolactate decarboxylase in 200 mM-acetate buffer, pH 5.0 the optical rotation of the reaction mixture was found to be laevorotatory with a value of –0.035 ° at 589 nm. This is in agreement with previously reported observations that α-acetolactate decarboxylase decarboxylates the D(+) isomer of α-acetolactate (11, 13) at a high rate forming the L(-) isomer of acetoin resulting in a nett laevorotatory effect on the assay solution.

The HPLC analysis of the total amount of α-acetolactate present (section *2.2.1*) confirmed that α-acetolactate decarboxylase decarboxylates only one isomer of α-acetolactate at a high rate (11, 13). Addition of acid to the enzymatically decarboxylated solution resulted in total disappearance of the peak corresponding to α-acetolactate (Figure 6). During the enzymatic decarboxylation no racemisation of the D- to the L-isomer was observed and the same was the case even after prolonged incubation at pH 4.0.

The colorimetric method and the HPLC method showed very similar results if the duration of the assay was kept below approx. 40 minutes, that is, before the formation of a substantial blank value occurs.

The kinetic constants were determined from the rates of decarboxylation of α-acetolactate over a range of (D,L)-α-acetolactate concentrations from 2 to 16 millimolar as shown in Figure 7. The K_m value calculated for one isomer, (D)-α-acetolactate was 4.8 mM and k_{cat} was approximately 5.570 S^{-1}, assuming a molecular weight of 48,000.

3.4. Metal requirement

The effect of metal ion chelating agents on the activity of pure α-acetolactate decarboxylase was investigated. Figure 8 shows the time dependent inhibition by four such agents: 1,10-phenanthroline produced rapid total inhibition,

Table III.

Reconstitution of α-acetolactate decarboxylase activity by divalent metal ions. Solutions of 1.3 μg of α-acetolactate decarboxylase in 500 μl of 200 mM-acetate buffer, pH 5.0 containing BSA (1mg · ml^{-1}) and chelating agent (0.25 mM) were incubated at 37 °C for 10 min (1,10-phenanthroline) or 20 min (EDTA). 10 μl aliqouts were then transferred to a volume of 0.8 ml containing 1 μmole of the relevant metal ion in the above buffer and incubated for a further 10 min at 37 °C after which the amount of ALDC activity was determined. A no-enzyme blank was used for each assay to account for the effect of metal ions on the absorbance at 520 nm.

Assay	% of native activity	
	1,10-phenanthroline	EDTA
Enzyme – chelating agent	100	100
Enzyme + chelating agent	6	27
" + Ba^{2+}	1	21
" + Ca^{2+}	4	27
" + Co^{2+}	10	45
" + Cu^{2+}	2	1
" + Fe^{2+}	–	53
" + Mg^{2+}	–	26
" + Mn^{2+}	0.1	54
" + Zn^{2+}	12	72

whilst EDTA inhibited the enzyme at a lower rate, while the remaining two chelators had only a slight effect.

These findings, together with earlier results (9) imply that α-acetolactate decarboxylase activity is dependent on a metal atom, activity being lost on removal of this metal by complexing agents. The inhibitory effect of 1,10-phenanthroline was shown to be due to its chelating properties (19) as incubation of the enzyme with 1,10-phenanthroline in the presence of excess Zn^{2+} ions did not inhibit activity.

Attempts were made to reconstitute the activity of the purified enzyme inhibited with metal ion chelating agents by subsequent incubation with divalent metal ions (Table III). When 1,10-phenanthroline was the chelating agent only Zn^{2+} and Co^{2+} ions had a slight stimulatory effect on enzyme activity, whereas the EDTA inhibited enzyme was reactivated by Co^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} ions (in order of increasing effectiveness). However, full activity was not regained with any of the metal ions used and incubation of the phenanthroline treated enzyme with metal ions for longer periods than 10 minutes resulted in no further increase in activity. Other attempts to reverse the inhibition by 1,10-phenanthroline were made, such as incubation with combinations of metal ions or carrying out the experiment in the presence of dithiothreitol (to prevent oxidation of exposed -SH groups (19)), but none proved effective.

The stimulatory effect of Zn^{2+} ions on the phenanthroline and EDTA treated enzyme again suggest that this ion may be a constituent of α-acetolactate decarboxylase. However, metal ion analysis of the enzyme would have to be carried out to confirm this since reconstitution of 100% activity is not possible.

Among the divalent metal ions only Cu^{2+} ions proved to be inhibitory. This may in part be due to complex formation between Cu^{2+} and acetolactate catalysing the non-enzymatic decarboxylation as described by DE MAN (2) but it seems likely that α-acetolactate decarboxylase is directly inhibited by Cu^{2+} ions also.

The enzyme was found to be sensitive to inhibition by p-hydroxy-mercuribenzoate which implies the involvement of sulphydryl groups in the active site of the enzyme.

It has been suggested by BRANEN and KEENAN (1) that thiamine pyrophosphate was essential for the activity of α-acetolactate decarboxylase in crude extracts of L.casei. This was not confirmed in our laboratory as only addition of zinc could stabilize partially purified α-aceto-

lactate decarboxylase from L.casei DSM 2547 in solutions at pH 4.2 containing yeast cells (9), addition of thiamine pyrophosphate had no effect.

4. DISCUSSION

The α-acetolactate decarboxylase produced by Lactobacillus casei DSM 2547 is shown to be quite different from the enzyme described by LØKEN and STØRMER (12) from A.aerogenes, since it has higher specific activity (375 against 4.3 kU · mg^{-1}), lower molecular weight (48,000 against 73,000), lower isoelectric point (4.7 against 5.6), lower pH optimum (5.0 against 6.2-6.4) and higher K_m (4.8 against 3.4 mM).

Furthermore, the α-acetolactate decarboxylase from L.casei is found to be zinc dependent while the enzyme from A.aerogenes does not require any cofactor (12). The pure α-acetolactate decarboxylase is unstable in diluted solutions, where partially purified preparations were stable. This can be explained by impurities stabilizing the enzyme as seen by BSA's stabilizing effect on the pure α-acetolactate decarboxylase.

The low pH optimum and high specific activity of the α-acetolactate decarboxylase from L.casei would have made this enzyme suitable as a maturation aid in beer production. However, yeast cells were found to inactivate the enzyme by removal of zinc (9). Further investigations are in progress with the aim of isolating an enzyme exhibiting a good stability and activity in fermenting wort.

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REFERENCES

1. BRANEN, A.L. & T.W. KEENAN: Biosynthesis of α-acetolactate and its conversion to diacetyl and acetoin in cell-free extracts of Lactobacillus casei. Can. J. Microbiol. 8, 480-485 (1972)
2. DE MAN, J.C.: The formation of diacetyl and acetoin from α-acetolactic acid. Rec. trav. chim. Pays-Bas 78, 480-486 (1959)
3. DEBUSSOLO, J.M.: Reversed-phase liquid chromatography of proteins and peptides. Int. Biotech. Lab. 2, 14-31 (1984)
4. EGGLETON, P., S.R. ELSDEN & N. GOUGH: The estimation of creatine and of diacetyl. Biochem. J. 37, 526-529 (1943)
5. FRIDOVICH, I: Acetoacetate decarboxylase. The Enzymes, Ed. Boyer, P.D., Academic Press. Vol. 6, 3. ed., 255-270 (1972)
6. GODTFREDSEN, S.E. & M. OTTESEN: Maturation of beer with α-acetolactate decarboxylase. Carlsberg Res. Commun. 47, 93-103 (1982)
7. GODTFREDSEN, S.E., M. OTTESEN, P. SIGSGÅRD, K. ERDAL, T. MATHIASEN & B. AHRENST-LARSEN: Use of α-acetolactate decarboxylase for accelerated maturation of beer. EBC congress, Lecture No. 17 (1983)
8. GODTFREDSEN, S.E., H. LORCK & P. SIGSGÅRD: On the occurrence of α-acetolactate decarboxylase among microorganisms. Carlsberg Res. Commun. 48, 239-247 (1983)
9. GODTFREDSEN, S.E., A.M. RASMUSSEN, M. OTTESEN, T. MATHIASEN & B. AHRENST-LARSEN: Application of the acetolactate decarboxylase from Lactobacillus casei for accelerated maturation of beer. Carlsberg Res. Commun. 49, 69-74 (1984)
10. GODTFREDSEN, S.E., A.M. RASMUSSEN, M. OTTESEN, P. RAFN & N. PEITERSEN: Occurrence of α-acetolactate decarboxylases among lactic acid bacteria and their utilization for maturation of beer. Appl. Microbiol. Biotech. 20, 23-28 (1984)
11. HILL, R.K., S. SAWADA & S.M. ARFIN: Stereochemistry of valine and isoleucine biosynthesis IV. Bioorganic Chemistry 8, 175-189 (1979)
12. LØKEN, J.P. & F.C. STØRMER: Acetolactate decarboxylase from Aerobacter aerogenes. Purification and Properties. Eur. J. Biochem. 14, 133-137 (1970)
13. JUNI, E.: Mechanisms of formation of acetoin by bacteria. J. Biol. Chem. 195, 715-726 (1952)
14. Pharmacia Fine Chemicals: Polyacrylamide Gel Electrophoresis. Laboratory techniques 27-28 (1980)
15. SPECKMAN, R.A. & E.B. COLLINS: Diacetyl biosynthesis system in Streptococcus diacetylactis and Leuconostoc citrovorum. J. Bacteriol. 95, 174-180 (1968)

16. STØRMER, F.C.: 2,3-Butanediol biosynthetic system in Aerobacter aerogenes. Methods Enzymol. 41, 518-533 (1975)
17. STØRMER, F.C.: The pH 6 acetolactate-forming enzyme from Aerobacter aerogenes. Kinetic studies. J. Biol. Chem. 243, 3735-3739 (1968)
18. UMBARGER, H.E. & B. BROWN: Isoleucine and valine metabolism in Escherichia coli. J. Biol. Chem. 233, 1156-1160 (1958)
19. VALLEE, B.L. & A. GALDES: The metallobiochemistry of zinc enzymes. Adv. Enzymol. 56, 283-430 (1984)
20. WATT, D. & L.O. KRAMPITZ: α-Acetolactic acid, an intermediate in acetylmethylcarbinol formation. Fed. Proc. 6, 301-302 (1947)
21. WESTERFELD, W.W.: A colorimetric determination of blood acetoin. J. Biol. Chem. 161, 495-502 (1945)

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