COMPLETE AMINO ACID SEQUENCE OF α -ACETOLACTATE DECARBOXYLASE FROM *BACILLUS BREVIS*

by

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The complete amino acid sequence of acetolactate decarboxylase (EC 4.1.1.5) from *Bacillus brevis* has been determined by sequencing of the intact enzyme and of peptides obtained by cleavage with cyanogen bromide, *Staphylococcus aureus* V8 protease and trypsin, respectively. Determination of the C-terminal part was made by treatment with carboxypeptidases Y and M II. The enzyme has a molecular weight of 29.093 and consists of 260 amino acid residues arranged in a single peptide chain without disulphide bonds.

1. INTRODUCTION

The rate limiting step in beer maturation is the non-enzymatic oxidative decarboxylation of α -acetolactate to diacetyl. One way of accelerating beer maturation is to add acetolactate decarboxylase to fermenting or maturing beer and thereby rapidly convert α -acetolactate to acetoin which contrary to diacetyl does not give an off-flavour to the beer (3, 4, 5, 6). An enzyme isolated from *Bacillus brevis* has been used successfully in laboratory experiments, but nothing is known about the mechanism by which the enzyme operates. As an initial step in elucidating the structure-function relationship of the enzyme the primary structure has been

determined by conventional amino acid sequencing using automated Edman degradation.

2. MATERIALS AND METHODS

2.1. Materials

Acetolactate decarboxylase (ALDC) was isolated from *Bacillus brevis* as described below. *S. aureus* V8 protease was from ICN Immuno Biologicals, Lisle, Ill., USA and trypsin type XI from Sigma, St. Louis, USA. Carboxypeptidase Y was from Carlbiochem, Copenhagen, Denmark and carboxypeptidase MII was purified in Department of Chemistry, Carlsberg Laboratory. Reagents and solvents used in sequencing

Abbreviations: ALDC = acetolactate decarboxylase; CPD-MII = carboxypeptidase II from malt; CPD-Y = carboxypeptidase Y from yeast; TFA = trifluoro acetic acid.

were from Applied Biosystems, Foster City, Cal., USA. S-Sepharose FF, PBE 96, Sephadex G 25 SF, Mono-P HR 5/20, Superose 12 HR 10/30 and Polybuffer 96 were from Pharmacia, Uppsala, Sweden. Biogel P60 and Bio-Rad Protein Assay were products of Biorad, Richmond, Cal., USA and Vydac C₁₈ obtained from The Separation Group, Hesperia, Cal., USA. All other chemicals were of the highest purity obtainable and "Milli Q" water was used throughout. Ultrafiltration equipment was from Amicon Corp., Danvers, Mass., USA.

2.2. Methods

2.2.1. Extraction and purification of ALDC

Bacillus brevis ATCC 11031 was grown on a complex medium composed of yeast extract, corn steep liquor, hydrolyzed casein, hydrolyzed soybean meal and glutamate. Cells were harvested from a continuous (d=0.2) fermentation broth by centrifugation on a SAMS centrifuge at a speed of 1200 liters per hour. The sludge was homogenized in a Manton Gaulin homogenizer at 550 bar and extracted at pH 7 with four volumes of water. Following clarification by filtration the extract was concentrated by ultrafiltration to 1.5% of the original fermentation broth volume. The concentrated extract was germ filtered and finally spray-dried. The activity yield was 6.5%.

120 g of spray-dried whole cell extract was suspended in 600 ml water and pH was adjusted to 4.6 with acetic acid (700 ml, 171.5 U/ml, 2.07 U/mg prot.). The mixture was kept at 50 °C for 30 minutes. After centrifugation the supernatant was dialyzed against 0.05 M ammonium acetate pH 4.8. The retentate was filtered (0.45 µ) and applied onto a column of S-Sepharose FF (800 ml, 5 cm diam., 10 ml/min) equilibrated with the same buffer. The column was washed with starting buffer (1 l) and the activity was eluted with an ammonium acetate gradient 0.05-1.5 M (total vol. 4 l). The enzymatically active fractions were pooled (840 ml). pH was adjusted to 6.0 and the pool was first concentrated on an Amicon hollow-fiber cartridge (H1P10-20) and finally in a stirred cell (PM 10 membrane) to 19 ml (2950 U/ml, 46.4 U/mg prot., step yield 46.7%).

17 ml was dialyzed against 0.025 M MES pH 6.1. After centrifugation and filtration (0.45 µ) the sample was passed through a 40 ml column of PBE 96. The flow-through was collected and concentrated to 9.5 ml in a stirred ultrafiltration cell (3072 U/ml, 134.7 U/mg prot., step yield 63.3%).

Buffer exchange to 0.025 M Tris pH 8.3 (acetic acid) was done on an 80 ml column of Sephadex G 25 SF. The protein fraction (30 ml) was applied on a Mono-P HR 5/20 at a flowrate of 0.5 ml/min. The column was washed with 0.025 M Tris pH 8.3 and then eluted with Polybuffer 96 (0.0075 mmol/pH/ml) pH 6.2 with acetic acid. The three most active fractions were pooled (3 ml, 4687 U/ml, step yield 51.8%, no prot. determination).

Polybuffer was removed by gel filtration on Superose 12 HR 10/30 (flowrate 0.5 ml/min, 0.2 M ammonium acetate pH 6.0). 0.3 ml was applied in each run. The pooled fractions contained 1192 U/ml, 1610 U/mg prot., step yield 84.4%. Total yield was 12.9%. The preparation was homogeneous as judged by SDS-PAGE.

Enzyme activity was determined as described earlier (6). The Bio-Rad Protein Assay was used with ovalbumin as protein standard.

2.2.2. Cleavage with cyanogen bromide

To 170 µg (6 nanomoles) of ALDC were added 2 mg of cyanogen bromide in 70% formic acid and the reaction mixture was left overnight in the dark at room temperature. The resulting peptides were separated on a C₁₈ column (Vydac 218TP54) using a Water's HPLC system with 0.1% TFA and a linear gradient in acetonitril from 0 to 60% in 90 min. Relevant peaks were collected, dried under vacuum (in a Savant Speed Vac Concentrator) and sequenced.

2.2.3. Digestion with *S. aureus* V8 protease

To 170 µg (6 nanomoles) of ALDC dissolved in 400 µl of 0.1 M NH₄HCO₃, pH 7.8 and *S. aureus* V8 protease was added in the ratio 1:20 (w/w). The mixture was left at 40 °C in a water-bath with stirring for 6 hrs. The resulting peptides were separated as described under 2.2.2.

2.2.4. Digestion with trypsin

To 170 µg (6 nanomoles) of ALDC dissolved in 400 µl 0.1 M NH_4HCO_3 , pH 7.8, was added trypsin in the ratio 1:100 (w/w) and the mixture left overnight with stirring at room temperature. The resulting peptides were separated as described under 2.2.2.

2.2.5. Amino acid analysis

The amino acid composition of the intact enzyme was determined by acid hydrolysis in 5.7 M HCl for 24, 48 and 72 hrs at 110 °C in sealed and evacuated tubes and in duplicate. In those cases where sufficient material was available, 24 hrs analyses were conducted with peptides obtained from cleavage with chemicals or enzymes as described in 2.2.2 to 2.2.4. A Durum 500 amino acid analyzer was used.

2.2.6. Amino acid sequencing

An Applied Systems Sequenator model 470A, equipped with an on-line HPLC, Applied Systems model 120A PTH Analyzer, was used for determining the amino acid sequences of intact protein and peptides. Program no. 03CPTH supplied by the manufacturer was used.

2.2.7. Digestion with carboxypeptidases

In order to determine the C-terminal sequence of ALDC, 4.5 nmol of the C-terminal peptide obtained by cleavage with cyanogen bromide was dissolved in a 50 mmol sodium acetate buffer pH 4-5 and first treated with CPD-MII for 30 min at room temperature (ratio 1:199 w/w), whereafter an aliquot was removed for analyses of free amino acids. The reaction mixture was subsequently treated with CPD-Y for 30 min (ratio 1:100) during which period aliquots were removed at suitable time intervals for amino acid analysis. In all cases the enzymatic reaction was stopped by addition of HCl to pH 2.

2.2.8. Sequence alignment and prediction of secondary structure

Sequence comparison with ALDC from *E.*

aerogenes was made manually while the prediction of secondary structure was done using the programme PREDICT from the Protein Sequence Database of PIR.

3. RESULTS AND DISCUSSION

The amino acid sequence of 260 residues shown in Figure 1 was obtained from sequencing of the intact enzyme and peptides obtained by cleavage with cyanogenbromide, *S. aureus*, strain V8 protease, and trypsin. Of the peptides sequenced only those necessary to obtain overlaps are included in the figure.

N-terminal sequencing of the enzyme gave rise to two sequences, one starting at Thr1 and the other starting at Thr4. The enzyme thus has a "ragged" N-terminal which conceivably is the result of proteolytic activity taking place during purification.

Amino acid sequences of peptides obtained by cleavage with cyanogen bromide which were of importance for the construction of the total sequence were Leu39-Met57, Leu123-Met144 and Val145-Lys166. In the first two cases the peptide was sequenced to the end and a peak always appeared in the same position as PTH-Thr on the HPLC chromatograms. It has not been shown whether this peak corresponds to PTH-homoserine or its lactone. However, since PTH-homoserine will be more hydrophilic than its lactone it is most likely PTH-homoserine which is observed eluting from the reversed phase column at this position. Sequences of peptides obtained from cleavage with *S. aureus* V8 protease were Ser14-Asp45 (indicating incomplete cleavage at Glu43), Met69-Pro89 (indicating incomplete cleavage at Glu87), Ser91-Glu106, Lys107-Leu119, Asn129-Gln153, Phe172-Thr202, Asp204-Glu222 and Phe229-Ala255. Finally, the following sequences of peptides obtained from cleavage with trypsin are included in Figure 1: Asn26-Ala36, Gly55-Met69, Leu85-Lys93, Thr108-Lys121, Gln153-Lys173, Gly177-Lys205 and Thr206-His235.

It is apparent that two overlaps are missing in the sequence: between Glu106 and Lys107 and between Lys121 and Leu123. (The Met in position 122 is inferred from cleavage with cyanogen bromide.) However, comparison with a corre-

Figure 1. The amino acid sequence of acetolactate decarboxylase from *Bacillus brevis*. → indicates start of sequencing and number of residues sequenced. ↓ indicates the alternative starting point in N-terminal sequencing of intact protein. Polymorphism was observed in positions 33 and 82.

Attempts to determine the C-terminal of ALDC on the intact protein failed. However, using the C-terminal cyanogenbromide fragment Val145-Lys260 (Fig. 1) gave the result shown in Figure 2. It is clear that Lys is the C-terminal residue followed by an Arg. However, it was difficult to place the Ser relative to the two Glu. Ser was placed in position 257 after comparison with the translated nucleotide sequence (1).

from the corresponding nucleotide sequence (1) shows identity except for three positions. Gln249 found in this study is Thr249 in the nucleotide sequence. Thr was found in position 33 with both methods, but in addition amino acid sequencing of the tryptic peptide starting at Asn26 showed a Gly in position 33. However, no Gly was observed when the *S. aureus* V8 peptide starting at Ser14 was sequenced. Finally, in one case where the CNBr peptide starting at Gly73 was sequenced, a Thr as well as a Val were found in position 82. This microheterogeneity thus indicates that more than one gene codes for ALDC in *B. brevis*.

Another nucleotide sequence corresponding

Figure 2. Determination of the C-terminal amino acid sequence of acetolactate decarboxylase by digestion with carboxypeptidase MII followed by carboxypeptidase Y. The liberated amino acid residues are given by the one letter code. For details see text.

to acetolactate decarboxylase from *Enterobacter aerogenes* (9) has also been sequenced. A comparison between that and the one described in the present investigation is shown in Figure 3. The two enzymes are of approximately the same length the *B. brevis* enzyme extending two residues further in the C-terminal end compared to the one from *E. aerogenes*. It has not been necessary to introduce gaps in order to obtain a homology of 31% (86 residues in identical positions). No similarity between the sequences is observed for the N-terminal 23 residues or the 15 C-terminal residues. On the contrary, strong homology is observed in the regions Gly55-Ile70, Lys93-Pro104, Arg148-Tyr157 and Asn192-Leu212. Nothing is known about which amino acid residues are involved in the catalytic function of ALDC. It is known, however, from well characterized families of enzymes that amino acid sequences around catalytic active residues are well preserved. It is therefore reasonable to assume that the functional amino acid residues of ALDC may be found in the homologous regions mentioned above.

If it is speculated that the reaction mechanism in ALDC is the same as found with acetoacetate decarboxylase where an intermediate Schiff-base is formed with an ϵ -amino group of an

essential lysyl residue (7, 8) it is noteworthy to compare the numbers and positions of the lysyl residues in the two ALDC's for which the amino acid sequences are known. While ALDC from *B. brevis* has 23 Lys and only 4 Arg the ALDC from *E. aerogenes* has only 5 Lys, but 15 Arg (Table I). When the two sequences are compared only position 93 has a Lys in both enzymes which is located N-terminal in a highly homologous sequence. Thus, if the two ALDC's operate by the mechanism suggested in part above, Lys93 is likely the essential amino acid.

Table I. Amino acid composition of ALDC from *B. brevis* and *E. aerogenes*.

Amino acid	<i>B. brevis</i>	<i>E. aerog.</i> ³⁾
Asx	27.10 ¹⁾	27 ²⁾
Thr	28.20	15
Ser	11.28	18
Glx	35.00	29
Pro	20.86	13
Gly	15.00	18
Ala	16.52	21
$\frac{1}{2}$ Cys	0	4
Val	19.15	14
Met	5.27	7
Ile	8.26	11
Leu	25.26	24
Tyr	6.74	7
Phe	15.76	15
His	9.69	12
Arg	3.24	15
Lys	23.38	5
Trp	0	1
Total	260	258

¹⁾From amino acid analysis.

²⁾From amino acid sequence.

³⁾From ref. (9).

One striking difference is seen between the two ALDC's in Figure 3. While the enzyme from *E. aerogenes* contains four Cys in positions 6, 8, 13 and 134, the one from *B. brevis* is devoid of Cys residues. It is not known whether the four Cys in the *E. aerogenes* enzyme are present as free Cys or as two disulfide bridges, but in ALDC from *B. brevis* the tertiary structure must be maintained by other means as is known from another group of bacterial enzymes, the subtilisins.

Figure 3. Comparison of the amino acid sequence of acetolactate decarboxylase of *Bacillus brevis* (B) with that obtained from nucleotide sequencing of the corresponding enzyme from *Enterobacter aerogenes* (E) (9). Residues in identical positions are boxed. The sequence of the *E. aerogenes* enzyme may be two Met residues longer in the N-terminal since the starting position of the amino acid sequence has not been determined.

Predictions of the secondary structure by the method of GARNIER et al. (2) indicate a highly ordered structure of ALDC from *B. brevis*. 54% is predicted as being α -helix and 34% as being β -sheets. The corresponding figures for ALDC from *E. aerogenes* are also 54% helix and 34% β -sheet.

The molecular weight calculated on the basis of the amino acid sequence is 29.093. In SDS-PAGE the enzyme appears to have a molecular weight of 35.000 and in gelfiltration experiments 70.000 indicating that under native conditions ALDC appears as a dimer. Since ALDC does not contain any carbohydrate the rather

large discrepancy between the calculated and experimentally determined molecular weight is difficult to explain.

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