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Received June 23rd, 1969

Biochim. Biophys. Acta, 191 (1969) 198–200

BBA 61200

Purification and mode of action of a lytic enzyme from *Bacillus cereus*

We have described earlier a lytic enzyme which is formed in ultraviolet-irradiated cultures of *Bacillus cereus*[1]. The enzyme is able to hydrolyze the isolated *B. cereus* cell wall[2]. After induction by ultraviolet irradiation, the enzyme activity greatly increases in the culture media, and we have attempted the isolation of the lytic enzyme.

The lytic activity is expressed in arbitrary units, 1 unit of the enzyme is defined as the amount which leads to a decrease of 0.001 unit of absorbance in 20 min at 20° when present in 4 ml of a cell-wall suspension having an initial absorbance of 0.5. Details of the test were described earlier[2].

B. cereus was grown on a casamine medium[1], induced by ultraviolet irradiation and harvested 90–100 min after induction. The cells were resuspended in one-tenth of the original volume and sonicated[1]. The disintegrated cells were recombined with the supernatant culture fluid and thus a crude extract was obtained. 2.5 l of the crude extract were mixed with 2.8 l of acetone (−5°) and centrifuged at 0°. The precipitate was discarded, and the enzyme precipitated from the supernatant by further addition of 2.15 l of cold acetone. The precipitate was dissolved in 250 ml of 0.01 M Tris–HCl buffer (pH 7.8). This was Fraction 1.

Further purification was achieved by adding 150 ml of acetone to the solution obtained in the previous step. The precipitate of the inactive proteins was removed by centrifugation, and the enzyme was precipitated from the supernatant by the addition of a new portion of 150 ml of acetone. The precipitate was dissolved in 25 ml of 0.01 M Tris–HCl buffer (pH 7.6) and dialyzed against the same buffer solution at 3–5° for 24 h (Fraction 2).

DEAE-cellulose chromatography was performed (Fig. 1) applying 45 mg protein obtained in the previous step to the column. Elution was performed by Tris–HCl

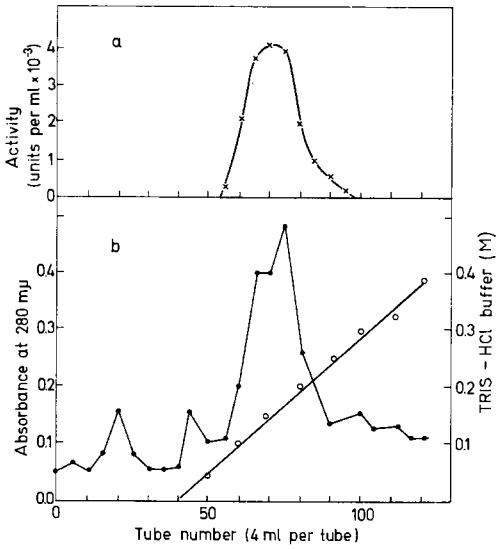

Fig. 1. DEAE-cellulose chromatography of the lytic enzyme. (a) Lytic activity of the eluted fractions. (b) Absorbance of the proteins and the concentration of the eluting buffer. The DEAE-cellulose column (1.5 cm × 20 cm) was equilibrated by 0.01 M Tris–HCl buffer (pH 7.6). The column was loaded by 15 ml (45 mg) of lytic enzyme obtained in the second acetone fractionation and then washed with 50 ml of equilibrating buffer. The elution of the proteins was achieved by increasing concentrations of Tris–HCl buffer (pH 7.6). ×—×, lytic enzyme activity; ●—●, absorbance at 280 mμ of the fractions; ○—○, concentration of Tris–HCl buffer.

buffer gradient at pH 7.6. Enzyme activity emerged together with the main protein peak when the buffer concentration reached 0.1 M.

Yields and the specific activity of the enzyme during purification are shown in Table I. The specific activity increased in this case by a factor of 15. Further purification steps did not result in any increase in specific activity, and the enzyme activity appears to be associated with only one protein fraction.

Enzymes attacking the cell wall may split either the glycosidic bonds of the polysaccharide chains built up from glucosamine and muramic acid or the peptide bonds connecting the polysaccharide chains.

The changes in amino groups and reducing sugar content were studied when the

TABLE I

YIELD AND SPECIFIC ACTIVITY OF THE LYTIC ENZYME DURING PURIFICATION

Step	*Total activity (units)* × 10^{-3}	*Specific activity (units/mg protein)* × 10^{-3}
Crude extract	570	1.1
Fraction 1	502	5.3
Fraction 2	405	9.0
DEAE-cellulose chromatography	245	169.0

TABLE II

CHARACTERIZATION OF THE EFFECT OF THE LYTIC ENZYME ON THE *B. cereus* CELL WALL

Reaction mixture a: 40 mg of cell wall were suspended in 10 ml of water, pH was adjusted to 7.8 by NaOH and the mixture was incubated with 1 mg of purified lytic enzyme at room temperature. Reaction mixture b: as Reaction mixture a but without lytic enzyme. Reaction mixture c: 40 mg of cell wall suspended in 10 ml of 1 M HCl and boiled for 60 min. Samples of 1 ml were taken from Reaction mixtures a and b and reacted with FDNB[3]; subsequently the DNP-peptides were hydrolyzed by 6 M HCl for 16 h. The DNP-amino acids were extracted[4] and separated by paper chromatography[5]. The spots of the separated DNP-amino acids were cut out and eluted. Their quantity was determined by the absorption measured at 360 mμ. Samples of 0.4 ml were taken to determine the reducing groups[6]. Reducing groups were expressed in glucose equivalent.

Reaction mixture	*Time (min)*	*μmoles of DNP-alanine per g cell wall)*	*mmoles of glucose equivalent per g cell wall*	$A_{620\ m\mu}$
a	0	6.4	0*	16.0
	60	82	0*	4.0
b	0	6.4	0*	16.0
	60	6.4	0*	16.0
c	0	—	0*	16.0
	60	—	1.2	1.0

* Reducing groups could not be detected.

purified lytic enzyme acted on the purified cell-wall preparation of *B. cereus*. As it is shown in Table II, no increase could be detected in the amount of reducing sugars, whereas the free amino group of alanine increased up to 10 times the original amount, and no other free amino group appeared.

The purified lytic enzyme seems to be associated with a single protein fraction. It liberates the amino group of alanine from the bacterial cell wall. Accordingly, the enzyme appears to be an amidase, which splits the peptides connecting the polysaccharide chains of the cell wall.

The author wishes to thank Prof. F. B. Straub for his interest and helpful discussion.

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Received July 14th, 1969