Occurrence of a Thermoacidophilic Cell-bound Exo-Pectinase in *Alicyclobacillus acidocaldarius*

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**ABSTRACT.** *Alicyclobacillus acidocaldarius* was able to degrade pectin under thermoacidophilic conditions of high temperature and acidity. Both extracellular and cell-bound pectolytic activities were found (28 and 72 % of total activity, respectively). When *A. acidocaldarius* was subjected to lysozyme or sonication, more than 50 % of the activity was found to be bound with the cell debris. The cell-bound enzyme presented principally exopectolytic activity. SDS-PAGE and zymogram showed that the estimated molar mass of the crude enzyme was 52 kDa. pI optimum was between 1.5 and 2.0 and the enzyme was thermostable at 70 °C for 1 h at pH 2.0.

Pectinases are used in food technology and are produced by various microorganisms: fungi, yeasts and bacteria (Fogarty and Kelly 1983; Pilnik and Rombouts 1979). Pectic enzymes have been intensively investigated (Pilnik and Rombouts 1979) but little is known about thermoacidophilic forms. Microorganisms growing under extreme conditions of pH and temperature are of biotechnological interest, because of their ability to produce enzymes with higher catalytic activity and stability than enzymes of mesophilic bacteria growing in neutral media. *A. acidocaldarius* isolated from acid hot springs (Darland and Brock 1971) and originally described as *Bacillus acidocaldarius* (Wisotzkey et al. 1992), is a thermoacidophilic bacterium which produces an unusual amylase (Kanno 1986; Koivula et al. 1993; Schwermann et al. 1994). Evidence on the presence of an unusual temperature- and acid-stable cell-bound exopectinase activity is shown in this paper.

**MATERIALS AND METHODS**

*Bacterial strain and medium.*** Alicyclobacillus acidocaldarius* ATCC 27009 previously known as *Bacillus acidocaldarius* ATCC 27009 was obtained from American Type Culture Collection (MD, USA). The strain was maintained in a sterile broth (M573 medium)—glycerol (60:40 %, V/V) solution at −20 °C.

*Detection of pectolytic activity.* 0.2 mL of culture grown overnight in M573 containing 2 % polygalacturonic acid was plated onto agar plates of M573 medium containing 2 % of polygalacturonic acid. After incubation at 55 °C, enzymic activity was detected by determining the diameter of the hydrolis zones surrounding the colonies, after staining the plates with ruthenium red.

*Pectinolytic activity assay.* For the determination of pectinolytic activity, the cells were grown in a medium containing 1 % partially methylated pectin (47 % esterified pectin — citrus pectin — Sigma, USA) and incubated at 55 °C with shaking for 3 d in 1-L conical flasks. Cultures were centrifuged (170 Hz, 10 min, 4 °C), and the supernatant and the resuspended pellet (in 2 mL water) were assayed.

Pectinolytic activity was determined by measuring the increase in reducing groups from citrus pectin by the DNS method (Miller 1959). An enzyme sample (0.2 mL) was mixed with 1 mL of 1 % citrus pectin in 50 mmol/L acetate buffer (pH 4) and the mixture adjusted to a final volume of 1.5 mL with distilled water. The reaction mixture was incubated at 50 °C for 1 h. One unit of enzyme activity was defined as the amount that liberated 1 mg galacturonic acid per hour under the specified conditions.

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Endopectolytic activity was determined by measuring relative changes in viscosity at 50 °C in a rotational viscosimeter (Grant Instruments, Cambridge, England). Reaction mixtures contained 10 mL of 1 or 2 % (W/V) citrus pectin solution (Sigma) in 50 mmol/L phosphate buffer (pH 2). Enzyme assays were started by addition with gentle mixing of 0.5 mL of sample (resuspended cell pellet) to the buffered pectin solution contained in the viscosimeter. One unit of endopectolytic activity was defined as the amount of enzyme that reduced the viscosity of 10 mL pectin solution by 50 % in 10 min at pH 2. A control was made without addition of the enzyme sample in order to eliminate the interference of low pH (2) with the viscosity change.

All control assays were performed using heat-denatured samples. Assays were run in duplicate and repeated three times.

Electrophoresis and molar mass estimation. Polyacrylamide gel electrophoresis (PAGE) was done in 7 % (W/V) gels copolymerized with 0.5 % pectin (Sigma) by the method of Laemmli (1970), and with 0.1 % sodium dodecyl sulfate (SDS). Samples were prepared with the Laemmli sample buffer and electrophoresis was performed in a vertical slab unit (model SE 6600, Hoefer Scientific Instruments). The samples were loaded in duplicate, symmetrically from both edges of the gel. Then the gel was cut in two halves, one half being used for protein staining, the other for the zymogram preparation.

For protein staining, the gel was laid overnight in a solution containing 0.2 % (V/V) Coomasie blue R-250 (Bio-Rad Laboratories), 50 % (V/V) methanol and 7.5 % acetic acid and then destained in 7.5 % acetic acid and 30 % methanol.

For the zymogram, the gel was used for in situ detection of pectinase activity after renaturation of the enzyme, using the techniques of Cruickshank and Wade (1980): the gel was incubated overnight, with shaking, in 50 mmol/L phosphate buffer (pH 2). Then, the gel was stained with a solution of ruthenium red at 0.05 % for 30 min. After staining, the gel was washed with distilled water. Pectinase action was evidenced by colorless or pale zones in the stained gel.

Pectinase activity at different pH values. Pectolytic activity was determined at several pH values (2.0–8.0) with 0.15 mol/L phosphate buffers (hydrogen dipotassium and dihydrogen potassium phosphate, and phosphoric acid). 0.2 mL of cell pellet was mixed with the phosphate buffer and incubated for 1 h at 50 °C and residual activities were determined. A control was run for each pH value in order to eliminate the interference of the pH with pectin hydrolysis.

Effects of temperature on enzyme stability. 0.2 mL of cell pellet was incubated in 50 mmol/L phosphate buffer (pH 2) at several temperatures (50-90 °C) for variable time periods. The samples were then cooled and the residual activities were determined.

Physical and enzymic treatment of cells. Two samples, each containing 2 mL of cell suspension, were centrifuged and pectolytic activity was measured in the pellet and the supernatant separately. Then the cell pellet was washed twice and treated with lysozyme or sonicated. The experiments were done in duplicate.

The cell pellet was subjected to lysozyme digestion as follows. The pellet was washed twice with STE buffer (0.1 mol/L NaCl, 10 mmol/L Tris-HCl pH 8, 1 mmol/L EDTA pH 8), resuspended in 1 mL STE buffer containing lysozyme (50 mg/mL) and incubated at 37 °C for 3 h. After treatment, the sample was centrifuged and pectolytic activity was measured in the pellet (cell debris) and the supernatant (cell extract) separately, before and after dialysis in 50 mmol/L phosphate buffer (pH 2), for 3 h.

Cell pellets were also sonicated three times at 20-s intervals in an ice bath, and then centrifuged. Enzymic activity was assayed in the cell debris and the cell extract separately.

RESULTS

Pectinase localization. Pectolytic activity was first detected on an M573 agar medium containing 2 % polygalacturonic acid after 3 d of growth. For the determination of pectolytic activity, the cells were grown in liquid M573 medium containing 1 % citrus pectin for 3 d. Then the culture was centrifuged and pectolytic activity was measured in the supernatant fluid and cell pellet. Two-thirds of pectolytic activity were present in the cell pellet, even after washing with distilled water, and the remainder was found in the supernatant.

When cell pellets were subjected to sonication or lysozyme digestion, 41 and 44 %, respectively, of the total pellet activity was released in the supernatant.

Properties of the cell bound pectolytic enzyme. To identify the cell-bound pectolytic enzyme by SDS-PAGE and to perform enzyme zymography, either intact cells or cell extract from the pellet...