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SHORT COMMUNICATION

MULTIPLE CELLULASES PRODUCED BY A SOFT ROT BACTERIUM, *ERWINIA CAROTOVORA*[1]

TSUNG-CHE TSENG

Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, Republic of China

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Erwinia carotovora is a soft rot pathogen which is known to produce *endo*-polygalacturonate *trans*-eliminase (*endo*-PGTE), phosphatidase C and protease. It has also been demonstrated that the purified *endo*-PGTE has an ability to cause maceration and cellular death of potato tissues (Mount *et al.*, 1970). Recent studies by Tseng and Mount (1973) revealed that phosphatidase C and protease produced by *E. carotovora* were able to burst cucumber protoplasts, and suggested that these enzymes may play a role in disease development.

Cellulose is generally regarded as the major structural constituent of the cell wall of higher plants. Many phytopathogens are able to produce cellulase in culture which can hydrolyze soluble cellulose derivatives, but not all of these organisms are able to attack or utilize native cellulose (Reese, 1956). The cellulase produced in culture has been referred to Cx enzyme when cellulose derivative is used as substrate; those enzymes which are capable of hydrolyzing or altering native cellulose have been designated as C_1 enzyme. Studies on cellulolytic enzyme produced by *E. carotovora* are meager. The current study was undertaken to examine the nature of multiple cellulase system produced by *E. carotovora*.

E. carotovora (Jones) Holland was used throughout this inverstigation. It was maintained on nutrient agar slants at 26°C; stock cultures were transferred at 2-week intervals. Cellulases were obtained from the culture filtrates of *E. carotovora* grown on the nutrient broth (Difco) supplemented with sodium polypectate (Sunkist Growers, Ontario Calif.). Cultures were grown on a shaker at 26°C for 24 hr in 500 ml flask containing 200 ml of the medium which had been autoclaved for 20 min at 121°C. Bacterial cells were removed by centrifugation (20,000 g for 20 min at 4°C) and the supernatant was used immediately as a crude enzyme source or lyophilized and stored at −20°C.

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Cellulase(Cx) activity was determined by following the rate of loss in viscosity of CMC (carboxymethylcellulose) in size 300 Fenzke-Ostwold viscometers at 30°C. The reaction mixture consisted of 4 ml of 0.6% CMC buffered at pH 4.5 with 0.05 M citrate plus 1 ml each of enzyme and water. The enzyme activity was reported in terms of relative activity (RA); the RA values were determined by multiplying the reciprocal of the time in min necessary for 50% reduction in viscosity at 30°C by 1,000 (Bateman, 1963). For measuring C_1 enzyme activity the procedures were the same as described except Whatman No. 1 paper or cotton fibers was used as the substrate.

Crude enzyme preparations were brought to 60% saturation with ammonium sulfate, allowed to stand for 30 min at 5°C, centrifuged for 15 min at 20,000 g, and the precipitate was saved. This routine was repeated for ammonium sulfate saturations of 80 and 95%. The precipitate from each fraction was dissolved in water, and assayed for cellulase activity.

Five ml of 0–60% ammonium sulfate fraction was subjected to column chromatography on diethylaminoethylcellulose (DEAE cellulose). Column of DEAE cellulose (Cl^- form) 2.3×23 cm was prepared and equilibrated with 0.05 M citrate buffer (pH 4.5). The column was eluted with 90 ml of the same buffer followed by a stepwise increase in NaCl concentration in the buffer until 0.4 M NaCl was reached. Five ml fractions were collected and assayed for cellulase activity.

The cellulose peak fractions (Cx–1 and Cx–2) from the DEAE cellulose column (Fig. 1) were combined and dialyzed for 24 hr against water at 5°C. This enzyme fraction was subjected to isoelectric focusing in a LKB 8101 Ampholine electrofocusing apparatus equipped with a 110 ml column (LKB-Produkter AB, Bromma, Sweden) containing pH 5–8 Ampholine carrier. Electrofocusing was carried out at 5°C for 19 hr, at that time the pH grident had formed and the current had stabilized (520 V, 0.4 mA). Five ml fractions were collected at a flow rate of 110 ml/hr, and the pH of each fraction was measured. Each fraction was dialyzed against water at 5°C for 24 hr and then assayed for cellulase activity. This procedure was used to determine the isoelectric point of the enzyme as well as the final step in its purification.

E. carotovora produced a considerable amount of cellulase on the nutrient broth-sodium polypectate medium has been demonstrated. Only Cx enzyme existed in the enzyme preparations. Upon ammonium sulfate fractionation of the clude enzyme, most of cellulase activity was located in the precipitate obtained between 0–60% saturation with ammonium sulfate. When this fraction was applied to DEAE cellulose, two peaks showing cellulase activity were eluted on DEAE cellulose column (Fig. 1). Further purification of the cellulases was performed by subjecting the combined cellulase fractions (Cx–1 and

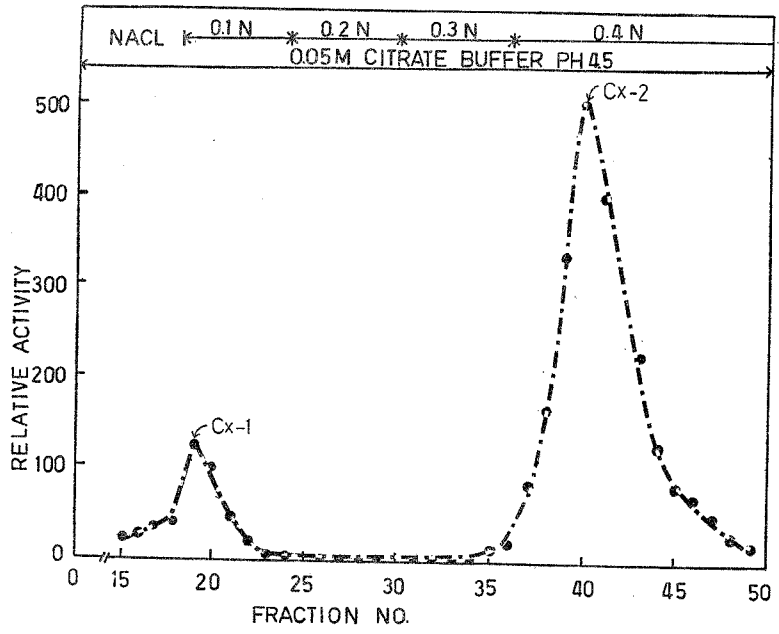

Fig. 1. Separation of Cx-1 and Cx-2 cellulases by DEAE cellulose column chromatography. Ammonium sulfate (0-60% saturation) fraction of a culture filtrate of *E. carotovora* were applied on top of column. Five ml fractions were collected. Cellulase activity was measured by the viscosity loss method.

Cx-2) from DEAE cellulose to isoelectric focusing. The results from Figure 2 revealed that the isoelectric points (pI) of Cx-1 and Cx-2 enzymes were estimated to be 6.76 and 2.23, respectively. However, the isoelectric point of Cx-2 enzyme was still uncertain, because the pI of 2.23 was not within the pH grident when a pH 5-8 Ampholine was used as a carrier.

Selby *et al.* (1963) have demonstrated two types of cellulase in the culture filtrate of *Myrothecium verrucaria*. It has been designated as A-enzyme and B-enzme. The A-enzyme is capable of attacking native cellulose. whereas B-enzyme results in hydrolysis of soluble cellulose derivatives. The present report shows that *E. carotovora* produces two cellulolytic enzymes which are able to hydrolyze a cellulose derivative, carboxymethylcellulose. These enzymes were separated by using DEAE cellulose column chromatography and isoelectric focusing. The fact that two cellulolytic enzymes were obtained in this study suggests that there are two isoenzymes in the culture medium. Further studies by using other methods such as polyacryamide gel electrophoresis and/or sucrose density-gradient ultracentrifugation are needed before confirmation of cellulase isoenzymes can be made.

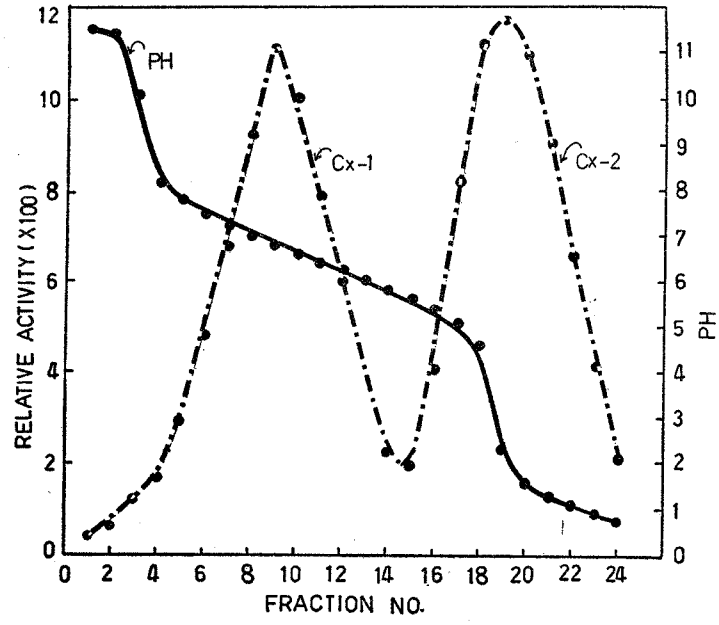

Fig. 2. Isoelectric focusing of dialyzed Cx-1 and Cx-2 cellulases from DEAE cellulose column chromatography (Fig. 1). A pH 5-8 Ampholine was used as a carrier. Five ml fractions were collected. Cellulase activity was measured by the viscosity loss method. Cx-1 and Cx-2 enzymes were estimated at pI 6.76 and 2.23, respectively.

Literature Cited

BATEMAN, D. F. 1963. The macerating enzyme of *Rhizoctonia solani*. Phytopathology **53**:1178-1186.

MOUNT, M. S., D. F. BATEMAN, and H. GRANT BASHAM. 1970. Induction of electrolyte loss, tissue maceration, and cellular death of potato tissue by an endopolypalacturonate *trans*-eliminase. Phytopathology **60**:924-931.

REESE, E. T. 1956. A microbiological progress report. Enzymatic hydrolysis of cellulose. Appl. Microbiol. **4**:39-45.

SELBY, K., C. C. MAITLAND, and K. V. A. THOMPSON. 1963. The degradation of fibrous cotton by the extracellulase of *Myrothecium verrucaria*. Biochem. J. **86**:9 p.

TSENG, T. C., and M. S. MOUNT. 1973. Effect of extracellular enzymes from *Erwinia carotovora* on cucumber protoplasts. Phytopathology **63**:208.

一種軟腐細菌 *Erwinia carotovora* 產生多型纖維分解酵素

曾 聰 徹

中央研究院植物研究所

作者在純化由軟腐細菌所分泌的酵素實驗當中，發現這種細菌會產生多型纖維分解酵素，並且確定所分離的酵素都屬於 Cx 型纖維分解酵素。