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Effective Production of Bacterial Pectinases*

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Abstract

Efficient production of extracellular pectin lyase and pectage lyase and the induction of temperate phage were observed by cultivating soft-rot *Erwinia* strains in minimal medium containing the extracts of a number of plant species. Production of both enzymes and the ratio of these enzymes depended upon the source of the extract and the strain of soft-rot *Erwinia*. An *Escherichia coli* transformant (HB012) which contains a pectate lyase gene of *E. carotovora* subsp. *carotovora* (EC1) was shown to excrete pectate lyase efficiently under similar growth conditions.

Keywords: pectate lyase, pectin lyase, DNA-damage, excretion

Abbreviations: EDTA, Ethylenediaminetetraacetic acid; LB, Luria-Bertani medium; Tris, Tris (hydroxymethyl) aminomethane

1. Introduction

Pectate lyase (E.C. 4.2.2.2, PAL) and polygalacturonase (E.C. 3.2.1.15, PG) have been considered to be the major pectinases produced by many plant pathogenic bacteria (Bateman and Miller, 1966). These enzymes need deesterification before the degradation of natural pectic substances, which

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of high methoxyl content. Mixture of PNL and PAL or PG should be able to degrade natural pectic substances in a cooperative manner without methyl esterase (E.C. 3.1.1.11) by specific cleavage of the esterified portion by PNL and the non-esterified portion by PAL or PG. However, the induction mechanisms of these enzymes have been shown to be totally different. Production of PAL is regulated by dual mechanisms: "Product induction" (i.e. PAL is induced by the breakdown products of pectate (Tsuyumu, 1977) and "self catabolite repression" (i.e. the inducer of PAL also mediates the repression of PAL after it is catabolized) (Tsuyumu, 1979). In fungi, it was shown that PG was also induced by the breakdown products of pectic substances (Cooper and Wood, 1975). On the other hand, PNL was shown to be induced by DNA-damaging agents such as mitomycin C, nalidixic acid, bleomycin, or UV light, but not by pectic substances (Tsuyumu and Chatterjee, 1984; Itoh et al., 1982; Kamimiya et al, 1972). However, since many plant species were shown to contain both DNA damaging agents (Tsuyumu et al., 1985) and pectic substances, some plant sources or their combinations may be expected to induce the production of both PAL and PNL.

2. Materials and Methods

Organisms

Erwinia chrysanthemi (EC183, KS612), *E. chrysanthemi* pv. *zeae* (ALE8251P) and *E. carotovora* subsp. *carotovora* (EC1) were used as the representatives of soft-rot *Erwinia* spp. EC183 and KS612 were kindly supplied by A.K. Chatterjee, and ALE8251P and EC1 are from the culture collection of Shizuoka University. *Escherichia coli* strains (C600, C600/pBR322) were the gifts from A. Miyajima. *Erwinia* strains were grown at 30°C in YP (0.5% yeast extract, 1% peptone, pH 6.8), while *E. coli* strains were grown at 37°C in LB (0.5% yeast extract, 1% tryptone, 1% NaCl, pH 7.0). When required, ampicillin and tetracycline were added at a concentration of 50μg/ml and of 30μg/ml, respectively.

DNA isolation and cloning PAL genes

Chromosomal DNA of EC1 and plasmid DNA were isolated essentially by the methods of Rodriguez and Tait (1983). Pst I fragments of EC1 DNA was ligated into dephosphorylated Pst I site of pBR322, and was transformed by the calcium chloride procedure of Mandel and Higa (1970) and spread on LB-tetracycline plates. Tetracycline resistant colonies were patched onto citrus pectin plate and PAL lyase activity was tested by the method of Hankin et al. (1971). Subcloning was done by isolating DNA fragments from 1.0% agarose (NA, Pharmacia) by electroelution in dialyzing bag according to the method of Maniatis et al., (1982).

Inoculation of plant disks by Erwinia strains

The disks (1.5 cm in diameter, 0.5 cm thickness) of potato or radish were placed on the filter paper which absorbed one ml of sterile distilled water. Filter paper (0.5×0.5 cm) was immersed in a suspension of overnight culture of *Erwinia* strains and placed onto the plant disk. After 24 hr incubation, degree of soft rot symptom was recorded, and the disks were homogenized in 1 ml of Tris-HCl buffer (50 mM, pH 8.0). The homogenate was centrifuged at 3,500 rpm for 15 min, the supernatant was used for the enzyme assays.

Induction study of pectinases and temperate phage by plant extracts

Chopped vegetable tissues (20 g) or industrial plant products (0.5 g) were immersed in 50 ml distilled water and autoclaved for 20 min at 120°C. The plant debris were removed by either filtration through cotton layer or centrifugation. Into this extract, salts solutions for minimal medium of Mikula et al. (1972) were added. After 24 hr cultivation in this medium, the cell suspension was either treated with chloroform for the determination of phage titer or centrifuged for the determination of the extracellular activities of PNL and PAL. Phage titer of KS612 which has been reported to be lysogenic with the temperate phage, Erch12 (Chatterjee and Brown, 1980), was determined by assaying for plaque-forming units using *E. chrysanthemi* (EC183) as the host.

Enzyme assays

Pectin and pectate lyases were assayed by measuring the increase in absorbance at 235 nm using spectrophotometer (Ultrospec 4050, LKB) connected to personal computer (PC-9801, NEC). The reaction mixture (0.6 ml) for pectin lyase contained 0.25 ml of 1 mg/ml of Link pectin (98% methylesterified), 0.25 ml of 50 mM of Tris-HCl buffer, pH 8.0, containing 0.5 mM EDTA and 0.1 ml of enzyme and water. For the assay of pectate lyase, the reaction mixture contained 0.25 ml of 1 mg/ml of sodium polygalacturonate, 0.25 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 0.5 mM $CaCl_2$, and 0.1 ml of enzyme and water. One unit of lyase activity is defined as the amount of enzyme that produces an increase in absorbance of 1.0 per minute at 235 nm. Cellular density was determined by reading the absorbance at 600 nm.

3. Results

When two strains of *E. chrysanthemi* (ALE8251P or EC183) or a strain of *E. carotovora* subsp. *carotovora* (EC1) was inoculated onto potato and radish disks, considerable amounts of PNL and PAL were produced in the homogenates of macerated plant disks (Table 1). Although ALE8251P and EC1 strains produced PAL far more than PNL, EC183 produced PNL to about the same order of PAL. The optimum temperature for the production of pectinases and for the development of soft-rot by ALE8251P and EC183 was 30°C, while it was from 20°to 25°C for that by EC1.

After a lysogenic strain of *E. chrysanthemi* (KS612) was grown in the minimal medium containing an extract of vegetable, phage titer increased drastically in six out of nine tested vegetable extracts (Table 2). Though the presence of DNA damaging agents in these vegetables was confirmed from this result, the phage titer was not parallel to the external activities of PNL. Also, the efficiency of each extract for the production of PNL was not parallel to that of PAL as expected from the difference in their induction mechanisms.

When several industrial plant materials which can be obtained economically and storageable were added into the culture of EC1 in minimal medium, the extracellular pectate lyase production increased considerably compared to the one in minimal-glycerol medium (Table 3). However, the production of PNL by EC1 at this concentration of plant sources was very poor. *E. coli* HB012 (a clone containing about 3 kb of EC1 DNA including a PAL gene) produced extracellular PAL more efficiently than EC1 did in some extracts.

4. Discussion

The extracellular activities of PNL and PAL were found to be high after the cultivation of *Erwinia* strains in the medium containing many different plant materials. It should be emphasized, however, that the extracellular activities of these enzymes are influenced by many factors such as the growth yield of bacteria, inhibitors or activators of these enzymes in the medium and cellular lysis of *Erwinia* strains. It has been reported that the extracts of a number of plant species and the metabolic products of the pathogen are known to inhibit pectic enzymes (Bateman and Miller, 1966). This may be the reason why phage titer and PNL production was not parallel (Table 2), in spite of the finding that mitomycin C induces both phage and PNL coordinately (Tsuyumu and Chatterjee, 1984). Also, the factors other than PNL and PAL have been shown to be involved in the maceration of plant tissues

Table 1. Pectin lyase (PNL) and pectate lyase (PAL) activities, and soft-rot (rot) developed in the inoculated plant disks at various temperatures.

Temperature °C	ALE8251P PNL	ALE8251P PAL	ALE8251P rot	EC183 PNL	EC183 PAL	EC183 rot	EC1 PNL	EC1 PAL	EC1 rot
(Potato tuber)									
15	–*	13	+**	1.3	–	+	–	138	+
20	–	129	++	34	198	++	5.1	215	++
25	3.0	132	+++	93	194	+++	6.8	152	+++
30	4.4	154	+++	103	340	+++	6.1	150	+++
37	2.3	80	+++	11	113	+++	–	14	++
(Radish root)									
15	–	52	+	–	12	+	–	157	+
20	–	216	++	18	19	+	–	139	+++
25	4.2	135	+++	16	21	++	–	144	+++
30	19.0	177	+++	15	14	+++	–	125	+++
37	6.3	135	+++	2.9	4.9	+++	–	186	+

* Enzymatic activities of PNL and PAL in the homogenate were assayed as mentioned in text. The activity was expressed as the units $\times 10^{-2}$ in one ml culture. – stands for not detectable due to low activity.

** Degree of soft-rot was expressed by the degree from – (no soft-rot) to +++ (soft-rot of whole disk).

Table 2. Induction of temperate phage (Erch 12), pectin lyase (PNL) and pectate lyase (PAL) by growing *E. chrysanthemi* (KS612) in the medium containing various plant extract.

Plant extract	Phage titer	PNL	PAL
Min + Gly	– *	4.5 **	–
Radish	+	32	63
Potato	–	22	127
Carrot	++	5.4	97
Sweet potato	++	9.0	432
Cabbage	++	29	432
Chinese cabbage	+	20	270
Cauliflower	++	1.9	4.2
Winter squash	++	9.0	14
Green onion	–	27	54

* Phage titer: ++, $\geq 1000\times$ higher than the titer in Min + Gly; +, $10\times \sim 1000\times$; ––; $\leq 10\times$.

** Enzymatic activities were expressed as mentioned in the legend of Table 1.

Table 3. Production of PAL and PNL by PAL-transformant (HB012) and by its donor (EC1) in the media containing various plant materials.

Plant***	HB012 A600	PAL (sup)	PAL /A600	EC1 A600	PAL (sup)	PAL /A600	PNL
Min + Gly	0.05	– *	–**	0.2	2	10	–
Rape-seed meal	0.25	16	64	1.9	160	84	1.0
Corn Bran	0.28	16	57	1.8	31	17	–
Corn glutten feed	0.92	130	141	1.8	35	19	0.5
Corn steep liquor	1.11	140	126	1.3	25	19	–
potato tuber	0.26	11	42	1.6	62	39	3.0
carrot root	0.25	10	40	1.7	130	76	3.0

* The activities of PNL and PAL in the supernatant were expressed as mentioned in the legend of Table 1.

** Specific activities of PNL and PAL were expressed as units $\times 10^{-2}$/A600.

***Plant materials are added at the concentration of 1% (W/V).

(Bateman and Miller, 1966). Quantitative discrepancy between the degree of soft-rot symptom and the activities of PNL and PAL in the plants infected with *Erwinia* strains (Table 1) may be due to these factors. Despite these variations, our data suggest that the extracts of many plant species contain the inducers of both PNL and PAL of soft-rot causing *Erwinia* strains. Appropriate combination of *Erwinia* strain and plant materials was suggested to provide an effective way to obtain the mixture of high PNL and high PAL. PAL was accumulated in the culture medium even by *E. coli* transformant (HB012) at high efficiency (Table 3). This result indicates that excretion systems of *Erwinia* PAL were effective for the excretion not only through the inner membrane but also through the outer membrane of *E. coli*, though the excretion system through the outer membrane has been known to be absent in *E. coli*. Such devices of *Erwinia* strains may be used for the construction of effective excretion vector for *E. coli*.

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