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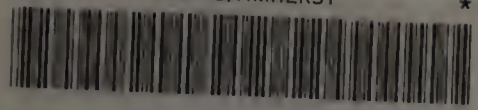
Stephen John Novak
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PRODUCTION OF EXTRACELLULAR PECTIC ENZYMES
FROM <u>ERWINIA CAROTOVORA</u> (ISOLATE 14) INCUBATED UNDER
AEROBIC AND ANAEROBIC CONDITIONS

A Thesis Presented

By

STEPHEN JOHN NOVAK

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

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Department of Plant Pathology
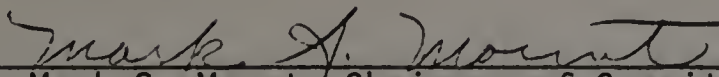
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Dr. Richard A. Rohde
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To my parents and Cathy

whose love and support has

made this work possible.

ACKNOWLEDGEMENTS

ABSTRACT
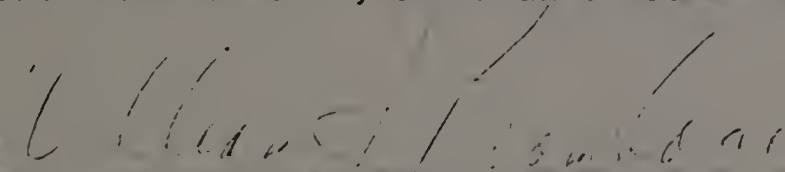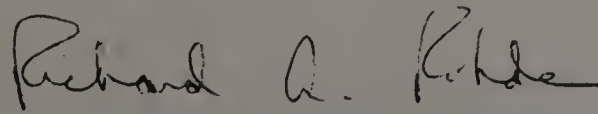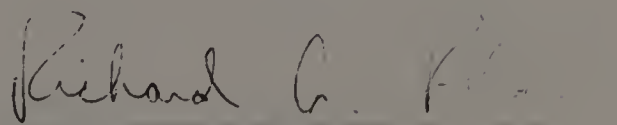
Cultures of _Erwinia carotovora_ isolate 14 were incubated under aerobic or anaerobic conditions at either 24 or 30°C. Cultures incubated under anaerobic conditions exhibited reduced growth when compared to those cultures incubated aerobically. However, the extracellular protein concentrations of these incubation treatments were similar. The level of production of extracellular endopectate lyase (PL) was dependent on temperature more than on any other environmental parameter. Highest levels of PL were produced in cultures incubated at 30°C either aerobically or anaerobically. The level of production of extracellular endopolygalacturonase (PG) were dependent on the availability of oxygen. Highest levels of PG production were observed for those cultures incubated at 24 or 30°C under aerobic conditions. Extracellular pectic enzyme activity values were adjusted to a level of production which would have been achieved by a 10^8 cells/ml culture. As a result, the production levels of extracellular pectic enzymes from EC14 were enormously increased in the anaerobically incubated cultures. Two possible mechanisms which may explain this phenomena are suggested.

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Introduction

A large and very diverse group of pathogens have been found
to possess the ability to degrade the constituents of the cell walls
of higher plants. These organisms utilize degradative enzymes in order
to gain access into a host and have been implicated in a wide variety
of plant diseases (5, 6, 7, 8, 21, 32, 35, 44, 56, 60), including soft
rots, dry rots, wilts, blight and leaf spots. By far, the most im-
portant of these enzymes in terms of the degree of tissue damaged and
dollars lost are the pectic enzymes (1, 5, 6, 7, 8, 9, 12, 32, 33,
34, 44, 51, 52, 55, 57).

Erwinia carotovora is a phytopathogenic bacterium which possesses
the ability to produce a wide variety of pectic enzymes (7, 8, 23, 30,
31, 32, 36). Bacterial soft rot of potato tubers in storage and
transit has been attributed most often to _Erwinia_ _spp_. (10, 49, 55,
61). Typically, in the soft rot disease of potato tubers caused by
E. carotovora, symptoms require the presence of sufficient levels of
pectic enzymes and follow a pattern of electrolyte loss, tissue
maceration, and cellular death (32, 55, 57).   Several investigators
have demonstrated that the production of extracellular levels of a
pectic enzyme, endo-polygalacturonate trans-eliminase (PGTE) is inte-
grally related to symptom initiation and soft rot progression (32, 57).
An avirulent mutant strain of _E. carotovora_ was produced by nitro-
soquanidine (NTG) treatment and was found to be deficient in the ability
to synthesize extracellular pectic enzymes and thus unable to cause

disease in a potential host (9).

Erwinia carotovora (Jones) Holland isolate 14 (EC14) has been shown to produce two extracellular pectic enzymes, endopectate lyase (PL) and endopolygalacturonase (PG) (32, 51, 52, 57). These enzymes, in either crude or purified form, induce soft rot conditions in plant tissues. Subsequently, an intracellular pectin depolymerase complex was isolated and characterized from this isolate (51, 52). This complex consisted of four enzymes which were designated PDI through IV. PDI was shown to possess dual activities and was determined to be identical to the extracellular enzymes produced by this bacterium. The regulatory mechanism(s) of pectic enzyme synthesis has also been partially characterized for this isolate (19, 34).

Soft rot development of potato tubers has been found to depend on a number of factors. Two of these factors are the environmental conditions surrounding the tuber, and the types and numbers of pectolytic microorganisms in or on the tuber (10, 26). The most critical of the environmental factors in determining soft rot development are the presence of a film of water around the tuber and the depletion of oxygen within the tuber (11, 15, 16, 25, 26, 27, 38, 44, 45, 50, 64).

To date little work has been conducted to determine the effects the environmental conditions have on the synthesis of pectic enzymes and how this is related to the rotting of potato tubers. The aim of this study was to determine the effects of temperature and aerobic versus anaerobic conditions on PL and PG production, in EC14, as it relates to soft rot progression.

C H A P T E R   I I

Literature Review

Bacterial soft rot of potatoes in storage or transit has been attributed most often to Erwinia spp.- particularly E. carotovora var. carotovora (Ecc) and E. carotovora var. atroseptica (Eca) (10, 49, 55, 61). Other pectolytic bacteria have also been found to be present and may contribute to the soft rot condition. These include: Pseudomonas spp., Bacillus spp., Clostridium spp., Aerobacter spp. and Flavobacterium spp. (44, 45, 46, 49).

The exact estimate of losses due to soft rot is difficult to determine, but some figures range as high as $50 to $100 x 10^6 annually on a worldwide basis (44). These figures will vary depending on the climatic conditions and the value of the crop which is being attacked.

A number of factors may influence the initiation and spread of decay in potato tubers; these include: (1) environmental conditions, particularly temperature, relative humidity, aeration and the presence or absence of a film of water on the surface of the tuber; (2) injury to the tuber during harvest or handling; (3) the amount of soil or plant debris brought into storage with the tubers; (4) the type and amount of chemicals applied to the plant or tuber during the growing season and in storage; (5) the types and numbers of microorganisms, particularly soft-rotting bacteria in or on the tuber; and (6) the inherent physiological condition and rate of metabolism of the tuber (10, 26).

The environmental factors associated with soft rot disease development have been studied extensively by many investigators(15, 16, 25, 27, 38, 44, 45, 49, 64). It has been determined that the presence of high relative humidity and temperatures greatly increase the development of soft rot symptoms. Tuber decay in rail shipments from the State of Washington accounted for 63% of the total tuber defects from 1965 to 1969 (15). Tubers which were kept dry and stored for 11 days over ranges of 10-21C and 2-20.5% O_2 levels were found to be free of any soft rot development. Tubers which were partially immersed in water did not show rot development when cooled to 4.4C in less than 24 hours after harvest and held for 8 days at 4 to 20.5% O_2 levels. When tubers were immersed in water at 10C or higher after harvest and held for 8 days at 4 to 20.5% O_2 levels soft rot symptom developed. Tubers which had been partially immersed and incubated for 1 day at 21°C developed rot in a low oxygen (4%) atmosphere when later stored for 8 days at 4.4°C (15).

However, the major environmental determinants involved in soft rot development are probably the presence of a film of water on the tuber surface and the depletion of oxygen within the tuber (11, 15, 16, 25, 26, 27, 38, 44, 45, 50, 64). This depletion of oxygen may be the result of a number of factors. When mature tubers are covered with a persistent film of water with an average thickness of about $3x10^{-2}$mm anaerobic conditions will occur after 6½ hours at 10°C or 2½ hours at 21°C (11). It has been determined that restricted aeration in closed containers, during transit, resulted in an increase in the incidence of tuber infection and decay as respiratory gases accumulated around

the potatoes (38).

A number of investigators have shown that low concentrations of O_2 will increase the rate of rotting of potato tubers (15, 16, 25, 27, 38, 44, 45, 50). The susceptibility of King Edward potatoes to soft rot was determined using varying concentrations of oxygen and carbon dioxide (27). Tubers were wounded, inoculated with E. carotovora var. atroseptica and stored at 10°C and 100% relative humidity in controlled concentrations of oxygen and carbon dioxide for up to 18 days. Extensive, soft spreading rots were produced in potatoes stored at 5% O_2 + 16% CO_2, in 1% O_2 + 20% CO_2, in 1% O_2 and in 1% N_2, the most extensive rots occurred at the 1% O_2 and N_2 atmospheres (27).

When 10 ml of six inoculum levels (1×10^6 to 5×10^9) were injected into potato tubers it was determined that the bacteria acted independently and that tubers were equally susceptible to decay. Greater amounts of decay were recorded at the 2% O_2 level as compared to the 6 at 10% O_2 levels (16).

The increased rate of rot which occurs at low O_2 concentrations is believed by some to be an inhibition of the resistance mechanisms of the tuber (16, 25, 49, 64). A decrease in the rate of suberization and periderm formation, as well as the wound healing response, of potato tubers is observed as a result of low (½ to 1%) levels of O_2 (25, 64). These conditions have been found to occur during transcontinental rail and truck shipments of fresh fruits and vegetables (25).

A possible sequence of events occurring under wet anaerobic conditions has been suggested (45). It is believed that when excess water

is absorbed the lenticels are opened; an oxygen deficiency will affect cell membrane integrity causing leakage of water and solutes from turgid cells. This can establish a continuous liquid phase between the cortex of the tuber and the lenticel, thereby allowing microorganisms in the lenticels to penetrate deeper into the tuber via this liquid stream.

The combination of the leakage of water and solutes from the tuber with the decrease in the resistence of the tuber under anaerobic conditions enables pectolytic bacteria to proliferate and soft rot disease to develop.

The cell wall provides the initial barrier that a possible pathogen must overcome if it is to invade a host. For this reason a discussion on the cell wall and its components is relevant. The major components of the cell wall are pectic polysaccharides, cellulose, hemicellulose, structural glycoproteins and lignin (2, 7, 20, 24, 32, 48). The cell wall can be viewed as a two phase system -- the first consisting of a dispersed phase of cellulose microfibrils and the second being a complex continuous matrix (7, 32). The cell wall has been divided into three functional-structural regions, the middle lamella, the primary cell wall and the secondary cell wall (2, 7, 32). The primary cell wall of various plants are probably similar in structure and function (32). It is the first wall region formed with definite organisation and consists mainly of pectic substances, cellulose and hemicelluloses and serves to support and protect the cell protoplast (7).

The secondary cell wall is added after cell elongation is completed (7). A great deal of variation occurs in the secondary cell wall from plant to plant and tissue to tissue (54). The principle component is cellulose, but pectic substances, hemicelluloses and lignin are also found in this region.

The middle lamella is the area where adjacent cells are linked into a tissue system (54). In herbaceous tissues the middle lamella is mainly composed of pectic substances. In lignified tissues, lignin is the major component of the middle lamella (32). A hydroxyproline rich glycoprotein has also been identified in plant cell walls (20, 24, 40). Although its occurrence and function are not well known it is thought that this may add to the structural integrity of the pectic component.

The major component of the pectic portion of the primary cell wall and the middle lamella is a high molecular weight polymer consisting of a backbone of α-1, 4-linked D-galacturonopyranose interspersed with 1, 2-linked rhamnopyranose (20, 54). Polymers of neutral sugars are also present which may serve as a bridge between the rhamnogalacturonan and hemicellulosic wall components. The carboxyl group of uronic acid may possess a certain degree of methylation (8). If over 75% of the uronide residues are methylated the polymer is referred to as pectin. Those polymers with a lower degree of methylation are referred to as pectinic acid. When no uronide residues are esterified with methyl groups this molecule is known as pectic acid. The uronic acid residues may also be acetylated at positions 2 and 3.

In some cases pathogens will utilize primarily mechanical means to gain access into a host. A large majority of pathogens, however, utilize a wide variety of enzymes which are capable of degrading cell wall constituents. These organisms possess the ability to produce cell wall degrading enzymes and have been implicated in a wide variety of plant diseases (5, 6, 7, 8, 21, 32, 35, 44, 56, 60). These include soft rots, dry rots, wilts, blights and leaf spots. Of this variety of cell wall degrading enzymes available in the arsenal of pathogenic organisms, probably the most important in terms of the degree of tissue damaged and dollars lost, are the pectic enzymes (1, 2, 5, 6, 7, 8, 9, 12, 32, 33, 43, 44, 51, 52, 55, 57).

Pectic enzymes can be classified by several criteria: (1) the mechanisms by which the 1, 4 glycosidic linkage is cleaved (hydrolytic or lyitic); (2) the optimal activity the enzyme has for a substrate; and (3) whether cleavage of the substrate occurs in a random (endo) or terminal (exo) manner (8, 48).

Hydrolase enzymes, such as pectin esterase and polygalacturonase, have an acidic pH optimum (pH 4.0 to 6.5) and do not require metal ions to exhibit activity (5, 6, 7, 8, 42, 46, 51, 52). In some cases fungal polygalacturonases have been reported to be inhibited by calcium ions (5). Pectin esterase hydrolyses the esterified methyl groups of the galacturonosyl chain producing uronic acid groups and methanol as reaction products. The activity of pectin methylesterase can be determined by measuring the amount of liberated methanol (23). The pectin chain length is not affected by this enzyme although removal of the methyl

group makes pectin more vulnerable to attack by other polysaccharide degrading enzymes (7, 8, 32).

Polygalacturonase has been shown to have a specificity for non-methoxylated polygalacturonic acid (7, 8, 32). Endopolygalacturonases result in several size products (diner to possible pentamer) while exoforms of this enzyme release di-galacturonic acid from the polymer (18). The endo-enzymes have been shown to induce soft rot symptoms when isolated from the organism and either purified or used directly (32, 55).

Trans-eliminase or lyase enzymes break the glycosidic linkage of the pectin polymer at carbon 4 and simultaneously eliminates the H^+ from carbon 5. This results in oligouronides which terminate in a modified carbon 4, 5 unsaturated galacturonyl unit and a loss in viscosity (7, 8, 32). This loss of viscosity and the formation of the double bond are used to assay lyase activities (5, 6, 47, 62). Polygalacturonic acid rather than methylated pectin appears to be preferred substrate. Some lyases have been shown to attack pectin, depending on the degree of methylation. The pH optimum for lyase enzymes is in the alkaline range (pH 7.5 to 9.5) (7, 8, 32). The lyase activity is stimulated by specific concentrations of calcium, although higher or lower levels of calcium can result in inhibition of enzyme activity (3, 28, 30). The presence of a chelating agent, such as ethylene diamine tetraacetic acid (EDTA) will also inhibit enzyme activity (32, 48). Other divalent cations have had no effect on lyase activity in E. carotovora, however, the activity of a lyase from Clostridium

multifermentens was greatly enhanced by other divalent cations such
as strontium and magnesium (28).

Phytopathogenic Erwinia species induce soft rot to develop be-
cause they are capable of producing a variety of pectic enzymes, in-
cluding intra and extracellular forms of lyase and hydrolase enzymes
(30, 33, 36, 51, 52). An E. carotovora isolate from soft rot spoilage
in a shipment of green peppers was grown in a semisynthetic pectin
medium and found to produce extracellular pectinesterase and polygal-
acturonase (23). The polygalacturonase had a wide pH range extending
from 3.9 to 7.9, with a broad optimum around pH 5.8 as measured by the
release of free reducing groups from the substrate. It was later
shown that galacturonic acid is converted by isomerization and reduc-
tion into D-hexonic acid which is further metabolized into triose-
phosphate and pyruvic acid (22). The pyruvic acid is then utilized
in the tricarboxylic acid cycle.

A polygalacturonic trans-eliminase (PGTE) was later isolated
from isolate 153 of E. carotovora (52). This enzyme had a pH optimum
of 8.5, was stimulated by the addition of up to 0.001 M $CaCl_2$
and was inhibited by 3×10^{-5}M EDTA, although activity was restored upon
the addition of 0.001M $CaCl_2$. Under the investigators assay conditions
a temperature optimum of 50°C was observed.

This same group successfully purified a polygalacturonase from
polygalacturonic acid trans-eliminase which was produced in culture
by isolate 153 of E. carotovora (36). This enzyme was purified 56 fold
by means of acetone precipitation, acetate buffer extraction, and

two successive treatments by column chromatography on carboxymethyl cellulose. This enzyme was shown to have a pH optimum between 5.2 and 5.4. The major end products were determined to be mono- and di-galacturonic acid and digalacturonic acid was not hydrolyzed further.

Isolate 153 was also shown to have an intracellular polygalactur-onic acid trans-eliminase which had properties similar to the extra-cellular polygalacturonic trans-eliminase (30). These trans-eliminases had a pH optimum of 8.5, was stimulated by calcium and had an endo mode of action. The major end product was determined to be unsaturated digalacturonic acid.

An oligogalacturonide trans-eliminase (OGTE) was later pruified from isolate 153 of E. carotovora (31). This enzyme degraded D-galacturonic acid into two molecules of deoxyketuronic acid, indicating an eliminase (lyase) reaction. OGTE attacks unsaturated digalacturonic acid at 400 times the rate that polygalacturonic acid is attacked. The rate of attack of oligouronides varies inversely with the chain length.

An endo-PGTE was purified from E. carotovora (Jones) Holland isolate 14, a soft rot bacterium, which induced electrolyte loss, tis-sue maceration and cellular death of potato tuber (33). This PGTE was purified 295-fold from crude culture filtrate by ammonia sulfate precipitation, column chromatography on DEAE-cellulose, and isoelectric point focusing. This enzyme was shown to have a pH optimum of 8.5 and required calcium for activity. An endo-PGTE, which was purified through isoelectric focusing was purified from this same bacterium and was found to macerate potato and cucumber tissue disks and induce

leakage of ^{86}Rb from potato disks (57). Death of host cells was shown to be caused by the inability of the cell wall to support the protoplast, when exposed to high water potentials, which results in cell lysis(1, 17).

An intracellular pectic enzyme complex of E. carotovora was discovered, purified through isoelectric focusing, and partially characterized (51, 52). This complex was comprised of at least four pectate depolymerases which were designated PDI, PDII, PDIII, and PDIV. PDI had an isoelectric point (PI) of 9.4 and possessed the ability to depolymerize a sodium polypectate (NaPP) substrate in both an endo-PGTE manner at pH 8.5 in the presence of Ca^{++}, and in an endo-PG manner at pH 6.0 in the presence or absence of divalent cations. PDI is the only pectic enzyme that is found outside the cell; the extracellular form has been designated PDIa. PDIa was found in a culture filtrate of nutrient media as well as in rotted potato tubers. When purified PDI was reacted with potato tuber disks at pH 6.0 or 8.5 tissue maceration and cellular death occurred.

Further work has led to a partial characterization of the regulatory mechanism governing lyase synthesis in E. carotovora isolate 14 (19, 34, 65). As had been demonstrated earlier the synthesis of lyase is inducable and subject to catabolite repression after cells growing in NaPP were supplemented with glucose (19). Exogenously supplied adenosine 3'-5' cyclic monophosphate (cAMP) reversed repression of intracellular PGTE activity. A decrease in cAMP was correlated with glucose repression of PGTE activity. As PGTE activity increased in

induced cultures, the specific concentration of cAMP increased.

Various analogues of cAMP were unsuccessful in reversing glucose

repression of PGTE. "Self" catabolite repression of pectate lyase

(16) activity in E. carotovora strain EC1 was observed when high con-

centrations of the inducer accumulated in the medium (59). Repression

of the induction by high concentration of inducer was relieved by

consumption of the inducer and by the addition of $2x10^3$McAMP. Subse-

quently, a cAMP-deficient mutant of E. carotovora was isolated from a

nitrosoquanidine-treated B-galactosodase constituative strain (34).

This mutant was unable to use α lactose, L-arabinose, D-galactose,

L-rhamnose, D-xylose, raffinose, D-cellobiose, glycerol and sodium

polypectate as a sole carbon source unless exogenous cAMP was supplied.

The synthesis of PGTE was found to be under the direct control of the

cAMP regulatory mechanism. Only low levels of PGTE were detected when

this mutant was grown on a casein hydrolysate-minimal salts medium,

even in the presence of the inducer. PGTE levels would increase to

those of the parent strain when NaPP and cAMP were added to the medium.

This is a similar regulatory mechanism to that found in Escherichia coli.

Pectic acid was added to a minimal medium in which strain EC1 of

E. carotovora was growing (58). Time course samples were extracted,

washed, sonicated and assayed. An increase in Pectic Acid lyase (PAL)

was observed, but a long lag phase was evident before the onset of in-

duction. This suggested that pectic acid was not inducing directly.

The true inducer was sought in time-course induction experiments.

A rapid induction of PAL activity was observed specifically when $2x10^{-3}$M

purified unsaturated digalacturonic acid was added to a minimal media as the sole carbon source. No induction was observed after the addition of di-galacturonic acid, which is the major product of the cleavage of pectic acid by E. carotovora PG enzymes.

Both unsaturated and saturated digalacturonic acid (UDG and DG) or their metabolites, have been found to induce endopectate lyase in E. chrysantheni (13, 14). Oligogalacturonate lyase (OGL) converts MDG and UDG into 4-deoxy-5 keturonic acid (DK) which was found to be a highly effective inducer of PL synthesis. These investigators postulate that DK (or its precursor) is the actual inducer and that OGL is the primary generator of the apparent PL inducer and its high rate of activity on UDG promotes self-catabolite repression of PL. An OGL-deficient mutant was poorly induced unless supplied with deoxyketuronic acids or a substrate that could be converted by another intracellular enzyme to DK (13).

To date little work has been conducted on the effect environmental conditions have on the synthesis of pectic enzymes and how this is related to the rotting of potato tubers.

The growth rate in vitro of Erwinia spp. was reduced when cells were grown in low oxygen atmospheres (1 to 3%) (29, 63). The mean growth rate of E. carotovora was reduced to 50-64% of that in air at 3% O_2 levels. At 0% O_2 growth was between 3 and 12.5% of that in air. In the absence of CO_2 Erwinia spp. did not grow at any concentrations of O_2 during a 24-hour period (63).

With reduced growth rates decay develops because only small

conditions on endo-PG and endo PL production, from E. carotovora var. carotovora, as it related to soft rot progression. Most investigators believe that PL is the primary cause of tissue maceration in soft-rotted tubers. However, the observation that rotted tubers tend to have a lower pH than normal (near 6.0 the optimal pH for PG activity) and recent work, in controlled environmental chambers, with whole tubers under anaerobic conditions, has led us to believe that PG may play a larger role in soft rot development than previously thought (personal communication - Arthur Kelman).

C H A P T E R   I I I

MATERIALS AND METHODS

Culturing of Erwinia carotovora

All experiments were conducted using isolate EC14 of Erwinia caro-
tovora (Jones) Holland, which was originally obtained from R. S. Dickey
of Cornell University. EC14 was maintained on LB agar slants consisting
of 1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar.
Slants were inoculated and incubated at 30°C for 24 to 48 hours then
stored at 4°C. Subculturing was performed every three to four weeks.

Bacteria were grown in a culture medium, 50 ml in 250 ml Erlenmeyer
flasks containing 1% NaPP and Difco nutrient agar (8g/l). To some of
the culture flasks 0.05% L-cysteine was added to serve as a reducing
agent. Media was inoculated, by loop, from a slant and incubated over-
night (8 to 12 hours) on a rotary shaker set at 150 revolutions per
minute at room temperature (25 to 27°C). The overnight culture was
used to inoculate 50 ml of the same medium in 250 ml culture flasks.
A 10% inoculation rate was used.

Aerobic and Anaerobic Incubation of E. carotovora

Inoculated culture flasks were incubated either aerobically,
free standing in an incubator, or anaerobically. Anaerobic conditions
were obtained using a Gas-Pak anaerobic chamber and hydrogen and carbon
dioxide generator envelopes (Beckton, Dickinson and Co. Cockeysville,

17

Md., 21030). The 0.5% palladium Gas-Pak catalyst pellets were changed
before each experiment and a Gas-Pak aerobic indicator strip was
placed in each chamber before it was sealed. Aerobic culture flasks
and the anaerobic chamber which contained the culture flasks to be
incubated anaerobically were placed side by side in the same incubator
and incubated statically at either 24 or 30°C. Duplicate cultures
were grown for each experiment. All cultures were incubated for 5 days.
This was the time required, as determined previously in this laboratory,
for wounded but uninoculated whole potato tubers to reach rot condi-
tions when placed in the Gas-Pak anaerobic system (personal communica-
tion - Patrick D. Colyer). At the end of the incubation period bac-
terial populations of each culture were determined. A dilution series
for each culture was performed in sterile distilled H_2O and 0.1 ml
of each dilution was plated in triplicate, on Difco nutrient agar
plates (23 g/1), which were supplemented with 0.4% casamino acids.
Dilution plates were incubated at 30°C for 24 to 48 hours at which time
the number of colonies was determined.

Preparation of Crude Enzyme Samples

After incubation bacterial cells were removed from the culture
medium by centrifugation (12,000 g at 4°C for 20 min.) using a GSA
rotor in a Sorvall RC2-B refrigerated centrifuge. The supernatant was
retained and filtered through a Nalgene filter unit. Protein concen-
trations of the filtrate were determined using the Bio-Rad protein assay
(Bio-Rad Laboratories, Richmond, CA). Bovine serum albumin was used

as a protein standard. The filtrates were then placed in 250 ml Erlenmeyer flasks and stored at 4°C until assayed for enzyme activity.

Enzyme Assays

Endopectate lyase (PL) activity was measured by the periodate-thiobarbituric acid assay (TBA) (47, 62). Endopolygalacturonase (PG) was determined using the Nelsons reducing group analysis (37). The ability of these enzymes to reduce the viscosity of a NaPP solution was determined using the viscometric assay (56).

Preparation of substrates. For the TBA assay and the viscometric assay for endoPL the substrate consisted of 1.4% NaPP in 0.05 M tris-HCl buffer (pH 8.3) containing, at a final concentration, 0.5 mMEDTA and 1.0 mM calcium chloride. For the Nelsons reducing group analysis and the viscometric assay of endoPG the substrate consisted of 1.4% NaPP in 0.05M PO_4 buffer (pH 6.0) containing, at a final concentration, 0.5mM EDTA. The NaPP solutions for the assays were twice the final concentration and were used to make the above mentioned two substrates. In this way a constant concentration of NaPP could be maintained for each substrate prepared.

Viscometric Assay. In the viscometric assay 5.0 ml of substrate plus 1.0 ml of the crude enzyme preparation were added to an Ostwald-Fenske 300 viscometer, mixed and measured for depolymerization of NaPP over time. The readings for the viscometric assay were conducted at 30°C, which was maintained using a constant temperature water bath equipped

with a heating unit and a mixer. Relative activity was expressed as the reciprocal of the time in minutes for 50% loss in viscosity times 10^3 (5, 6). Specific activity is the relative activity per milligram of protein per ml of enzyme solution. Autoclaved enzyme was used as a control.

TBA Assay. In this assay 100 ml of crude enzyme preparation was incubated with 100 ml of substrate at 30°C for the time required to reduce the viscosity of a NaPP solution by 50% as determined by the viscometric assay. An optical density of 0.3, measured at 548 nm in a Bausch and Lomb Spectronic 20 colorimeter, indicated the formation of 0.1 umoles of unsaturated product. A unit of activity was expressed as the amount of enzyme yielding an increase in absorbance of 0.1 in 1 hour, or the amount of enzyme yielding 1.0 umole of unsaturated product in one hour. Specific activity was expressed as units of activity per mg of protein per ml of enzyme solution.

Nelsons reducing group analysis. For determining the amount of reducing sugar released from NaPP, 100 ml of crude enzyme preparation was incubated with 250 ml of substrate at 30°C for the time required to reduce the viscosity of a NaPP solution by 50% as determined by the viscometric assay. A standard curve was prepared using D-galacturonic acid in concentrations ranging from 5 to 500 mg. Absorbance was measured at 500 nm in the Spectronic 20 colorimeter. Specific activity was expressed as the micromoles of product formed per mg of protein per ml of enzyme solution.

In Vivo Pectic Enzyme Isolation

An enzyme isolation was also performed from potato tissue which had been inoculated with E. carotovora isolate 14 and incubated anaerobically at 24 C.

Preparation of Inoculum. A culture of EC14 was grown in 1% NaPP and nutrient broth and incubated overnight on a rotary shaker set at 150 rpm at room temperature. The culture was then centrifuged (12,000g at 4 C for 20 min.) using a GSA rotor in a Soruall RC2-B refrigerated centrifuge. The supernatant was discarded and the bacterial cell pellet was retained and resuspended in 50 ml of sterile distilled H_2O and the bacterial population of this suspension was determined. A dilution series was performed in sterile distilled H_2O and 0.1 ml of the diluted cell suspension was plated, in triplicate, on nutrient Agar supplemented with 0.4% casamino acids. Dilution plates were incubated at 30 C for 24 hours, at which time the number of colonies was determined.

Preparation and Assay of Crude Enzyme Samples. The EC14 cell suspension was then used to inoculate potato pieces which had been surface sterilized in 10% Clorox for 30 sec., patted dry on paper towels and placed in beakers which had been sterilized. The

beakers were sealed in the Gas-Pak anaerobic chamber and the chamber

was incubated at 24°C for 5 days at which time the rotted potato

tissue and surrounding liquid were strained through several layers

of cheesecloth, and centrifuged at 12,000 g at 4°C for 30 min. The

supernatant was filtered through Nalgene filter units, placed in 250 ml

Erlenmeyer flasks and stored at 4°C. Protein concentrations were de-

termined and enzyme assays were conducted as previously described.

Chemicals

All the chemicals used in these experiments were obtained from

the Sigma Chemical Co., P. O. Box 14508, St. Louis, MO 63178, unless

otherwise indicated. The sodium polypectate used in this study was

that which was obtained from Sigma Chemical Co. prior to 1970.

C H A P T E R   I V
RESULTS

Maintenance of Anaerobic Culture Conditions

Cultures incubated anaerobically at 24°C maintained anaerobic
conditions throughout the incubation period. Anaerobic conditions,
for those cultures incubated anaerobically at 30°C, were slightly
aerobic on the last two days of the incubation period for the second
and third replicates of this culture treatment. A light-blue color
on the indicator strip signified that complete anaerobic conditions had
been lost, but that the level of O_2 present was still less than atmos-
pheric. It is believed that the loss of anaerobic conditions in these
cultures did not have a significant qualitative or quantitative effect
on the enzymes produced. This is because: (1) the anaerobic conditions
were lost so late in the incubation period; (2) the availability of O_2
for the bacterial cells incubated statically in the culture medium is
probably not high; and (3) the bacterial population counts for the 30°C
anaerobic cultures were not significantly different from those of the
24°C anaerobic cultures (Table 1).

Erwinia Carotovora Populations

The population of EC14 incubated at 24°C under aerobic conditions
was higher than that of cells incubated under anaerobic conditions
at 30°C (Table 1). Populations of bacteria anaerobically incubated were

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TABLE 1

Means of bacterial populations of <u>Erwinia carotovora</u> isolate 14
Culture incubated aerobically or anaerobically.[a]

Culture Conditions	Populations (cells/ml)[b]
24°C Aerobic	1.41×10^8
24°C Aerobic with L-cys.	1.40×10^8
24°C Anaerobic	2.95×10^4
24°C Anaerobic with L-cys.	6.86×10^3
30°C Aerobic	3.72×10^7
30°C Aerobic with L-cys.	3.64×10^7
30°C Anaerobic	9.80×10^4
30°C Anaerobic with L-cys.	8.85×10^4

[a]Means were based on three replicates for cultures grown in
1% NaPP Nutrient broth medium without L-cysteine and two replicates
for those cultures grown in 1% NaPP Nutrient broth medium with 0.05%
L-cysteine. Each replicate consisted of two duplicate cultures.

[b]Bacterial populations were determined by plating dilutions
on nutrient agar with 0.4% casamino acids. Plates were incubated
at 30°C for 24 to 48 hours, after which time the number of colonies
was determined.

markedly reduced when compared to the populations of aerobically in-
cubated cultures. The 30°C anaerobic cultures had larger populations
than those bacteria incubated under anaerobic conditions at 24°C.
This may be due to the loss of anaerobic conditions which occurred
late in the incubation period of the second and third replicates for
this treatment. A reduction in bacterial populations was observed for
cultures supplemented with 0.05% L-cysteine.

Because bacterial populations for anaerobic culture conditions
were lower than the populations of aerobically cultured bacteria, the
data is presented in two ways. In the first method data for extra-
cellular protein concentrations and enzyme activity are presented un-
altered; just as it was collected and calculated. The second method
involved adjusting all extracellular protein concentrations and enzyme
activities to the levels which would have been produced if that bac-
terial population had been 10^8 cells/ml. This was performed in order
to estimate the enzyme activities and the amount of protein produced
had the population levels of the anaerobic cultures reached those of
the aerobic cultures.

Protein Concentrations

The protein concentrations for each culture condition of EC14 were
found to be fairly similar (Table 2). Cultures incubated at 24°C
generally had slightly higher protein concentrations than cultures
incubated at 30°C. The amount of protein produced by cultures supple-
mented with 0.05% L-cysteine was not markedly different from the protein
concentrations of cultures grown without L-cysteine. When protein

TABLE 2

Extracellular protein concentrations for each culture of Erwinia
carotovora isolate 14 incubated aerobically or anaerobically.[a]

Culture Conditions	Protein (mg/ml) in replicate[b]		
	1	2	3
24°C Aerobic	0.135	0.095	0.155
24°C Aerobic with L-cys.	0.142	0.132	-
24°C Anaerobic	0.135	0.128	0.135
24°C Anaerobic with L-cys.	0.138	0.13	-
30°C Aerobic	0.10	0.108	0.138
30°C Aerobic with L-cys.	0.085	0.116	-
30°C Anaerobic	0.125	0.112	0.133
30°C Anaerobic with L-cys.	0.12	0.10	-

[a]Bacterial cultures were grown in 1% NaPP Nutrient broth with
and without L-cysteine supplementation.

[b]Protein concentrations were determined using the Bio-Rad Protein
assay. Bovine serum albumin was used as a protein standard. Each
replicate consisted of two duplicate cultures.

concentrations were adjusted to 10^8 cells/ml a large increase in the

protein concentrations of the anaerobically incubated cells resulted

(Table 3). A slight increase was also noted for bacteria incubated under

aerobic conditions at 30°C.

Enzyme Activities

<u>Viscometric Assay</u>. The ability of the crude extracellular enzyme pre-

parations (endopectate lyase, PL; endopolygalacturonase, PG) to reduce

the viscosity of a NaPP solution was measured using the viscometric

assay (5, 6) (Table 4). The activities for the depolymerization of the

acidic substrates were not dramatically affected by incubation at either

24 or 30°C. The activities remained fairly constant for both of these

temperature treatments. The highest specific activities recorded for

the depolymerization of the acidic substrate occurred for the 30 and 24°C

aerobic cultures, with the 30°C culture being slightly higher. A slight

decrease in the specific activity values of the viscometric assay was

observed for enzyme solutions obtained from cultures which had contained

0.05% L-cysteine. When all activity values for the viscometric assays

of the various culture conditions were adjusted to 10^8 cells/ml a large

increase was observed for the 24 and 30°C anaerobic cultures, when com-

pared to the aerobic cultures (Table 5).

A marked difference in the specific activities for the de-

polymerization of the alkaline substrate was observed for the two

temperatures tested (Table 4). All activity values, for the crude

enzyme solution prepared from 30°C cultures, were higher than those

TABLE 3

Extracellular protein concentrations for each culture of Erwinia
carotovora isolate 14 incubated aerobically or anaerobically and
adjusted to 10^8 cells/ml.[a]

Culture Conditions	Protein (mg/ml) in replicate;[b]		
	1	2	3
24°C Aerobic	0.095	0.0675	0.110
24°C Aerobic with L-cys.	0.101	0.0946	-
24°C Anaerobic	4.57×10^2	4.36×10^2	4.58×10^2
24°C Anaerobic with L-cys.	2.02×10^3	1.89×10^3	-
30°C Aerobic	0.269	0.292	0.373
30°C Aerobic with L-cys.	0.234	0.319	-
30°C Anaerobic	1.27×10^2	1.15×10^2	1.36×10^2
30°C Anaerobic with L-cys.	1.36×10^2	1.13×10^2	-

[a]Bacterial cultures were grown in 1% NaPP nutrient broth with and
without L-cysteine supplementation.

[b]Protein concentration were determined using the Bio-Rad Protein
assay and values were adjusted to 10^8 cells/ml. Bovine serum albumin
was used as a protein standard. Each replicate consisted of two
duplicate cultures.

TABLE 4

Means of viscometric assay values of crude extracellular enzyme
preparations of Erwinia carotovora isolate 14 incubated aerobically
or anaerobically.[a]

| Culture Conditions | Enzyme Activity | | | |
| | Relative Activity[b] | | Specific Activity[c] | |
	pH 6.0	pH 8.3	pH 6.0	pH 8.3
24°C Aerobic	7.91	12.2	70.63	180.06
24°C Aerobic with L-cys.	5.81	9.98	42.44	73.39
24°C Anaerobic	5.75	12.5	43.89	95.29
24°C Anaerobic with L-cys.	5.64	9.34	42.33	70.64
30°C Aerobic	7.60	24.25	73.23	235.85
30°C Aerobic with L-cys.	5.23	18.06	52.98	185.50
30°C Anaerobic	5.97	18.72	50.12	147.49
30°C Anaerobic with L-cys.	5.30	13.23	49.75	125.36

[a]The alkaline substrate consisted of 1.4% NaPP in 0.05 MTris-HCl
buffer at pH 8.3 plus 0.5 nM EDTA and 1.0 mM calcium for measuring the
pectate lyase activity. The acidic substrate contained 1.4% NaPP in
0.05 M PO_4 buffer at pH 6.0 plus 0.5 mMEDTA for measuring polygalactur-
onate activity. Five ml of substrate and 1.0 ml of crude enzyme solu-
tion were added to Ostwald-Fenske 300 viscometers, mixed and the
depolymerization of NaPP was measured over time. Means were based on
the first two replicates of these experiments, each replicate consisted
of two duplicate cultures.

[b]Relative activity is the reciprocal of the time in minutes to
reach 50% reduction in viscosity times 10^3(5, 6).

[c]Specific activity is the relative activity per milligram of
protein per ml of enzyme solution.

TABLE 5

Means of viscometric assay values of crude extracellular enzyme preparations of Erwinia carotovora isolate 14 incubated aerobically or anaerobically and adjusted to 10^8 cells/ml.[a]

Culture Conditions	Enzyme Activity			
	Relative Activity[b]		Specific Activity[c]	
	pH 6.0	pH 8.3	pH 6.0	pH 8.3
24°C Aerobic	5.6	8.67	50.20	127.97
24°C Aerobic with L-cys.	4.15	7.13	30.31	52.42
24°C Anaerobic	1.95×10^4	4.24×10^4	1.49×10^5	3.23×10^5
24°C Anaerobic with L-cys.	8.22×10^4	1.36×10^5	6.17×10^5	1.03×10^6
30°C Aerobic	20.43	65.19	1.97×10^2	6.34×10^2
30°C Aerobic with L-cys.	14.36	49.68	1.46×10^2	5.10×10^2
30°C Anaerobic	6.09×10^3	1.91×10^4	5.11×10^4	1.50×10^5
30°C Anaerobic with L-cys.	5.99×10^3	1.49×10^4	5.62×10^4	1.42×10^5

[a]The alkaline substrate consisted of 1.4% NaPP in 0.05 M Tris-HCl buffer at pH 8.3 plus 0.5 mM EDTA and 1.0 nM calcium for measuring the pectate lyase activity. The acidic substrate contained 1.4% NaPP in 0.05 M PO_4 buffer at pH 6.0 plus 0.5 mM EDTA for measuring polygalacturonate activity. Five ml of substrate and 1.0 ml of crude enzyme solution were added to Otswald-Fenske 300 viscometers, mixed, and the depolymerization of NaPP was measured over time. Means were based on the first two replicates of these experiments, each replicate consisted of two duplicate cultures.

[b]Relative activity is the reciprocal of the time in minutes to reach 50% reduction in viscosity times 10^3 per 10^8 cells/ml (5, 6).

[c]Specific activity is the relative activity per milligram of protein per ml of the enzyme solution per 10^8 cells/ml.

values obtained for the 24°C cultures. This increase was so high that even the enzyme solution prepared from 30°C anaerobic culture approached the values for 24°C aerobic cultures. All aerobic cultures had higher activity than their anaerobic counterparts. Cultures which were supplemented with 0.05% L-cysteine had decreased specific activity values. However, the specific activity values of anaerobic cultures which had been adjusted to 10^8 cells/ml were 100 to 10,000 x greater than those for the aerobic cultures at 24°C and 100 x greater than those for the aerobic cultures at 30°C (Table 5).

TBA Assay. The PL specific activity values, as measured by the thiobarbituric acid (TBA) assay (47, 62) (Table 6) agreed with the results obtained in the viscometric assay (Table 4). All PL specific activity values obtained from crude enzyme solutions of 30°C cultures were higher than those values obtained from 24°C cultures. Unlike the viscometric assay, this also included the 24°C aerobic cultures. The PL activity values were higher for cultures incubated under aerobic conditions than for cultures incubated anaerobically. Also, cultures which were not supplemented with L-cysteine had higher activity values than cultures which were supplemented with 0.05% L-cysteine. When PL activity values were adjusted to 10^8 cells/ml an appreciable increase was obtained for anaerobically incubated cultures (Table 7). The PL activities of cultures incubated under anaerobic conditions at 24 and 30°C were about 100 x greater than the activity values obtained for cultures incubated under aerobic conditions at 24 and 30°C. Cultures incubated anaerobically

TABLE 6

Means of pectate lyase (PL) activities of crude extracellular enzyme preparations of _Erwinia carotovora_ isolate 14 incubated aerobically or anaerobically.[a]

Culture Conditions	PL Activity[b]	
	u moles Product[c]	Specific Activity[d]
24 °C Aerobic	1.22	10.56
24°C Aerobic with L-cys.	0.744	5.46
24°C Anaerobic	0.940	7.15
24°C Anaerobic with L-cys.	0.547	4.14
30°C Aerobic	5.007	48.79
30°C Aerobic with L-cys.	2.771	28.58
30°C Anaerobic	2.792	23.54
30°C Anaerobic with L-cys.	1.665	15.72

[a]Means were based on the first two replicates of these experiments, each replicate consisted of two duplicate cultures.

[b]Reaction mixtures contained 100 ul of crude enzyme solution plus 100 ul of 1.4% NaPP in 0.05mM Tris-HCl buffer pH 8.3 and 0.5 nM EDTA with 1.0 mM calcium and analyzed by the TBA assay (47, 62).

[c]Expressed as the umoles of unsaturated product formed per ml of enzyme solution per hour.

[d]Expressed as the umoles of product formed per ml of enzyme solution per hour per milligram of protein per ml.

TABLE 7

Means of calculated pectate lyase (PL) activities of crude extracellular enzyme preparation of <u>Erwinia carotovora</u> isolate 14 incubated aerobically or anaerobically and adjusted to 10^8 cells/ml.[a]

Culture Conditions	PL Activity[b]	
	u moles Product[c]	Specific Activity[d]
24°C Aerobic	0.867	7.50
24°C Aerobic with L-cys.	0.531	3.90
24°C Anaerobic	3.19×10^3	2.42×10^4
24°C Anaerobic with L-cys.	7.97×10^3	6.03×10^4
30°C Aerobic	13.46	1.31×10^2
30°C Aerobic with L-cys.	7.62	7.86×10^1
30°C Anaerobic	2.85×10^3	2.40×10^4
30°C Anaerobic with L-cys.	1.88×10^3	1.78×10^4

[a]Means were based on the first two replicates of these experiments; each replicate consisted of two duplicate cultures.

[b]Reaction mixtures contained 100 ul of crude enzyme solution plus 100 ul of 1.4% NaPP in 0.05 mM Tris-HCl buffer pH 8.3 and 0.5 mM EDTA with 1.0 nM calcium and analyzed by the TBA assay (47, 62).

[c]Expressed as the umoles of unsaturated product formed per ml of enzyme solution per hour per 10^8 cells/ml.

[d]Expressed as the umoles of product formed per ml of enzyme solution per hour per milligram of protein per ml per 10^8 cells/ml.

at 24°C had slightly higher activity values than those incubated under anaerobic conditions at 30°C.

Nelson reducing group analysis. The specific activity of PG in crude extracellular enzyme solutions was measured using the Nelsons reducing group analysis (37) (Table 8). These values agreed with the activity values obtained, for the same enzyme solutions, in the viscometric assay (Table 4). The umoles of reducing product formed by the 24°C incubated cultures were higher than the amounts of product formed in the corresponding cultures incubated at 30°C. However, the specific activity values obtained for 30°C cultures were higher than those of the 24°C cultures. The 24 and 30°C aerobic cultures possessed specific activity values which were twice as high as those of anaerobically incubated cultures at the same temperature. Once again cultures which were supplemented with 0.05% L-cysteine had reduced specific activity values when compared to cultures without L-cysteine. When data for PG specific activity were adjusted to 10^8 cells/ml the activity values of cultures incubated under anaerobic conditions increased by about 1000-fold for 24°C cultures and about 10-fold for 30°C cultures, when compared to cultures incubated under aerobic conditions at these same temperatures (Table 9). Cultures incubated anaerobically at 24°C had slightly higher specific activity values than anaerobically incubated cultures at 30°C.

In Vivo Enzyme Isolation

A 1.08×10^9 cells/ml suspension of E. carotovora isolate 14, in

TABLE 8

Means of polygalacturonase (PG) activities of crude extracellular
enzyme preparation of Erwinia carotovora isolate 14 incubated
aerobically or anaerobically[a]

| Culture Conditions | PG Activity[b] | |
	umoles Product[c]	Specific Activity[d]
24 °C Aerobic	9.895	88.36
24 °C Aerobic with L-cys.	5.46	39.95
24 °C Anaerobic	5.77	44.32
24 °C Anaerobic with L-cys.	5.28	39.87
30 °C Aerobic	9.35	88.86
30 °C Aerobic with L-cys.	4.56	45.65
30 °C Anaerobic	5.305	45.20
30 °C Anaerobic with L-cys.	4.79	45.41

[a]Means were based on the first two replicates of these experi-
ments, each replicate consisted of two duplicate cultures.

[b]Reaction mixtures contained 100 ul of crude enzyme solution
plus 250 ml of 1.4% NaPP in 0.05 M PO_4 buffer pH 6.0 and 0.5 mM EDTA
and assayed by the Nelsons reducing group analysis (37).

[c]Expressed as the umoles of free reducing sugar released from
NaPP per ml of enzyme solution per hour.

[d]Expressed as the umoles of free reducing sugar released per
milligram of protein per ml of enzyme solution.

TABLE 9

Means of calculated polygalacturonase (PG) activities of crude extracellular enzyme preparation of Erwinia carotovora isolate 14 incubated aerobically and anaerobically and adjusted to 10^8 cells/ml.[a]

Culture Conditions	PG Activity[b]	
	u moles Product[c]	Specific Activity[d]
24°C Aerobic	7.03	62.00
24°C Aerobic with L-cys.	3.90	28.54
24°C Anaerobic	1.96×10^4	1.50×10^5
24°C Anaerobic with L-cys.	7.69×10^4	5.81×10^5
30°C Aerobic	25.13	238.87
30°C Aerobic with L-cys.	12.54	125.58
30°C Anaerobic	5.41×10^3	4.61×10^4
30°C Anaerobic with L-cys.	5.41×10^3	5.13×10^4

[a]Means were based on the first two replicates of these experiments; each replicate consisted of two duplicate cultures.

[b]Reaction mixtures contained 100 ul of crude enzyme solution plus 250 ul of 1.4% NaPP in 0.05 M PO_4 buffer pH 6.0 and 0.5 mM EDTA and were assayed by the Nelson reducing group analysis (37).

[c]Expressed as the umoles of free reducing sugar released from NaPP per ml of enzyme solution per hour per 10^8 cells/ml.

[d]Expressed as the umoles of free reducing sugar released per milligram of protein per ml of enzyme solution.

sterile distilled H_2O, was used to inoculate potato tuber tissue slices.

These slices were then incubated under anaerobic conditions at 24 C.

When the incubation period was completed a crude extracellular enzyme

solution was prepared as previously described.

The protein contraction of the crude enzyme solution was deter-

mined to be 0.735 mg/ml. This solution was found to reduce the viscosity

of a 1.4% NaPP solution (pH 8.3 l. by 50%, in 3.6 minutes. The enzyme

activity of this crude enzyme solution was 373.25 (Table 10). The

umoles of unsaturated products, as measured by the TBA assay (47, 62)

were 16.67 (Table 10).

The viscosity of a 1.4% NaPP solution (pH 6.0) was reduced to

50%, by the above mentioned extracellular enzyme solution, in 28

minutes, with a corresponding activity of 47.91 (Table 10). The amount

of free reducing groups released from NaPP, as measured by the Nelsons

reducing group analysis (37), was 19.11 umoles (Table 10).

TABLE 10

Activities of pectate lyase and polygalacturonase isolated from macerated potato tuber tissue inoculated with Erwinia carotovora isolate 14 and incubated anaerobically at 24°C

Assay Performed	Enzyme Activity[a]	
	pH 6.0	pH 8.3
TBA Assay[b]	-	16.67
Nelsons Reducing Sugar[c] Analysis	19.11	-
Viscometric Assay[d]	47.91	373.25

[a]The alkaline substrate utilized in enzyme activity assays consisted of 1.4% NaPP in 0.05 M Tris-HCl buffer at pH 8.3 plus 0.5 mM EDTA and 1.0 mM calcium. The acidic substrate utilized in enzyme activity assays contained 1.4% NaPP in 0.05 M PO_4 buffer at pH 6.0 plus 0.5 mM EDTA.

[b]Reaction mixture for the thiobarbituric acid assay (47, 62) contained 100 ul of crude extracellular enzyme solution plus 100 ul of the alkaline substrate. Enzyme activity is expressed as the umoles of unsaturated product formed per ml of enzyme solution per hour.

[c]Reaction mixture for the Nelsons reducing sugar analysis (37) contained 100 ul of crude extracellular enzyme solution plus 250 ul of the acidic substrate. Enzyme activity is expressed as the umoles free reducing sugar released from NaPP per ml of enzyme solution per hour.

[d]In the viscometric assay 5.0 ml of substrate and 1.0 ml of crude extracellular enzyme solution were added to Ostwald-Fenske 300 viscometers, mixed, and the depolymerization of NaPP was measured over time. Enzyme activity is expressed as the reciprocal of the time in minutes to reach 50% reduction in viscosity times 10^3 per milligram of protein per ml of enzyme solution (5, 6).

C H A P T E R   V

DISCUSSION

Environmental conditions and the types and numbers of microorgan-
isms in or on the potato tuber have been found to be major determinants
in the development of soft rot of potato tubers (10, 15, 16, 25, 26,
27, 38, 44, 45, 50). The question arises as to whether the increase
in the development of soft rot occurs as a result of a reduction in the
resistance response of the tuber during anaerobic conditions or an
increase in the metabolism of pectic enzyme synthesis in the pathogen
under anaerobic conditions. This investigation sought to determine
if the production of the pectic enzymes necessary for soft rot develop-
ment were affected by either temperature or more importantly the avail-
ability of oxygen.

To date most investigators have believed that extracellular PL is
the primary cause of tissue maceration and cellular death in potato
tubers rotted by EC 14 (33, 57). However, the observation that the
rotted tuber tends to have a lower pH than normal (rear pH 6.0 -
the optimal pH for PG activity) and recent studies in controlled en-
vironmental chambers, with whole potato tubers under anaerobic conditions,
has led us to believe that extracellular PG may play a larger role
in soft rot development than previously thought.

A reduction in the population of EC 14 occurred when this bacterium
was incubated under anaerobic conditions. Wells (63) and Menely and
Stanghellini (29) reported that the growth rate, in vitro of Erwinia

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spp. was reduced when cells were grown in low oxygen atmospheres (HO 3%). In light of these data a reduction in the population of anaerobically incubated EC 14 cultures is not surprising. What is surprising, however, is the observation that extracellular protein concentrations for EC 14 cultures incubated aerobically and anaerobically were approximately the same.

To determine the importance of PL or PG in the development of soft rot in potato tubers the levels of production of these enzymes were studied under a variety of culture conditions. Our results indicate that extracellular PL levels from EC 14 were higher than that of the PG levels produced from EC 14. Perombelon and Ghanaker (43) determined that in seven of nine strains of E. carotovora var. carotovora the production of PL was as great at 15°C as at 30°C. The remaining two strains, however, produced less PL at 15°C than at 30°C. The synthesis of PL, for our strain of E. carotovora was shown to be dependent on temperature, more than on any other environmental parameter. Higher levels of PL activity were found for cultures incubated either aerobically or anaerobically at 30°C compared to those cultures incubated at 24°C. These results indicate that our strain of EC 14 may be similar to the two strains described above. Also, this laboratory has always incubated the EC 14 strain of E. carotovora at 30°C and the regulatory mechanism(s) involved in the production of extracellular PL levels may have become selective for this temperature.

Endo PG production was dependent on the availability of oxygen. PG activities were higher for cultures incubated aerobically at 24 and

30 C than for those cultures incubated anaerobically at the same tem-
peratures. Because PL activities were reduced at 24 C the levels of
PG, which remained constant for aerobically incubated cultures, becomes
more important in the maceration of the pectic portion of the cell wall.
It is believed, however, that the low pH values observed in soft-
rotted potato tuber tissue cannot be attributed to PG levels alone.
This pH reduction may be the result of various organic acids which are
liberated from cell walls which have been degraded by soft rot organisms.

When pectic enzymes were isolated from anaerobically treated,
macerated potato tuber tissue, 0.735 mg/ml of protein was detected in
the crude extracellular enzyme solution. The viscometric assay results
indicated, once again, that PL was produced in higher amounts and was
more effective in reducing the viscosity of a NaPP solution, than was
PG. PL from the in vivo enzyme preparation was able to cause a reduc-
tion in viscosity of 50% 20 times faster than any crude enzyme solution
prepared from in vitro cultures. While PG did not reduce the viscosity
of a NaPP solution as rapidly as PL, a 50% reduction in viscosity did
occur at a rate 4.5 times faster than that obtained for in vitro enzyme
solutions.

When all activity values were adjusted to the levels which would
have been obtained by a 10^8 cells/ml culture, a marked increase in the
activity values obtained for all anaerobically incubated cultures resulted.
It was also observed that more protein was produced, on a per cell basis,
in anaerobic than in aerobic cultures. This raises the question of what
mechanism(s) is involved in controlling these phenomena. Two

possibilities are evident: either the bacteria are transporting

existing intracellular proteins out of the cell more efficiently

or the synthesis of all extracellular proteins by the cell are greatly

increased. A combination of these two possibilities may also take place.

In this scenario bacteria which are placed under anaerobic conditions

determine that they are in a stressful situation and are capable

of utilizing one, or both, of these mechanisms to insure a greater

chance of survival.

The second of these two mechanisms involves an acceleration

of the bacterial metabolisms once it is placed under stress conditions.

This would increase the amount of pectic enzymes each bacterium pro-

duces, and enable a smaller number of cells to initiate soft-rot

symptoms. Anaerobic conditions may also increase the amount on non-

pectolytic enzymes synthesized. Several researchers have reported that

extracellular enzymes such as protease, phosphotidase, galactanase,

mannase, galactosidase and xylanase may also be involved in the patho-

genesis of soft rot conditions (21, 56, 57, 60).

Intracellular endo PL enzymes were characterized from nonpathogenic

klebsiella oxytoca and yersinia enterocolitica (4). The klebsiella and

yesinia PLs were shown to have significant physical and chemical

differences when compared to a PL obtained from a pathogenic strain

of E. chrysantheni. These enzymes were not transported out of the

cell and did not macerate plant tissue unless pectin esterase (PE) was

endogenously supplied. When placed under anaerobic conditions enzymes

such as these, or the intracellular pectin depolymerase complex described

by Stack et al. (52), may be transported out of the cell and aid the bacterium in obtaining nutrients. The mechanism by which certain enzymes, and not others, are transported out of the cell is not clearly understood, but a recognition response at the membrane level may be involved. Recognition might be regulated by the ionic charges or anti-genic properties of the enzyme.

When assessing the importance of endo PG or PL, in pathogenesis, it is crucial to keep in mind the effects of the end products of both of these enzymes. Collmer (13, 14) showed that unsaturated and satur-ated di-galacturonic acid (4DG and DG) can be further degraded by oligogalacturonate lyase (OGL), which was not assayed in this study, into 4-deoxy-5 keturonic acid (DK). DK, or its precursor, was found to be an effective inducer of PL synthesis in E. chrysanthemi. Stack (51, 52) found that, at pH 6.0, the cleavage of NaPP by PDIa (endo PG) resulted in the production of saturated products only. Traces of. saturated digalacturonic acid were produced and may prove (not tested with EC 14) to be an effective inducer of PL. If a system of in-ducer formation, similar to that found for E. chrysantheni, is observed for E. carotovora, the importance of PG activity in soft rot development will be greatly increased.

In order to more fully understand the occurrence and prevention of soft rot of potato tuber further research is required. New areas of investigation will include studying the role of the intracellular pectic enzyme complex, produced by EC 14, in determining PL synthesis. Stack (51, 52) determined that two of the enzymes identified in the intracellular

enzyme complex of EC 14, PDII and III, exhibited exo modes of action and were able to produce unsaturated digalacturonic acid which has been shown to be an effective inducer of PL synthesis in E. carotovora. The effects the environmental conditions necessary for soft rot development, have on these regulatory mechanisms should be a focus of further research. By obtaining this information post-harvest physiologists and disease physiologist can cooperate in determining the storage methods which will maximize post harvest life and minimize the losses which occur as a result of soft rot diseases.

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