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Full Length Research Paper

The influence of carbon source and calcium on the production of proteases by *Erwinia chrysanthemi*

N. T. Mazomba* and L. V. Mabinya

Department of Biochemistry and Microbiology, School of Biological and Environmental Sciences, University of Fort Hare, Alice, 5700, South Africa.

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***Erwinia chrysanthemi* (*Ec*) is the causative agent of soft rot diseases in many plants. In order to cause disease, it produces hydrolytic enzymes, which are involved in the degradation of plant tissue. However, the role of proteases in its pathogenicity to plants is not well understood. *E. chrysanthemi* targets the host's middle lamella, which was mainly made of pectic substances containing calcium, for colonization. In this study, it is reported that a pectic substance, such as sodium polypectate (NaPP) may be preferable, not only for the production of hydrolytic enzymes, but for the production of protease as well. Furthermore, since the middle lamella contains high levels of calcium, it was thought that it may play an important role in the production of proteases by this organism. Both predictions were supported by the results presented in this study. When *E. chrysanthemi* subsp. *chrysanthemi* (*Ecc*) was grown in HXC medium supplemented with NaPP and calcium, it produced the highest levels of protease as compared to NaPP alone. These results suggest that proteases produced by *E. chrysanthemi* may be induced in part by calcium.**

Key words: *Erwinia chrysanthemi*, protease, phytopathogenicity, extracellular enzyme.

INTRODUCTION

Erwinia chrysanthemi (*Ec*) causes soft rot diseases in various plants, including maize (Chatterjee and Starr, 1980). One of the major symptoms of this disease is the maceration of parenchymatous tissue brought about by the degradation of pectic substances in the middle lamella. For example, pectate lyase (EC 4.2.2.2), an enzyme that cleaves the bond between α-1,4-linked galacturonosyl residues, plays a major role in the development of the disease (Andro et al., 1984).

Ec produces a number of other extracellular enzymes including proteases (Lettoffe et al., 1989). The role of proteases in the pathogenicity of this organism has not been well studied. In order to appreciate and understand the possible role of proteases, it is important to consider the sequence of events from when the pathogen enters the host to the development of a full-blown soft rot disease. Numerous studies have been conducted on the production of proteases by *E. chrysanthemi*. However, most have included media that is quite different from the natural environment these bacteria reside (Barras et al., 1986; Wandersman et al., 1986; Delepelaire and Wandersman, 1989; Dahler et al., 1990; Wolff et al., 1994; Shevchik et al., 1998). Moreover, the studies have generally emphasized the biochemical properties of the proteases produced and mechanisms involved in their production, and not on their possible role in phytopathogenicity of *E. chrysanthemi*.

The importance of studying a bacterial pathogen in an environment that mimics its natural habitat has been reported by numerous investigations (Potts, 1994; Heithoff et al., 1997). However, this is not simple to achieve, as there are a number of parameters one must consider to reproduce a natural environment in controlled setting such as a laboratory. Such parameters include the potentially numerous factors involved in host-pathogen interactions and the unique host environment that contribute to pathogen survival. Therefore, employing media that mimics the natural habitat is likely to elucidate more specifics of a host-plant interaction than those which do not. This paper reports the production of proteases by *E.*

*Corresponding author. E-mail: nmazomba@ufh.ac.za.

chrysanthemi subsp. *chrysanthemi* (Gray, 1985) in response to various media components, especially with regards to a media that mimics a 'natural environment' in which the pathogen finds itself in the host plant.

MATERIALS AND METHODS

Organism and inoculum

A local isolate of *E. chrysanthemi* pv *zeae* (Mildenhall, 1974) was used in this study, and has been deposited, as strain SR260, in the collection of Professor A. Kelman, of the Department of Plant Pathology, University of Wisconsin, Madison, USA. In this study, it is designated as *E. chrysanthemi* subsp. *chrysanthemi* (*Ecc*) (Gray, 1985). Stock cultures were maintained on nutrient agar (Difco Laboratories, Michigan), at 20°C, and subcultured monthly. Other stock cultures were stored in water perms at room temperature. This strain has been the subject of various investigations in our laboratory and elsewhere in South Africa (Gray et al., 1986; Prior et al., 1994).

An 8 h old culture in a modified M63 medium with glycerol (0.2% w/v) as carbon source was used as the inoculum for all the experiments.

High-calcium *Xanthomonas campestris* (HXC) medium

The high-calcium *X. campestris* (HXC) medium contained the following ingredients: 1.0 g K_2HPO_4; 0.5 g $(NH_4)_2$ SO_4; 0.3 g $MgSO_4.7H_2O$; 1.1 g $CaCl_2.2H_2O$; 4.0 g yeast extract; 10.0 g carbon source; and 1000 ml distilled water. The pH was adjusted to 7.0. Carbon source for the medium was glycerol, glucose or NaPP as indicated.

Modified M63 medium

M63 medium (Miller, 1972) was modified by the addition of yeast extract, a carbon source and CaCl2.2H2O. Each liter of the modified M63 medium contained (g): K2HPO4, 13.6; (NH4)2SO4, 2.0; MgSO4.7H2O, 0.3; CaCl2.2H2O, 1.1; FeSO4.7H2O, 0.005 and yeast Extract. The pH was adjusted to 7.0. Carbon source for the medium was glycerol or glucose as indicated.

Growth conditions

Growth of the bacterial cultures was carried out in Erlenmeyer flasks (250 ml) containing 25 ml medium. The flasks were shaken at 200 rpm in an incubator-shaker (New Brunswick Scientific Company Inc., USA), at 30°C for 16 h.

Crude protease and pectate lyase solution

Ecc culture suspensions grown for 16 h in the indicated medium was centrifuged (27200 x *g*, 4°C, 10 min) in a Beckman J2-21 centrifuge (Beckman Instruments, USA), and the supernatant was used as a crude enzyme, that is, either protease or pectate lyase solution.

Preparation of partially digested polygalacturonic acid

A 2% (w/v) solution (100 ml) of polygalacturonic acid (ICN Pharmaceuticals, Inc., USA) in 50 mM ammonium acetate buffer (pH 8.5), was pre-warmed to 30°C. Digestion was started by adding 5 ml of crude pectate lyase solution of about 5 U/ml. The precipitation of the pectate by Ca^{2+} ions was monitored periodically by taking samples of the mixture and mixing them with an equal volume of 20 mM $CaCl_2.2H_2O$ solution. When no further precipitation was noted, the enzyme-pectate mixture was placed in ice-bath to stop the reaction. The solution was passed through an Amberlite IR-120 (H^+) (BDH, England) column (5.7 × 1.5 cm) and filtered through a glass fibre filter (GF/A, Whatman Ltd., England). It was then freeze-dried for 24 h. The powder (1.6 g) was stored desiccated at -20°C. Before use, the powder was dissolved in water to a concentration of 5% (w/v), and the pH of the solution was adjusted to 7 using 3 N KOH solution. The solution was filter sterilized (0.45 µm Millex-GV filter unit, Millipore, Massachusetts) and stored at -20°C.

Azocasein assay

A modified version of the method described by Braun and Schmitz (1980) was used. Azocasein (Sigma, USA, 1.5% w/v in water, 0.25 ml) was added to 0.25 ml of buffer (0.1 M Imidazole-HCl, pH 6.2) and the mixture was equilibrated at 37°C for 10 min. Crude enzyme solution (0.5 ml) was added to the substrate-buffer mixture, and after incubation for 30 min, the reaction was stopped by adding 0.5 ml of 1.5 M $HClO_4$. Assay blanks were prepared by adding 0.5 ml of 1.5 M $HClO_4$ before addition of crude enzyme solution. The reaction mixture was centrifuged at 8000 x *g* for 2 min, the supernatant (0.5 ml) was mixed with 0.5 ml of 1 M NaOH and the absorbance of the mixture was determined at 440 nm. One unit of the enzyme was defined as the amount of enzyme that catalyzes the hydrolysis of 1 µg of azocasein per minute at 37°C. An extinction coefficient of 35 $g^{-1}(ml\ cm)^{-1}$ for azocasein solution (1% w/v in 0.1 M NaOH) at 440 nm, was used to calculate the enzyme activity.

Pectate lyase assay

A modification of the method described by Dave and Vaughn (1971) was used to measure pectate lyase activity. Enzyme reaction was initiated by mixing crude enzyme solution (10 µl) with 990 µl of substrate (0.4% w/v polygalacturonic acid, Pfaltz and Bauer Inc., USA) in 50 mM Tris-HCl buffer (pH 8.5) containing 1 mM $CaCl_2.2H_2O$ at 30°C. The increase in absorbance at 230 nm was continuously monitored in a recording spectrophotometer for 1 to 1.5 min. The initial linear slope of the progress curve was used to calculate enzyme activity. One unit of the enzyme was defined as the amount of enzyme that catalyzes the production of 1 µmole of unsaturated uronide per minute. A molar extinction coefficient of 4600 $M^{-1}cm^{-1}$ (Nagel and Anderson, 1965) was used to calculate the enzyme activity.

RESULTS AND DISCUSSION

Several studies on *E. chrysanthemi* (Barras et al., 1986; Wandersman, et al., 1986; Delepelaire and Wandersman, 1989; Dahler et al., 1990; Wolff et al., 1994; Shevchik et al., 1998) have shown that it produces proteases, although its role in phytopathogenicity is not clearly under-stood. Most of the studies carried out on *E. chrysanthemi* proteases have concentrated on the isolation and characterization of the enzyme protein. Little effort has been made on correlating the production of protease and its role in plant pathogenicity. Dahler et al. (1990) applied culture fluids of *E. chrysanthemi* mutants

Table 1. Comparison of protease activities produced by *Ecc* grown for 16 h on different media supplemented with glycerol, succinate or NaPP as carbon source.

Medium	Carbon source[a]	*Ecc* protease activity (U/ml)[b] assayed on azocasein
GlySMM	Glycerol	1.31 ± 0.001
M63	Glycerol	1.23 ± 0.002
Boethling medium	Succinate	1.45 ± 0.003
HXC	Glycerol	2.17 ± 0.006
HXC	NaPP	21.00 ± 0.012

[a]Glycerol, succinate and NaPP were added to a final concentration of 1% (w/v); [b]*Ecc* protease assay was carried out using the azocasein method and the activity is presented as U/ml ± standard error of the mean.

grown on LB to the surface of potato tuber disks or inoculated whole plants by placing a mass of bacterial cells on the stem below the youngest fully extended leaf. However, they did not find any relationship between the protease produced by their mutants and their involvement in plant pathogenicity. Shevchik et al. (1998) suggested that the protease produced by the strain of *E. chrysanthemi* may be involved in the post-translation modification of pectate lyase, but made no mention of its possible role in pathogenicity.

What is noticeable about many of these studies is that the media used to grow *E. chrysanthemi* are rich media such as Luria broth (Wandersman et al., 1986; Delepelaire and Wandersman, 1989; Dahler et al., 1990; Wolff et al., 1994; Shevchik et al., 1998), whose constituents are very different from what the phytopathogen would encounter in the plant tissue. That is why an attempt was made in this study to optimize conditions that mimic the environment of the plant tissue, and see how *E. chrysanthemi* would behave with regards to production of protease.

Various growth media were compared for the production of protease by *Ecc* and the results are presented in Table 1. The activity of protease in *Ecc* cultures grown in GlySMM, M63 and Boethling media was similar, whereas the activity in HXC medium with glycerol as carbon source was almost twice that of these media. However, the highest production of proteases was in the cultures grown in HXC with NaPP as carbon source. The activity was about 15-fold that obtained with GlySMM, M63 and Boethling media and 10-fold that of HXC with glycerol as carbon source.

Other ingredients of the HXC-NaPP medium were varied. Firstly, a comparison of nitrogenous sources was carried out and the results are shown in Table 2. Yeast extract supported the highest production of protease by *Ecc*.

Table 3 lists a comparison of the protease activity produced by *Ecc* in HXC media with different amounts of calcium and magnesium. In the first three media, magnesium was added to 0.75 mM, while calcium was added at 0, 0.75 and 7.5 mM, respectively. The corresponding protease activity in the cultures was 27.76, 21.42 and 28.28 U/ml, respectively. The fourth row shows the protease activity in HXC medium without magnesium at 29.43 U/ml. Protease production was similar in the first (27.76 U/ml), the third (28.28 U/ml) and the fourth (29.43 U/ml) medium, while the production in the second medium was lower (21.42 U/ml).

All *Ecc* protease activity results so far were expressed either in terms of net absorbance at 440 nm or in the units of enzyme activity per unit volume (per ml) of supernatant. This way of expressing enzyme activity is not related to the protein content of the crude enzyme solution, nor can it be used to determine the stage of growth at which the enzyme is produced. In addition, growth could not be followed since after calcium was mixed in the medium containing NaPP, a 'lump' was formed which lasted until the end of the growth period. To quantify the enzyme activity in relation to growth, it was attempted to determine the protein content of the supernatant using the Lowry method (Lowry et al., 1951). However, the protein content in the supernatant was so low that it was not possible to detect protein in a reproducible manner using this method.

NaPP was modified so that it was not 'precipitable' with calcium at or above its final concentration in the medium. It was partially digested using pectate lyase produced by *Ecc* to the extent that 10 mM calcium failed to precipitate the NaPP. The partially hydrolyzed NaPP was used as carbon source for the growth of *Ecc*. The results of the effect of various carbon sources and calcium on the production of protease by ECC are presented in Table 4. Without calcium, glycerol as carbon source supported the least growth as shown by the absorbance at 600 nm, followed by glucose. The highest growth obtained was when the cells were grown in partially hydrolyzed NaPP. A similar trend was found with growth in the media with the three carbon sources and calcium. However, with all three carbon sources, growth was higher when calcium was added than when it was not added.

Without calcium, glycerol as carbon source also gave the lowest production of protease, followed by glucose. The production of protease on the two NaPPs,

Table 2. The effects of various complex nitrogenous sources on protease production by *Ecc* grown in HXC medium supplemented with NaPP (1%, w/v). Growth was allowed for 16 h.

Complex nitrogenous source[a]	ECC protease activity (U/ml)[b] assayed on azocasein
None	0.57 ± 0.003
Tryptone	0.91 ± 0.002
Peptone	1.03 ±0.002
Casein hydrolysate	1.14 ± 0.001
Casamino acids	1.26 ± 0.004
Protone	1.83 ± 0.002
Soytone	7.77 ± 0.001
Proteose peptone	10.80 ± 0.004
Beef extract	13.60 ± 0.003
Yeast extract	34.50 ± 0.005

[a]Each nitrogenous source was added to a final concentration of 0.4% (w/v); [b]protease activity (U/ml) ± standard error of the mean.

Table 3. Comparison of *Ecc* protease production on HXC-medium supplemented with NaPP (1% w/v). Growth was allowed for 16 h.

Calcium[a] added (mM)	Magnesium[a] added (mM)	Activity (U/ml)[b] assayed on azocasein
0.00	7.5	27.76 ± 0.016
0.75	7.5	21.42 ± 0.014
7.50	7.5	28.28 ± 0.010
7.50	0.0	29.53 ± 0.025

[a]Calcium and magnesium added as explained in the text; [b]protease activity (U/ml) ± standard error of the mean.

Table 4. Effects of calcium and various carbon sources on growth and protease production by *Ecc*. Growth was allowed for 16 h.

Carbon source[a]	Growth (A_{600})[b]		Protease activity (U/ml)	
	-Ca[c]	+Ca[d]	-Ca	+Ca
Glycerol	3.98	4.72	0.14	0.75
Glucose	4.65	6.24	1.14	2.25
Partially hydrolyzed NaPP	5.32	7.02	25.36	39.62
Unhydrolyzed NaPP	N/D[e]	N/D	25.09	27.55

[a]Glycerol and glucose were added to a final concentration of 0.2% (w/v) and NaPP was added to 1% (w/v); [b]absorbance of the culture was measured at 600 nm; [c]calcium was omitted from the medium; [d]calcium (7.5 mM) was present in the medium; [e]Not done.

hydrolyzed and unhydrolyzed were similar. With calcium however, the production of protease was higher for all four carbon sources than when no calcium was added. Another noticeable result is that protease activity on partially hydrolyzed sodium polypectate as carbon source was higher with calcium than without calcium, whereas with unhydrolyzed sodium polypectate, the activities were not significantly different (Table 4).

Table 5 shows the effect of the two carbon sources, that is, unhydrolyzed and hydrolyzed NaPP, on the production of protease and pectate lyase. While protease production was greater on the partially hydrolyzed NaPP, the production of pectate lyase was greater on the unhydrolyzed NaPP.

Results from the work done on other phytopathogens such as *Erwinia carotovora* (Marits et al., 1999), *Pseudomonas syringae* (Bashan et al., 1986) and *X. campestris* (Dow et al., 1998) have suggested that extracellular proteases from these organisms may play a role in disease development. Tseng and Mount (1974) found that when purified protease and phosphatidase from *E. carotovora* were inoculated on cucumber disks,

Table 5. Comparison of protease and pectate lyase production by *Ecc* grown on HXC medium with partially hydrolyzed and unhydrolyzed NaPP (1% w/v) and calcium (7.5 mM). Growth was allowed for 16 h.

Substrate	Protease activity (U/ml)	Pectate lyase activity (U/ml)
Partially hydrolyzed NaPP	37.98	4.36
Unhydrolyzed NaPP	26.9	8.96

they caused cellular death. Heilbronn et al. (1995) showed *in vitro* degradation of potato lectins by a protease produced from the potato pathogen, *E. carotovora.* Marits et al. (1999) isolated a mutant of *E. carotovora* that produced normal levels of pectate lyase, polygalacturonase and cellulase but was deficient in extracellular protease production. They inoculated potato tubers and tobacco seedlings with a culture of the mutant and found that it was less virulent than the wild type. Dow et al. (1990) have shown that mutants of *X. campestris* deficient in protease activity are considerably less virulent in tests in which the bacteria are introduced into mature turnip leaves through vein endings. This is a procedure that mimics natural black rot infection in which bacteria enter leaf veins through openings in the leaf margin called hydathodes. Dow et al. (1990) induced protease production by adding leaf cell walls to the growth medium, which led them to deduce that the proteases produced by *X. campestris in vitro* are the major proteases produced by the organism *in planta*.

Dow et al. (1998) showed that the proteases produced by *X. campestris* are able to degrade various hydroxyproline-rich glycoproteins (HRGPs). These proteins are thought to be building blocks of the cell wall and play a role in immobilizing bacterial cells after infection (Bowles, 1990). Thus, Dow et al. (1998) concluded that proteases of *X. campestris* might be involved in possible proteolysis of structural proteins in plant cell walls and thus allow spread of the bacteria to overcome some host defense mechanisms.

Initial efforts to produce high levels of protease for further purification studies by *Ecc* grown in GlySMM medium and media described by Wandersman et al. (1986) and Boethling (1975) were not successful. Very low levels of enzyme activity were obtained with these media (Table 4). This led to a systematic investigation of the influence of a number of factors together with a variety of carbon and complex nitrogenous supplements on protease production by *Ecc*.

It was reasoned that, as *E. chrysanthemi* targets the middle lamella for colonization, that a pectic substance, such as NaPP may be preferable, not only for pectate lyase production, but for the production of other enzymes as well. Furthermore, because of the presence of high levels of Ca^{2+} in the middle lamella, it was thought that Ca^{2+} might also play an important role in protease production by this organism.

Both predictions were supported by the results presented in this study. When *Ecc* was grown in GlySMM, M63 and Boethling media, little protease was produced. However, when HXC medium supplemented with glycerol was used, an improvement in the production of proteases was noted which was further enhanced by using NaPP as carbon source (Table 1).

The nature of the complex nitrogenous supplement also has a significant effect on protease production by *Ecc*, with the most effective supplement (yeast extract), increasing protease levels by about 60-fold over a medium without a complex nitrogenous source (Table 3). In some respects, the results are somewhat surprising, as a plant protein product, soytone, was initially thought to be the most appropriate supplementary complex nitrogenous source for protease production by *Ecc*. Although soytone stimulated protease production, this was far lower that obtained with yeast extract. These data all suggest that factors, other than the protein-derived products, may play a role in stimulating protease production by *E. chrysanthemi*; vitamins, of which yeast extract is known to be a good source, especially vitamin B complex.

Table 4 shows the effect of adding calcium to the HXC-NaPP medium. When calcium was not added, the production of proteases was similar to when calcium was present in equal amounts as magnesium. Also, the production of protease was not significantly different in HXC-NaPP without magnesium. However, when calcium is present in low amounts (0.75 mM) as compared to magnesium (7.5 mM), there was lower production of proteases. These results indicate that there may be a competition in the use of calcium and magnesium by the bacterium in the process of growing and producing proteases, and that calcium is the most preferred cation.

Thus, a combination of NaPP and Ca^{2+} increases the levels of protease produced by *Ecc*. It also appears that for the *Ecc* proteases to be produced (Tables 4 and 5), a divalent cation is needed. It does not seem to matter if the NaPP is unhydrolyzed or partially hydrolyzed as regards protease production, although partially hydrolyzed NaPP leads to lowered pectate lyase production (Table 5). The latter result is not surprising, as it is reported that pectate lyase is susceptible to catabolite repression by the degradation products of pectic substances (Hugouviex-Cotte-Pattat et al., 1996).

These studies illustrate an important aspect of microbial nutrition as regards enzyme production. Heithoff et al. (1997), although with regards to genetic studies, said that in order to identify new virulence-associated factors, one must grow bacterial cultures and identify those factors within the infected host or in a synthetic medium

mimicking the host internal milieu. Substrates, complex nitrogenous materials and the ionic conditions that the organism will meet in its natural environment, that is, in the soil or in the plant, may have a profound influence on its growth and on the production of extracellular enzymes. Knowledge of the organism's preferred habitat may therefore provide important clues as to the best carbon source for enzyme production. The influence of NaPP on pectate lyase production can be rationalized in terms of the products of NaPP being inducers of the enzyme. The mechanism by which Ca^{2+} and NaPP influence protease production is not too clear. Tardy et al. (1997) found that the activity of the five major *E. chrysanthemi* pectate lyases PelA, B, C, D and E, show an absolute requirement of Ca^{2+} ions. None of the other tested cations (Ba^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+} and Zn^{2+}) could substitute for Ca^{2+} to activate the five enzymes. The work of Shevchik et al. (1998) is interesting in that, it may explain why there is a concomitant production of protease by *E. chrysanthemi* in media that were thought to induce production of pectate lyase. It remains to be seen whether proteases produced by other strains of *E. chrysanthemi* (or any other phytopathogen) do play such a role.

During the bacteria's ingress into plant tissue, it will encounter protein molecules in the plant tissue as functional enzymes, as well as structural components. In the cytoplasm of plant cells, there are many more proteins that play different roles. Considering the likely abundance of proteins as enzymes, as constituents of cell membranes and as structural components of plant cell walls, their degradation by proteolytic enzymes secreted by plant pathogenic bacteria, can profoundly affect the organization and function of the host cells. However, the nature and extent of such effects in disease development is currently not clear.

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