Pectic Enzyme Activities of Bacteria Associated with Rotted Onions (*Allium cepa*)

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The aerobic bacteria associated with soft rot in onions (*Allium cepa*) were isolated and identified as a *Vibrio* sp., *Micrococcus epidermidis*, *Pseudomonas cepacia*, an *Acinetobacter* sp., a *Xanthomonas* sp., *Bacillus polymyxa*, and *Bacillus megaterium*. With the cup-plate assay method, no pectin hydrolase could be detected from any of these isolates when they were cultured in pectin medium, but lyase and pectinesterases were detectable. Onion tissue cultures showed pectin hydrolase activity for *P. cepacia* and *B. polymyxa* and lyase and pectinesterase activities for all of the isolates, usually at higher levels of activity than those of the pectin medium culture filtrates. In both culture media, *Vibrio* sp. showed the highest lyase and pectinesterase activities. In the viscometric test, all of the isolates achieved at least a 50% decrease in viscosity for lyase enzyme, with *M. epidermidis* and *Vibrio* sp. recording viscosity decreases as high as 83%. The ability to cause soft rot in onion bulbs was demonstrated by *P. cepacia* and *Xanthomonas* sp. Benzoic acid at a concentration of 0.8 mg/ml caused total suppression of enzyme production, whereas sodium benzoate at this concentration reduced pectinesterase production by 71% and lyase production by 72%. The possible use of these preservatives in the control of soft rot in onions is noted.

Onions are usually stored under ambient conditions of up to 30°C with varying relative humidity. Under such circumstances, they may undergo microbial spoilage. Prominent among these storage diseases are white rot caused by *Sclerotium cepivorum* (9), neck rot caused by *Botrytis allii* (10), and bulb end rot caused by *Fusarium* spp. (1). Equally significant are soft-rot diseases of bacterial origin, particularly those associated with *Pseudomonas allicola* (6, 15), *Pseudomonas marginalis* (14), and *Pseudomonas cepacia* (8, 17).

Enzymatic maceration of plant tissues resulting in soft rot is an important factor in plant pathogenesis. Among the pectic enzymes known to be active in maceration are endo-polygalacturonase (EC 3.2.1.15), endo-pectate lyase (EC 4.2.2.1), and endo-pectin lyase (EC 4.2.2.3) (7).

Although Ulrich (17) reported the production of endo-polygalacturonase and polygalacturonate transeliminase by *P. cepacia* and the involvement of endo-polygalacturonase in onion tissue maceration, there is still not enough information on the pectic enzyme complex of other bacteria associated with onion maceration. The present paper, apart from exploring the pectinase activities of such bacteria, determines the effect of some common food preservatives on pectic enzyme production.

MATERIALS AND METHODS

Source of onions. Onions (*Allium cepa*) with clearly visible soft-rot disease symptoms as well as healthy specimens were obtained from onion storage heaps in the Nsukka area of Nigeria.

Isolation of bacteria from diseased onions. Isolations were made from 100 diseased onions by cutting out 1 g of rotted tissue from each onion. Samples from 10 onions (10 g) were pooled and homogenized in sterile 0.1% peptone water by using a Waring blender (three 30-s bursts). A loopful of the homogenate was then streaked onto freshly prepared nutrient agar (Oxoid Ltd.) plates in triplicate and incubated under aerobic conditions at 30°C for 48 to 72 h. Pure cultures of all morphologically contrasting colonies were subcultured onto nutrient agar slants and kept for identification and enzyme studies. Mixed cultures were purified by repeated streaking onto nutrient agar plates. Identification of isolates was based on morphological and biochemical characteristics according to Schmidt-Lorenz (16) and Buchanan and Gibbons (2).

For each sample that yielded more than one bacterial type, only the numerically predominant isolate as seen on the agar plates was studied in detail.

Production of pectic enzymes. (i) Growth of isolates in pectin medium. The medium consisted of (in grams per liter): pectin (Sigma Chemical Co.), 5; yeast extract (Difco Laboratories), 5; Na₂HPO₄·2H₂O, 7.2; KH₂PO₄, 3.5. The final pH was adjusted to 7.0 to 7.1 by using 2 M NaOH. The pectin medium was dispensed in 50-ml portions per 100-ml flask, and

the flasks were sterilized at 121°C for 15 min and then inoculated in duplicate, each with 1 ml of an 18-h nutrient broth culture of each isolate. A flask serving as the control was inoculated with the same amount of sterile nutrient broth. All cultures were incubated at 30°C for 8 days. The cultures were harvested by centrifugation at $23,000 \times g$ at 3°C for 2 h. The supernatant solution was used for assay of enzyme activity after it had been dialyzed twice for 24 h at 1°C against 50 volumes of distilled water.

(ii) Growth of isolates in onion tissue. The isolates were cultured in fresh onion tissue and immersed in sterile mineral medium consisting of the following: $(\text{NH}_4)_2\text{SO}_4$, 2 g; KH_2PO_4 , 4 g; Na_2HPO_4 , 6 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg; MgSO_4 , 0.2 g; CaCl_2 , 1 mg; H_3BO_3 , 10 μg ; MnSO_4 , 10 μg ; ZnSO_4 , 70 μg ; CuSO_4 , 50 μg ; $(\text{NH}_4)_2\text{MoO}_3$, 10 μg ; and distilled water, 1 liter (pH 7.4).

To prepare the onion tissue, a fresh onion bulb was immersed in ethanol and flamed to sterilize the outer surface. The charred surface was removed, and the bulb was aseptically cut into bits of the same size. These were transferred to 100-ml conical flasks, each containing 15 ml of sterile mineral medium, and the flasks were then inoculated with 1 ml of a 4-day-old nutrient broth culture of each isolate in duplicate. A flask inoculated with sterile nutrient broth served as a control. After incubation for 8 days at 30°C, cultures with fungal contamination were discarded. Tissues in most of the other flasks were degraded to a large extent; the remaining tissue was dispersed either by strong agitation or mechanically. All cultures of a given organism were pooled, large tissue fragments were separated by centrifugation at $2,000 \times g$ for 10 min, and the supernatant solution was decanted and further centrifuged at $32,000 \times g$ at 5°C for 10 min. The resultant supernatant solution was dialyzed twice for 24 h at 1°C against 50 volumes of distilled water and assayed for enzyme activity.

Cup-plate assay of pectic enzymes. (i) **Pectin hydrolases and lyases.** The method for the assay of hydrolases and lyases was based on that described by Dingle et al. (5) for polygalacturonases.

Hydrolase activity was assayed at a pH of 5.2, and the composition of the medium (percent, wt/vol) was as follows: pectin, 1; ammonium oxalate, 0.5; thiomersal (BDH), 0.01; Ionagar no. 2 (Oxoid), 1.5; 0.2 M sodium acetate buffer, pH 5.2. Assay for lyase activity was carried out at pH 8.6, and the medium contained (percent, wt/vol): pectin, 1; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.002; thiomersal, 0.01; Ionagar no. 2, 1.5; 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.6. Both assay media were heated to 100°C for 45 min and dispensed in 15-ml amounts into 9-cm petri dishes. After solidification of the gel, the open dishes were dried at 50°C for 45 min, and wells of 8 mm in diameter were cut in the agar gel with a cork borer. Four wells were made in each plate and filled with 200 μl of the enzyme preparations. After incubation at 30°C for 24 h, the plates were flooded with 5 M HCl. Undegraded pectin was precipitated, whereas the breakdown of pectin was indicated by a clear zone around the cup. The results were recorded as the diameter in centimeters of the cleared zone minus the cup diameter

and represent the mean values of four measurements on two different plates.

Pectinesterases. The method used was a slight modification of that described by McComb and McCready (11). The modification was in the use of cups made in agar as described by hydrolase and lyase in place of filter-paper disks. The agar gel for the assay contained (percent, wt/vol): pectin, 1; thiomersal, 0.01; Ionagar no. 2, 1.5; 0.2 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.6. To avoid non-enzymatic de-esterification of the substrate, the crude enzyme preparation was adjusted to pH 7 with 0.1 M NaOH. The cups were then inoculated, incubated, and developed as described for hydrolases and lyases.

Viscometric assay of hydrolase and lyase activities. Enzyme activity based on change in the viscosity of reaction mixtures at 30°C was determined in an Ostwald viscometer with a water flow time of 9 s. The composition of the reaction mixture for assay of hydrolases activity was: pectin, 0.3% (wt/vol); NaCl, 0.1 M; 0.025 M sodium acetate buffer, pH 5.2 (14). For lyase activity, the reaction mixture was made up of: pectin, 0.25% (wt/vol); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.25 mM; 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.6 (13). The assay mixtures for both hydrolase and lyase activities were prepared by mixing 16.5 ml of the substrate solution with 1.5 ml of the enzyme solution. The substrate control contained 16.5 ml of the substrate solution and 1.5 ml of distilled water. The water control contained only distilled water. The tubes were incubated at 30°C, and viscometric determinations were made at regular time intervals for 120 min, starting with the tubes containing the enzyme preparations. Enzyme activity is expressed as the percentage of decrease in viscosity and was calculated according to the formula: $D = [100 \times (T_s - T_t)] / (T_s - T_w)$, where D is the percentage of decrease in viscosity, T_s is the flow time of substrate control, T_t is the flow time of test solution, and T_w is the flow time of water.

Effect of benzoic acid and sodium benzoate on enzyme production in *Vibrio* sp. To select the working concentrations of these preservatives, it was necessary to determine their minimal inhibitory concentrations. These were found to be 1.0 mg/ml for benzoic acid and 3.0 mg/ml for sodium benzoate. Since the tolerance level of benzoic acid and sodium benzoate in foods is 1.50 mg/ml, only concentrations below this tolerance level were tested. Sodium benzoate and benzoic acid were separately incorporated at concentrations of 0.1, 0.2, 0.4, 0.6, and 0.8 mg/ml into 15 ml of sterile mineral medium containing bits of onion tissue. Each concentration was inoculated in triplicate with 1 ml of a 4-day-old culture of a *Vibrio* sp. (PS1). The flasks were incubated at 30°C for 8 days, debris was removed by centrifugation, and the supernatants were assayed for enzyme activity by the cup-plate method.

Phytopathogenicity test. The isolates were tested for ability to cause onion soft rot by inoculating 48-h-old Nutrient agar cultures into wells made in healthy onion bulbs. The inoculated onions were kept at room temperature (25 to 28°C) for 3 weeks and then examined.

RESULTS

Bacterial isolates from rooted onions. Six bacterial genera consisting of seven species were isolated and identified. These were a *Vibrio* sp. (PS1), *Micrococcus epidermidis* (PS2), *P. cepacia* (PS3, PS6), an *Acinetobacter* sp. (PS4), a *Xanthomonas* sp. (PS5), *Bacillus polymyxa* (PS7), and *Bacillus megaterium* (PS8).

Pectic enzymes produced in pectin medium. With the cup-plate assay method, no hydrolase activity was detectable from any of the isolates (Table 1). All of the isolates, however, exhibited lyase activity. The highest lyase activity was recorded for a *Vibrio* sp. (PS1), followed closely by *M. epidermidis* (PS2). A *Xanthomonas* sp. (PS5) demonstrated the least activity. For pectinesterase, a *Vibrio* sp. again showed the highest activity, followed by *P. cepacia* (PS3). No pectinesterase activity was observed in cultures of *Bacillus polymyxa* (PS7) or *Bacillus megaterium* (PS8).

Pectic enzymes produced in onion tissue medium. In contrast with enzyme extracts from a pectin medium which showed no hydrolase activity for any of the tested bacterial isolates (Table 1), the onion tissue cultures (Table 2) exhibited some hydrolase activity for three isolates, *P. cepacia* (PS3), *P. cepacia* (PS6), and *Bacillus polymyxa* (PS7). For the rest of the isolates, no activity was detectable. All isolates produced both lyase and pectinesterase enzymes; the highest activity was recorded for a *Vibrio* sp. Generally, the levels of activity were comparatively higher in extracts from onion tissue cultures than in pectin medium cultures.

Viscometric assays of hydrolases and lyases. With the viscometric assay method, some hydrolase activity was detected for all

isolates (Table 3). These activities were very low for most of the tested isolates. The highest activity (18%) was recorded for *P. cepacia* (PS6). For lyase activity (Table 4), *M. epidermidis* (PS2) and a *Vibrio* sp. (PS1) showed the highest

TABLE 2. Activities of pectin enzymes produced in onion tissue^a

Culture supernatant from:	Activity (cm) ^b		
	Hydro-lase	Lyase	Pec-tinesterase
<i>Acinetobacter</i> sp. (PS4)	0.0	1.1	1.8
<i>B. megaterium</i> (PS8)	0.0	0.9	1.0
<i>B. polymyxa</i> (PS7)	0.2	1.3	0.8
<i>M. epidermidis</i> (PS2)	0.0	1.2	1.5
<i>P. cepacia</i> (PS3)	0.2	1.3	1.1
<i>P. cepacia</i> (PS6)	0.3	1.3	1.0
<i>Vibrio</i> sp. (PS1)	0.0	1.7	2.1
<i>Xanthomonas</i> sp. (PS5)	0.0	1.0	1.5
Control	0.0	0.0	0.0

^a Assay was by the cup-plate method.

^b For hydrolase activity, pectin at pH 5.2 was used; for lyase and pectinesterase activities, pectin at pH 8.6 was used.

TABLE 3. Percentage of decrease in viscosity of pectin (d.e. 8%) by using pectin hydrolase in culture filtrates

Culture supernatant from:	% Viscosity decrease of (min):			
	30	60	90	120
<i>Acinetobacter</i> sp. (PS4)	0.0	0.0	3.0	3.0
<i>B. megaterium</i> (PS8)	0.0	0.0	3.0	3.0
<i>B. polymyxa</i> (PS7)	3.0	12.0	15.0	15.0
<i>M. epidermidis</i> (PS2)	0.0	0.0	6.0	6.0
<i>P. cepacia</i> (PS3)	3.0	3.0	12.0	12.0
<i>P. cepacia</i> (PS6)	3.0	12.0	18.0	18.0
<i>Vibrio</i> sp. (PS1)	3.0	3.0	6.0	6.0
<i>Xanthomonas</i> sp. (PS5)	0.0	0.0	3.0	3.0
Control	0.0	0.0	0.0	0.0

TABLE 4. Percentage of decrease in viscosity of pectin (d.e. 8%) by using pectin lyase in culture filtrates

Culture supernatant from:	% Viscosity decrease at (min):			
	30	60	90	120
<i>Acinetobacter</i> sp. (PS4)	33.0	33.0	50.0	50.0
<i>B. megaterium</i> (PS8)	17.0	33.0	50.0	50.0
<i>B. polymyxa</i> (PS7)	33.0	67.0	67.0	67.0
<i>M. epidermidis</i> (PS2)	67.0	83.0	83.0	83.0
<i>P. cepacia</i> (PS3)	33.0	33.0	67.0	67.0
<i>P. cepacia</i> (PS6)	33.0	67.0	67.0	67.0
<i>Vibrio</i> sp. (PS1)	50.0	67.0	67.0	83.0
<i>Xanthomonas</i> sp. (PS5)	33.0	50.0	67.0	67.0

TABLE 1. Activities of pectic enzymes produced in pectin medium^a

Culture supernatant from:	Activity (cm) ^b		
	Hydro-lase	Lyase	Pec-tinesterase
<i>Acinetobacter</i> sp. (PS4)	0.0	0.9	0.8
<i>B. megaterium</i> (PS8)	0.0	0.4	0.0
<i>B. polymyxa</i> (PS7)	0.0	0.8	0.0
<i>M. epidermidis</i>	0.0	1.8	0.8
<i>P. cepacia</i> (PS3)	0.0	1.0	1.5
<i>P. cepacia</i> (PS6)	0.0	0.5	0.7
<i>Vibrio</i> sp. (PS1)	0.0	1.9	2.0
<i>Xanthomonas</i> sp. (PS5)	0.0	0.2	0.8
Control	0.0	0.0	0.0

^a Assay was by the cup-plate method.

^b For hydrolase activity, pectin at pH 5.2 was used; for lyase and pectinesterase activities, pectin at pH 8.6 was used.

activity (83%), whereas *Bacillus megaterium* (PS8) and an *Acinetobacter* sp. (PS4) recorded the lowest activity (50%). All of the isolates achieved at least a 50% decrease in viscosity at 120 min, indicating a high level of lyase activity in the onion isolates.

Effect of benzoic acid and sodium benzoate on enzyme production. In onion tissue medium, a *Vibrio* sp. (PS1) showed the highest lyase and pectinesterase activities (2.1 and 1.7 cm, respectively). Incorporation of various levels of benzoic acid and sodium benzoate resulted in various degrees of reduction of enzyme production as shown by a decrease in zone diameter (Table 5). Benzoic acid at a level of 0.1 mg/ml in the growth medium reduced pectinesterase and lyase activities by 62 and 59%, respectively, whereas at a level of 0.6 mg/ml, the activities were reduced by 90 and 82%, respectively. At 0.8 mg/ml, there was no detectable activity for any of the enzymes. With sodium benzoate, total inhibition of enzyme production could not be achieved at the levels tested. However, significant decrease in the quantities of the two enzymes was recorded. In the presence of 8.0 mg

of sodium benzoate per ml in the growth medium, pectinesterase activity was reduced by 71%, whereas lyase activity decreased by 72% (Table 5). These compounds are known to suppress bacterial growth and to interfere with enzymatic processes. The low levels of pectinesterase and pectin lyase found in culture filtrates from cultures containing these compounds are caused mainly by suppression of bacterial growth.

Phytopathogenicity of isolates. The results of the phytopathogenicity test showed that three isolates, *P. cepacia* (PS3), *P. cepacia* (PS6), and a *Xanthomonas* sp. (PS5), caused extensive soft rot of onion bulbs. The rot caused by the *Xanthomonas* sp. is shown in Fig. 1. The other isolates, a *Vibrio* sp. (PS1), *M. epidermidis* (PS2), an *Acinetobacter* sp. (PS4), *Bacillus polymyxa* (PS7), and *Bacillus megaterium* (PS8), caused no experimental soft rot. The rots in the original samples from which they were isolated were either caused by other agents or by these same organisms in association.

DISCUSSION

The involvement of endo-polygalacturonase from *P. cepacia* in onion maceration has been reported by Ulrich (17). This study contains part of the limited information on the role of bacterial pectic enzymes in soft rot in onions. In the present study, some hydrolase (polygalacturonase) activity was detectable for *P. cepacia* (PS6) and *Bacillus polymyxa* (PS7), although at very low levels.

Production of pectin lyases, particularly the endotype, has been associated with bacteria more than with any other microbial group (3). All of the isolates examined in our study demonstrated considerable pectin lyase activity, thus confirming already existing observations. The high viscometric activity of this enzyme may be

TABLE 5. Effect of benzoic acid and sodium benzoate on enzyme production in *Vibrio* sp.

Concn in growth medium (mg/ml)	Activity (cm) with pectin at pH 8.6 ^a			
	Pectinesterase		Lyase	
	Benzoic acid	Sodium benzoate	Benzoic acid	Sodium benzoate
0.1	0.8 (62)	0.9 (57)	0.7 (59)	0.8 (53)
0.2	0.7 (67)	0.9 (57)	0.6 (65)	0.8 (53)
0.4	0.5 (76)	0.7 (67)	0.6 (65)	0.6 (65)
0.6	0.2 (90)	0.6 (71)	0.3 (82)	0.6 (65)
0.8	0.0 (100)	0.6 (71)	0.0 (100)	0.5 (71)

^a The numbers in parentheses represent the percentage of decrease in enzyme activity.

FIG. 1. Maceration of onion tissue by *Xanthomonas* sp. (PS5). (A) Control; (B) onion inoculated with *Xanthomonas* sp. (PS5). The arrow indicates the area of soft rot in the onion tissue.

an indication that it is the endotype. Ishii (7) has observed the rapid maceration of onion tissue by endo-pectin lyase from *Aspergillus japonicus*. The high activity level of pectin lyase in all of the isolates suggests at first sight their participation in the same way as in the pectin lyases from *Aspergillus japonicus*. Our observations, however, show that apparently only the lyases of *P. cepacia* and the *Xanthomonas* sp. were important in soft-rot development.

Apart from the pectin lyases, equally high activities were recorded for pectinesterase from all of the isolates. This observation is consistent with the idea of Codner (4) that all pectin-degrading systems may require the participation of pectinesterase, which is believed to be a rate-determining factor.

The control of microbial soft-rot disease of onions calls for either the total suppression of microbial growth or the inhibition of their activities. A number of fungicides, basamid, benlate, and bromafume (12) and benomyl, thiophenatemethyl, and dichlozoline (9), prevent white rot of onions caused by *S. cepivorum*. Whether these preparations will arrest bacterial soft rots equally well is unknown. The preservatives used in this investigation, benzoic acid and sodium benzoate, had minimal inhibitory concentrations of 1.0 and 3.0 mg/ml, respectively. The high minimal inhibitory concentration of sodium benzoate makes its application for the preservation of onions at such a level unacceptable, since its tolerance level in foods is 1 mg/ml. Sodium benzoate may, however, be applied at a sub-minimal inhibitory concentration level (0.8 mg/ml) to achieve considerable growth suppression and thus reduce pectin enzyme production by soft-rot bacteria.

Benzoic acid with minimal inhibitory concentration within the tolerance level may offer itself for use in the suppression of bacterial growth on onions. Even at a concentration below the tolerance level, total absence of pectic enzyme was observed with the tested organism. One of the problems associated with the use of benzoic acid and benzoates in food preservation is their negative effect on flavor at concentrations near and

above the tolerance level. It may therefore be prudent to aim at the inhibition of pectic enzymes rather than at that of whole organisms.

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