Eliminative Split of Pectic Substances by Phytopathogenic Soft-Rot Bacteria

Abstract. Culture filtrates of *Erwinia carotovora* and *Bacillus polymyxa* split pectic substances to yield a product that absorbs at 230 mμ, and which reacts with thiobarbituric acid to form a substance that absorbs at 547 mμ. This product is believed to be a C-4,5-unsaturated oligouronide. The preferred substrate is polygalacturonic acid rather than pectin; the enzyme, provisionally named polygalacturonic-*trans*-eliminase, is activated by calcium.

Soft-rot bacteria damage the host plant by digesting the pectic and possibly other components of the plant cell wall (*1*). The bacterial phytopathogen in which this capacity has been most extensively studied is *Erwinia carotovora*, in which pectin esterase, polygalacturonase, "pectin depolymerase," and "protopectinase" have been reported (*2, 3*). In attempting purification of the hydrolytic enzymes of *E. carotovora*—pectin esterase and polygalacturonase—a different, and perhaps additional, mode of degradation of pectic substances by soft-rot organisms has been discovered (*4*). Culture filtrates of phytopathogenic *E. carotovora* and *Bacillus polymyxa* act upon polygalacturonic acid or pectin solutions to cause a decrease in viscosity and the concomitant appearance of ultraviolet-absorbing material which reacts with thiobarbituric acid to yield a strong absorbancy maximum at 547 mμ. This degradation, thus, is related to the *trans*-elimination reaction discovered by Deuel and co-workers (*5*) to occur when pectin is held in neutral or alkaline solutions, and found by them to be mirrored in an enzymic reaction mediated by a commercial (presumably fungal) pectinase which possessed what they termed pectin-*trans*-eliminase activity (*6*). Pectin-*trans*-eliminase breaks the glycosidic linkage of pectin at C-4 and simultaneously eliminates the H from C-5. The viscosity-reducing split results in oligouronides which terminate in a modified C-4,5-unsaturated galacturonyl unit. These unsaturated compounds are responsible for the ultraviolet absorption and for the specific color reaction with thiobarbituric acid. The analogous action of bacterial preparations on mucopolysaccharides has been presented convincingly by Linker and collaborators (*7*). It is the purpose of this communication (*8*) to report that phytopathogenic soft-rot bacteria degrade polygalacturonic acid eliminatively in a similar fashion and to present a preliminary characterization of the crude enzyme.

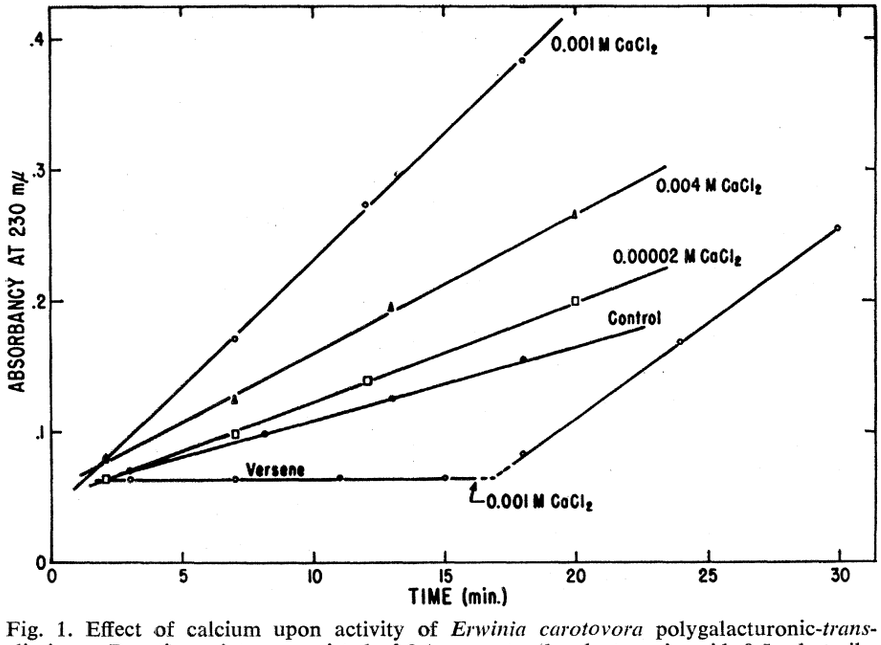

Fig. 1. Effect of calcium upon activity of *Erwinia carotovora* polygalacturonic-*trans*-eliminase. Reaction mixture consisted of 0.1 percent polygalacturonic acid, 0.5 ml sterile, dialyzer culture filtrate, in 0.1*M tris*-HCl buffer at *p*H 8.5, total volume 5.0 ml, 30°C. Top to bottom curves: 0.001*M* $CaCl_2$; 0.004*M* $CaCl_2$; 0.00002*M* $CaCl_2$; control, no added calcium; $3 \times 10^{-5}M$ ethylenediaminetetraacetic acid (Versene) at start, 0.001*M* $CaCl_2$ at 15 minutes. The reaction was followed by measuring absorbancy at 230 mμ.

Erwinia carotovora strain EC 153 (*3, 9*) was grown on a shaker at 30°C in an ammonium-salts medium (*3*) containing 1 percent of either citrus pectin (N.F. grade of Sunkist Growers, Inc.) or polygalacturonic acid (No. 491 of Sunkist Growers, Inc.) neutralized with NaOH. After about 15 hours, the cells were removed by centrifugation, the supernate was passed through a Millipore filter, and the cell-free filtrate was used as a crude enzyme preparation. For some experiments, the crude enzyme was dialyzed at 4°C against 0.001*M p*H 8.0 *tris*-HCl buffer (three successive 2-hour treatments, with 25 volumes of fresh buffer each time). Similar crude enzyme preparations were made from phytopathogenic *Bacillus polymyxa* (potato-rotting strains from Z. Volcani, Rehovot, Israel); these bacilli were grown in a solution containing 1 percent pectin or polygalacturonic acid, 0.5 percent Bacto Casamino acids, and 0.1 percent Bacto yeast extract. The enzymic activity of these crude preparations was followed in a model DU or DK2 Beckman spectrophotometer by measuring the absorbancy of the reaction mixture (enzyme; 0.1 percent polygalacturonate or pectin; 0.1*M tris*-HCl buffer at *p*H 8.5; 30°C) at either 230 mμ (absorption maximum for product from polygalacturonate) or 235 mμ (pectin). Samples of the reaction mixture were treated with thiobarbituric acid according to the procedure of Neukom (*10*) and the absorbancy was measured at 547 mμ. Suitable controls guarded against possible artifactual increases in absorbancy which could result from the instability of pectic substances in alkaline or neutral solutions.

Cell-free culture filtrates of pectin- or polygalacturonate-grown phytopathogenic *Erwinia* and *Bacillus* render solutions of pectin and polygalacturonic acid considerably less viscous; there is a progressive increase in ultraviolet absorbancy (at 230 mμ with polygalacturonate, or 235 mμ with pectin) which, after treatment of samples with thiobarbiturate, is seen to be paralleled by a rise in absorbancy at 547 mμ. Because this is similar to the action reported (*6*) for commercial pectinase, we believed at first (*4*) that the bacterial enzyme was identical with the fungal pectin-*trans*-eliminase. Subsequent observations have confirmed the essential similarity, but also have indicated one important point of difference; namely, polygalacturonic

acid rather than methoxylated pectin appears to be the preferred substrate for the crude bacterial enzyme. To avoid commitment at the present time concerning the relationship with fungal pectin-*trans*-eliminase, we shall refer to this enzyme as bacterial (or *Erwinia*, or *Bacillus*) polygalacturonic-*trans*-eliminase.

Culture filtrates of *E. carotovora*, grown on a shaker in a synthetic medium with polygalacturonic acid as the sole carbon source, exhibited maximum polygalacturonic-*trans*-eliminase activity when the culture entered the stationary phase of growth, that is, after approximately 15 hours at 30°C. There was a loss of enzyme activity on incubation of cultures beyond 16 hours. Although good growth was obtained in glucose, filtrates prepared from such glucose-grown cultures failed to show polygalacturonic-*trans*-eliminase activity. The enzyme system is stable for periods up to 1 month when stored at *p*H 8.0 at 4°C. Using *tris*-HCl buffer in the range of *p*H 6.8 to 8.5 and glycine-NaOH buffer at *p*H 8.8 and 9.3, bacterial polygalacturonic-*trans*-eliminase activity was observed to be optimal at *p*H 8.5. Under the conditions of our assay method, an optimum temperature of 50°C was recorded for the reaction. An absolute requirement for calcium could be demonstrated in the dialyzed enzyme preparations (Fig. 1). Added $CaCl_2$, up to 0.001*M*, increased the rate of the reaction; higher concentrations of $CaCl_2$ resulted in precipitation of the substrate with a consequent decrease in the reaction rate. The addition of $3 \times 10^{-5}M$ ethylenediaminetetraacetic acid (Versene) completely inhibited the reaction; upon addition of 0.001*M* $CaCl_2$ almost all the original activity was restored. At the time our first statement (*4*) on bacterial polygalacturonic-*trans*-eliminase was submitted, most of our work had been based upon pectin as substrate. Subsequent investigation revealed that the polygalacturonic-*trans*-eliminase of *E. carotovora* acts very much more readily on polygalacturonate than on methoxylated pectin. (Observed in a typical experiment: with pectin as substrate, the optical density at 235 mμ was 0.03 at 2 minutes, 0.045 at 6 minutes, 0.08 at 15 minutes, and 0.09 at 20 minutes; with polygalacturonate as substrate, the optical density at 230 mμ was 0.10 at 1 minute, 0.32 at 4 minutes, and 0.57 at 7 minutes.) It is not yet clear whether the attack on pectin was preceded by the action of pectin esterase which was present in the crude enzyme preparation.

Under the conditions we employed, prolonged incubation of the crude bacterial enzyme with polygalacturonic acid resulted in two major degradation products, one substance with R_F value corresponding to digalacturonic acid and one compound with R_F value between that of mono- and digalacturonic acid. This latter material is the unsaturated dimer, based upon the observed ultraviolet absorption and specific reaction with thiobarbiturate. Chemical characterization of this material is under way, as is further purification of the enzyme systems.

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Newton and Spectral Lines

The apparatus used by Isaac Newton in his premier investigations into the nature of color during the years 1668–1672 differs in no important respect from a modern prism spectroscope. According to his *Opticks*, Newton used a prism, an opening in his shutter, and a lens to focus the image of the opening on the prism, and onto a screen on the wall. He experimented with various shapes for the opening; at first, he used a round ¼-inch hole. However, the spectrum produced by this method was too narrow and he says (*1*, p. 70):

> Yet, instead of the Circular Hole, F., 'tis better to substitute an Oblong Hole shaped like a long Parallelogram, with its Length parallel to the Prism ABC. For if this Hole be an Inch or two long, and but a tenth or a twentieth Part of an Inch broad, or narrower; the Light of the Image . . . will be as simple as before, or simpler, and the Image will become much broader, and therefore more fit to have Experiments tried on its light than before.

He used a variety of prisms, including ones made both of solid glass and of glass plates filled with rainwater, which he saturated with "Saccharum Saturni" (lead acetate) to increase the refractive index. Although he complained in a number of instances about the poor quality of the glass in his solid prisms, he seems to have found a few fairly good specimens whose defects were sufficiently localized so that he could mask them with black paper (*1*, p. 88):

> I took another Prism of clear white Glass; but the Spectrum of Colours which this Prism made had long white Streams of faint Light shooting out from both ends of the Colours, which made me conclude that something was amiss; and viewing the Prism I found two or three little Bubbles in the glass, which refracted the Light irregularly. Wherefore I covered that part of the glass with black Paper, and letting the Light pass through another Part of it which was free from such Bubbles, the Spectrum of Colours became free of those irregular Streams of Light, and was now such as I desired.

He used lenses of focal length 6, 8, 10, or 12 feet, and of sufficiently high quality "as may serve for optical uses." He thus had a spectroscope in nearly modern form, and with it generated solar spectra nearly 10 inches in length. With such an instrument Newton should have obtained both the dispersion and resolution necessary to produce distinguishable absorption lines in the solar spectrum.

Why, then, did he not report the presence of spectral lines? Were the lines really not visible, or did he observe but not report them, and if so, why?

H. Kayser, in the introduction to his *Handbuch der Spectroscopie* (*2*), discusses these questions and suggests that Newton did not see the lines because of internal inhomogeneities and surface imperfections of his prisms. More recently, R. C. Lord (*3*) has concluded after careful calculations that the visibility of the *d* lines would be borderline and thinks that Newton's failure might be ascribed to his use of a slit that was too wide.

The answers to these questions should, in part, be provided by experiment.