*Acta Botanica Neerlandica* **14** (1965) 315–322

# ON THE GELATION OF PECTIN BY PLANT EXTRACTS AND ITS INHIBITION

## SOME CONSIDERATIONS IN RELATION TO HOSTPARASITE INTERACTIONS

A. FUCHS

*(Research Unit for Internal Therapy of Plants, T.N.O., Laboratory of Phytopathology, Agricultural University, Wageningen, The Netherlands)*

*(received May 17th, 1965)*

ABSTRACT

Gelation of pectin by plant extracts is due to demethylation by pectin methylesterase; in the presence of Ca⁺⁺-ions Ca-pectate gels are formed. Pectin methylesterase is inhibited to some extent by 1,2-dihydroxy-anthraquinone-sulphonic acid-3, 1,4-dihydroxy-anthraquinone-sulphonic acid-2, and 8-hydroxy-quinoline sulphate, but much stronger by *m*-digallic acid and tannic acid. A *trans*-eliminative breakdown of pectin can interfere with the action of pectin methylesterase.

In host-parasite complexes, the degradation of pectin (either hydrolytically or *trans*-eliminatively) can be catalyzed by many different enzymes of plant, fungal or bacterial origin. Various substances, occurring naturally in higher plants, can greatly affect the activity of these enzymes. Therefore, in order to obtain quantitative data on their respective activities in host-parasite systems, thorough purification of all pectolytic enzymes from such systems is indispensable.

## INTRODUCTION

Pectolytic and cellulolytic enzymes are generally assumed to play an important role in the infection process of fungal and bacterial diseases. In order to investigate this role more closely, usually both pectolytic and cellulolytic enzyme activities are estimated in healthy and diseased susceptible and resistant plant varieties as well as in fungal and bacterial culture filtrates. In most of such studies crude enzyme extracts are prepared, and enzyme activities are measured in buffered mixtures of crude extracts with added substrate. Fungal culture filtrates are often found to exhibit high polygalacturonase activities, or at least an enzyme activity resulting in a loss in viscosity of solutions containing pectic substances. With extracts of healthy and diseased plants these decreases in viscosity are often small, and occasionally even increases in the viscosity of extracts containing pectin or pectic acid as a substrate are reported (see e.g. STRIDER and WINSTEAD, 1961; WOOD, 1961; DEESE and STAHMANN, 1962a, b and c; KUĆ, 1962; MATTA and DIMOND, 1963), sometimes even leading to a complete gelation of the extract-pectin mixture. From such findings quite often the conclusion is drawn, that polygalacturonase activity in the host-parasite complexes is negligible or absent.

It has long been known, that multivalent cations, e.g. Ca·· (DEUEL and STUTZ, 1958), can cause gelation of pectic substances (polygalacturonic acid chains showing variable degree of methylation) by giving rise to the formation of heteropolar principal valent gels (cf. SCHILT, 1961). In mixtures of plant extracts and high-methoxyl pectin, the substrate is first demethylated by pectin methylesterase, producing additional free carboxyl groups in the polygalacturonic acid chains, which in the presence of Ca··-ions form firm Ca-pectate gels.

In this paper, some experiments will be described on the gelation of pectin by plant extracts, and its inhibition by certain compounds. Moreover, some comments will be made on the estimation of pectolytic enzymes in host-parasite complexes.

## MATERIALS AND METHODS

Crude extracts from plants, grown under greenhouse conditions unless otherwise indicated, were obtained by disintegrating the plant material in a Turmix homogeniser; the homogenates were then filtered through cheese cloth and the crude filtrates centrifuged for 10 minutes at 1600 × g. To the supernatants, chloroform and thymol were added to prevent microbial growth; they were then stored at 2° until further use.

In later experiments, acetone precipitations were carried out by adding three parts of acetone (—21°) to one part of plant extract. After centrifugation at 2600 × g, the acetone-insoluble material was dissolved in distilled water for use in the assays.

The pectic substances used were samples of citrus pectin (Exchange Citrus Pectin), a sample purified from this one according to KERTESZ (1957), pectin N.F. (Light & Co. Ltd.), low-methoxyl pectin (Exchange L. M. Pectin) and Na-polypectate (N.B.Co.). The samples were chromatographically pure, except for the first one, which contained glucose.

In most experiments, pectin methylesterase activity was measured by a very simple and rapid gelation test: 1 ml of extract was incubated at 30° with 1 ml of an aqueous solution of either citrus pectin or pectin N.F. and 1 ml of 1/15 M Sørensen phosphate buffer in a test tube with an exact inner diameter of 15.5 mm. The gelation was considered complete as soon as the test tube could be turned upside down without the contents falling out, measurements being made every 5 minutes.

In later experiments, the exact time required for the enzymatic reaction to decrease the pH of an extract-substrate mixture from 8.0 to 7.0 was determined to measure pectin methylesterase activities more accurately. A comparable method has recently been described by SOMOGYI and ROMANI (1964). Like the first, this method also has definite disadvantages in measuring pectin methylesterase activities quantitatively, since enzyme activity may vary within this pH interval. Because the pH-optima of pectin methylesterases of higher plants proved to be rather broad within the selected interval, this method has been chosen as one of the more accurate ones.

## Results

To make sure that gelation of pectin could be used as a method for measuring pectin methylesterase activity, it was necessary to examine whether gelation of pectic substances could take place in the absence of pectin methylesterase, but in the presence of inorganic ions. To this end, 1 ml of aqueous solutions containing mono- (K, Na, or $NH_4$) or bivalent ions (Ba, Ca, Cu, Hg or Mg), all as 0.01 M chloride, and 1 ml of 1/15 M Sørensen phosphate buffer pH 6.8 were added to 1 ml of 4% aqueous solutions of each of the substrates mentioned under "Materials and methods". The monovalent ions, Hg··, and Mg·· did not cause any gelation at all with any of the substrates tested. Ba··-ions, however, caused gelation of all substrates within 24 hours; with purified pectin and Na-polypectate, gelation was accomplished even within 10 minutes. In the presence of Ca··, there was an immediate and complete gelation of Na-polypectate and an incomplete gelation of low-methoxyl pectin, but no gelation took place with the higher methylated pectins. With Cu··, gelation of all pectic substances tested occurred within 10 to 30 minutes.

All extracts from green plants examined so far, except that from potato, showed pectin-gelating activity. On the other hand, extracts from etiolated cucumber seedlings, grown in the dark on wetted filter paper, did not cause gel formation.

Heating of active extracts for 10 minutes at 100° proved to destroy the activity entirely. When the extracts were dialysed for 2 days at 2° the activity was also lost. When, however, heated undialysed extracts and non-heated dialysed extracts from the same plant were added to each other, the activity was completely restored. Addition of Ca··-ions to the dialysed extracts also entirely restored their gelating activity.

From these results it may be inferred, that gelation of pectin depended upon the presence of both pectin methylesterase and Ca··-ions. Addition of Ca··-ions to extracts not causing gelation (potato, etiolated cucumber seedlings) caused pectin to form a stable gel after some time; evidently, it was the lack of bivalent ions, and not the absence of pectin methylesterase, which prevented gelation of pectin in these cases. The observation that the pH decreased from 6.8 to 4.2–4.5 (even in buffered reaction mixtures) after 48 hours of incubation of dialysed extracts from barley, rye, broad bean, French bean, or cucumber, and pectin as the substrate, supports the assumption, that Ca··-ions are not necessary for pectin methylesterase activity.

The gelating activity of the plant extracts was found to be pH-dependent; for every extract investigated with the gelation test, the optimal pH appeared to be between 6.8 and 7.2. The results of two experiments are given in Table 1.

No gelation occurred in extract-substrate mixtures at final pectin concentrations of 0.13%; at concentrations from 0.27% to 1.33% there was complete gelation, sometimes even within 5–10 minutes.

Dilution of extracts resulted in an increased gelation time. Since the chronometric method of determining gelation activity was a rather

TABLE 1

Gelation time of pectin as a function of pH. Reaction mixture: 1 ml of 4% pectin, 1 ml of 1/15 M Sørensen phosphate buffer, 1 ml of extract of cucumber or French bean. Temperature of incubation: 30°

| pH | gelation time in minutes | |
|---|---|---|
| | cucumber | French bean |
| 4.5 | | 30 |
| 5.6 | 105 | 25 |
| 5.9 | 75 | 25 |
| 6.5 | 15 | 15 |
| 6.8 | 10 | 15 |
| 7.2 | 15 | 15 |
| 7.7 | 20 | 20 |
| 8.3 | 25 | 25 |

inaccurate one, it is not surprising that reaction velocity was not linear with extract concentration.

Subsequently, the gelation reaction was followed at 30° in reaction mixtures, containing 1 ml of extracts from various non-etiolated plants, 1 ml of 2% pectin N.F., 1 ml of 1/15 M Sørensen phosphate buffer pH 6.8, and 1 ml of 0.01 M solutions of either of the following substances: benzamide, butylgallate, (+)-catechin, *m*-digallic acid, 1,4-dihydroxy-anthraquinone-sulphonic acid-2 (= rufianic acid), ellagic acid, (-)-epicatechin, gallic acid, glycine, 8-hydroxy-quinoline sulphate, methylgallate, Na-dimethyldithiocarbamate, phenylthiourea, phloroglucinol, propylgallate, protocatechuic acid, tannic acid (= pentadigalloylglucose), 1, 2, 5, 8-tetrahydroxy-anthraquinone (= quinalizarin), L-*threo*-$\beta$-phenylserine, and urea; 2,4-dichloro-phenoxyacetic acid, indole acetic acid and 2,4,6-trichloro-phenoxyacetic acid were used in concentrations of $5 \times 10^{-3}$ M. These substances were selected, partly because their alleged inhibitory action on pectin methylesterase activity has been investigated earlier (cf. GROSSMANN, 1962) and partly because they have been found to exert a systemic action in different fungal plant diseases, in the etiology of which pectolytic enzymes might play a part (VAN ANDEL, 1958; KAARS SIJPESTEIJN and PLUIJGERS, 1962). Others, such as gallic acid, *m*-digallic acid, and several alkylgallates, were selected since they are either constituents of compounds which proved to be inhibitory (tannic acid) or because they are derivatives of such compounds. Of all the substances examined, only four inhibited gelation in our test system (Table 2). This inhibition was particularly pronounced in the presence of tannic acid and *m*-digallic acid. Extracts from different plants and extracts from various tissues of the same plant responded differently to the inhibitory action of the various substances. In general, the inhibition seemed lower with extracts from leguminous plants than with those from others, although with two of the tomato extracts examined, the inhibitory effects were also relatively small.

TABLE 2

Gelation time of pectin in the presence of (a) *m*-digallic acid, (b) 1.4-dihydroxy-anthraquinone-sulphonic acid-2, (c) 8-hydroxy-quinoline sulphate, (d) tannic acid. Control: distilled water. Reaction mixtures: 1 ml of 2% pectin N.F., 1 ml of 1/15 M Sørensen phosphate buffer pH 6.8, 1 ml of extract, 1 ml of distilled water or of 0.01 M aqueous solutions of above-mentioned compounds. Temperature of incubation: 30°; ∞, no gelation

| source of extract | | gelation time in minutes, unless otherwise stated | | | | |
|---|---|---|---|---|---|---|
| | | (control) | (a) | (b) | (c) | (d) |
| cucumber (var. Lange Gele Tros) | seedlings | 5 | ∞ | 15 | 10 | ∞ |
| | seedlings | 15 | ∞ | 35 | 20 | ∞ |
| | cotyledons of seedlings | 20 | 4 days | 50 | 25 | ∞ |
| | hypocotyls of seedlings | 45 | ∞ | 2 days | 60 | ∞ |
| cucumber (var. Vios) | seedlings | 30 | ∞ | 75 | 50 | ∞ |
| | cotyledons of seedlings | 10 | < 12 h | 30 | 15 | ∞ |
| | hypocotyls of seedlings | 15 | ∞ | 60 | 15 | ∞ |
| barley (var. Balder; whole plant) | | 15 | ∞ | 20 | 15 | ∞ |
| rye (var. Petkuser; whole plant) | | 35 | ∞ | 90 | 45 | ∞ |
| wheat (var. Leda; whole plant) | | 50 | ∞ | 75 | 55 | ∞ |
| wheat (var. Leda; whole plant) | | 35 | ∞ | 75 | 75 | ∞ |
| broad bean (var. Con Amore; whole plant) | | 15 | 3 days | 30 | 30 | ∞ |
| broad bean (var. Con Amore; leaves) | | 5 | 3 days | 15 | 10 | ∞ |
| French bean (var. Beka; whole plant) | | 10 | 195 | 15 | | |
| French bean (var. Beka; stem) | | 30 | ∞ | 45 | 35 | ∞ |
| pea (var. Big Ben; whole plant) | | 5 | < 12 h | 5 | 5 | ∞ |
| pea (var. Big Ben; whole plant) | | 10 | < 12 h | 10 | 10 | ∞ |
| tomato (var. Ailsa Craig; whole plant) | | 5 | < 12 h | 40 | 5 | ∞ |
| tomato (var. Bonner Beste; whole plant) | | 5 | 195 | 10 | 5 | ∞ |
| tomato (var. Moneymaker; whole plant | | 5 | 195 | 10 | 5 | ∞ |
| tomato (var. Moneymaker; whole plant) | | 20 | ∞ | 90 | | |

The inhibitory action of tannic acid, its constituent *m*-digallic acid as well as that of 8-hydroxy-quinoline sulphate and a number of anthraquinone derivatives has also been investigated utilizing the more accurate method of measuring pectin methylesterase activity (see "Materials and methods"). The assay system contained 10 ml of

2% pectin N.F., 1 ml of 2 N Na-acetate, 1 ml of 2% K-oxalate and 10 ml of 0.01 M aqueous solutions of the above-mentioned compounds (or 10 ml of distilled water in the controls); after adjusting the pH to 8.0 and bringing the volume to 30 ml, 10 ml of tomato extracts were added. These had been prepared by acetone precipitation from different varieties and were adjusted to pH 8.0 before use. The reaction rates are expressed in per cent of control, calculated from the inverse of the reaction time needed to decrease the pH from 8.0 to 7.0 (Table 3). Tannic acid and *m*-digallic acid show by far the greatest inhibitory effects; 8-hydroxy-quinoline sulphate and the two dihydroxy-anthraquinone-sulphonic acids tested inhibited the reaction to a much smaller extent.

TABLE 3

Activity of pectin methylesterases from different tomato varieties in the presence of inhibitory substances (final concentration 2.5 × $10^{-3}$ M); control = 100

| compound | | Ailsa Craig | Bonner Beste | | Moneymaker |
|---|---|---|---|---|---|
| tannic acid | | 0.0005 | <0.002 | | |
| *m*-digallic acid | | 0.3 | <1 | | |
| 8-hydroxy-quinoline sulphate | | 52 | 64 | 52 | 57 |
| 1,2-dihydroxy-anthraquinone-sulphonic acid-3 | | 16 | 22 | | |
| 1,4-dihydroxy-anthraquinone-sulphonic acid-2* | BDH | 37 | 39 | 43 | 29 |
| | BDHa | | 42 | | |
| | OCI | 63 | 76 | 83 | 40 |
| | Schuchardt | | 50 | | |
| | 924a | | 50 | | |
| anthraquinone-sulphonic acid-1 | | 79 | 100 | | |
| anthraquinone-sulphonic acid-2 | | 106 | 118 | | |
| anthraquinone-disulphonic acid-1, | | 599 | 112 | | |
| anthraquinone-disulphonic acid-1,8 | | 91 | 82 | | |
| anthraquinone-disulphonic acid-2,6 | | 91 | 107 | | |
| 1,2-dihydroxy-anthraquinone | | 91 | 100 | | |
| 1,2,5,8-tetrahydroxy-anthraquinone | | | 105 | | |

* Different samples have been used.

In some experiments with non-dialysed extracts the gelation of pectin was found to be incomplete, the viscosity decreasing later on and reaching its initial value after 24 hours. Thus, not only demethylation of methoxyl groups seemed to occur, but also a cleavage of the pectin chain, leaving units too small to effect gelation. After heating 1 ml of these enzyme-substrate mixtures with 10 ml of 0.01 M thiobarbituric

acid and 5 ml of 0.5 N HCl (cf. ALBERSHEIM *et al.*, 1960*a*), spectrophotometric measurement of the samples revealed an absorption maximum at 547 m$\mu$, indicating the presence of breakdown products with a double bond between C-4 and C-5 of the galacturonic acid unit. This phenomenon was studied in more detail with dialysed extracts using experimental conditions identical with those used in the gelation tests. Especially with extracts from French bean, but also with those from barley and rye, this type of pectin degradation occurred. From these observations it can be concluded, that in these instances a pectin *trans*-eliminase was present, as has been described by ALBERSHEIM *et al.* (1960*b*) and discovered also in peas (ALBERSHEIM and KILLIAS, 1962). Evidence from preliminary experiments suggests, that pectin is more readily cleaved than pectic acid; if this is true, then pectin methylesterase could be considered to interfere with the action of pectin *trans*-eliminase. These findings are at variance with the results of NAGEL and VAUGHN (1961) obtained with bacterial *trans*-eliminases; here, pectic acid was broken down more readily than pectin.

The presence of pectin-cleaving enzymes in these plant extracts has further been substantiated by the observation, that addition of $Ca^{++}$ to mixtures of pectin and dialysed extracts from barley or rye after incubation for 24 and 48 hours did not result in gel formation. If, however, $Ca^{++}$ was added already after ½, 2 and 6 hours, gelation occurred within 5 minutes. Apparently, the pectin chain was degraded slowly to such small units as to prevent gelation after prolonged incubation.

## DISCUSSION

From our experiments, although preliminary, it has become clear, that many factors can affect de-esterification and gelation of pectic substances by plant extracts.

Usually, the role of pectolytic enzymes in host-parasite interactions is studied with only partially purified, or even crude, enzyme preparations. Although it even seems questionable to use results from studies in vitro for an interpretation of the much more complex reaction mechanisms in vivo, it should at least be realized in such studies, that polygalacturonases co-occur with pectin methylesterases and pectin *trans*-eliminases in higher plants (cf. HOBSON, 1964), and that fungal pathogens also produce a great variety of pectolytic enzymes.

As has been shown above, the strongest inhibitors of pectin methylesterase found are tannins and related compounds such as *m*-digallic acid. Interestingly, the latter occurs naturally, a.o. in tea leaves (ROBERTS and WOOD, 1951) and *Acacia arabica* (ENDRES and HILAL, 1963) and has recently been recognized as a synergist of auxins (ZINSMEISTER and HÖLLMÜLLER, 1963; ZINSMEISTER, 1964). On the other hand, many extracellular fungal pectolytic enzymes are known to be inhibited to a great extent by crude plant extracts; especially polyphenolic substances exert an appreciable inhibition (cf. BYRDE, 1963).

With crude extracts, inhibition of enzyme activities by naturally occurring products, or the concerted action of different pectolytic enzymes, can completely obscure the reaction mechanisms. Thus, it becomes increasingly clear, that in order to gain exact information on the role and activity of pectolytic enzymes in host-parasite interactions, thorough purification of extracts from diseased plants is indispensable.

Investigations have been undertaken to purify pectolytic enzymes from host tissues and from pathogenic fungi as well as from host-parasite complexes. Results of these studies will be published elsewhere.

## REFERENCES


ALBERSHEIM, P. and U. KILLIAS. 1962. Arch. Biochem. Biophys. **97**: 107.
————, H. NEUKOM and H. DEUEL. 1960*a*. Arch. Biochem. Biophys. **90**: 46.
————, ———— und ————. 1960*b*. Helv. chim. acta **43**: 1422.
ANDEL, O. M. VAN. 1958. Tijdschr. PlZiekt. **64**: 307.
BYRDE, R. J. W. 1963. Perspectives of Biochemical Plant Pathology. Conn. Agric. Exp. Sta. Bull. **663**: 31.
DEESE, D. C. and M. A. STAHMANN. 1962*a*. J. Agric. Food Chem. **10**: 145.
———— and ————. 1962*b*. Phytopathology **52**: 247.
———— and ————. 1962*c*. Phytopathology **52**: 255.
DEUEL, H. and E. STUTZ. 1958. Adv. Enzymol. **20**: 341.
ENDRES, H. und M. HILAL. 1963. Phytochemistry **2**: 151.
GROSSMANN, F. 1962. Phytopath. Z. **44**: 361.
HOBSON, G. E. 1964. Biochem. J. **92**: 324.
KAARS SIJPESTEIJN, A. and C. W. PLUIJGERS. 1962. Meded. Landbouwh. Gent **27**: 1199.
KERTESZ, Z. I. 1957. Methods in Enzymology **3**, New York: 27.
KUĆ, J. 1962. Phytopathology **52**: 961.
MATTA, A. and A. E. DIMOND. 1963. Phytopathology **53**: 574.
NAGEL, C. W. and R. H. VAUGHN. 1961. Arch. Biochem. Biophys. **93**: 344.
ROBERTS, E. A. H. and D. J. WOOD. 1951. Biochem. J. **49**: 414.
SCHILT, R. 1961. Pectinen en pectinesplitsende enzymen. Thesis, Utrecht.
SOMOGYI, L. P. and R. J. ROMANI. 1964. Anal. Biochem. **8**: 498.
STRIDER, D. L. and N. N. WINSTEAD. 1961. Phytopathology **51**: 765.
WOOD, R. K. S. 1961. Ann. appl. Biol. **49**: 120.
ZINSMEISTER, H. D. 1964. Planta **61**: 130.
———— und W. HÖLLMÜLLER. 1963. Ber. dtsch. bot. Ges. **76**: 251.