ACTA BOTANICA NEERLANDICA, Vol. 3 (1), 1954

ON THE SOFTENING OF FRUITS OF MESPILUS GERMANICA

BY

P. A. ROELOFSEN

Laboratory of Technical Botany, Techn. Univ., Delft, Netherlands

(Received Dec. 2nd, 1953)

INTRODUCTION

In 1928 SLOEP (9) came to the conclusion that the rapid physiological softening of the medlar fruit (*Mespilus germanica L.*) is due to an enzyme which apparently can dissolve cell wall pectin (protopectin), but cannot depolymerize nor demethylate dissolved pectin and hence is different from polygalacturonase (PG), depolymerase (DP) and pectin esterase (PE). It is called protopectinase (PP).

Although SLOEP's publication has often been mentioned in literature, her demonstration of a separate PP seems to have been neglected. For instance, KERTESZ in his recent book (4 p. 335) states: "whenever the macerating action of protopectinase can be demonstrated, the enzyme action invariably proceeds to hydrolyze the pectinic acids from protopectin into nonpectic polyuronides and galacturonic acid."

SLOEP's conclusion should have received more attention, for, if true, this means the occurrence of a specific PP at least in one instant.

Therefore we repeated her experiments with the medlar fruit, supplementing them in several respects. Our results contradicted hers in one — rather important — point and this compelled us to reject her conclusion that a specific PP has been demonstrated in the medlar fruit.

The absence of PG and DP in apples and pears had induced KERTESZ (3) to suggest that the softening of ripening fruits might be due to oxydation of cell wall pectin by dehydroascorbic acid or by peroxydes, both of which may arise from oxydative processes with atmospheric oxygen as hydrogen acceptor. SLOEP's experiments were performed in air. With a view on KERTESZ' suggestion, it seemed desirable to repeat them in oxygen-free atmosphere.

EXPERIMENTS AND RESULTS

a. SOFTENING OF FRUIT TISSUE

In the middle of October of the years 1951–1953, fruits were gathered from two trees growing in our botanical garden and stored at 3° C in a refrigerator until used. Many kept hard until December so that each

fall six weeks were available for the experiments. At room temperature the fruits soften or mould much earlier.

First, the following observations of SLOEP were repeated and confirmed. If slices of ripe but hard fruits are held in a chloroform-saturated atmosphere at 30–35° C, they soften within one hour, at the same time showing enzymatic browning. No softening or browning occurs with slices which are not killed, or with slices from fruits, which had been heated either in water of 80° C for 10 min. or in hot air. Neither does softening occur with unripe fruits, gathered in September.

In addition, the following supplementary observations were made.

1. Softening also occurs after killing the fruit tissue by other, not too rigorous means, viz. freezing, vapour of toluene, methanal, acetic acid or ammonia, as well as by a too acid or too alkaline condition, caused by H–, resp. OH-ion-exchanges (see section e).
2. The pH of the press juice is 3,5–4,0 and does not change during softening.
3. Softening also occurs in a nitrogen atmosphere. Fruit slices were put in a vacuum jar and the air was completely replaced by oxygen-free nitrogen, which was accomplished by alternately filling and evacuating. Then, some chloroform was admitted in order to kill the slices. They softened as usual but did not discolour. Evidently, oxygen is not necessary for softening. Hence, it cannot be due to oxydation of cell wall pectin.
4. During softening cell wall pectin is attacked, which is evident from the following experiment.

Ripe fruits were peeled and cut in halves, which were collected in two identical 50 g portions: A and B. Portion A was immediately boiled in ethanol and B after having softened in a chloroform saturated atmosphere for 15 hrs at 30° C. After filtering off the ethanol, both portions were extensively disintegrated either by means of a blendor or by grinding in a mortar with addition of quartz sand until upon microscopical examination, mainly unicellular fragments were found. Then, the samples were analysed for water soluble pectin and, subsequently, acid soluble pectin.

Soluble pectin was extracted from the A and B portions by shaking with 500 ml distilled water at room temperature for 2 hrs. This was repeated three times. Then the residue was centrifuged and extracted 4 times at 80-90° C with 100 ml 0,05 n HCl for 2 hrs with constant stirring. Pectin was determined in the combined water- or acid-extracts, using the Ca-pectate method of Carré and Haynes. The results are given in table 1.

TABLE I

Soluble and insoluble pectin fractions in fresh (A) and softened (B) fruit halves.

Type of pectin	Ca-pectate in % of dry weight of fruit tissue							
	macerated in blendor				ground with sand			
	exp. 1		exp. 2		exp. 3		exp. 4	
	A	B	A	B	A	B	A	B
Water-soluble fraction	5,17	6,90	5,53	6,97	6,07	8,60	4,20	7,47
Acid-soluble fraction .	3,47	2,03	3,40	2,17	3,67	2,30	5,0	2,53
Total	8,64	8,93	8,93	9,14	9,74	10,9	9,2	10,0

Softening causes a small — probably apparent — increase of total pectin and a large increase of the soluble fraction. This is in line with the view that softening is due to solubilization of cell wall pectin, but of course is no proof.

5. This view is confirmed by the observation that Pectasin W, a commercial fungal enzyme preparation containing active pectic enzymes, readily softens the tissue of heated as well as of fresh slices of medlar fruit.

So far, SLOEP's concept of the liberation of an enzyme which attacks cell wall pectin as soon as the parenchyma cells die, may be upheld.

b. ABSENCE OF PECTIN-DEPOLYMERIZATION ACTIVITY

SLOEP inferred the absence of depolymerization-activity from the observation that softened overripe fruit tissue, which had acquired a jelly-like condition as a result of PE-activity, stayed in this condition for a year, when preserved with chloroform. Pieces of Capectate gel, kept in press juice of such fruits, did not change either.

We too observed that gelatinous press juice, preserved with toluene, shows syneresis, but otherwise keeps unchanged for a long time.

In addition, we have tried to detect depolymerization activity by investigating if any galacturonic acid was produced during softening and if the viscosity of a pectin solution decreased after addition of press juice or an extract of fresh fruits or of softened ones.

1. Test for galacturonic acid.

Slices from ripe fruits were halved. One portion of 6 g was ground and extracted with boiling 65% ethanol. The other portion was extracted after having softened in chloroform vapour for 15 hrs at 30° C. The extracts were evaporated and the residues were extracted with 2 ml pyridine at 45° C. This dissolves sugars, uronic acids and uronides, but leaves inorganic salts in the residue. After evaporating the pyridine, the residue was dissolved in water and baker's yeast was added to ferment the sugars. After filtrating and drying, the residue was re-dissolved in a known volume of water. Then, quantities equivalent to 12-125 mg of dry fruit tissue were applied as spots on filter paper and chromatographed (8).

No galacturonic acid or uronides could be detected.

2. Viscometry. We used two methods for determining the viscosity.

Method of DEUEL & WEBER (2). Pectin solution: 4 g low-methoxyl (4%) apple pectin is dissolved in 500 ml water, then 500 ml 0,02 m. Na-oxalate and 50 ml 1 m. acetate buffer solution of pH 4,6 are added. The mixture is heated at 70° C for 1 hr, preserved with toluene and stored for at least one day before use.

Determination. In three 50 ml vials 25 ml. pectin solution of 30° is mixed with 10 ml of the enzyme solution to be tested. Immediately and after ½-4 hrs, 3 m. eq. alkali are added and the total volume is adjusted to 50 ml. After saponification during more than 6 hrs at room temperature, the solution is centrifuged and the flow time is determined at 30° C with a Höppler or an Ostwald viscometer.

Na-pectate method. Because the alkali used for saponification in the previous method, depolymerizes the pectin in proportion of its methoxyl content (10), PE tends to increase the viscosity. To eliminate this effect, we also used a 95% solution of Na-pectate, adjusted to pH 4,5-5,6. The fruit extract was provided with

0,1% sodium-hexa-metaphosphate in order to prevent the formation of Ca-pectate at the moment it was mixed with the pectate solution. In these cases the flow time was determined with an Ostwald viscometer at intervals during the reaction. In a control experiment, heated fruit extract was added.

Using these methods, we have tested juice from ripe hard fruits, from "chloroform-softened" ones and from naturally softened overripe ones. To this end, fruit mash was diluted with an equal volume of water, pressed through cloth and centrifuged.

We also used juice from mashes, which had been adjusted with NaOH to pH 8,0 and to which 0,25 m. NaCl had been added. It is known that under these conditions more PE and DP may be obtained from tomato and other plant materials. On standing, the pH of such a press juice spontaneously decreased to about 6,5 (PE activity). It was used as such and also after re-adjustment of the pH to 4,0.

Furthermore we also tested tannin-free extracts from fresh fruits and softened ones, obtained as follows. The tissue was frozen with solid CO^2, disintegrated, extracted with cold (–20° C) acetone in order to remove tannins and dried. The powder was extracted with water at room temperature or at 3° C, both at its natural pH of 6,5 or at pH 8, either with or without addition of 0,25 m. NaCl. The pH of the extract was kept at 6,7 or adjusted to 4,0.

Since shaking with n-butanol may facilitate enzyme extraction (5), we also used press juice that had been treated with n-butanol, as well as a water-extract of press residue, prepared with a water-butanol mixture. (For these experiments we used fruits that had been stored for several months at -5° C).

Only in one experiment with juice from naturally softened fruits, a small decrease of viscosity was observed. Even if considered significant, this might have been due to an unnoticed mouldy fruit. Although all fruits were carefully scrutinized for occurrence of external or internal mould growth, one is never quite sure, since internal mouldiness is difficult to detect. Mouldiness often occurs in naturally softened fruits. Apparently fungi have an easy access through the top of the fruit, which is the opening of the urn-shaped receptacle.

Apparently, no DP or PG can be obtained from fresh or from physiologically softened medlar fruits by these methods. Usually, these enzymes are typically exo-cellular and may easily be extracted. Therefore it is quite understandable that SLOEP deduced from her experiments that they are absent. In that case, the enzyme responsible for the softening, must be a protopectinase, which is liberated as soon as the protoplasm dies, and is destroyed by a temperature of 80° C.

c. ATTEMPTS TO EXTRACT THE SOFTENING AGENT

According to SLOEP the supposed protopectinase readily diffuses out of softened medlar fruit tissue and can be demonstrated in the juice. She observed maceration of potato-sections and of the collenchyma in transverse sections of *Lamium*-stems, kept in such juice. This did not occur if the juice had been heated. Furthermore, slices

of softened fruits, if put on potato-slices, rapidly softened the latter.

To our astonishment we could not reproduce these observations. Since it was not clear whether SLOEP had used naturally softened overripe fruits, or "chloroform-softened" ones, we tried both, the former after having been scrupulously examined for mouldiness.

Thin potato slices or thicker slices from heated medlar fruits, or several sections (5 μ thick) of *Lamium* stems, were put either between slices from fresh medlar fruits, which were subsequently softened in chloroform vapour, or between slices from naturally softened fruits.

Furthermore, slices of potato or heated medlar fruits as well as *Lamium* sections, were kept in mash, press juice, or extract from fresh, or artificially softened, or naturally softened medlar fruits. The press juices and the extracts were the same as were tested on depolymerization activity (section *b*).

In every case controls were run with heated mash, juice or extract and microbial growth was always prevented by adding chloroform or toluene.

Although most of these experiments were repeated three times, viz. in the fall of the years 1951–1953, never any macerating effect could be observed. However, if overripe *mouldy* fruits were used, maceration did occur. We are compelled to assume that SLOEP had accidently used internally moulded fruits for her experiments on the maceration of *Lamium* and potato tissue, but healthy ones for her experiments which demonstrated the absence of depolymerization.

Here we are confronted with the very remarkable fact that the macerating enzyme apparently does diffuse from the dead protoplasm into the cell wall, but cannot reach tissues which are in contact with the softened tissue and cannot be extracted from softened or unsoftened tissue. Apparently, the enzyme that attacks the cell wall pectin as soon as the cell contents die, is either strongly adsorbed to the cell wall or is inactivated very soon.

In the previous section we reasoned that it must be protopectinase since we failed to demonstrate any depolymerization activity. However, if we likewise fail to obtain the supposed protopectinase *in vitro*, then the macerating enzyme might as well have been a depolymerizing one, PG or DP, differing in one respect from the pectic enzymes known so far, viz. great lability for inactivation by substances likewise occurring in the medlar fruit.

If the enzyme is a protopectinase, then it might merely become strongly adsorbed to the cell wall. This possibility does not apply for a depolymerizing enzyme, for in that case it very probably will continue to depolymerize the pectin to which it is adsorbed and galacturonic acid should have been found in the softened tissue. If it is active for a short time only, this might suffice for solubilization of cell wall pectin, but not for liquefication of pectate gel, neither for production of galacturonic acid in detectable amounts. With mould PG the monomer does not appear until 90 % of the glycosidic bonds have been split (7).

In any case, the softening of the medlar fruit can no more be cited as an example of the occurrence of protopectinase.

d. ATTEMPT TO FIND AN INHIBITOR IN MEDLAR FRUIT TISSUE

In the acetone-precipitate of pear juice, WEURMAN (11) has found a thermolabile inhibitor of mould PG, a fact which could be confirmed by us. In addition, he could demonstrate the occurrence of PG or DP-activity in pears of "ideal ripeness".[1] He supposed an inhibition in other stages, but — unfortunately — found that the pear-inhibitor was inactive with the pear-enzyme, which brings doubts as to its role in the softening process of pears.

Since the presence of a strong inhibitor in the medlar fruit might explain our results described in the sections b and c, it still seemed appropriate to find out whether an inhibitor of mould PG also occurs in the medlar fruit.

Therefore, we examined the decrease of viscosity of a mixture of 10 ml 1% Na-pectate, 1 ml 1% Pektasin W enzyme solution and 1 ml extract of medlar fruit tissue. For the blank experiment the extract was heated to the boiling point. The extract was prepared by mixing 4 ml water with 1 g dry medlar fruit powder, obtained by extracting medlar fruit tissue with cold acetone as described in section *b*. The pH of the Na-pectate mixture was adjusted to 5,6 and the decrease of the flow-time was followed at 30° C with an Ostwald viscometer.

No inhibition by medlar fruit extract was observed. Of course, this does not exclude the presence of an inhibitor specific for the macerating enzyme of the medlar fruit.

e. MIGHT THE SOFTENING BE DUE TO ION-EXCHANGE?

Plant tissues may be macerated and extraction of pectin may be enhanced by Ca-binding salts, such as polyfosfates and oxalates, as well as by cation-exchangers (1). A similar exchange might be the cause of the liquefication of a soft agar gel by H-saturated Amberlite ion exchanger (6).

Soluble Ca-binding salts cannot be the cause of the softening of the medlar fruit, for in that case the maceration agent should have been easily extractable. However, it is not impossible that killed protoplasm becomes an ion-exchanger as a result of some unknown enzymatic process which is a part of the autolysis which sets in as soon as the cells die. Admittedly, it is not very likely and furthermore it would still be difficult to explain why this ion-exchange process does not occur with *Lamium*-sections suspended in a mash of softened fruits and apparently, is confined to the interaction between the contents and the cell wall of the same cell.

To test this possibility, a layer of synthetic ion-exchange resin was applied between two slices of a medlar fruit that had been heated at 80° C for 10 min. We used Amberlite IRC 50, which has weak acid groups and Amberlite IR 120 as well as Dowex 50, which possess strong acid groups. The resins had been saturated either with acid

[1] Using a Na-pectate solution as substrate and its viscosity as a criterion, we failed to demonstrate depolymerization activity in juice of apples, ripe pears and overripe pears. Neither do these juices macerate *Lamium* sections.

or 5 % NaCl or a 0,5 molar acetate buffer of pH 4. Moreover, the OH-saturated anion-exchangers Ionac A 293 and Dowex 2 were tried.

In no case did softening occur. Softening was evident if the H or OH saturated exchangers were applied on *living* medlar fruit tissue, but evidently, this must have been the result of a killing of the tissue and not of an ion exchange with the cell wall pectin.

Therefore, there is no indication that the softening of the medlar fruit might be due to ion-exchange.

The fact that we performed the experiments described in section *e*, may serve to demonstrate that in our opinion one does not even know with certainty that the softening of the medlar fruit is due to a pectic enzyme, let alone whether this be PP, DP or PG. We only know that in all probability some enzyme system is involved. Since the softening of ripe fruits is a very common and all-important phenomenon, it is to be hoped that the completely negative results of our experiments with the medlar fruit, will not discourage other investigators to pay more attention to the strikingly rapid softening of this fruit, which seems to be an ideal object for investigating the process of softening in ripening fruits in general.

Summary

The claim (9) that the rapid softening of dying tissue of the medlar fruit (*Mespilus germanica* L.) has been proven to be due to protopectinase, has to be cancelled. It can merely be upheld that some enzyme system is involved and that in all probability it is not due to oxydation of pectin, nor to ion-exchange. The softening is accompanied by an increase of soluble and a decrease of insoluble pectin, but no galacturonic acid is produced.

Acknowledgement

The author is much indebted to Miss L. van Dijke for much assistance in performing the experiments.

References

1. Beohner H. L., A. B. Mindler, Ind. Eng. Chem. *41*, 448, 1949.
2. Deuel H., F. Weber, Helv. Chim. A. *28*, 1089, 1945.
3. Griffin J. H., Z. I. Kertesz, Botan. Gaz. *108*, 279, 1946.
4. Kertesz, Z. I., The pectic substances, 1951.
5. Morton, R. N., Nature *166*, 1092, 1950.
6. Olmsted, J. M., V. J. Koningsberger, Proc. Kon. Ac. v. Wetensch. C 54, 3, 1951.
7. Rahman, M. B., M. A. Joslyn, Food Res. *18*, 308, 1953.
8. Roelofsen, P. A., Bioch. bioph. acta *10*, 410, 1953.
9. Sloep, A. C., Onderzoekingen over pectinestoffen, etc. Thesis Delft 1928.
10. Vollmert, B., Makromol. Chemie *5*, 110, 1950.
11. Weurman, C., Pectine-omzettingen en pectine-fermenten in Doyenné Boussoch peren; Thesis Amsterdam 1952.