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Histochemical Determination of Polysaccharides in Fruit Pulp Cell Walls[1]

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Well known procedures of fixation, dehydration and paraffin embedding when applied to fruit pulp result in severe shrinkage and stretching of the cell wall. A method to safely perform such steps when dealing with fruit pulp is described. Furthermore, to determine the polysaccharide content, which are abundant into the fruit pulp cell wall, there are not many available method: neither these are sufficiently specific nor completely reliable as e. g. the sulfuric acid-potassium iodide for cellulose and ruthenium red for pectins (Mericle and Whaley 1952). A different approach jointly based on selective polysaccharides extractions and PAS reaction (Hotchkiss 1948) was developed by Boroughs and Bonner(1953).

Later Jensen (1960) transferred this method to histochemistry, but it was scarcely consistent and reliable because, by Jensen's (1960) own admission, during some steps the slices are easily removed from the slide. Furthermore when applied to fruit pulp even an other step (pectin removal), which apparently did not pose problems on root tips, was critical and loosened the slices from the slide. In this paper we describe the modifications of the Jensen's (1960) method which allowed us to analyze the polysaccharides content according to the Boroughs and Bonner (1953) procedure without losses of histological material.

Materials and methods

Cubic pieces of about 5 mm were cut out of fruits with a scalpel and promptly immersed into dry ice-cold absolute alcohol. Alternatively a very deep (–80°C) freezer may be used. They are kept in the alcohol, which is changed every 24 hours, for 3 days. Then the blocks, still in alcohol, are transferred to a deep freezer (—22°C) where they can be kept for several days. After this treatment the tissues are well dehydrated, without shrinkage, but not yet fixed. Subsequent fixation is a prerequisite for staining. However, as fixatives contain water, a rehydration is necessary and it should be gradually performed at cold temperature (∼—20°C). Pieces are passed through a stepwise scale of cold ethanol down to 50% alcohol. Less concentrated alcohols solidifying at this temperature, rehydration may be completed at

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room temperature without tissue damage.

Two fixatives were tested, with equal success:

a) The standard Navashin's mixture.

b) FAA (formaldehyde acetic acid-alcohol) (Jensen 1962), modified by using 30% ethanol. A 24 hr treatment at room temperature with either of these fixatives gives satisfactory results.

Dehydration is again performed: this time through a graded series of alcohols at room temperature as described by Jensen (1962), but the length of each step is increased to 24 hrs.

The infiltration is performed, as usual, with paraffin (Paraplast) and 10 μ slices can easily be cut with a rotary microtome. The slices are affixed to gelatinized slides which are prepared as previously described (Jona 1963), but with modified concentrations: gelatine 0.5% w/v and chromalum 0.2% w/v both in dist. H_2O. After paraffin removal, the slices are ready for selective extractions which are basically as described by Jensen (1960), but modified as follows:

1. *Removal of pectic substances*

The slides are treated overnight in a Coplin jar with 1% w/v pectinase (N. B. Co., tomato) in dist. H_2O at 30°C. The pH should be adjusted to 4 for maximal enzyme efficacy and low enzyme decay. After a thorough washing with dist. H_2O, the slides are treated with 0.5% aqueous ammonium oxalate solution for 2 hrs at 90°C. for complete removal of pectic substances.

2. *Removal of hemicellulose*

After the removal of the pectins the slides are thoroughly washed with dist. H_2O and dried, then one lot is set aside to be stained later while the other slides are coated with a cellulosic film by dipping them for few seconds into a 0.5% ethyl cellulose (B. D. H.; cellulose ethyl ether; degree of substitution 2.42 to 2.53; ethoxy content 47.5 to 49.0%) solution in a 4:1 mixture of toluene and absolute ethanol. After complete evaporation of the solvent, the slides are extracted in Coplin jars for 12 hrs with 4% aqueous NaOH at room temperature.

3. *Removal of non-cellulosic polysaccharides*

The slides treated as described in 2, are divided into two groups. One is again set aside to be stained later; the other, after renewal of ethyl cellulose coating, is treated in Coplin jars for 12 hrs at room temperature with 17.5% aqueous NaOH. Before renewal and after extraction the slides are dried and ethyl cellulose coating is removed by a 5 min rinsage in 4:1 toluene abs. ethanol mixture.

The slides are then gradually hydrated through a graded series of alcohol and rinsed in H_2O in order to remove NaOH crystals. Finally all the slides (non-extracted and extracted, as described in 1–2 and 3, are oxidized for 10 min in 0.5% aqueous periodic acid (HIO_4) and then stained with Schiff reagent for 15–60 min at room temperature.

Differentiation is performed as described by Jensen (1962) with 2.0% potassium bisulfite in dist. H_2O.

Results and discussion

Three main results are obtained by the above method. The first is the perfect degree of integrity of the cell wall achieved by the gentle removal of water from the cells of the fruit pulp. The method which may be considered a freeze substitution is more limited in its scope than the usual procedure (Jensen 1962): while usually the purpose is to avoid loss of water-soluble substances, we used it only to gently remove big amounts of physiological water without damaging the tissues. Ethyl alcohol, though considered to be the simplest fixative, does not act as such at this temperature. Consequently, at the end of the dehydration process, fixation is a prerequisite for further treatment of the cell walls. Being the fixatives more or less rich in water, hydration, followed by a new dehydration, are necessary before paraffin infiltration: both should be performed very gradually in order to avoid damaging cell walls. Vacuum was therefore, avoided and complete and deep penetration of the reagents was achieved by increasing the length of treatments.

Table 1. Extinction values of PAS stained cell walls

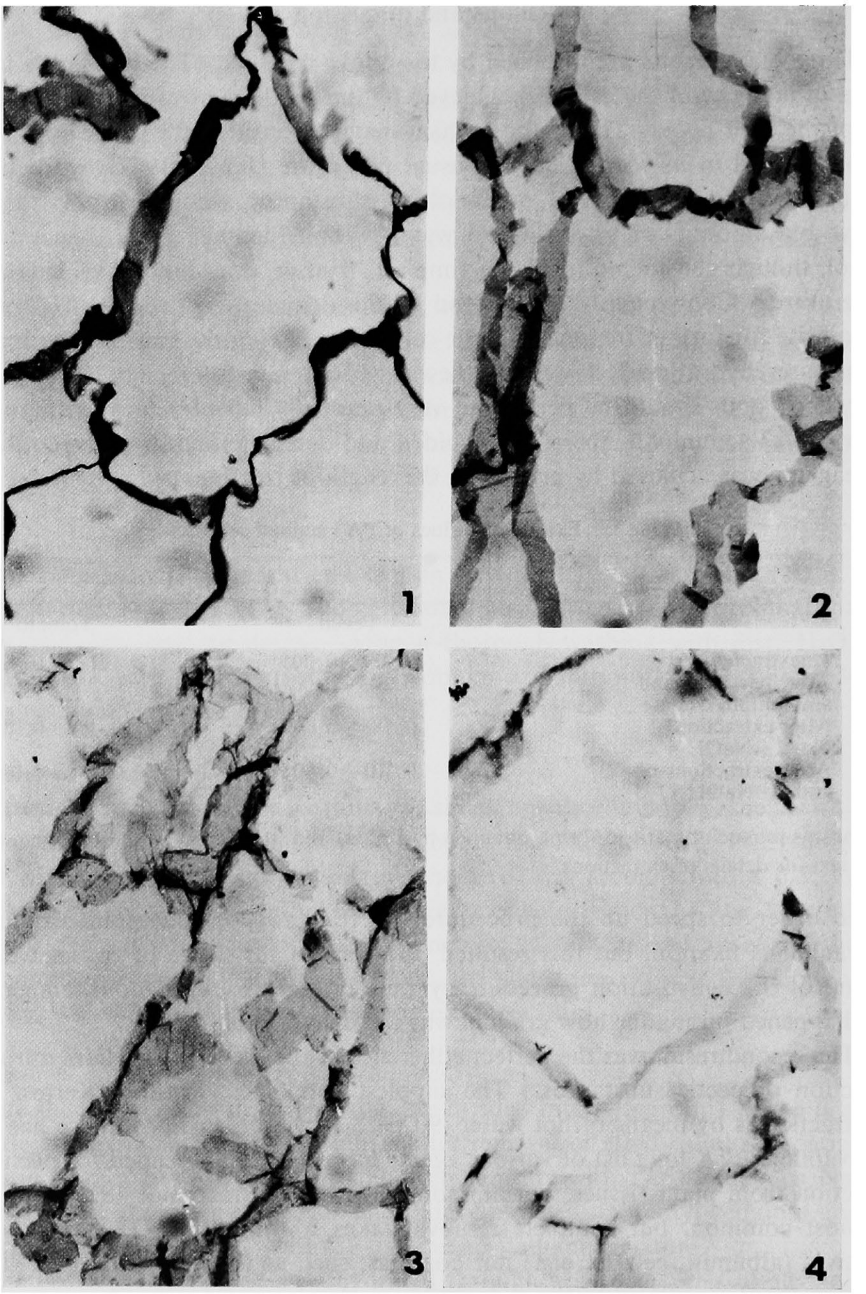
Treatments	Species	
	Peach	Tomato
1 Unextracted	0.576±0.005	1.127±0.021
2 After pectinase and ammonium oxalate	0.205±0.009	0.555±0.010
3 After extraction No. 2+NaOH 4%	0.188±0.005	0.483±0.019
4 After extractions no. 2 and 3+NaOH 17.5%	0.101±0.006	0.348±0.013

Light was monochromatic (547 mμ, obtained by a filter) and was measured with photomultiplier. See text for details of extractions.

In order to speed up the procedure, we tried to postpone cold dehydration after aqueous fixation, but this resulted in a serious shrinkage of tissues when, at the end of the dehydration process, they were brought back at room temperature. This happened no matter how gradual was the dehydration process.

The second result was the perfect adherence of the slices to the slides during the extraction of pectic substances. The simplest and oldest method (Kertesz 1951) of extraction is by means of hot water (90°C). However, this procedure has many disadvantages. A long list of various agents has been recommended for pectic acid extraction from plant tissues, hot ammonium oxalate (Braconnot 1826 a, b) being the most common, but complete removal takes a long time (12 hrs). Neither adhesives (albumin, gelatin, etc.) nor coatings, such as celloidin or ethyl cellulose, can keep the slices affixed to the slide during such a long and strong treatment. Removal by pectinase was tried but the results were not completely satisfactory. Pectic substances are partially removed, as it appears from PAS stain intensity, but a lighter stain can be obatined only with an additional hot ammonium oxalate treatment, the length of which may be decreased from 12 to 2 hrs, thus leaving the slices undamaged on the slide.[1]

[1] *Note added in proof*: The sequential order of the two treatments for removal of pectins is critical: its inversion results in total loss by detachment of the slices.



Figs. 1–4. PAS stained grape tissue. 280×. All pictures are taken exactly at the same speed, developed and printed exactly in the same conditions. Consequently darkeness intensity exactly reflects the amount of stained polysaccharides for evaluations of content of cell wall. 1. unextracted. 2, after pectinase and ammonium oxalate treatment for pectic substances removal. 3, after pectinase, ammonium oxalate and 4% NaOH treatment for hemicellulose removal. 4, after pectinase, ammonium oxalate, 4% NaOH and 17.5% NaOH for non cellulosic polysaccharides removal.

Such a result appears very clearly from comparison of Figs. 1 and 2 as well as from Table 1. A better firmness however and a higher degree of adhesiveness of gelatine is achieved by increasing the concentration of chromalum from standard levels (Jona 1963).

According to one widely held theory, in the process of hardening, coordinately held water molecules are displaced by amino and carboxyl groups of the protein. The hardening of gelatine containing dichromate seems to be due to the reduction of dichromate, producing a chromic salt; pH 4 is critical for optimum hardening (Kirk and Othmer 1946).

However through the NaOH treatments, despite all the above described procedures, a 100% loss of slices was registered. Even Jensen (1960), in outlining the the method, stressed the possibility of the loss of slices and recommended great care in handling them. Furthermore, he suggested that they be treated while "placed on thin glass rods on the bottom of a large Petri dish". Nevertheless, slides completely immersed in the alkaline solution result in slices removed from their place with no way to recover them and to remove the NaOH solution. On the other hand, if only a few drops of NaOH solution are administered above the slides, this causes a non-uniform evaporation, leading to a non homogeneous treatment of the slices. In addition, the previous celloidin coating of the slides, which Jensen (1962) suggested, proved to be of little help because its main component, nitrocellulose (Sass 1958), does not resist the attack of either weak and strong alkalies (Kirk and Othmer 1946), thus leading to a total loss of almost all the slices.

The method suggested by Beatty *et al.* (1974), which modifies a procedure previously devised by Wilson (1961), according to which the sections are retained on slides by glass wool is, by their own admission, unsatisfactory after 17.5% NaOH extraction either because some glass wool remaining on the slides is mounted beneath the coverslip (Jensen 1962) or because the tissue adheres to the fiberglass cloth (Beatty *et al.* 1974). Consequently the third main result involved the use of ethyl cellulose whose degree of moisture-vapor permeability is comparable to that of nitrocellulose (Kirk and Othmer 1946) while its use requires less work and waste of staining solution than the glass wool method. Ethyl cellulose, which is fairly resistant to weak and strong alkalies (Kirk and Othmer 1946) proved to be successful during the successive removal of hemicellulose and non cellulosic polysaccharides, as described by Jensen (1962), with no losses of slices from the slide (see Figs. 3 and 4).

However it was found useful and safe to renew the ethyl cellulose coating after the first NaOH treatment and before the subsequent alkaline extraction, as some damage was produced even to the ethyl cellulosic film. Though, for sake of uniformity, ethyl cellulose was washed away before staining, no losses of tissue were observed throughout staining.

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Summary

For histochemical investigations of fruit pulp, before fixation tissue should be dehydrated in absolute alcohol at dry ice temperature (−78°C) to avoid cell shrinkage. Cell wall polysaccharides can be removed selectively without section losses provided the sections are mounted on slides coated with a solution made with 0.5 g gelatin, 0.2 g chrome alum and 100 ml H_2O. Pectic acids are removed by overnight pectinase treatment at 30°C, followed by 2 hrs in 0.5% aqueous ammonium oxalate at 90°C. Hemicellulose and noncellulosic polysaccharides are removed by 12 hr treatments respectively with 4% and 17.5% aqueous NaOH at room temperature, with slices kept affixed by a coating made up by dipping the slides into a 0.5% ethyl cellulose solution in a 4:1 mixture of toluene and absolute ethanol.

After the extraction the coating is dissolved away and periodic acid Schiff staining is subsequently used to detect and evaluate remaining cell wall polysaccharides.

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