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LIGHT MICROSCOPY PREPARATION TECHNIQUES FOR STARCH AND LIPID CONTAINING SNACK FOODS

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Abstract

Many processed foods lack the structural integrity associated with biological tissue so that the conventional methods of preparation and staining used in light microscopy may introduce misleading artifacts.

Taking as examples of starch-based processed foods, potato chips (UK potato crisps) and three distinct potato snack foods, methods for preparing and demonstrating the constituents present in cryosections of whole and masticated products are discussed. To show constituents in their true relative locations vapor staining and polarized light are used. Iodine vapor staining indicates the extent of starch gelatinization in the dry snack and it is also used to show the structural changes that occur on mastication. Osmium tetroxide vapor colours the liquid fats and polarized light indicates the presence of crystalline fats and intact starch granules.

Key Words: Light microscopy, potato chips, fabricated snack foods, cryosections, gelatinized starch, lipids, iodine vapor staining, osmium tetroxide vapor staining, birefringence.

Introduction

All microscopy is subject to artifacts and for one to have confidence in the validity of the results it is important to recognize the artifacts associated with a chosen technique and to minimize or avoid them.

In light microscopy preparations must be sufficiently thin to transmit light and if the relative position of constituents is important then sections must be cut. For this the modern cryostat has many advantages (Bancroft, 1975). Fixed and unfixed material can be sectioned and embedding can often be avoided because many foods are moist and when frozen this moisture acts as the support needed for sectioning. Dry foods present a challenge because the unrestricted addition of water may greatly alter the very structure which is being examined. Fortunately dry foods do not have to be fully hydrated to permit sectioning. Provided that the sample size is small proprietary aqueous embedding media can be used to permeate the specimen sufficiently to give support without causing obvious swelling.

Staining presents the next opportunity to introduce artifacts. Aqueous iodine-potassium iodide solution is the classic method for demonstrating starch but many foods contain starch in a swollen gelatinized state and the addition of water further disrupts already fragile starch granules. Even intact starch granules may become detached from the slide during aqueous staining unless they happen to be firmly attached to the slide or surrounding material.

Although the iodide ion is necessary for iodine staining of starch because it forms an essential component of the starch-iodine complex, added iodide ions are not essential because iodine in the presence of water provides sufficient of these (Holló and Széitli, 1968). Clearly some moisture must be added to allow staining of the starch in otherwise dry preparations but the amount needed is small. This is the basis of iodine vapour staining (Little, 1957).

For the lipid staining the standard technique of using alcoholic solutions of oil soluble dyes such as Oil Red O or the more sensitive Sudan Black B are often satisfactory but they carry the risk of dissolving small droplets of lipid and of displacing low melting point lipids.

Aqueous osmium tetroxide avoids some of these problems but if the sections involved are sensitive to water then this method too is unsuitable. Osmium tetroxide is widely used as a fixative and users are aware of the hazardous nature of this toxic material which is exacerbated by its volatility. This volatility suggested the use of osmium tetroxide as a vapor stain for fat.

Complementary to vapor staining is the use of polarization...
microscopy. Its value in observing starch based snack foods lies in its ability to demonstrate such ordered structures as intact starch granules, crystalline fats and cellulose derived from potato or cereal grain structures.

Materials and Methods

Reference materials: raw potato tissue sampled from the interior of a maincrop potato (variety, Majestic) commercial potato flakes prepared by a drum drying process. The reference materials are included to illustrate the response of potato starch to cooking. Starch derived from raw potatoes and potato flakes are primary ingredients for snack manufacture.

Potato-based snack products: four distinctly different commercial snacks were examined:
1. A standard potato chip.
2. A stacking chip based on potato solids and cereal starch.
   This was similar in appearance to the potato chip but the moulding of the snack during manufacture produces uniformly saddleshaped items that stack together.
3. Product A: a puffed snack based on potato solids and cereal starch, initially cooked under pressure and extruded as a rounded pellet which is dried and later puffed by frying.
4. Product B: a puffed product based on potato starch and potato granules extrusion cooked and directly fried. Product B had the appearance of short lengths of spaghetti.

Section preparation.
Potato tissue was sectioned by hand using a single edged razor blade. All other materials were sectioned at 10μm using a Slee retracting microtome in a Bright cryostat at −20°C. The sections were collected on clean microscope slides. Prior to sectioning one of three procedures was used:

a) Potato flakes were broken into pieces about 2 mm in diameter and the pieces were mixed with Tissue-Tek OCT embedding medium and placed directly on the cryostat chuck (specimen holder).

b) Each snack product was broken into pieces 2-5 mm in diameter which were placed in a perforated stainless steel cage. The cage was placed in a beaker, covered in Tissue-Tek and evacuated slowly in a vacuum oven (without heat) to remove air trapped within the product's structure. When no more air bubbles were seen to escape the vacuum was slowly released. Individual fragments were then mounted on cryostat chucks.

c) Each snack product was masticated until thoroughly softened and a sample (ca 5 mm diameter) of the resulting pulp (masticate) was mounted on a cryostat chuck and covered with Tissue-Tek.

After procedures a), b) or c) the mounted specimens were quickly frozen in liquid nitrogen and then transferred to the cryostat. They were left to warm to cryostat temperature (−20°C) before sectioning.

Storage of sections prior to staining.
The slides holding the 10μm sections were stored at −20°C. This preserves the fat present in the sections and it ensures that when the slides are brought to ambient temperature a film of moisture will be present to aid iodine vapor staining.

Methods of staining

1. Demonstration of starch using iodine vapor.
The method is based on that used by Little (1957). An environment of saturated iodine vapor was produced in a small covered staining dish by placing in it a small watch glass containing finely ground elemental iodine, covering the dish and leaving for 5 min. (Caution! iodine is poisonous, do not inhale the vapor).

Individual slides were taken from the −20°C deep freeze cabinet and a film of moisture was allowed to form on each slide before quickly placing it in the covered iodine vapor dish. The slide was positioned so that the section could be observed from the outside. Optimum staining took about 1 min. Excess staining was avoided as this obliterated structural detail.

Mounting iodine stained sections.
A semi-permanent mounting medium based on Hinchman's mountant, (Hinchman, 1973) was used. The medium was prepared by warming 80 ml of a low viscosity corn syrup (Morsweet supplied by CPC United Kingdom) with 14 ml distilled water and 6 ml Lugol's iodine in 100 ml water. This mountant was found to preserve iodine staining for at least 6 weeks.

2. Demonstration of lipid material using osmium tetroxide vapor.
Osmium tetroxide is a toxic material its volatile nature making it especially hazardous. All the staining was done in a fume cupboard and gloves and goggles were worn when handling the 2% OsO₄ solution. A covered slide staining dish was used the base of which was covered with filter paper and two lengths of glass rod (5 mm diameter) were positioned on the base so that the slides would not touch the paper. A 5 ml vial of 2% OsO₄ solution was poured onto the filter paper, a slide was then positioned in the grooves of the staining dish and the lid was replaced. Sections were allowed to stain for 20 min.

Mounting osmium stained slides
Aquamount, a commercial aqueous mountant obtained from BDH Chemicals Ltd., Poole, Dorset, England, was used as a temporary mountant. The mounts were photographed immediately because the osmium stained oil was still mobile.

Polarization microscopy
Stained and unstained sections were examined for birefringence. Glycerol or Aquamount were used as mountants for unstained sections.

Results

Reference materials (Figs 1 and 2)
A comparison of these iodine stained sections shows the effect of heat processing on the starch present in potato cells. With the raw potato tissue (Fig. 1) many of the starch granules were lost during sectioning when individual cells were cut but the appearance of raw starch and the way it fills intact potato cells can be seen in two of the cells. The cryostat section of the potato flake shows how well the cooked potato cells retain their gelatinized starch during the processing of the flake. Although the starch loses birefringence during cooking the outlines of individual granules can still be seen.

Potato-based snacks (Figs 3-14)
Potato chip (Figs 3-6). In its whole state the potato chip was seen to be composed of mainly intact potato cells each filled with gelatinized starch. As with the potato flake, iodine vapor staining demonstrates individual swollen starch granules within each potato cell (Fig. 3). Osmium tetroxide vapor reveals the extent of fat penetration (Fig. 4), liquid fat is closely associated with cell walls and inter cellular spaces. Fig. 5 shows the presence of crystalline fat revealed by polarized light and the birefringence of the potato cell walls. The masticated chip sections when stained with iodine vapor show blocks of potato cells which have sheared away from one another during mastication. These cells still retain gelatinized starch which stains strongly.
Figs 1 and 2. Reference materials stained with iodine:
Fig. 1 — potato tissue, intact cells (arrows) contain raw starch granules.
Fig. 2 — potato flake, cooked potato cells filled with gelatinized starch.

Figs 3-6. Potato chip:
Fig. 3 — iodine stained, gelatinized starch granules visible within cells.
Fig. 4 — OsO₄ stained, oil associated with cell walls (arrows) and intercellular oil (i).
Fig. 5 — unstained in polarized light, birefringent cell walls (arrows) and crystalline fat (f).
Fig. 6 — masticated chip, iodine stained, intact cells (i) starchy matrix (sm) and oil globules (arrows).
Figs 7-12. Potato-based snacks, iodine vapor stained:

Fig. 7 — stacking chip, starchy matrix (sm) entrains potato cells (p) and air cells (a).

Fig. 8 — stacking chip masticate viewed in polarized light, starchy matrix (sm) potato cells (p) and fat (f). Arrows show fat coating potato cells and dispersed in matrix.

Fig. 9 — product A starchy matrix of gelatinized granules (sm) contains cereal particles (cp) and air cells (a).

Fig. 10 — product A masticate, single starchy phase contains small voids (v) and some oil (arrows).

Fig. 11 — product B starchy matrix (sm) contains air cells (a) fat (f) and potato starch (arrows).

Fig. 12 — product B polarized light shows birefringent fat (f) and potato starch (arrows).
Potato-based snack foods

The methods described here to show the microstructure of potato snack foods could be applied to other starch and lipid containing foods. The value of iodine vapor staining is that it provides a sensitive test for assessing the extent of starch gelatinization without causing swelling. Osmium tetroxide vapor is especially useful for locating low melting point lipids when the use of lipid soluble colorants applied in solvent solutions would lead to movement and loss of the lipids.

However, the loss and movement of lipid material does not always accompany the use of oil soluble dyes. If a comparison of sections colored with an oil soluble dye such as Oil Red O or Sudan Black B shows similar results to sections stained with osmium tetroxide vapor then the oil colorant is to be preferred because of its greater safety in use.

The methods are intended to supplement rather than replace existing techniques e.g. in many instances a dilute solution of iodine in potassium iodide (the standard method for demonstrating starch) is quite satisfactory and the solution provides its own mountant which is convenient. However, if polarized light shows that the starch present has lost birefringence and is therefore susceptible to swelling in aqueous solutions, then the iodine vapor staining method and the Hinchman mountant are recommended.

Polarization microscopy provides an excellent means of discerning structure without introducing staining artifacts. This study shows it in use to demonstrate starch, cellulose and solid fats but it can also reveal other crystalline food constituents such as sucrose, lactose and calcium carbonate and oxalate.

Acknowledgements

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References


**Discussion with Reviewers**

**Reviewer IV:** What artifacts are possibly present due to tempering the sample at $-20^\circ C$ prior to cryosectioning?

**Author:** Tempering i.e. storage prolonged until an equilibrium of ice crystal formation is reached would almost certainly damage the specimen. Good cryostat technique involves very rapid freezing of a small specimen which is then brought to cryostat temperature and promptly sectioned. Liquid nitrogen (boiling point $-190^\circ C$) provides rapid freezing but there would be considerable damage to both knife and specimen if an attempt were made to section the specimen at temperatures much below $-30^\circ C$ (Bancroft 1975). It is therefore important to allow the block to warm to sectioning temperature which takes about 10 minutes.

When material has been damaged by freezing (ice crystal artifact) the block is difficult to section and the sections show an uneven or torn appearance.

**Reviewer IV:** What artifacts may be induced by vacuum degassing prior to sectioning?

**Author:** A sudden application of reduced pressure could cause damage to fragile aerated samples. It is important to protect the specimen by enclosing it in a metal cassette and to evacuate slowly. A vacuum oven is useful because it carries a vacuum gauge so that the vacuum can be applied and later released in known easy stages.

The aim is to replace most of the air in the specimen with embedding medium and so ease subsequent sectioning but not all the air is removed. This can be demonstrated by very prolonged iodine vapor staining of the sections which show the extent of Tissue-Tek penetration which is colored yellow by the iodine. (With normal staining times the Tissue-Tek is barely visible.)

**Reviewer IV:** Why are air cells present in some structures after vacuum degassing?

**Author:** The air cells seen in the sections are an intrinsic feature of the fabricated snacks. These air cells can be seen if the products are broken and observed with a stereomicroscope using oblique illumination and focussing up and down.

**Reviewer IV:** What moisture content is present in these samples?

**Author:** The dry snacks contain only 1-2% of moisture, it is difficult to estimate the moisture content of the embedded material. The softness corresponds to a dry snack containing 10% additional moisture but the presence of the infiltrated Tissue-Tek makes comparison difficult.

**Reviewer V:** Why was crystalline osmium not used for more effective permeation of lipid components?

**Author:** The amount of osmium tetroxide required for vapor staining is quite small. The 5 ml ampoules of 2% osmium tetroxide which were used contain only 100 mg of osmium tetroxide. This amount of the solid would need careful weighing and would involve more handling of this very toxic material. It is therefore for reasons of safety that a solution was used. In laboratories used to handling osmium tetroxide the aqueous solution could of course be replaced by an equivalent amount of the crystalline chemical.

**Reviewer V:** Can you please discuss the implications of birefringence being induced in fat globules due to osmium complexing with lipids?

**Author:** In my experience with food lipids I have never observed osmium induced birefringence in fat globules. Fat globules often display birefringence but this is due to the unstained crystalline fat they contain and can be observed in unstained sections. Osmium staining will of course aid in the location of fat droplets and with partially crossed polars it will enhance the appearance of any birefringent material present by adding contrast to the image but this is not induced birefringence.

**Reviewer V:** Glass knives are routinely used for ultra low temperature microtoming. Have these been used on your samples? Does the level of magnification and/or resolution using the light microscope eliminate the problem of ice crystal damage that may be inherent in the preparation techniques now being used (with steel blade, ice crystal damage is certainly present at the ultra structural level)?

**Author:** Steel knives were used through this work. Provided that the specimen has been correctly frozen, ice artifacts are not a problem. This may be for the reasons you suggest but I believe that section thickness is an important factor. For much work in light microscopy 10μm sections are used, a thickness which is greatly in excess of the size of the ice crystals present. With ultra thin sections the way the knife deals with individual ice crystals is likely to be more critical.