ANAEROBIC COLUMN CHROMATOGRAPHY OF NON-OXIDIZED COMPONENTS OF PLANT TISSUE EXTRACTS

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SUMMARY

A new method of anaerobic chromatography of plant tissue extracts is described which employs liquid nitrogen grinding of tissues. Nitrogen gas is used to remove free oxygen from the frozen, ground powders. After thawing the tissue is subjected to anaerobic centrifugation and then the clarified sap is applied to cellulose columns and chromatographed with buffer solutions under nitrogen gas pressure. The anaerobic method is compared with the conventional aerobic method using extracts from healthy Turkish tobacco leaves. Differences in the elution of proteins and nucleic acids are noted. The application of the method to tissues infected with tobacco mosaic virus is also described.

INTRODUCTION

A cellulose cation exchanging adsorbent column has been used to separate tobacco mosaic virus from other constituents in tobacco plant extracts[1]. In further experiments using either cation or anion exchanging cellulose adsorbents in studies of plant tissue extracts, a number of highly colored substances were eluted. Dark brown zones remained on the columns which could only be removed with concentrated sodium hydroxide-sodium chloride solutions. Even this treatment failed to remove certain darkly-colored adsorbed materials. These colored materials were assumed to be products of the browning reactions that occur, almost immediately, when living plant cells are disrupted. Their formation is presumed to be the result of the action of oxidative enzyme systems on protoplasmic constituents. These oxidative changes must be prevented if the components of living cells are to be isolated and studied in as near their natural state as possible.

An anaerobic method for the preparation of leaf extracts in an atmosphere of nearly pure nitrogen has been described[2]. Cytoplasmic proteins prepared in this way are colorless in solution. This method was not adopted for the preparation of materials for chromatography because of the limitations and inconveniences imposed by the necessity of undertaking the operations within the limited space of a nitrogen box. Instead, methods were developed for the use of laboratory equipment in normal atmospheres.

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EXPERIMENTAL METHODS

Liquid nitrogen grinding of tissue

A grinding method was devised in which liquid nitrogen was used as a substitute for water. An Omnimixer was modified for liquid nitrogen grinding by replacing the conventional lid of the 200-ml bowl with another machined from aluminum, giving 0.025 inch of looseness of fit in the lid threads. The lid was perforated with 16 3/8-inch diameter holes to permit the rapid escape of gaseous nitrogen during the grinding operation. A filter paper disc was cut to fit inside the lid to pass the gaseous nitrogen, while retaining the grinding plant material and the liquid nitrogen.

A 10-inch long stainless steel shaft and shaft collar were inserted between the grinding chamber and the driving motor to give thermal isolation between these units. Ten to fifty g of fresh leaf tissue were placed in the mixer bowl along with buffering chemicals. Liquid nitrogen was then added and the grinding unit was assembled. Ten to twenty sec of grinding were usually sufficient to yield a fine preparation. The inexpensive medium grade of liquid nitrogen (10 % oxygen) gave excellent results. The use of liquid nitrogen for grinding has several advantages. (1) All of the advantages of grinding with a liquid such as water are retained, yet there is no dilution of the sample because of the high volatility of the liquid nitrogen. (2) Undesirable chemical reactions during the grinding and immediately subsequent operations are avoided because of the extremely low temperatures (—193°C). All free water is frozen so that it cannot participate in chemical reactions. (3) The extreme brittleness of the tissue is conducive to rapid fine grinding. (4) If desired, water or buffer solution can be added to the liquid nitrogen and ground in a frozen state with the plant tissue.

Removal of free oxygen

Free oxygen must be removed from the frozen ground powders while they are still near liquid nitrogen temperatures. Several methods of deoxygenation by scrubbing with 99.996 % nitrogen gas were devised. A method of scrubbing the frozen powders in capped centrifuge tubes was finally adopted as a standard procedure because it provided the simplest way of preparing anaerobic clarified and concentrated extracts that could be transferred directly to chromatographic columns.

The metal cap assemblies of Model E Spinco preparative rotor D tubes were specially modified. The upper part of the cap was replaced with a similar unit machined from brass and fitted with two nipples spaced 180° apart providing exterior hose attachments and extending halfway through the rubber gaskets below. Two holes were drilled through the rubber gaskets corresponding to the position of the nipples above. Four 1/8-inch diameter holes were drilled through the basal part of the aluminum centrifuge cap assembly, spaced 90° apart, and located the same distance from the center of the cap as the nipples. A piece of 1/8-inch outer diameter aluminum tubing was fitted in one of these holes and was long enough to reach to the bottom of the plastic tube when the cap was in position. These modifications are illustrated in Fig. 1.

The frozen ground plant material was packed into the plastic tubes while it was still in a liquid-nitrogen-paste condition. The cap was assembled and tightened with one of the nipples positioned over the aluminum tube. Pure nitrogen gas was then

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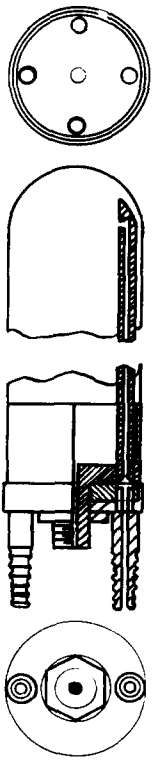

Fig. 1. Modification of centrifuge tube assembly for the removal of oxygen from liquid-nitrogen-ground plant powders by scrubbing with pure nitrogen gas.

passed in through the nipple and the aluminum tube to the bottom of the centrifuge tube. The gas traveled upward through the frozen ground powder to escape from the outlet nipple. If this scrubbing operation were done in a cold room at 3 to 5°, gas could be passed through the tube for 20 to 40 min before the ground material thaws. A periodic shaking and tapping of the tube assemblies during this scrubbing operation prevents a channeling of the gas flow. Scrubbing was stopped when the ground material had thawed. The nipple-bearing cap-unit with rubber gasket was rotated 45° and locked by tightening the rubber gasket-compressing nut to seal the tube against atmospheric oxygen. The tubes were then ready for centrifuging.

Low speed centrifuge clarification

The Spinco Model E centrifuge could not be used because of the protruding nipples on the caps. Instead, the tubes were centrifuged in the 823A rotor of a refrigerated International centrifuge. The tubes were balanced and cushioned in the centrifuge cup assemblies by the addition of water and were positioned in the rotor so that the internal aluminum tubes would rest against the closest plastic tube wall when under centrifugal force. After centrifuging at 1° for 30 min at 2900 × *g*, Turkish tobacco leaf material had a straw-colored supernatent lying above bright green sedimented material. Any failure in the oxygen removal was evidenced at this time by a dark brown color in the supernatant liquid.

Preparation of chromatographic columns

Chromatographic columns packed with cellulose adsorbents having cation or anion exchanging surfaces[3,4], may be used for separation of components of anaerobic plant extracts.

The columns were packed with care to avoid trapping of air bubbles and to attain uniform structure and level top surfaces. They were then preconditioned by the passage of the initial, oxygen-free buffer for about 24 h before the application of the sample. When anion exchanging adsorbents were used the pH of the initial buffer was higher than the values of the isoelectric points of proteins that were to be separated. In experiments with extracts from Turkish tobacco plants 0.02 *M* dibasic sodium phosphate with 0.001 *M* ethylenedamine tetraacetic acid (EDTA) was used as the initial buffer. This had a pH of 7.7. All of the solvents used for the development of the columns were deoxygenated by bubbling pure nitrogen gas through them. They were applied to the column by means of nitrogen gas pressure. All of the air in mixing flasks and in the upper part of the chromatographic tube was displaced by pure nitrogen gas 24 h before the application of the sample.

Application of clarified anaerobic sap

The clarified supernatant from the anaerobic centrifuge run was applied to the

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column by means of nitrogen gas pressure. The nipples on the centrifuge cap were connected in series in a nitrogen line leading to the top of the chromatographic tube assembly. After passage of pure nitrogen gas through the assembly for 2 or 3 min to remove any oxygen admitted while making the connections, the centrifuge tubes were tilted upward to force the supernatant fluid into the chromatographic tube. The supernatant was forced in slowly to prevent any disturbance of the packed upper surface of the adsorbent column. Nitrogen gas pressure was used to drive the sample on the column and then to apply the developing solvents. Columns were usually developed with buffers having changing pH and salt values[4].

Chromatographic procedure

The elution procedure finally adopted for diethylaminoethyl (DEAE) cellulose anion exchanging adsorbents[3] was to start with 1 l of pH 7.7 phosphate–EDTA buffer in a magnetically stirred mixing flask. The developing solvent was removed from the bottom of the flask with a siphon. A constant volume of 1 l of liquid was maintained in the mixing flask by adding oxygen-free acid buffer in a dropwise manner at the top of the flask. This buffer had a pH value of 2.95; it consisted of a 0.1 *M* phosphate, 0.5 *M* sodium chloride and 0.001 *M* EDTA.

The rate of elution was regulated by varying the nitrogen pressure. Pressures from 5 to 10 lbs/sq. in gave elution rates varying from 10 to 50 ml/h. Several components moved rapidly through the column. The first detected materials were eluted with the passage of 35–40 ml of developing solvent when the packed column had a diameter of 20 mm and a height of 130 mm. The elution of components could be followed visually by observing the column with visible and ultraviolet light.

The progressive elution of components was also indicated on the chart of a recording spectrophotometer. The absorption spectra from 200 mμ to 2 μ was recorded for each 10 ml fraction collected. Each fraction was also assayed for protein content by the Folin phenol procedure[5], for ribonucleic acid (RNA) content by the orcinol reaction[6] and for deoxyribonucleic acid (DNA) by the STUMPF reaction[7].

EXPERIMENTAL RESULTS

Aerobic versus anaerobic chromatography of healthy Turkish tobacco leaf tissue extracts

Similar lots of healthy Turkish tobacco leaf tissue were chromatographed under aerobic and anaerobic conditions using identical elution procedures.

Aerobic chromatography

Under aerobic conditions no bands were visible in ultraviolet light and the recording spectrophotometer failed to detect any characteristic absorption spectra that might be identified with eluted components. The assays for RNA and DNA gave only slight indications of nucleic acids which were too low to be plotted. The Folin protein determinations are recorded in Fig. 2. It was evident that protein had been eluted by the aerobic procedure, but the eluted protein could not be characterized with the spectrophotometer and appeared to contain no nucleic acids.

Anaerobic chromatography

Under anaerobic conditions chromatographic bands visible in ultraviolet light

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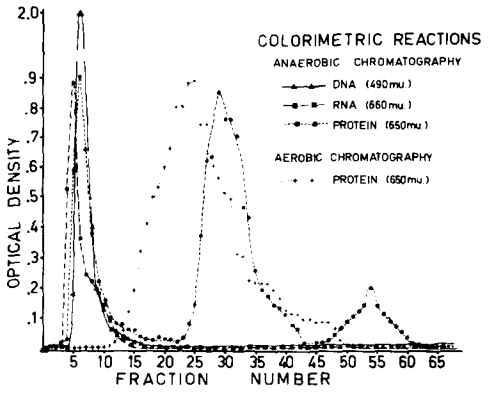

Fig. 2. Colorimetric assay of aerobic and anaerobic chromatographic fractionation of healthy Turkish tobacco tissue on a DEAE anion-exchanging cellulose adsorbent. DNA was estimated by the STUMPF reaction, RNA was estimated by the orcinol reaction, and protein was established by the Folin phenol reaction.

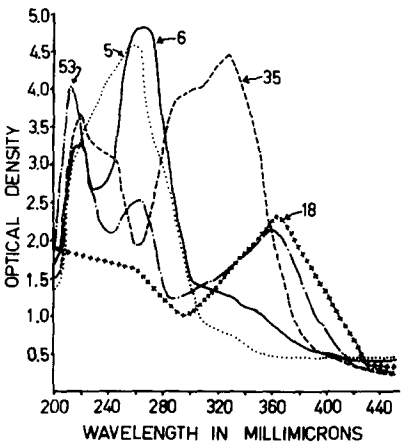

Fig. 3. U.v.-absorption spectra of five fractions isolated from healthy Turkish tobacco leaves by means of anaerobic chromatography on a diethylaminoethyl cellulose adsorbent.

could be seen moving down the column and could be correlated with the progressive rise and decline of characteristic absorption spectra on the spectrophotometer recording. The assay for protein with the Folin reagent (Fig. 2) indicated that protein had been eluted in three bands.

Component 1. The first band, collected in fractions 4 to 13, was designated as Component 1 but was recognized to be a mixture of more than one eluted substance. The colorimetric assays showed a maximum RNA concentration in fraction 5, a maximum DNA concentration in fraction 6, and a maximum protein concentration in fraction 6. The spectrophotometric absorption curves of fractions 5 and 6 were also quite different; that of 5 being more typical of nucleic acid and that of 6 more like a nucleoprotein. The ultraviolet curves are given in Fig. 3 and the near infrared curves are shown in Fig. 4. Because both ultraviolet curves have absorption maxima at 260 mμ, this wavelength was chosen for a density plot of Component 1 in Fig. 5.

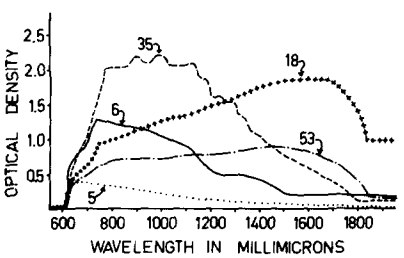

Fig. 4. Near infrared adsorption spectra of five fractions isolated from healthy Turkish tobacco leaves by means of anaerobic chromatography on a diethylaminoethyl cellulose adsorbent.

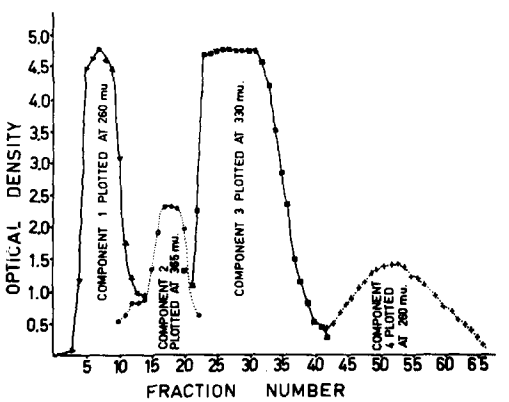

Fig. 5. Elution of certain components from healthy Turkish tobacco leaf tissue chromatographed under anaerobic conditions on a diethylaminoethyl cellulose adsorbent as determined by recorded densities at the absorption maxima of the eluting components.

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Component 2. The low level of protein in fractions 13 to 23 (Fig. 2) suggested that something other than protein was being eluted. The spectrophotometer record showed that a component having absorption maxima at 365 mμ in the ultraviolet and at 1650 in the near infrared was eluted in these fractions. The characteristic absorption spectra of this substance (fraction 18) are shown in Figs. 3 and 4. This substance was designated as Component 2 and was plotted at its absorption maximum of 365 mμ in Fig. 5.

Component 3. The second eluting protein band was shown to be carried in fractions 24 to 40 by the Folin test (Fig. 2). The characteristic absorption spectra of this protein are shown in Figs. 3 and 4 where fraction 35 was chosen as a representative example. In the u.v., absorption maxima occur at 220 and 330 mμ with a pronounced minimum at 260 to 265 mμ. This protein appears to fluoresce a greenish-yellow color when viewed with u.v. light. The minimum at 260 to 265 may be the result of fluorescence at these wavelengths. The near infrared spectrum is also interesting because a number of maxima appear to exist in the 750- to 1400-mμ range. This was the most abundant protein isolated from the leaf tissue. It was plotted as Component 3 in Fig. 5 at its absorption maxima of 330 mμ. The flat-topped curve indicates that the limit of response was reached by the spectrophotometer in recording the elution of Component 3.

Component 4. Components 1, 2, and 3 were eluted from DEAE cellulose with pH 7.7 buffer, but Component 4 (the third protein band) was not eluted by this buffer. Component 4 was eluted only when buffers had values of pH 6.5 and lower. This third eluting protein band was shown to be carried in fractions 46 to 63 by the Folin test (Fig. 2). The characteristic absorption spectra of this protein are shown in Figs. 3 and 4 where fraction 53 was chosen as a representative example. In the u.v. absorption maxima occur at 212, 260–265, and 360 mμ. The absorption is general throughout the near infrared with a maximum at about 1500 mμ. This protein is readily identified by its characteristic triple absorption maxima in the ultraviolet. It was plotted in Fig. 5 at its 260 mμ maximum and was designated as Component 4.

An analysis of elution as revealed by spectrophotometric data, presented in Fig. 5, showed that 4 major components could be distinguished in the first 65 fractions.

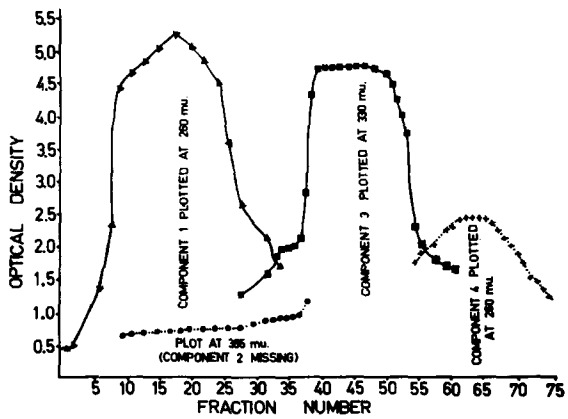

Fig. 6. Elution of certain components from tobacco mosaic virus-infected Turkish tobacco leaf tissue chromatographed under anaerobic conditions on a diethylaminoethyl cellulose adsorbent as determined by recorded densities at the absorption maxima of the eluting components.

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Three of these, components 1, 3, and 4, appeared to be identical with the bands detected by the Folin protein test (Fig. 2). Component 2 did not react with the protein, RNA and DNA reagents.

Anaerobic chromatography of extracts of virus infected tobacco leaf tissue

Identical anaerobic methods were used for the chromatography of Turkish tobacco leaves taken from plants systemically infected with tobacco mosaic virus. In two experiments Components 1, 3, and 4 could be identified by their chromatographic behavior and by their characteristic absorption spectra. Component 2 appeared to be missing (Fig. 6). Under the conditions of these experiments, some of the virus (as determined by infectivity tests) appeared to be eluted with Component 1. Other virus was adsorbed on the cellulose and was only eluted much later in the elution schedule (following Component 4) at lower pH and higher salt values.

DISCUSSION

The anaerobic techniques described in this paper successfully prevented any visible oxidation (browning) of the plant tissue extracts prepared for chromatography. The prevention of oxidation during the preparative stages and during chromatography was further verified by the elution of distinct bands of protein and nucleic acid from tobacco leaf tissue extracts under anaerobic but not under aerobic conditions. Apparently the avoidance of oxidative reactions permits more host components to retain their physical and chemical integrity so that they can be differentially eluted from ion-exchange columns.

In these experiments the anaerobic chromatography separated the reacting components of the oxidative enzyme systems. The eluted, isolated components appeared to be rather stable in the presence of oxygen.

The methods described permit the use of laboratory equipment in normal atmospheres. Thus, the limitations and inconveniences of working within the limited space of a nitrogen box are avoided.

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