TOBACCO CURING

Protein Changes During Curing of Burley Tobacco, and Pectin Methylesterase of Cured Leaf

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Ultracentrifugal studies of the soluble proteins of burley tobacco leaves indicate that the rapidly sedimenting, high molecular weight component disappears quickly during curing, whereas the remaining nondialyzable fraction, which includes pectin methylesterase, remains relatively unchanged. The pectin methylesterase found in cured burley tobacco was also studied. A sensitive assay for this enzyme is described, based upon colorimetric microdetermination of the liberated methanol.

The POSTHARVESTING CHANGES in tobacco leaves which are important from the standpoint of smoking quality include changes in physical structure, development of aroma and flavor, and elimination or conversion of compounds that give unpleasant taste. The leaf proteins are generally believed to contain substances which are detrimental to smoke taste and it is known that they are partially degraded during curing. In order to understand better the chemical processes that occur during the curing and aging of tobacco, the proteins and enzymes present in the leaf tissue have been studied. Further knowledge of the nature of tobacco leaf proteins and the mechanism of post-harvesting transformations would find wide application in the cultivation, selection, and processing of cigarette types. Previous reports by Vickery and others of the nitrogen fractions and enzymatic activities in the cured leaf and proteolysis during curing have been thoroughly summarized by Frankenburg (*5, 6*).

Studies by Wildman and coworkers (*13, 15*) have shown that two major protein fractions are present in green tobacco leaves. Fraction I is characterized by a sedimentation constant of about 18 Svedberg units (18 S) and is fairly homogeneous, as judged by both electrophoresis and ultracentrifugation. Fraction II is characterized by a sedimentation constant of approximately 4 S and is definitely heterogeneous in composition. No physiological function has been clearly associated with Fraction I, but considerable enzymatic activity has been found in Fraction II (*15*).

Axelrod and Jagendorf (*2*) have shown that, during leaf autolysis, the soluble protein content decreases sharply, whereas phosphatase, invertase, and peroxidase activities remain relatively constant, the net result being a large increase in the specific activities of the enzymes. Enzymatic activity has been reported in flue-cured bright tobacco as well as in air-cured types (*4, 7, 11*). Recently, Barrett (*3*) found marked differences in enzyme activity of bright and burley, an air-cured type.

Investigations of changes in protein fractions during curing are discussed here, and evidence is presented that the rapidly sedimenting, high-molecular-weight protein component of leaf cytoplasm [Fraction I in the nomenclature of Singer, Eggman, Campbell, and Wildman (*13*)] is rapidly broken down during curing, whereas the remainder of the proteins, which includes many enzymes, is relatively more stable. The enzyme activity of pectin methylesterase in the protein fractions was followed by colorimetric measurement of the methanol liberated.

Methods

Ultracentrifugal studies were carried out in a Spinco Model E ultracentrifuge at 25° ± 1° C. All samples were kept refrigerated, except during ultracentrifugal analysis. The analytical runs were conducted routinely at 59,780 r.p.m.

Peak areas and areas at the meniscus were measured from enlarged tracings of the sedimentation patterns and recorded arbitrarily in units of square centimeters. The linear magnification from the centrifuge cell to tracings was approximately tenfold. The details of the procedure varied slightly, according to the nature of the pattern of each component of interest. To obtain Fraction I values, the centroidal ordinate of each peak was drawn and the area under the curve to the right of the ordinate was measured and doubled to obtain the total peak area. As a general rule, five exposures were made, and because, in the case of Fraction I, the peak areas appeared to be independent of sedimentation time, the results were averaged. In the case of Fraction II, the patterns were symmetrical, and the total peak areas were measured directly. These values generally decreased slightly with sedimentation time, and the recorded value for each run was obtained by extrapolating the results to the time at which the rotor reached maximum speed. "Peak" areas at the meniscus were determined by superimposing tracings from a run on the pure solvent (0.1M phosphate buffer, pH 8.0) over those for each solution and measuring the area encompassed by the two curves. The results were averaged as in the case of Fraction I.

Soluble protein was determined by adding 5 ml. of extract, after preparative centrifugation, to 5 ml. of 0.4N trichloroacetic acid in tared, 12-ml. centrifuge tubes, centrifuging after standing at least 30 minutes, washing the precipitate twice with water, and drying at 90° to 100° C. to constant weight. Total homogenate nitrogen was determined by the Kjeldahl method as modified to include nitrate nitrogen (*1*).

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Total particulate nitrogen was determined by micro-Kjeldahl digestion, followed by nesslerization.

Methanol Determination A quantitative colorimetric micromethod for methanol determination was developed for use in pectin methylesterase determinations based upon the procedure described by Holden (*8*). For accurate results with the small amounts of substance being determined, it was necessary to standardize exactly the times of addition of all reagents. Good agreement with Beer's law was obtained when the various steps were carried out at the time intervals described (Figure 1).

Procedure. The reagents described by Holden were employed (*8*). The phenylhydrazine hydrochloride was recrystallized from 83% ethanol.

The samples to be analyzed were placed in a volume of 5.0 ml. in individual colorimeter tubes. With each analysis, a reagent blank and a methanol standard—e.g., 1 μmole—were included. One milliliter of permanganate reagent was then added at timed intervals to each tube. After gentle swirling, the tubes were allowed to stand at room temperature for exactly 10 minutes. One milliliter of oxalic acid reagent was then added at timed intervals, the gentle swirling was repeated, and the tubes were placed in warm water (ca. 65° C.) until complete decolorization occurred.

At timed intervals, 2 ml. of phenylhydrazine reagent were added and the contents were mixed. Exactly 5 minutes later, 1 ml. of ferricyanide reagent was added, followed by immediate mixing. Exactly 1.5 minutes later, 3 ml. of concentrated hydrochloric acid were added, the contents were thoroughly mixed, and the color intensity was read immediately at 520 mμ against a water blank. It was convenient to develop and read the color at this stage in groups of three tubes. By a suitable time schedule, the tubes can be analyzed in a continuous fashion.

The reagent blank value was subtracted from the absorbance of each sample, and the amount of methanol present calculated from the absorbance of the standard.

The cherry-red color formed had one sharp peak at 520 mμ in the range 400 to 650 mμ. This color is dependent upon the formation of formaldehyde by permanganate oxidation and is specific for methanol and other formaldehyde-liberating substances under the conditions described. When compared on a molar basis (methanol = 100), the following relative amounts of color development were obtained: ethanol <0.1, 1-butanol <0.5, 1-hexanol <0.8, acetaldehyde <0.1, formic acid (not oxidized) <0.1, glucose <2.0, xylose <3.0. Acrolein produced a red color, but crotonaldehyde did not. Difficulties with interferences by sugars and any other nonvolatile formaldehyde- or methanol-liberating compounds were avoided by distillation of samples before methanol determination.

Pectin Methylesterase Determination Methanol could not be measured directly after incubation of pectin with tobacco extracts, for both pectin and substances present in the tobacco extracts interfered. It was therefore necessary to distill the samples before this determination. If the pH was too high or too low, pectin itself was broken down during the distillation to yield interfering substances, probably mainly methanol. Tris(hydroxymethyl)aminomethane was found to have buffering capacity around pH 3, and the volume of hydrochloric acid added was just sufficient to bring the pH to 3.2. By this procedure, only very small quantities of interfering compounds were liberated from pectin during the distillation. Only about 95% recovery was obtained in these distillations of small amounts of methanol (<2 μmole), but there was linearity between methanol added and recovered, and therefore by multiplying by a correction factor of 1.075, the amount present in the reaction mixture could be calculated.

Procedure. Citrus pectin (Eastman Kodak, practical) was purified by passage of a 1% solution successively through Amberlite IR 100 and IR 4 columns, followed by precipitation and washing with ethanol, acetone, and ether (*10*). The ash content was thus reduced from 2.5 to 0.8%.

Erlenmeyer flasks (25 ml.), containing 0.2 ml. of 2.0% pectin, 0.15 ml. of 0.2*M* magnesium sulfate, and sufficient 0.1*M* tris(hydroxymethyl)aminomethane buffer (pH 8.0) to make the total volume 2 ml. after the enzyme addition, were chilled in an ice bath and the chilled enzyme solution was added.

The stoppered flasks were then incubated for 10 to 20 minutes at 30° C. in a Dubnoff incubator shaker (40 cycles per minute shaking speed), removed from the bath, and placed in ice. The reaction was then stopped by immediate addition of 4 ml. of 0.02*N* hydrochloric acid and 2 ml. of water. Each flask was then connected to a 5-ml. volumetric pipet suitably bent for use as an air condenser, and 5 ml. of distillate were collected in calibrated colorimeter tubes placed in an ice bath. Methanol content was then determined. Blanks should always be run for both pectin and enzyme. Pectin methylesterase specific activity is expressed as micromoles of methanol liberated per hour per milligram of protein under standard assay conditions.

Linearity between methanol formation and enzyme concentration was found with the above procedure; the time course of this reaction was also linear (Figures 2 and 3).

Results

Protein Changes during Curing. The results of Wildman and coworkers (*13*) have been confirmed in general in experiments with extracts of green burley

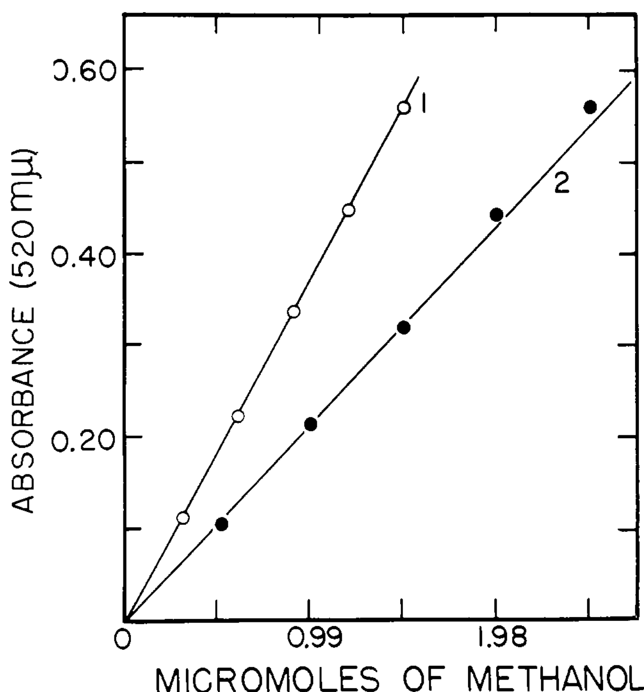

Figure 1. Effect of methanol concentration on color formation

Absorbances measured in 1.5 × 19 cm. colorimeter tubes in 1 and in 1-sq. cm. cuvettes in 2

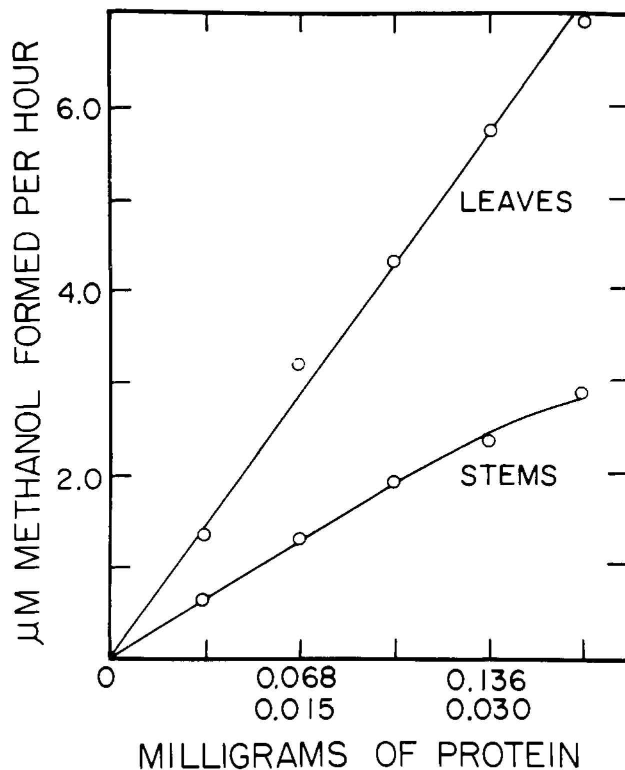

Figure 2. Effect of pectin methylesterase concentration on rate of methanol liberation from pectin

tobacco leaves. Ultracentrifugal analysis of extracts of cured tobacco leaves, however, revealed the complete absence of Fraction I, although appreciable amounts of Fraction II were present. This fact, coupled with preliminary findings that the pectin methylesterase specific activity of cured leaves was about twice that of green leaves, suggested a possible relationship between the increase in pectin methylesterase specific activity and the disappearance of Fraction I during curing.

To determine the rate of disappearance of Fraction I and its relation to the pectin methylesterase specific activity, a curing experiment was set up. Eight adjacent leaves from each of eight greenhouse tobacco plants (burley type, crossed with other species of *Nicotiana*, supplied by the Greeneville, Tenn. Experiment Station) were divided into eight samples, in which each plant and stalk position was proportionately represented as described in the statistical method of Vickery, Leavenworth, and Bliss (*14*). These samples were then air-cured in a closed room maintained at a temperature of 25–26° C. and at a relative humidity of 65 to 70%. After various time intervals the samples were frozen in liquid nitrogen, then homogenized for 3 minutes in cold 0.1M disodium hydrogen phosphate buffer (initially pH 8.0), filtered through cheesecloth, and centrifuged 30 minutes at 18,000 × g (relative centrifugal force) [separation into particulate and soluble (cytoplasmic) fractions]. The pH of the supernatant fluid from the first sample was 7.3.

These conditions were such that maximal pectin methylesterase activity was extracted. Extracts prepared with buffers of lower or high pH values contained less pectin methylesterase activity, but buffers of pH 8.5 and 9.0, contained more soluble protein. Various unbuffered solutions such as water, 0.88M sucrose, and 0.154M potassium chloride extracted less pectin methylesterase and soluble protein. The prepared extracts were run immediately to investigate the Fraction I content, and a portion of the remaining sample was dialyzed overnight against two portions of 0.1M disodium hydrogen phosphate buffer (pH 8.0) at 4° C. No significant changes in volume occurred during this process. A second run was made on the material freed of dialyzable components to sediment Fraction I, and the supernatant fluid from this was then analyzed in a synthetic boundary cell (*12*) against a portion of the dialysis buffer to investigate the content and sedimentation behavior of Fraction II (Figure 4).

Total homogenate nitrogen values were determined on the whole extracts after filtration through cheesecloth, and total particulate nitrogen on the material centrifugable at 18,000 × g. The soluble fractions were analyzed for pectin methylesterase activity and soluble protein. The results, summarized in part in Figure 5, show a rapid decrease of area on the sedimentation diagram of Fraction I and of soluble protein, with an accompanying increase in pectin methylesterase specific activity. The milligrams of particulate nitrogen per milligram of homogenate nitrogen varied considerably but did not increase, the ratios being 0.36, 0.24, 0.17, and 0.23 at 0, 4, 7, and 11 days, respectively. Pectin methylesterase activities (micromoles of methanol formed per milligram of total homog-

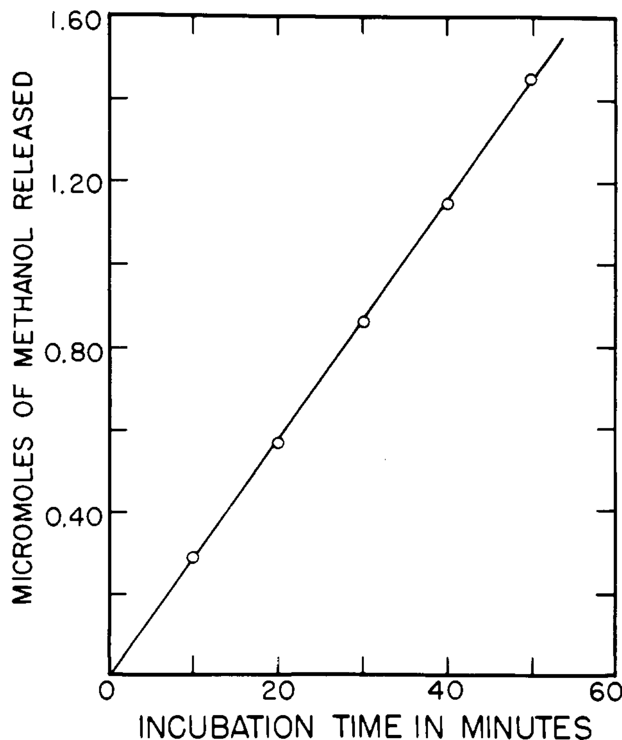

Figure 3. Time course of pectin methylesterase action

Substrate mixture and enzyme incubated separately for 5 minutes at 30° before being mixed. Final protein concentration, 0.045 mg. per ml.

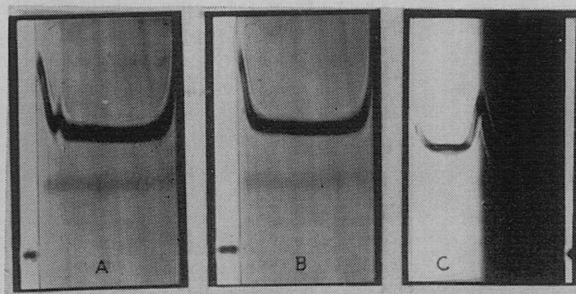

Figure 4. Sedimentation diagrams of cured burley tobacco extracts

Sedimentation from left to right. Thin vertical line at left in each frame is meniscus.

A. Small peak represents Fraction I and area at meniscus Fraction II. Sample prepared from tobacco cured 4 days and dialyzed against 0.1*M* Na_2HPO_4 (pH 8.0) prior to ultracentrifugal analysis. Photograph taken approximately 4 minutes after rotor reached full speed (59,780 r.p.m.) and at bar angle of 35°

B. Same conditions as *A*, shows absence of Fraction I after 14 days of curing

C. Fraction II. Obtained in synthetic boundary cell using supernatant fluid from run shown in *B* with portion of dialysis buffer as supernatant solvent. Operation conditions same as for *A* and *B*

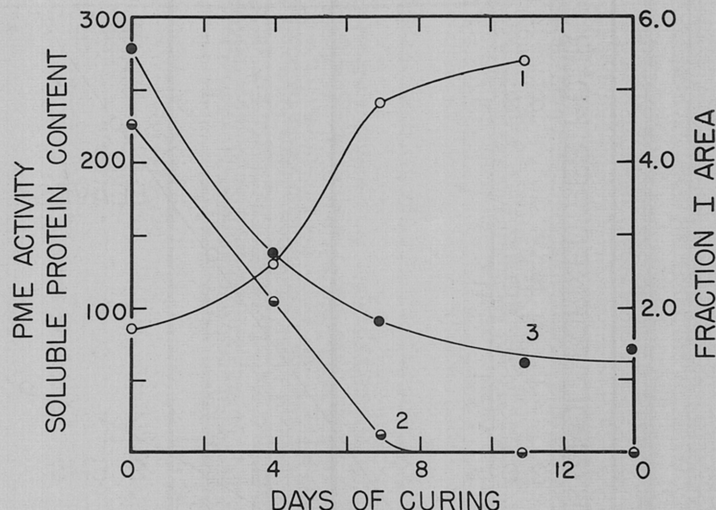

Figure 5. Effect of curing on pectin methylesterase, area of Fraction I, and soluble protein

1. PME specific activity
2. Sq. cm. of Fraction I per mg. of total homogenate N per ml.
3. Mg. of soluble protein per mg. of total homogenate N × 10^2

enate nitrogen) were 238, 175, 222, and 162 at 0, 4, 7, and 11 days, respectively.

The Fraction II concentration did not change appreciably during curing, as shown by the ratios of Fraction II area to total homogenate nitrogen (Table I). The "peak" areas at the meniscus, which represent base line elevations (solvent-corrected) attributable to the presence of both Fraction II and components of low molecular weight, diminished on dialysis during the period of decomposition of Fraction I, but leveled off after the disappearance of this fraction. This would point to the formation of dialyzable compounds of low molecular weight during curing.

The sedimentation constant of each fraction remained essentially constant during curing, the average value for Fraction I being 17.9 S and that for Fraction II, as determined with the synthetic boundary cell, being 1.9 S.

The authors were not able to demonstrate this decrease of Fraction I in experiments with extracts prepared from green burley leaves frozen in liquid nitrogen, lyophilized, and stored at −30° C. Incubation of such homogenates, after filtration through cheesecloth, at 30° C. and pH 6.7 for periods of time extending to 24 hours caused no significant decrease in the relative concentration of this component. The sedimentation constants of Fraction I in these extracts were also essentially the same as those found above.

Studies on Pectin Methylesterase. Pectin methylesterase specific activities with the cured burley leaves representing several grades and crops varied from 142 to 199 in the crude extracts of pH 8. Cured burley stems (leaf midribs) were also found to contain pectin methylesterase (see Figure 2). An extract of one sample of flue-cured bright tobacco leaves was found to have a pectin methylesterase specific activity of 27, which is indicative of the stability of this enzyme in the intact leaves when maintained at a temperature around 80° C. for 18 to 24 hours, as normally practiced in flue-curing.

The pH optimum of 8 reported by Holden (*9*) was confirmed. Pectin methylesterase was found in the supernatant fluid after 90 minutes' centrifugation at 18,000 × g. Analysis of an extract of green burley tobacco leaves after removal of Fraction I by ultracentrifugation showed that better than 95% of the enzyme activity was present in the unsedimented Fraction II.

Table I. Changes in Protein Fractions During Curing

Item[a]	Curing Time, Days 0	4	7	11	14
1. Leaf-buffer ratio, g./ml.	97/390	31/200	16.5/150	11/200	11/200
2. Soluble protein, mg. dry weight/ml.	0.88	0.98	0.76	0.44	0.54
3. Total homogenate N, mg. N/ml.	0.314	0.729	0.828	0.727	0.752
4. Fraction I area	4.5	2.1	<0.2	0	0
5. Fraction II area	7.0	[b]	6.0	4.1	6.4
6. Fraction II area/soluble protein	2.5	[b]	6.6	6.8	8.9
7. Area at meniscus (undialyzed)	7.3	[b]	7.6	6.3	7.7
8. Area at meniscus (dialyzed)	7.6	[b]	5.2	3.4	4.4
9. % decrease of meniscus area on dialysis	0		32	46	43

[a] Units of area are sq. cm. of peak in sedimentation diagram per mg. of nitrogen in 1 ml. of homogenate; in 6, sq. cm. per mg. of soluble protein in 1 ml. of homogenate.
[b] Dialyzed solution containing Fraction II accidentally lost.

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