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PEROXIDASE ACTIVITY DURING CURING AND STORAGE OF CIGAR-FILLER TOBACCO

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Abstract—During the usual air curing of cigar-filler tobacco the peroxidase activity decreases rapidly for 6 weeks and then becomes stabilized at a level approximately one-fifth the original. Detectable activity persists during storage for as long as 5 years. The presence of enzymatic activity demonstrates that protein is present in the fully cured and stored leaf.

INTRODUCTION

PENNSYLVANIA cigar-filler tobacco is air-cured on the stalk. After curing the leaves are removed from the stalk and stored under ambient conditions until subjected to fermentation. During this storage period, which may be as long as 5 years or more, changes take place within the leaf which appear to facilitate the fermentation process.

When tobacco is air-cured the initially green leaves show transitory increases in respiration and in some enzyme activities during the first 2 or 3 days.[1,2] For the next 12–15 days respiration steadily decreases until at the end of this time the cells are essentially dead.[3,4] Many of the changes in chemical composition which occur after this period are well documented.[3–6] The question is often raised as to the importance of enzymes in producing these changes.

Changes in enzymatic activity have been studied during both flue-curing[1,7–12] and air-curing[1,2] of tobacco. Several enzymes have been shown to be active throughout the entire curing period. After air-curing of burley tobacco, Barrett[1] showed that an oxidase, catalase, protease, invertase, cellulase, tyrosinase and peroxidase were still active. In contrast, after flue-curing, complete loss of enzymatic activity has been reported by several workers.[2,11] Spencer and Weston,[9] on the other hand, found amylase activity present after flue-curing. These conflicting results are probably due to variations in the temperature attained during the cure. Air-curing does not involve elevated temperatures and thus the enzymes are not as readily inactivated. The brown pigments so prevalent in air-cured tobacco are present to

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only a small extent in flue-cured. This is believed to be due to heat inactivation of the enzymes responsible for browning before the cells are autolyzed.[8] Autolysis of the cell allows these enzymes to mix with substrates normally not available to them in the intact cell.

In this study, Pennsylvania cigar-filler tobacco was examined for peroxidase activity during a full curing season. Samples of similarly cured leaves from previous crops, which had been held in storage without further processing, were also analyzed. Peroxidase was chosen because of its stability and ease of assay. In addition any fluctuation in microbial populations during the curing or storage periods would not be expected to be reflected in the peroxidase assay, since this enzyme is not normally considered to be of microbial origin. A change in activity would thus reflect a change within the leaf.

RESULTS AND DISCUSSION

The variation in peroxidase activity observed during the 1967 cure is shown in Fig. 1a. Only minor differences were detected between the three sets of leaves into which the stalk was divided and for this reason the results have been combined. As expected, there was a rapid

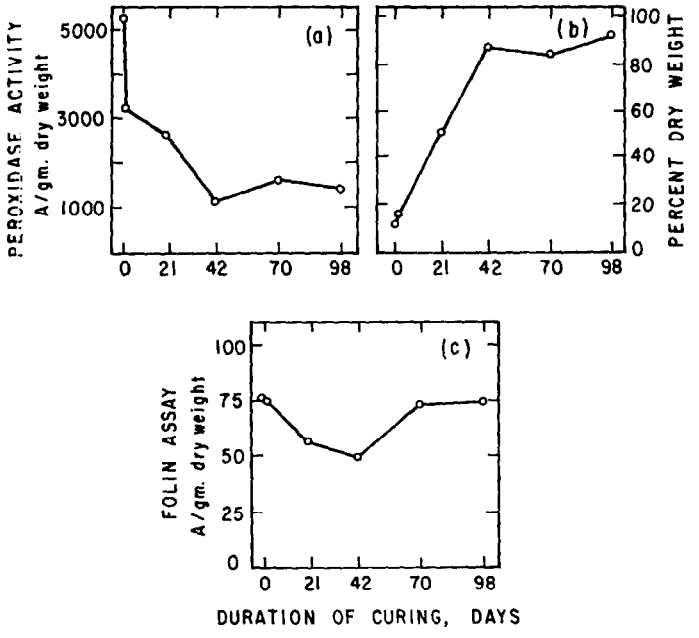

FIG. 1. BEHAVIOR OF PEROXIDASE, DRY WEIGHT AND FOLIN ASSAY DURING AIR-CURING.

loss of activity over the first few weeks of curing. After the sixth week the decrease lessened and the remaining activity appeared to level off. These changes correlated with the decrease in moisture content of the leaf (Fig. 1b). This supports the suggestion of Weston[9] that drying stabilizes the secondary structure of the enzyme, preserving its activity.

The results of the peroxidase assays on the cured samples are presented in Table 1. Measurable activity was found in the 1962 and later crops, indicating that this enzyme is particularly stable. A plot of the log of the activity vs. the crop year gave a straight line, indicating that

TABLE 1. PEROXIDASE ACTIVITY IN STORED CIGAR TOBACCO

Crop year	1967	1966	1965	1964	1963	1962	1961	1960
Activity*	1260	190	120	N.A.†	4·07	0·85	0·0	0·0

* Units of activity were absorbance per minute per g dry wt.
† N.A. = Sample of this crop not available.

the loss of activity follows first-order kinetics in much the same way as radioactive decay does. This loss of activity over a long period of time is likely due to physical factors, such as change in temperature and moisture content of the leaf.

Disc electrophoresis on polyacrylamide gel was performed on each of the samples obtained during the 1967 cure. The gels were stained for protein (amido black) and for peroxidase activity. The sample of green leaf contained the large number of protein bands usually obtained with this tissue. There were no visible protein bands present on the gel when an equivalent amount of fully cured leaf was prepared and run in the same way. The green tissue contained two major anionic bands of peroxidase activity. This pattern did not change during curing. (The staining procedure for peroxidase activity on the gel is considerably more sensitive than that used for protein.) Over the first year of storage, however, a qualitative change in the pattern was found. One of the two anionic bands was no longer present in the 1966 and earlier crops. A procedure for double disc electrophoresis, developed by Racusen,[13] revealed three anionic and three cationic bands of peroxidase in the fully cured 1967 crop.

Protein determinations were performed by the Lowry method[14] on each of the samples taken during curing. These data (Fig. 1c) point to the difficulty involved in assaying the cured leaf for protein. It is well documented that protein content of the leaf decreases during the curing period.[4, 6] However, the Lowry assays indicated only a small initial drop followed by an increase after 6 weeks of curing. These results actually reflect an increase in substances, probably phenolic, which cause a falsely inflated value for protein. All of the usual spectrophotometric methods for determining protein would be expected to suffer from similar interference. The existence of enzymatic activity demonstrates that intact protein must be present in both the cured and stored leaf. To determine the true protein concentration, a method not subject to the above interference will be necessary.

The existence of peroxidase activity during curing and storage does not prove that enzymatic mechanisms are responsible for the changes that occur, but is compatible with this hypothesis.

EXPERIMENTAL

Sampling

Leaf samples were obtained during the curing of the 1967 crop of cigar-filler tobacco in Lancaster County, Pennsylvania. Several stalks of tobacco were taken at 2 days, and at 3, 6, 10 and 14 weeks after harvest. This crop was described by the grower as being "fully cured" approximately 6 weeks after harvest. Each stalk was divided into top, middle and bottom leaves and each group assayed separately for peroxidase and protein. Midribs and major veins were removed in all samples. For the first 6 weeks the leaves had a high moisture content and were cut into approximately 20-mm squares before processing. The older, dryer leaves were ground in a Wiley Mill through a 60-mesh screen. Extraction and assay were done as rapidly as practical after receiving the samples. Any interim storage was done at 4°. Green tobacco taken directly from the field was used as a control.

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Samples of previously cured crops (from 1960–1966) were obtained from the General Cigar Company, Lancaster, Pennsylvania. Each of these was extracted and tested for peroxidase activity using the procedures described below. Protein determinations were not done on these samples.

Preparation of Samples for Assay

Each leaf sample was ground in a mortar and pestle with phosphate buffer (0·05 M, pH 7·0, 5 ml/g dry weight). This suspension was filtered through cheesecloth and then centrifuged at 10,000 × *g* for 15 min. The supernatant was passed through a column of Sephadex G-50, which had been pre-equilibrated with the extraction buffer, to remove the low molecular weight materials. That portion of the eluate containing the high molecular weight fraction was used for assay.

Protein Assay

Protein was determined by the method of Lowry,[14] using bovine serum albumin as a standard.

Peroxidase Assay

Peroxidase activity was measured by the method of Racusen and Foote,[15] using guiaicol as the substrate. One unit of activity was defined as that amount of enzyme required to increase the absorbance of the solution by one unit per min under the specified conditions (0·05 M phosphate buffer, pH 7·0, 0·001 M guiaicol, 0·003% v/v H_2O_2, 25°).

Disc Electrophoresis

Disc electrophoresis of each sample was performed by the method of Davis.[16] The gels were stained for protein with amido black. Peroxidase activity was located by immersing the gels in a solution containing 0·05 M phosphate buffer (pH 7·0), 0·003% H_2O_2 and 0·001 M guiaicol. The time required for complete reaction depended upon the amount of enzyme present in the gel.

Double Disc Electrophoresis

Double disc electrophoresis was performed on samples of the 1967 crop by the method of Racusen.[13] Peroxidase activity was located on the gels in the same manner as mentioned above.

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