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STUDIES ON PROTEIN METABOLISM IN HIGHER PLANT LEAVES—II.

VARIATION IN AMINO ACID COMPOSITION OF PROTEIN IN AUTOLYZED TOBACCO LEAVES

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Abstract—Chlorophyll and protein contents, amino acid composition of total and subcellular fractions, and protease activities were determined during the process of curing of tobacco leaves. About 50 per cent of the initial protein had been diminished in the yellowing stage. Chloroplast protein diminished rapidly and supernatant protein more slowly. Variations in amino acid composition in chloroplast and supernatant protein in this stage were larger than those in total protein, although the variation of the latter was also appreciable. The changes in basic amino acids, tyrosine and phenylalanine showed opposite tendencies between in chloroplast and supernatant protein. Protease activity was increased with progress of curing, especially in the supernatant fraction.

INTRODUCTION

IT IS well known that excision of plant leaves from the stem initiates an immediate rapid reduction in total protein content.[1–3] Similar results were also found in cured tobacco leaves. Vickery[4] indicated that the reduction was accelerated under flue-curing condition, and roughly more than one half of the protein of the leaf disappeared during the yellowing stage. Subsequently, the protein nitrogen remained substantially constant.

Along with the reduction, qualitative changes occurs in leaf protein. Pogall *et al.*[5] found that components of cytoplasmic proteins with higher molecular weights were rapidly broken down, whereas smaller components were relatively more stable. Zelitch's study on enzyme activities also confirms the existence of qualitative changes in the enzyme proteins.[6]

The accumulation of free amino acids and amides at the expense of protein also has been indicated by several investigators.[7–9] Ranjan *et al.*[10] also found degradative changes in excised leaves. These changes may be related to the change of protease activities. Recently, Tomita and Tamaki[11] showed the high increase of protease activity during flue-curing process, accompanying a decrease of protein.

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These changes in nitrogenous compounds may result in changes of amino acid composition in leaf protein. Recently, we investigated the amino acid composition of tobacco leaf protein in various cell fractions as a function of age.[12] As a continuation of this study, the variation in amino acid composition of tobacco leaf protein in various cell fractions during the curing process was investigated. In addition, qualitative and quantitative alterations in protease activity were also examined.

RESULTS

Change in Protein and Chlorophyll in Various Fractions during Curing

Variation of fresh weight, dry weight, and the protein and chlorophyll content of each sample during curing are tabulated in Table 1. The decrease of fresh weight per unit area with the progress of curing was mainly attributed to loss of water, even when the relative humidity was maintained over 90 per cent. Dry weight also decreased, but only 20 per cent was lost during 68-hr curing.

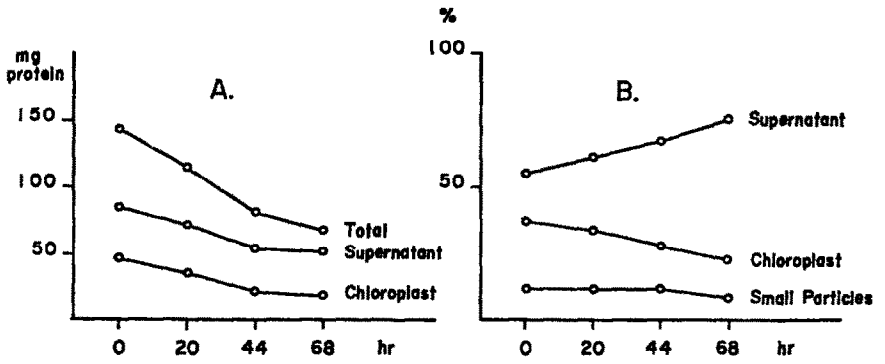

FIG. 1. CHANGE IN PROTEIN CONTENT DURING CURING.

A—relative rate of loss of proteins of subcellular fractions in cured leaves; B—Distribution of protein in each subcellular fraction as % of total protein in these fractions during curing.

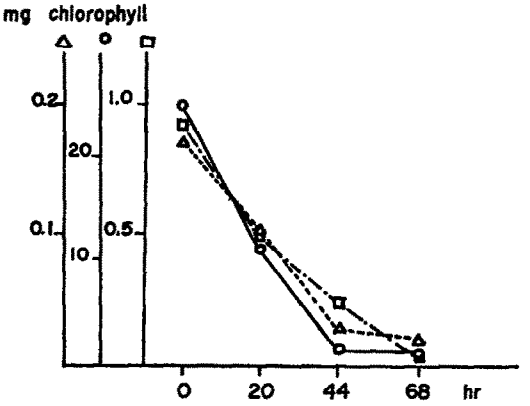

FIG. 2. RELATIVE RATES OF LOSS OF CHLOROPHYLL IN CURED LEAVES.

○—○—total chlorophyll content; △--△—content of chlorophyll/total protein; □-·-□—content of chlorophyll/chloroplast protein.

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Changes in the protein and chlorophyll content per unit area in each fraction are shown in Figs. 1 and 2, respectively. As shown by Vickery,[4] the results in Table 1 and Fig. 1 indicate that a rapid decrease in the protein of the leaf takes place with the progress of curing. The loss was found to be approximately linear with time (44-hr curing) up to a limiting point at which about 50 per cent of the initial protein had been diminished. More detailed examination of protein breakdown has been carried out with separate determination of chloroplastic and cytoplasmic protein fractions. Although the loss of protein occurred in both fractions, chloroplastic protein was lost more rapidly than the other.

TABLE 1. NITROGENOUS FRACTIONS OF LEAVES CURED FOR VARIOUS PERIODS

	Period of curing (hr)			
	0	20	44	68
Fresh weight (g)	13·1	10·7	7·94	4·36
Dry weight (g)	2·17	2·03	1·97	1·75
Protein content in original filtrste (mg)	143·8	114·6	78·5	66·5
Chlorophyll content in original filtrate (mg)	25·2	12·7	1·76	1·11
Protein content in 200 *g* supernatant fraction (mg)	102·8	90·0	48·4	39·9
Protein content in chloroplast fraction (mg)	33·2	26·4	11·8	8·22
Protein content in supernatant fraction (mg)	58·9	55·8	32·0	30·0
Protein content in small particle fraction (mg)	10·5	7·75	5·05	1·72
Chlorophyll content in chloroplast fraction (mg)	3·13	1·33	0·286	0·090

Chlorophyll also disappeared rapidly (Fig. 2) and was completely lost during 44-hr curing. The ratios of chlorophyll to total and chloroplast protein were also decreased during the process.

Changes in Amino Acid Composition of Protein in Various Fractions during Curing

Amino acid compositions of each protein fraction are shown in Table 2. Sixteen commonly occurring amino acids were detected and no unusual amino acid was found. Levulinic acid, as shown previously,[12] was also detected among the amino acids.

In the total protein, lysine, glutamic acid and proline showed appreciable changes, while the other amino acids varied very little with the progress of curing. Larger variations were observed in the separated fractions. Figure 3 depicts changes of amino acid composition of each protein fraction before and after curing (68 hr). In the chloroplast protein, an increase was found in the basic amino acids and glutamic acid, and a decrease in proline, glycine, leucine and phenylalanine with the progress of curing. The decrease in phenylalanine was especially significant. On the other hand, in the supernatant protein, aspartic acid, serine and proline showed an increase, and basic amino acids, glutamic acid and tyrosine showed a decrease. Previously, it was shown that the relative content of basic amino acids in the supernatant and the chloroplast protein were 13·9 and 11·3, respectively. The ratios of tyrosine to phenylalanine were also 0·91 and 0·55 in the supernatant and in the chloroplast, respectively. These values characterize both proteins and are not affected by growth or other factors.[12] On the other hand, the curing led to a steady decrease in the relative content of

TABLE 2. AMINO ACID COMPOSITIONS OF LEAF PROTEIN FRACTIONS IN DIFFERENT PERIOD OF CURING

	Period of curing (hr)											
	0			20			44			68		
	Orig.	Super.	Chl.	Orig.	Super.	Chl.	Orig.	Super.	Chl.	Orig.	Super.	Chl.
Lysine	5·74	6·59	5·55	6·26	6·96	6·25	6·79	6·67	6·69	6·45	6·32	6·71
Histidine	2·44	2·19	2·07	2·01	2·14	2·07	2·11	2·04	2·26	—*	—*	2·24
Arginine	4·69	4·99	4·43	4·55	4·67	4·60	4·80	4·63	4·74	4·77	4·58	4·87
Aspartic acid	8·77	9·54	8·83	9·44	9·90	8·76	8·99	10·23	8·82	9·36	10·78	8·88
Threonine	5·10	5·37	4·92	5·16	5·28	4·97	5·16	5·40	5·14	5·23	5·61	5·04
Serine	5·47	5·23	5·84	5·61	5·41	5·84	5·86	5·60	5·98	5·96	5·86	6·10
Glutamic acid	10·28	11·22	9·81	10·65	11·18	10·27	10·90	10·95	10·45	10·45	10·37	10·80
Proline	6·31	5·80	6·59	5·71	5·57	6·06	5·46	5·64	5·86	5·87	6·31	5·90
Glycine	9·55	9·09	9·92	9·21	8·69	9·20	9·10	8·90	8·74	9·20	9·36	8·80
Alanine	9·42	9·65	9·61	9·13	9·41	9·41	9·27	9·28	9·20	9·05	9·20	9·46
Valine	7·35	7·49	6·91	7·33	7·49	7·03	7·45	7·49	7·17	7·26	7·20	7·04
Methionine	1·71	1·68	1·90	—†	1·71	1·87	1·61	1·64	2·02	1·94	1·52	2·01
Isoleucine	5·25	5·05	5·59	—†	5·48	5·82	5·86	5·49	5·95	5·70	5·37	5·83
Leucine	10·41	8·65	10·49	—†	9·05	10·46	9·65	9·09	10·09	9·74	8·47	9·75
Tyrosine	2·86	3·51	2·41	—†	3·28	2·64	2·86	2·89	2·52	2·74	2·70	2·58
Phenylalanine	4·47	3·99	5·15	—†	4·03	4·80	4·13	3·98	4·48	4·28	4·06	3·99

Following abbreviations were used in this Table; Orig. = original filtrate, Super. = supernatant fraction, Chl. = chloroplast fraction. The values are given as a percentage of the content (mole) of each individual amino acid of the total of amino acids detected by the analysis.

* The values are about 2·0–2·3%, but could not be determined accurately because unidentified contaminants overlapped the histidine position.

† Sum of the values of these positions was calculated as 25·0%.

basic amino acids in the supernatant which dropped 12·9 after 68-hr curing, from an initial value of 13·8. On the contrary, in the chloroplast fraction, the content increased from 12·0

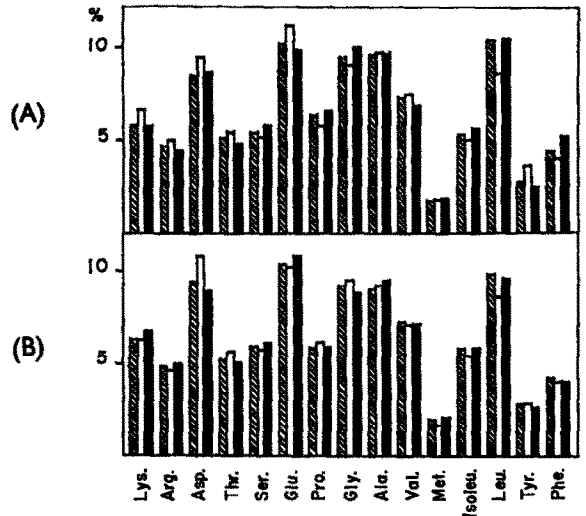

FIG. 3. COMPARISON OF THE AMINO ACID COMPOSITION OF THE LEAF PROTEIN FRACTIONS BEFORE (A) AND AFTER (B) CURING.

■—total protein; □—supernatant protein; ■—chloroplast protein.

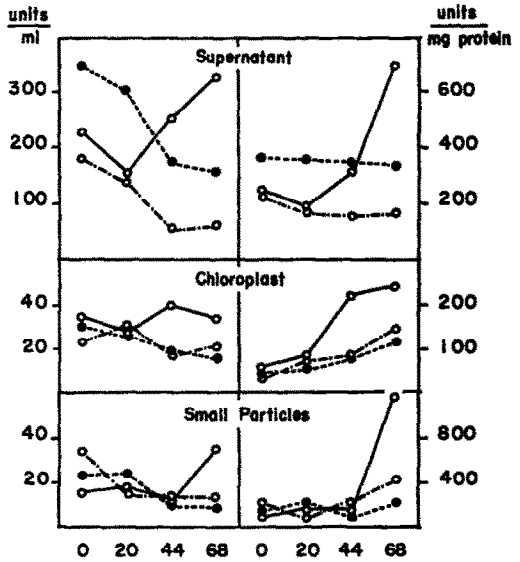

FIG. 4. CHANGES IN PROTEOLYTIC ACTIVITIES AT VARIOUS pHs DURING CURING.

o—o—pH 5·5 in McIlvain buffer; ●—●—pH 7·0 in phosphate buffer; o—o—pH 8·5 in borax buffer.

to 13·8. The ratio of tyrosine to phenylalanine also showed considerable difference before and after curing in both fractions. The initial values were 0·88 and 0·48 in the supernatant and the chloroplast, respectively, and 0·66 and 0·66 after curing.

Changes in Protease Activity in the Fractions during Curing

The decrease of protein after curing suggests that proteolytic systems have been activated in the leaves. Recently, more than three kinds of proteases having different pH optima were found in tobacco leaves in our laboratory. To determine the qualitative and quantitative changes in the protease during curing, the activity was measured at each optimum (pH 5·5, 7·0 and 8·5) in the fractions. These results are plotted in Fig. 4, as total and specific activities. The protease activities were mainly found in the supernatant fraction and less than 10 per cent was observed in the particulate fractions. The protease activities at pH 7·0 and 8·5 decreased but the specific activities remained almost constant. Those in the chloroplast fraction changed little.

DISCUSSION

It has been reported that protein hydrolysis in excised leaves is only shown in yellowing stage of curing, after which the protein content remained constant.[4] Acceleration of yellowing by incubation at higher temperature also induced rapid degradation of protein, although the total losses of protein were almost the same between both leaves incubated at high and low temperature. Now, the question arises as to whether or not there are any correlations between the decomposition of chlorophyll and protein. Michel[13] showed that there was a direct correlation between chlorophyll and chloroplast protein quantity, leaves of high protein content being similarly high in chlorophyll content. He concluded that decomposition of the chlorophyll–protein complex must lead to total loss of chlorophyll. Our results also show a parallel correlation between the chlorophyll and protein content during curing in the chloroplast fraction (Fig. 2), although the ratio of chlorophyll to protein on the whole was decreased. In more precise examination, however, such a parallel correlation between chlorophyll and total or supernatant protein was not found. These results prompt us to suggest that the decomposition of the chlorophyll–protein complex initiates the decomposition of chlorophyll but that the degradation of chlorophyll is not associated with that of the protein segments.

A second question may arise from the observed rates of protein decomposition in the chloroplast and the supernatant fractions. Despite lower protease activity, the chloroplast protein was lost more rapidly than the supernatant one. This suggests an outflow of the chloroplast protein to the supernatant fraction after breakdown of chlorophyll protein complex. This suggestion may have additional support from previous studies on amino acid composition. As previously reported,[12] chloroplast protein was characterized by a lower content of basic amino acids and the lower value of the ratio of tyrosine to phenylalanine, in comparison with those of the supernatant protein. These characters were not changed, even when the chloroplasts were obtained from leaves of various ages. However, with progress of yellowing these differences between the fractions were reversed. In this process, release of chlorophyll from protein–chlorophyll complex increase the solubility of the protein moiety, and these proteins may then be free to pass into the supernatant fraction. At the same time, a main component of the original supernatant protein, comprising 50 per cent of the fraction, is decreasing during this stage.[5, 14] Pogall *et al.* observed a 50 per cent decrease in supernatant protein, which was ultracentrifugally homogeneous, during air curing.[5] It was suggested that this protein corresponded to Fraction 1, detected in tobacco leaves by Wildman and co-workers.[15, 16] Recently, we also found a decrease of the Fraction 1 protein during the

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flue-curing using an immunochemical technique.[14] Complete decomposition of the Fraction 1 protein and release of solubilized chloroplast or grana protein might change the amino acid composition in the supernatant fraction to give the chloroplastic character.

Qualitative changes in proteases were also found in the yellowing process. One protease having maximum activity at pH 5·5 increased substantially. Proteases with optima at pH 7·0 and 8·5 remained essentially constant. The former is mostly found in the supernatant fraction. These observations may indicate that protease 5·5 has some relation to Fraction 1 protein.

EXPERIMENTAL

Materials

Tobacco plants (*Nicotiana tabacum* L., "Bright Yellow") were obtained from Utsunomiya Tobacco Experiment Station. The leaves in 9–10th positions from the top of the plants nine weeks after transplantation and one week after topping were removed and forty leaves of similar size were chosen. These leaves were divided into four groups and of which one was used to represent the initial time. The other three were cured in a chamber maintained at 40° and 90–92 per cent relative humidity for 20, 44 and 68 hr, respectively.

Samples

Seventy-seven discs, 3 cm diameter, were cut from each group. Seventy pieces were dipped in 60 ml of buffer (0·05 M phosphate containing 1% sodium ascorbic acid, pH 7·0) for 10 min before homogenation. The other 7 pieces were used for dry weight determination.

The protein fractions were prepared as described previously.[12] For the supernatant fraction of 68-hr cured leaves, the method had to be slightly modified in order to obtain a precipitate. Here, it was necessary to concentrate the fraction to $\frac{1}{5}$ of the initial volume by the aid of Carbowax-6,000, according to the method of Kohn.[17] From the concentrate, precipitates were obtained by addition of trichloroacetic acid followed by heating for 1 min.

Analytical Methods

Methods for the determination of protein and of chlorophyll were essentially the same as those used previously.[12] The protein contents of the supernatant fractions of 44 and 68-hr cured leaves were determined after concentration with Carbowax or after 40-hr dialysis. The latter gave values about 5 per cent larger than the former.

The hydrolytic condition and method of amino acid analysis were also the same as previously described.[12]

Protease activity was determined by the release of non-protein nitrogen from casein according to the method of Hagiwara[18] with slight modifications. One volume of enzyme solution was incubated with 4 volumes of 0·6% casein in 0·1 M McIlvain buffer at pH 5·5, in 0·1 M phosphate buffer at pH 7·0 or in 0·1 M borax buffer at pH 8·5. The mixture was incubated for 2 hr at 30° in the presence of cysteine. The reaction was stopped by addition of trichloroacetic acid and the nitrogen content in the supernatant was determined by the method of Folin.[19] A sample at the start of the incubation was taken as a control.

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