Chlorophyllase of Tobacco Plants I. Preparation and Properties of Water Soluble Enzyme

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Received September 26, 1962

When tobacco plants reach the mature state, farmers put them into the state of nitrogen starvation, cutting the supply of manure. In this stage, the leaf turns yellow, and there seems to be at least two possible ways to advance this change: 1. changes in nitrogenous compounds, especially in proteid components of chloroplasts, 2. destruction of chlorophylls.

We studied the biological destruction of chlorophylls. Willstätter and Stol¹⁾ showed that chlorophylls could be hydrolyzed in aqueous acetone solution by the dried powder of leaves free from the pigments. This reaction was enzymic and this enzyme was named "chlorophyllase". This enzyme activity has been observed in various plants²⁻⁷⁾ and many attempts have been made without success to isolate the enzyme in a soluble state^{1,2)}. However, recently Ardao and Vennesland⁸⁾ found that the soluble chlorophyll-lipoprotein complex obtained from spinach chloroplasts by digitonin treatment had chlorophyllase activity, and Holden⁹⁾ succeeded in preparing soluble chlorophyllase from sugar beet (*Beta vulgaris* var. *saccharifera*), but she failed to get the enzyme in a soluble state from tobacco leaves.

We succeeded to obtain chlorophyllase in a soluble state from tobacco leaves by a simpler method than the Holden's. This paper deals with the preparation of soluble chlorophyllase from tobacco leaves and some properties of the enzyme.

Materials and Methods

Plant materials:

Tobacco leaves (*Nicotiana tabacum* var. Bright Yellow) were obtained from Utsunomiya Tobacco Experiment Station.

Preparation of chloroplasts:

As chlorophyllase locates in chloroplasts⁴⁾, we intended to prepare the enzyme from chloroplasts: Laminae of tobacco leaves were homogenized with twice weight of ice-cold 0.35 M NaCl for 30 sec. The homogenate was squeezed through three folds of gauze and centrifuged at $500\times g$ for 3 min. From the resultant supernatant, chloroplasts were sedimented by centrifugation at $1,500\times g$ for 12 min. The chloroplasts were suspended in 0.35 M NaCl and recentrifuged at $1,500\times g$ for 12 min. The washing procedure was repeated twice.

Extraction of chlorophyllase from chloroplasts:

Autolysis and digestion with lipase and proteolytic enzymes have been used for bringing into solution several enzymes associated with cell nuclei and mitochondria, while treatment with detergent has enabled some separation of component from chloroplasts¹⁰⁻¹³⁾. These methods have limited application and are more or less undesirable for enzyme purification.

Morton¹⁴⁻¹⁶⁾ has shown that the controlled use of *n*-butanol enables to make ready solution of a wide range of enzymes from insoluble particles into distilled water or

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dilute buffers by removal of lipid and other materials from cell structure.

Sissakyan and his co-workers¹⁷⁻¹⁹⁾ applied the Morton's method to plant cell. These Russian researchers extracted the proteid component from sugar beet leucoplasts and chloroplasts. We followed the Morton's method with a little modification: Fifteen grams of chloroplasts were suspended in 60 ml of water (pH 9.0, adjusted with NaOH) or in the same volume of 1% NaCl solution. Slow addition of 120 ml of *n*-butanol with vigorous mechanical stirring at room temperature formed an emulsion. The emulsion was rapidly stirred and warmed to 37° for 10 min. by immersion of the vessel in a water-bath held at 40°. By centrifugation ($1,000\times g$ for 5 min.), the materials were separated into three distinct layers; a clear yellow aqueous zone overlaid by light particles, and a surface layer of excess *n*-butanol containing dissolved chlorophylls. The upper layer was readily removed by suction. The lower aqueous phase was removed by suction through a narrow glass tube and filtered. The filtrate was dialyzed against distilled water for 24 hrs., to remove the *n*-butanol contained in it.

Method of determining chlorophyllase activity:

Many methods have been proposed for the determination of chlorophyllase activity. They might be divided into three groups. The first group was that described by Willstätter and Stoll¹⁾ and used by Mayer²⁾ and Peterson and Mackinney³⁾. The pigment is extracted first into ether and then into alkali. The second method used by Weast and Mackinney⁵⁾, Ardao and Vennesland⁶⁾ and recently by Holden⁹⁾ is based on the insolubility of chlorophyllides in light petroleum. The chromatographic method described by Sironval²⁰⁾ and used by Gage and Aronoff⁷⁾ is the third method and seems to be most accurate. This method was used in the present investigation. For the substrate of the enzyme the acetone extract of tobacco leaves was used. The leaves were ground in 80% aqueous acetone (3 ml/g. of leaf), the extract was filtered and stored at 0° for several days. Before use, it was necessary to filter the acetone extract to remove carotenoids and others which had precipitated. The content of chlorophyll was determined by the method of Arnon²¹⁾.

One ml of protein solution was incubated with 20 ml of chlorophyll solution (approximately 100 mg/l) and 4 ml of 0.02 M Na-citrate. The flask

was sealed, covered with aluminium foil to exclude light and agitated occasionally at room temperature. One ml of this reaction mixture was transferred to Whatman No. 1 paper (15×40 cm) and chromatographed in the dark at 20°, using the descending method with a solvent system of benzene-petroleum ether-acetone (10:2.5:2), in an atmosphere of petroleum ether. After development, bands corresponding to chlorophylls a and b were cut off and eluted quantitatively with 80% acetone.

The activity of enzyme was expressed as a percentage of the broken chlorophylls

Fig. 1. Paper chromatograms of the reaction mixture, (a), at 0 min. and (b), at 60 min.

1, carotenoides; 2, pheophytines; 3, chlorophylls; 4, pheophorbides; 5, chlorophyllides.

by 1 g of protein in 60 min.

Determination of protein concentration:

Protein concentration was determined according to the method of Lowry *et al.*²²⁾, using casein as a reference standard.

Results

Partial purification of enzyme:

The enzyme was precipitated with acetone and ammonium sulphate. The results of precipitation are shown in Table 1. The precipitation with ammonium sulphate gave better result. Partially purified preparation had low amylase and polyphenol oxidase activity.

Effect of extraction medium:

Protein was extracted from chloroplasts with two kinds of solvents; water adjusted to pH 9.0 with NaOH and 1% aqueous solution of NaCl. The amount of protein extracted with these solvents and the enzymic activities of the resultant solutions are shown in Table 2. There was no difference between the amounts of extracted protein in both solvents, but stronger enzymic activity was obtained with 1% NaCl solution. In this paper, we used 1% NaCl for all the experiments as extraction medium, unless otherwise mentioned.

Table 1. Partial purification of enzyme.

Fraction	% of recovery	Total unit	Activity per g of protein
1% NaCl extract	100	2,091	8,500
Acetone ppt. (0-67%)	54	345	2,600
(67-87%)	1.1	36	17,100
(NH ₄) ₂ SO ₄ ppt. (0.4 sat.)	15.8	152	7,100
(0.6 sat.)	19.7	117	4,600
(0.9 sat.)	26.3	1,061	29,850

Table 2. Extraction of enzyme.

Extract	Extracted protein from 40 g of chloroplast (mg)	Activity per g of protein
1% NaCl extract	62.4	7,650
H ₂ O extract	62.0	5,560

Properties of enzyme:

(1) Effect of pH. Fig. 2 presents the effect of pH on the activity of the partial purified enzyme. McIlvaine's Na₂HPO₄-citric acid buffer was used for the whole range. The optimum pH lay between 7.0 and 7.5.

(2) Effect of substrate concentration. Fig. 3 shows how the concentration of substrate affects the enzyme activity. Over 0.5×10^{-4} M (concentration of chromatographically pure chlorophyll a), the enzyme activity was rather reduced.

(3) Effect of time. As shown in Fig. 4, the chlorophyllase activity increased

Fig. 2. Effect of pH on the enzymic activity.

Fig. 3. Effect of substrate concentration: Relative velocity is measured from decrease of optical density at 663 m μ for 20 minutes.

linearly during the first 30 minutes and then was suppressed in around 100 minutes.

(4) Seasonal changes of chlorophyllase activity and chlorophyll content. Leaves in the stalk position 19th from the bottom of the plants were sampled for enzyme activity and chlorophyll content determination. The sampling was started on the 30th day after germination and discontinued on the 145th day. During this period, 9 samplings were performed.

As shown in Fig. 5, the enzyme activity (curve I) reached maximum on the 56th day, and the maximum chlorophyll content (curve II) was found on the 91st day. There was a clear parallel relationship between the two curves. This finding may suggest that the biosynthesis of chlorophyll is closely related to chlorophyllase activity.

Fig. 4. Enzyme stability.

Fig. 5. Seasonal changes of enzyme activity and chlorophyll content: (I), enzyme unit per 1 g of protein; (II), chlorophyll content per 1 g of chloroplasts.

—, untopped plants;
 , topped plants.

Discussion

There have been only a few studies on chlorophyllase and this might be, in part, due to the difficulty to isolate the enzyme in a water soluble form. This property is resulted from the fact that an active protein of the enzyme may be linked with lipids. Ardao and Vennesland⁸⁾ suggested that the chlorophyllase of mature spinach leaf might be present in a chlorophyll-lipoprotein complex. Holden⁹⁾ also said that the linkage between the enzyme protein and lipids, either physically or chemically, might be responsible for the insolubility of chlorophyllase. In our research, we used *n*-butanol which removed the lipids and liberated the active protein into aqueous solution. The liberated protein was free from chlorophyll components and was slightly yellow.

The first step on the enzymic breakdown of chlorophyll *in vivo* has been thought to be removal of the phytol chain to yield water soluble chlorophyllide which then can undergo further degradations^{6,8,9)}. Then at the beginning of this study, the increase in chlorophyllase activity had been predicted to be a main cause of the yellowing of mature tobacco leaves. However, the present results have disproved this assumption with tobacco plants, and a similar result was reported with chlorosis in a soybean mutant⁷⁾. The decrease of the chlorophyll content in the mature leaf seemed to be caused by the decreasing activity of the enzyme in synthetic way and not by the increasing enzyme activity towards the opposite direction.

On the role of chlorophyllase in the synthesis of chlorophylls in higher plants, there have been few clear-cut demonstrations^{3,23,24)}, except that of Willstätter and Stoll¹⁾.

Younger tobacco leaves being low in the chlorophyll content had a stronger chlorophyllase activity than old ones. Sudina²⁴⁾ also observed an increase of the enzyme activity before chlorophyll formation took place during greening of etiolated hemp leaves. In the present work, the acetone extract of tobacco leaf was used as substrate of chlorophyllase. The young leaf extract, after incubation with chlorophyllase, gave a higher chlorophyll content than the original extract. On the other hand, the same treatment of the mature leaf extract decreased the chlorophyll content. It was reported that the content of phytol of young maize leaf was higher than older one²⁵⁾, and that after a short time illumination an etiolated hemp seedling contained much chlorophyllide²⁴⁾. If these two observations are true for tobacco plants, with this high content of phytol and chlorophyllide in the young tobacco leaf extract, chlorophyllase could act in a reverse way to synthesize chlorophyll. On this point, however, a further study is required to give any definite conclusion, and such a study is now in progress.

Summary

1. Water soluble chlorophyllase preparation was made from chloroplasts of tobacco plants, using *n*-butanol and aqueous solution of sodium chloride as the solvents.
2. Some properties of the enzyme were studied.
3. Seasonal changes of enzyme activity and chlorophyll content were studied. Chlorophyllase activity varied in advance of the variation of chlorophyll content.
4. Possible participation of chlorophyllase in the biosynthesis of chlorophyll was discussed.

We wish to express our thanks to Miss K. Kodaira and Miss H. Fukushima of our laboratory for their technical assistance.

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摘 要

清水 碩・玉置 英之助：タバコのクロロフィラーゼ I. 水溶性酵素の調製とその性質

タバコの葉が成熟期に達したとき、窒素肥料をあたえないで窒素飢餓の状態におかれると、葉は黄色になってゆく。このときどのような生理的な変化がおこって、葉緑素が分解してゆくかを、クロロフィラーゼ活性の変化を中心に観察した。この酵素はクロロフィルをフィトールとクロロフィリッドとに加水分解する。後者は水溶性であるために、以後の変化を受けやすくなるのだといわれてきた。クロロフィラーゼは、これまで水溶性の状態を得ることが困難であるとされていたが、われわれは *n*-ブタノールをもちいて、タバコの葉緑体から活性の大きな酵素を得ることができた。そこで一定の着位の葉について、この酵素の活性の変化と、それと同時に葉緑素の量の変化とを、いろいろな時期に調べた。その結果、若い葉での酵素活性は大きい、古くなるにつれて低下すること、これに比例して葉緑素の量の増減することがわかった。したがって、タバコにおいてはクロロフィラーゼはむしろ葉緑素の生成に関係し、これを分解し、葉を黄変させることに大きな役割を果たしているようには思われない。(日本専売公社中央研究所)