ACTION OF HYDROLYTIC ENZYMES ON SPINACH CHLOROPLAST FRAGMENTS

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SUMMARY

The effect of lipase and protease action on the low-temperature fluorescence spectra of spinach chloroplast fragments is described and the results are discussed. Attempts to fractionate chloroplast fragments treated with the hydrolytic enzymes into the two photochemical systems of photosynthesis were unsuccessful.

Investigation of the structural organization of chloroplast lamellae has long been hampered by the lack of suitable means of disrupting these structures, but recently successful fractionation has been achieved after desintegration of chloroplasts with detergents, notably digitonin (BOARDMAN & ANDERSON 1964) As the use of this detergent was found to give variable results in this laboratory and other detergents, such as Triton X 100 and sodium dodecyl sulfate, cause considerable destruction of chlorophyll complexes, we have tried to fragment chloroplasts with hydrolytic enzymes.

Suspensions of chloroplast fragments were prepared according to THOMAS *c.s.* (1967) in 0.01 M phosphate buffer, pH 7.4, which was used throughout. For the measurement of fluorescence spectra at 77° K and of chlorophyll concentrations, see BRIL *c.s.* (1969). The enzymes used were: 1) Steapsin (hog pancreas lipase, type II from Sigma Chemical Cy). 50 mg of the powder was thoroughly homogenized in 5 ml buffer and centrifuged for 10 min. at 10.000 × g to remove insoluble material. The clear supernatant was then added to the chloroplast suspension. 2. *Streptomyces griseus* protease (type VI from Sigma Chemical Cy). The enzyme dissolved in buffer was added to chloroplast suspensions to give a final concentration of 0.5 mg/ml. Incubation was carried out at 20° for periods as indicated and the reactions terminated by immersion of the mixtures in crushed ice. Final concentrations of chlorophyll in the incubation mixtures amounted to appr. 150μg/ml.

In *figs. 1a* and *1b* representative examples of the effects of the two hydrolytic enzymes on the fluorescence spectra at 77° K of samples from the same batch of chloroplast fragments are shown. The spectrum of untreated spinach lamellae shows three maxima at about 685,695 and 735 nm respectively. It was established in control experiments without enzymes that incubation at room temperature for periods up to two hours does not lead to measurable changes. By far the greatest changes occur in the presence of lipase. Immediately upon incubation there is a relative decrease of the two short-wave bands during the first 20–30

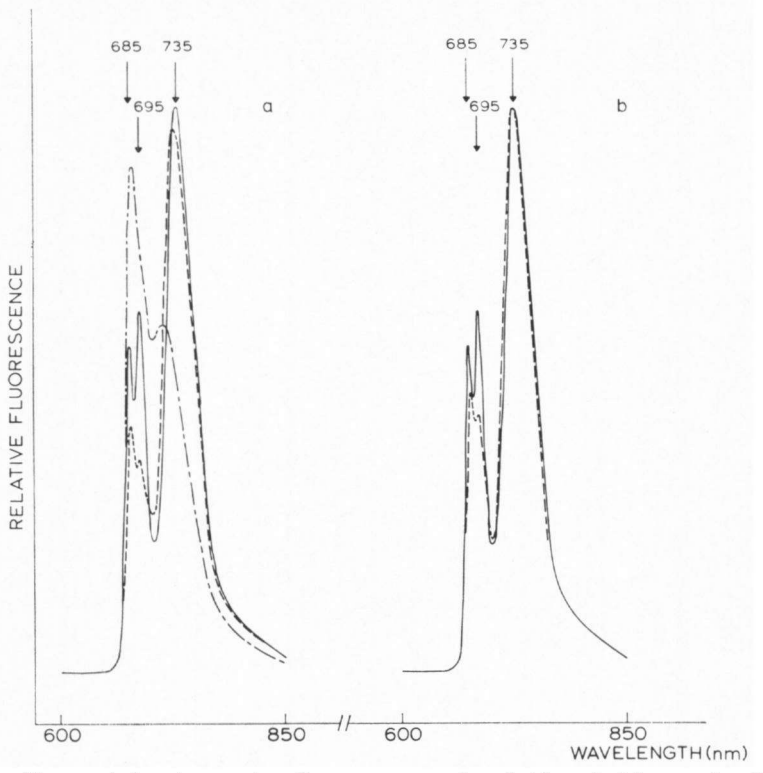

Fig. 1a. Changes in low-temperature fluorescence spectra of chloroplast fragments with time induced by lipase. ——— untreated; – – – – after 20 min.; –·–·–· after 90 min.

Fig. 1b. Changes in low-temperature fluorescence spectra of chloroplast fragments with time induced by protease. ——— untreated; – – – – after 30 min.

minutes, particularly of the 695 nm band. This initial decrease is then followed by an increase, during which both bands merge into an apparent single band with a maximum located at about 688 nm. Concomittant with these changes in the short-wave bands, an immediate and continuous shift up to 20 nm of the 735 nm band towards shorter wavelengths takes place, accompanied by an, initially slow, but gradually accelerated decrease in intensity of this band. Upon prolonged incubation only a shoulder at the long-wave side of the previously mentioned 688 nm band remains.

In comparison only minor changes occur upon proteolysis. Those of the short-wave bands are similar to the initial changes induced by lipase, be it smaller in magnitude. The 735 nm band remains virtually intact, although a slight shift of a few nm towards shorter wavelengths can be measured. No further changes take place after 30 minutes, not even after a second addition of enzyme. Washing of the chloroplasts prior to incubation to remove cytoplasmic proteins only slightly increases the rate at which changes take place.

Centrifugation of both reaction mixtures for 5 min. at 5000 x g and 15 min.

at 20.000 x g, respectively, yields two fractions, the emission spectra of which, except for some differences in the mutual heights of the emission bands, do not differ essentially from those of the unfractionated mixtures. In both cases some chlorophyll remains in the supernatant, which fluoresces with a maximum at 676 nm, characteristic for solubilized pigment. (BRIL *c.s.*) Although it might be inferred from the fluorescence data that only a limited part of the lamellar proteins is accessible to the protease, the fact that the enzyme removes some chlorophyll from its native state, together with the observation that Hill activity measured as DCPIP reduction is rapidly lost in its presence (as is also the case with lipase), indicates that protease action is more profound than can be deduced from the changes in the fluorescence emission spectrum alone (*cf.* BAMBERGER & PARK 1966).

The 5000 x g fraction is enriched in chlorophyll *b*, which is not necessarily due to enzyme action, as we have obtained similar results with untreated chloroplast fragments, in agreement with the findings of SHLYK & PRUDNIKOVA (1967). Differences in C_a/C_b ratios are therefore not an indication that a possible fractionation in photochemical systems has been achieved (*cf* ANDERSON & VERNON 1967).

The results indicate that hydrolytic enzymes are no useful agents for the disruption of chloroplasts. It is difficult to explain the changes in the fluorescent properties of the lamellae in terms of composition and structure. As the shape of the fluorescence spectrum with respect to the mutual heights of the short-wave emission bands relative to each other and to the 735 nm band was found to be influenced by a number of conditions such as simple washing, pH, ionic strength and the presence of urea, and is also dependent on growth conditions, it is likely that the initial changes induced by the enzymes are primarily due to structural changes rather than to destruction of pigment complexes responsible for their emission. In this respect it is reminded that lipase causes chlorophyll *a* dichroism at about 680 nm to decrease, whereas protease tends to increase this dichroism (THOMAS & VAN HARDEVELD 1968). On the other hand, the change in the 735 nm band leading to its ultimate disappearance in the presence of lipase is due to the destruction of a long-wave form (or forms) of chlorophyll *a*, as the change of this emission band is accompanied by a shift of the absorption maximum of the lamellae to shorter wavelengths.

The apparent greater effectiveness of lipase in comparison with that of a protease of such a broad specificity as the enzyme used in the present study is of interest. It not only demonstrates the significance of lipids for the integrity of native chlorophyll forms, at least of a long-wave form of chlorophyll *a*, but the easy access of the enzyme to its substrate also suggests the occurrence of lipids at the surface of the membranes. The possibility that lipids are exposed after a preliminary removal of proteins mediated by some proteolytic activity of the crude lipase extract is not excluded, but seems unlikely, considering the immediate onset of the alterations of the 735 nm emission band upon addition of lipase.

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