PLANTACYANIN FROM SPINACH

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1. Introduction

We have recently described a small water-soluble copper-containing protein from cucumber peelings which we named plantacyanin or cusacyanin [1]. This protein of unknown biological function differs considerably in its properties from another small copper-containing protein, plastocyanin, which is widely distributed in photosynthetizing organisms where it functions as an electron carrier between two photosystems [2, 3].

Here we describe the isolation procedure of the protein from the leaves of spinach (*Spinacea oleracea*), the classical object of photosynthetic studies.

2. Materials and methods

Optical spectra were obtained on an Specord UV-VIS instrument at room temperature, in 10 mm cells. EPR-spectra were recorded on a Varian E-4 spectrometer at 113° K. Protein concentration was 2 mg per ml. Purity of the preparation obtained was checked by its spectral index (A_{280}/A_{593}) and electrophoretically [4]. Mol. wt of the protein was determined by the method of Weber and Osborn [5]. Copper content of the protein was analyzed as described by Felsenfeld [6]. Cellulose ion-exchangers were purchased from Whatman, Sephadex G-75 (fine) was obtained from Pharmacia.

3. Results and discussion

Plantacyanin was prepared as follows. Acetone powder (600 g) of spinach leaves was suspended in 25% sat. ammonium sulphate solution (30 l). The suspension was stirred overnight, the precipitate removed by centrifugation (10 000 *g*, 20 min) and the supernatant was brought to saturation with ammonium sulphate by addition of the solid. Throughout the purification the solution was kept at pH 6.0 to avoid protein precipitation at the isoelectric point, which is near neutrality for plantacyanin. The precipitate which formed overnight was collected by centrifugation, suspended in 1.5 l of cold distilled water and again subjected to the two steps of ammonium sulphate precipitation. The final precipitate was collected, dissolved in 0.01 M phosphate buffer (1 l, pH 6.0) and dialyzed against the same buffer. All the following stages were performed in the same buffer. Dialyzate was clarified by centrifugation and the supernatant was passed through a column of DE-32 cellulose (3 × 8 cm). Plastocyanin was completely adsorbed on this column, whilst plantacyanin was not. The effluent was applied to a CM-32 cellulose column on the top of which it gave a well-defined green band. The green mass was collected with a spatula and suspended in saturated KCl solution. The resulting green solution obtained after centrifugation was diluted 25-fold with water and again applied to a column of CM-32 cellulose. The described procedure was repeated, the green solution was dialyzed against phosphate buffer and subjected to gel-filtration through Sephadex G-75 (3 × 80 cm). The blue-green fraction after the Sephadex column was concentrated by ammonium sulphate precipitation or adsorption on CM-52 cellulose. From 600 g of acetone powder 30–35 mg of plantacyanin were obtained.

The protein contains 0.66% copper. If one molecule of plantacyanin contains one atom of copper the mol. wt of the protein should be at least 9700.

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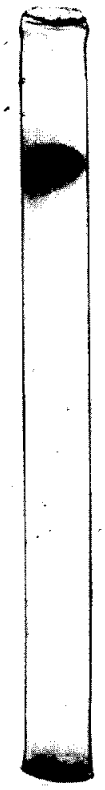

Fig.1. Electrophoretogram in polyacrylamide gel of spinach plantacyanin.

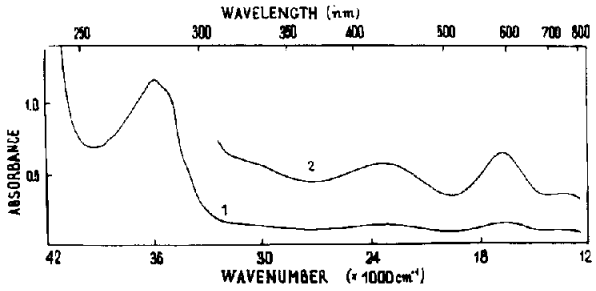

Fig.2. Absorption spectrum of oxidized plantacyanin in the UV- and visible regions (1). The visible spectrum of more concentrated protein sample was recorded separately (2).

SDS-polyacrylamide gel electrophoresis according to Weber and Osborn [5] shows that plantacyanin has an electrophoretic mobility somewhat higher than that of cytochrome *c* or plastocyanin, indicating a mol. wt of 9000. Mol. wt of plantacyanin from cucumbers estimated from gel-filtration data [1] is about 8000. Thus all the methods used gives a mol. wt of plantacyanin slightly less than 10 000.

Plantacyanin obtained according to the described procedure was found to be electrophoretically homogeneous (fig. 1). The optical spectrum of homogeneous plantacyanin differs considerably from that of plastocyanin from spinach [3] both in the u.v. and in the visible region. Plantacyanin, unlike plastocyanin, exhibits no fine structure in the 280 nm range; it shows absorbance at 330 nm, 400 nm (shoulder), 425 nm, 593 nm and 770 nm, the most intensive band being that at 593 nm (fig. 2). However the molar extinction of the band is 800 M^{-1} cm^{-1}, which is considerably lower than the molar extinction of the 597 nm band in the plastocyanin spectrum (4500 M^{-1} cm^{-1}). Plantacyanin absorbs more strongly at 400 nm than at 770 nm, whereas plastocyanin absorption at 400 nm is rather weak. Since the extinction coefficient at 593 nm is rather small, the absorbance ratio A_{280}/A_{593} for plantacyanin is much higher than the absorbance ratio A_{278}/A_{597} for plastocyanin. Thus plantacyanin from cucumbers has A_{280}/A_{593} equal to 12.8 [1] whilst the same ratio for spinach plantacyanin is 7.8. Addition of reducing agents such as ascorbate, hydroxylamine, dithionite results in disappearance of all the absorption bands except that due to protein. Reduced protein was not autoxidizable.

In general the spectrum of plantacyanin resembles that of the other copper protein, azurin [7], isolated from some micro-organisms. However spectra of these proteins are far from being identical, as their absorption maxima are differently located, and the molar extinctions for some main bands are also different. The shape of the plantacyanin spectrum is different from that of plastocyanin from spinach [3] and other plants [8], as well as from that of superoxide dismutase from spinach [9] and animal tissues [10]. The EPR-signal of plantacyanin has a rhombic shape (fig. 3), whereas azurins and plastocyanins have axial EPR spectra [7, 8]. An EPR signal of rhombic shape is exhibited also by stellacyanin, a small protein from *Rhus vernicifera* latex [11]. As with the stellacyanin signal shape, the EPR signal shape of plantacyanin does not change in acid media to pH 2.0. However it should be noted that stellacyanin has smaller hyperfine splitting in the

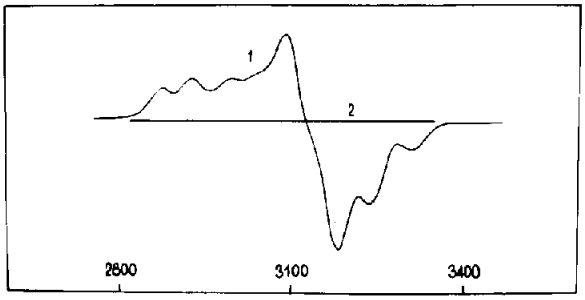

Fig.3. The EPR spectra of oxidized (1) and reduced (2) plantacyanin solution at pH 6.0. Microwave frequency, 9.12 GHz, modulation amplitude, 5 G, microwave power, 10 mW.

low field region of the EPR spectrum and the optical spectra of both proteins are also different. This comparison seems to indicate that the copper environment in plantacyanin is not the same as that in small copper-containing proteins from non-photosynthetizing organisms. It differs also from the copper environment in plastocyanin, an electron carrier protein from photosynthetizing organisms. Therefore it can be expected that the biological function of plantacyanin differs distinctly from that of plastocyanin.

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