COPPER-CONTAINING PROTEINS FROM *CUCUMIS SATIVUS*

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1. Introduction

In research on copper metabolism in plants it is desirable to isolate and to study copper-containing proteins from the same source. However at present the number of such investigations is limited. Usually only 1 or 2 copper-containing proteins may be isolated in highly purified form from the same source. The data available indicate that cucumber (*Cucumis sativus*) seems to be a good source of copper-containing proteins. A procedure of purification and some properties of the copper-containing enzyme, ascorbate oxidase (EC 1.10.3.3, L-ascorbate : O_2 oxidoreductase) from cucumber peelings was described [1]. Later we succeeded in isolating from this source 2 other copper-containing proteins: acidic-plastocyanin and basic plantacyanin [2]. The preliminary comparison of the copper content of these 3 copper-containing proteins with that of initial extract indicated that the content of copper in these proteins is ~70–80% of the total copper of the initial extract. Thus, other water-soluble copper-containing substances are expected to be present in the extract. In this connection it was interesting to study the occurrence of other copper-containing proteins in extract, obtained from cucumber peelings.

In this communication the presence of a fourth copper protein in the extract of cucumber peelings is reported. The procedure of purification of the 4 water soluble copper-containing proteins from the same initial extract as well as their main physico-chemical properties are described.

2. Materials and methods

Molecular weights of the proteins were determined by gel-filtration [3] and electrophoretically [4]. Copper content of the proteins was assayed as in [5] as well as by double integration of EPR spectra using Cu^{2+}-EDTA as standard. Isoelectric points of the proteins were determined by isoelectrofocusing [6]. Ascorbate oxidase activity was measured as in [7]. EPR spectra were recorded on a Varian E-4 instrument at 77 K. Other conditions of EPR spectroscopy were: microwave power, 10 mW; microwave frequency, 9.12 GHz; modulation amplitude, 6.3 G. Optical spectra were obtained in 10 mm cells on a 'Specord UV-VIS' (GDR) at room temperature. Yields of copper-containing proteins were evaluated from generally accepted values of molar extinctions of main absorption bands in visible region [8].

Cellulose ion-exchangers (DE-32 and CM-32) were purchased from Whatman, Sephadexes were obtained from Pharmacia. Chemicals used were reagent grade. Buffers were prepared on quartz distillate. Several different varieties of cucumber were used in this work. The results obtained were practically not dependent on the variety used.

3. Results and discussion

Acetone powder (500 g) obtained from 16 kg cucumber peelings was suspended in 25 l ammonium sulphate of 15% saturation (in terms of 700 g/l for the full saturation) adjusted to pH 6.0. The suspension was mixed at 6°C for 48–50 h and then filtrated through 4 layers of nylon sieve (100 mesh). The extract was brought to the full saturation of ammonium sulphate. The precipitate formed overnight was collected by centrifugation at 10 000 × *g* for 20 min and dissolved in 800 ml cold distilled water. The solution

was dialyzed for 2 days against 30 l 0.01 M sodium phosphate buffer. During this time the buffer was changed twice. The dialyzate was centrifuged at 20 000 × *g* for 20 min and further fractionated chromatographically on DE-32 and CM-32 cellulose exchangers.

The dialyzate was passed first through DE-32 cellulose column (5 × 8 cm), equilibrated with 0.01 M phosphate buffer (pH 6.0). The green band formed at the top of the column was found to be plastocyanin. This protein was further purified to electrophoretically homogeneous state using gel-filtration and anion-exchange chromatography [2]. Fractions eluted without retention on DE-32 cellulose were diluted twice by distilled water, pH was adjusted to 6.0 and then introduced into a column with CM-32 cellulose (3 × 8 cm), equilibrated with 0.01 M buffer (pH 6.0). In the course of the introduction the formation of several coloured bands was observed. In fig.1 the pattern of coloured bands on the charged column is schematically shown. The charged CM-32 cellulose column was washed by linear gradient (0.02–0.22 M) phosphate buffer (pH 6.0) of total volume 1100 ml. Three copper-containing fractions all absorbing at 600 nm, were eluted from this column with 0.03 M, 0.05 M and 0.2 M buffers (fig.2). It was established that these fractions exhibit EPR spectra characteristic for stellacyanin, ascorbate oxidase and plantacyanin, respectively.

The further purification of stellacyanin from the fraction eluted by 0.03 M buffer was carried out using CM–Sephadex chromatography as in [9]. It was found that gel-filtration through Sephadex G-75 of the preparation obtained led to two fractions which had

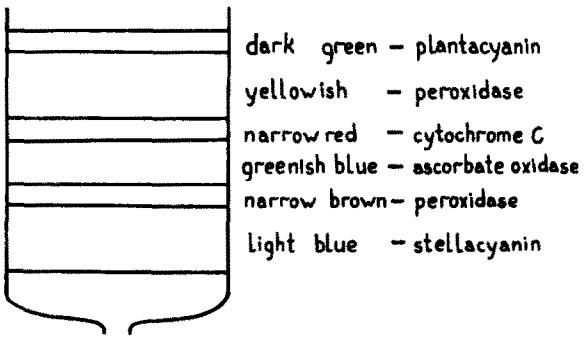

Fig.1. Schematic representation of the localization of coloured protein bands on CM-32 cellulose column.

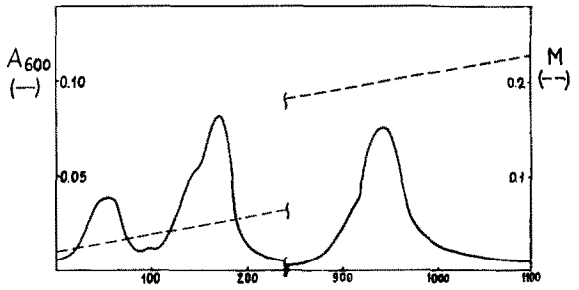

Fig.2. Typical elution pattern of 3 copper-containing proteins adsorbed on CM-cellulose.

completely identical optical and EPR spectra, although their molecular weights were different. Ascorbate oxidase (the crude fraction eluted from CM-cellulose by 0.05 M buffer) was further purified according to [10]. Plantacyanin which was eluted by 0.2 M buffer was purified essentially as in [2] with the introduction of the additional step of chromatography on CM–Sephadex C-50.

Thus, 4 water-soluble copper-containing proteins were isolated from the same initial extract of cucumber peelings. At pH 6.0, three of them were basic and one was acidic.

More than 10 isolations were done by the above procedure. For the complete reproducibility it is necessary to follow exactly all conditions and steps described above. Particularly, it is important to keep the duration of the extraction time, concentration of ammonium sulphate and conditions of chromatographies on DE- and CM-celluloses.

The 4 proteins were obtained in electrophoretically homogeneous state and they had minimal values of the spectral ratio, A_{280}/A_{600}. Besides, there is at least one more copper-containing fraction which was not adsorbed on DE- and CM-celluloses at conditions used. This fraction is not yet studied.

Yields and main physico-chemical properties of the 4 proteins are presented in table 1. EPR spectra of these proteins are compared in fig.3. From proteins isolated only ascorbate oxidase contains > 1 copper atom/mol. The content of different types of copper [8] in ascorbate oxidase of *Cucumis sativus* was found to be similar to that isolated from *Cucurbita pepo* [11]. As it can be seen from fig.3, plastocyanin and one of the copper species of ascorbate oxidase

Table 1
Comparison of some properties of the four copper-bearing proteins from cucumber peelings

	Ascorbate oxidase	Plastocyanin	Stellacyanin	Plantacyanin
Mol. wt	140 000	10 000	20 000 and 40 000	9000
Cu content (per molecule)	8	1	1	1
Isoelectric point	8.0	4.1	7.5	10.6
λ_{max} of the major visible band	610 nm	597 nm	605 nm	593 nm
A_{280}/A_{600} [a]	32	1.4	6	10
Yields from 500 g acetone powder (mg)	80–100	30–35	10–15 [b]	20–25

[a] A_{600} means absorption at major visible band of proteins
[b] The quantity obtained for both molecular forms of stellacyanin

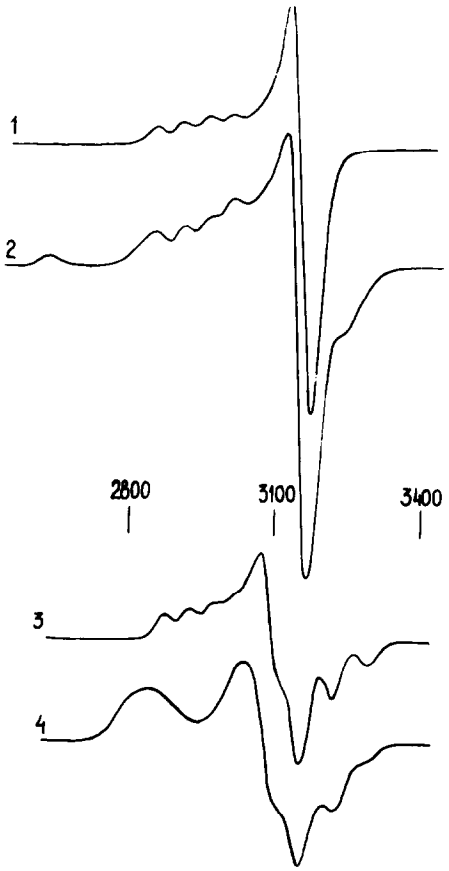

Fig.3. The comparison of EPR spectra of the 4 copper-containing proteins: 1-plastocyanin, 2-ascorbate oxidase, 3-plantacyanin, 4-stellacyanin.

(type I copper) have EPR spectra of axial symmetric shape with practically similar parameters ($A_{\parallel}$ = 50 G; $g_{\parallel}$ = 2.23; g_m = 2.06). The copper atom of type I with axial symmetric EPR spectrum is also present in umecyanin [12] and azurin [13]. On the other hand, the copper in plantacyanin and stellacyanin should be considered as type I with rhombic shape of the EPR spectrum. The type I copper with rhombic symmetry was also observed in rusticyanin [14].

Three from the 4 proteins were obtained from cucumber previously however stellacyanin was obtained from cucumber for the first time. It is worth mentioning that stellacyanin was obtained earlier only from laquer tree latex simultaneously with the blue copper-containing oxidase, laccase [15,16]. Thus, cucumber peelings are one more source from which stellacyanin and blue oxidase (ascorbate oxidase) may be obtained simultaneously. These observations allows us to consider two possibilities that stellacyanin is the copper-carrier protein acting as a precursor of blue oxidases or it is the product of their degradation. Besides, bearing in mind that EPR spectra of the type I copper of ascorbate oxidase are similar, the suggestion may be considered that plastocyanin serves as one of ascorbate oxidase subunits. Generally, ascorbate oxidase may be considered as a copper-containing enzyme formed from simpler copper proteins following their aggregation or polymerization, or, vice versa, relatively small copper-containing proteins isolated may be formed following disaggregation or digestion of

ascorbate oxidase. The answer to these questions, probably, may be given from the comparison of amino acid compositions of small copper-containing proteins and subunits of ascorbate oxidase as well from the study of the copper transfer between the proteins isolated. Work is in progress to prepare subunits of ascorbate oxidase.

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