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CYTOCHROME *c* COMPLEXES WITH CHLOROPHYLLIN

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Abstract. Chlorophyllin *a* (CHLA), a food-grade derivative of chlorophyll *a*, is a potent inhibitor of carcinogenesis. Recently a growing interest in CHLA application as a natural colorant and diet stabilizing supplement is observed. The study presented herein is concerned with the interaction between CHLA and ferricytochrome *c* (ferriCY) in aqueous solution at physiological conditions, studied by means of spectroscopic methods – absorption and fluorescence in the visible range and ultraviolet circular dichroism (UV-CD). In a 0.1M solution of phosphate buffer (pH 7) CHLA undergoes dark transformation from 645 nm (red) to 735 nm (far red) absorbing form with half time of $16 \cdot 10^3$ s. This first order reaction is activated by ruby laser irradiation energy. FerriCY with CHLA in solution undergoes reduction and forms a complex with an additional band of 694 nm in the absorption spectrum. The data indicated 1:1 stechiometry of ferriCY-CHLA complex formed at single bond site with association constant $K_A = 1.1 \cdot 10^6$ M^{-1} and free energy $\Delta G = -33.8$ kJ mol^{-1}. In such a complex UV-CD spectra reveal a decrease in the α-helix structure from 22.4% (native ferriCY) to 16.05%. These studies indicate that CHLA, when binding with a protein, is an effective reductant (antioxidant).

Keywords: chlorophyllin *a*, ferricytochrome *c*, α-helix structure

INTRODUCTION

In many countries the use of colorants in foods is regulated by relevant legislation. This legislation is becoming increasingly restrictive and the number of colorants allowed is limited and strictly controlled. Moreover, public opinion is very sensitive to the application of additives in general, and colorants in particular, and there is a visible tendency to use natural colorants rather than artificial ones. A potentially useful natural colorant is chlorophyllin *a* (CHLA) (Calvo and Salvador 2000), water soluble chlorophyll *a* derivative. The grow-

ing interest in CHLA application and consumption is driven by the increasing number of papers proving CHLA-induced positive effects on human health. Recently the former has been proved (Dashwood 1997, Díaz *et al.* 2003) to protect against diseases, kidney tumour, hypertension, and skin aging processes. It has also been used as a diet stabilizing agent and a colorant supplement in juices (Calvo and Salvador 2000).

Although physiological and pharmacological effects of CHLA are widely observed, the underlying mechanism of CHLA activity is not clear. A significant number of studies suggest that CHLA ability to form complexes with a wide range of potential human carcinogens is the mechanism that protects against damage of both the cellular DNA and proteins (Ouameur *et al.* 2005). Proving this hypothesis, one paper (Boloor *et al.* 2000) indicated CHLA inhibition of membrane damage caused by γ radiation. This and other data (Kamat *et al.* 2000) suggest strong interaction of the cytochrome system with exogenously applied CHLA.

Taking into account all the results mentioned it is of considerable interest to study the interaction between CHLA and cytochrome *c* (CY) in physiological conditions *in vitro* and to examine the effects of pigment complexation on protein secondary structure.

MATERIALS AND METHODS

Chlorophyll *a* obtained after chromatographic purification of chlorophyll extracted from frozen spinach leaves was dissolved in benzene. Next the chlorophyll *a* at the concentration of 100 μM was diluted 1:14 with pentane. Saponification was carried out by the addition of 1 ml of a filtered 7% KOH solution in methanol to 50 ml of chlorophyll *a* solution. On vigorous shaking the solution turns brown and after a few minutes bluish crystals of CHLA precipitate. The crystals were separated, washed with pentane, and kept in darkness at 278 K. Before measurements the crystals were dissolved in 0.1 M phosphate buffer (pH 7). All the chemicals used were distilled or crystallized.

Horse heart cytochrome *c* purchased from POCH was dissolved in deionised water, oxidized with potassium ferrocyanide and dialyzed against water.

Before the measurement a protein solution (1-22.5 μM) was added to the CHLA solution, with constant stirring to ensure the formation of a homogeneous solution.

The absorption spectra in a visible range were recorded on Beckmann ACTA IV spectrophotometer.

The fluorescence measurements were performed using Perkin Elmer MPF-3 spectrofluorimeter.

UV-CD spectra were recorded with a Mark III JOBIN-YVON spectropolarimeter. Spectra were corrected for buffer signal and expressed as the difference $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ between the molar extinction coefficient ε_L, ε_R for left and right handed circularly polarized light, respectively.

Results displayed in figures are means obtained in three replications. The standard deviation do not exceed 3-4%.

All measurements were performed in darkness at 293 K.

RESULTS AND DISCUSSION

The absorption spectrum of CHLA is shown in Figure 1. In time the initial maximum in this spectrum decreases and shifts from λ = 645 nm to λ = 660 nm, simultaneously a new long wave far-red absorption maximum λ = 735 nm emerges. On the basis of these absorption changes, the half time of CHLA transformation from the red light absorbing form $CHLA_R$ into far-red light absorbing form $CHLA_{FR}$ can be calculated as $\tau = 16 \cdot 10^3$ s.

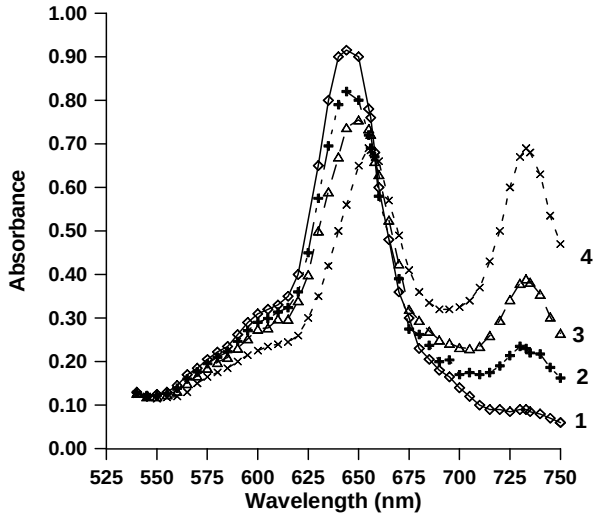

Fig. 1. Time dependence of the absorption spectrum for CHLA (19.0 μM). Measurements were made in time: 300 s (curve 1), half hour (curve 2), one hour (curve 3) and two hours (curve 4) after preparation of the solution

This $CHLA_R \rightarrow CHLA_{FR}$ transformation (Miedziejko 1989) is activated by the ruby laser irradiation (Fig. 2) as a function of energy dose. In the stationary state approximately 55% of CHLA still remains in the red light absorbing form ($CHLA_R$).

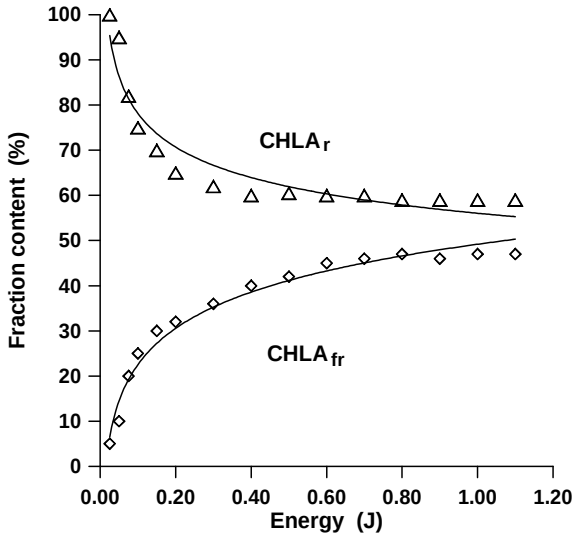

Fig. 2. Effect of ruby laser irradiation on the transformation $CHLA_r \rightarrow CHLA_{fr}$ as a function of energy dose

Obviously, the absorption spectrum of the CHLA with ferriCY solution undergoes considerable change over time (Fig. 3):

(1) the spectrum reveals fine structure in the green band (517, 548 nm), because ferriCY undergoes reduction,

(2) a new maximum at the wavelength 694 nm emerges, which is responsible for absorption of the CHLA-CY complex.

In order to reveal the number of bond sites in CHLA-CY complex the Scatchard equation (Scatchard 1949) was applied in the form:

$$\frac{\nu}{c} = K_A(n - \nu) \tag{1}$$

where: ν – binding parameter, c – quencher concentration, n – number of bond sites, K_A – association constant. We can calculate the binding parameter ν on the basis of fluorescence quenching data using the formula (Badley and Teale 1971, Plenzler and Miedziejko 1985):

$$\nu = \frac{F_o - F}{F_o - F_\infty} \tag{2}$$

where: F_0 – fluorescence of CHLA solution without CY, F – fluorescence of CHLA solution with CY at concentration c, F_∞ – saturated value (fluorescence of CHLA solution with CY extrapolated to concentration $c \rightarrow \infty$).

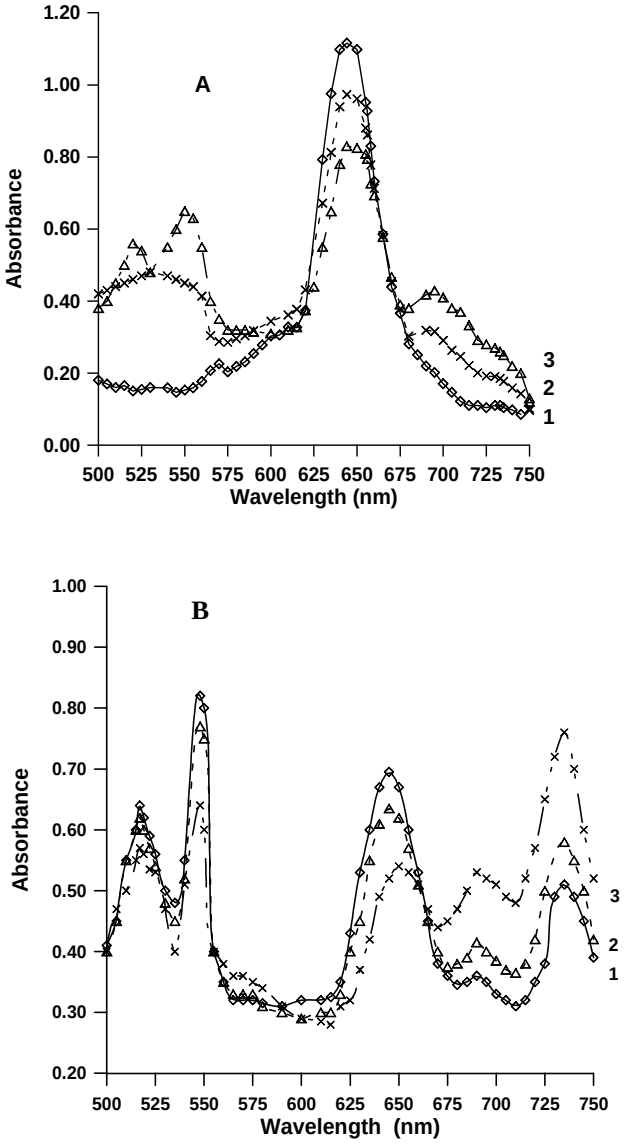

Fig. 3. Effect of ferriCY (22.5 μM) on the time dependence of the CHLA (21.0 μM) absorption spectra. Measurements were made in time: Part A: 180 s (curve 1), 300 s (curve 2) and 600 s (curve 3); Part B: half hour (curve 1), two hours (curve 2) and five hours (curve 3) after preparation of the solution

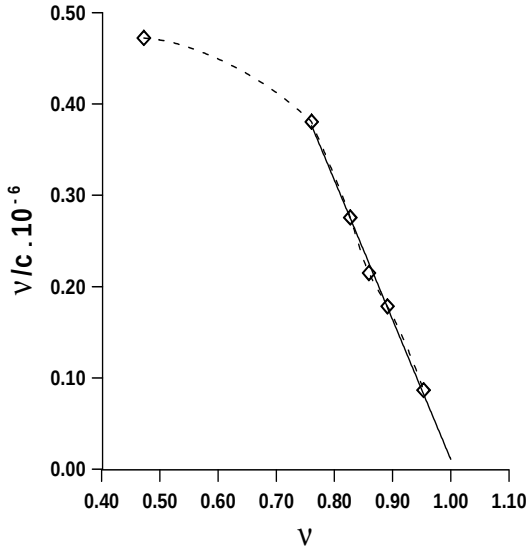

Fig. 4. Scatchard plot of the effect of ferriCY concentration on fluorescence quenching of CHLA (20.0 μM). Relative fluorescence intensities (F) at 665 nm, F_0 – without ferriCY, F_∞ – saturation value. Excitation wavelength λ = 425 nm. FerriCY concentration 1-11μM

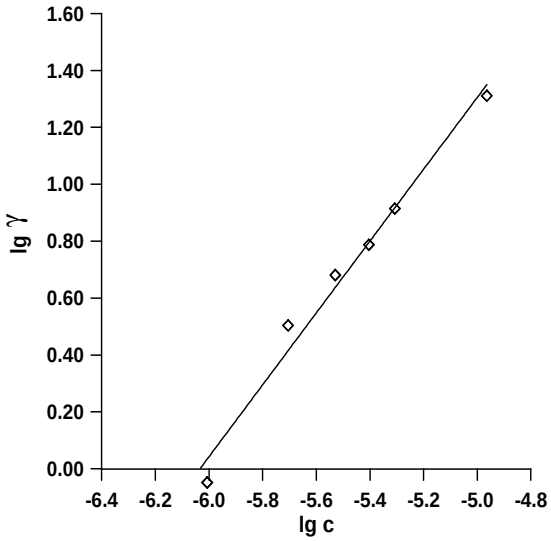

Fig. 5. Linear relationship between $\gamma = \lg (F_0 - F)/(F - F_\infty)$ and the lg c, where c is ferriCY concentration in μM. Relative fluorescence intensities (F) at 645 nm, F_0 – without ferriCY (20.0 μM), F_∞ – saturation value. Excitation wavelength 425 nm. FerriCY concentration 1-11 μM

The Scatchard plot for the CHLA fluorescence quenching by ferriCY (Fig. 4) shows n = 1 bond sites, which simply is the ν value for (ν/c) = 0.
Substituting Eq. (2) into Eq. (1) for n =1 we get the following relation:

$$\frac{F_o - F}{F - F_\infty} = K_A c \tag{3}$$

The CHLA-CY complex association constant derived from the plot (Fig. 5) of $\gamma = \lg (F_o - F)/ (F - F_\infty)$ against lg (c) (where c is concentration of ferriCY) equals $K_A = 1.1 \cdot 10^6$ M^{-1}.

The standard free energy of this binding process ($\Delta G°$) was calculated from the thermodynamic relationship $\Delta G° = -RT \ln K_A$ (Chipman *et al.* 1967, Plenzler and Miedziejko 1985) where R is the universal gas constant, T is the absolute temperature. The calculated energy value $\Delta G° = -33.8$ KJ mol^{-1} for the formation of CHLA-CY complexes indicated high affinity of binding. Therefore, the fluorescence quenching analysis clearly demonstrates that CHLA binds at or near one bond site on the ferriCY.

The peptide conformational changes induced by the ferriCY-CHLA complex formation were analysed on the basis of circular dichroism (CD) spectroscopy in the far–UV region (Chu and Fasman 1974, Greenfield and Fasman 1969).

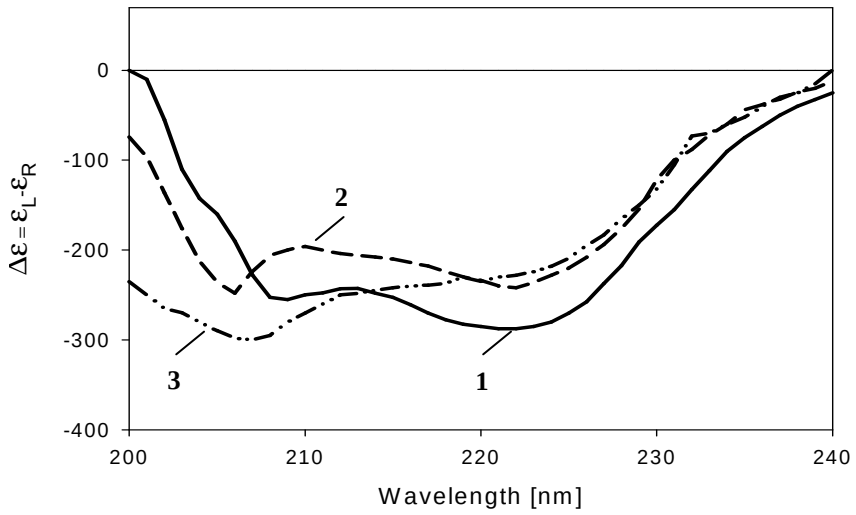

Fig. 6. UV- CD spectra of ferriCY (curve 1) and ferriCY with CHLA at the concentration of 0.3 μM (curve 2) and 1.3 μM (curve 3)

Generally, the CD spectroscopy detects the difference in the molar extinction coefficient $\Delta\varepsilon$ of left – versus right polarized light which arises due to structural asymmetry. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum which can contain both positive and negative signals. The far UV-CD spectra of native ferriCY and ferriCY interacting with CHLA are shown in Figure 6.

As a result of ferriCY-CHLA complex formation, the value of $\Delta\varepsilon$ at λ = 222 nm becomes less negative and simultaneously there is a gradual formation of a negative maximum at λ = 208 nm. These spectral changes (curve 2, 3 and 1 for comparison) depend on the CHLA concentration.

The results of $\Delta\varepsilon$ spectral changes at λ = 222 nm were expressed as the mean residue ellipticity MER_{222} and the α-helical content was calculated following the expression (Naeem and Khan 2004) :

$$\%\alpha_{helix} = \frac{MRE_{222} - 2340}{30300} \times 100 \qquad (4)$$

The calculated α-helical structure content values are: 22.4%, 17.62% and 16.05% for native ferriCY (curve 1), for ferriCY with CHLA solution at 0.3 μM (curve 2) and 1.6 μM (curve 3) concentration, respectively. Taking into account the decrease in the α-helical structure content, we can conclude that the conformation of protein in the case of CHLA-ferriCY complex is in a less hard (molten) state than in native ferriCY.

CONCLUSIONS

1. Chlorophyllin *a*, a food-grade derivative of chlorophyll, the ubiquitous green plant pigment widely consumed by humans, is a potent inhibitor of carcinogenesis. The above is a report on the absorption, fluorescence and CD spectroscopic results concerning the interaction between chlorophyllin *a* and cytochrome *c* in aqueous solution at physiological conditions. Structural information regarding the pigment bond sites, binding constant and the effects on the protein secondary structure are provided. The presented spectroscopic results can elucidate the nature of pigment-protein complexation *in vitro* and *in vivo.*

2. These studies indicate that CHLA is both an effective protein binder and a reductant (antioxidant).

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KOMPLEKSY CYTOCHROMÓW *c* Z CHLOROFILINĄ

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Streszczenie. Chlorofilina *a* (CHLA), pochodna chlorofilu *a* występująca w żywności, jest potencjalnym inhibitorem karcinogenezy. Ostatnio obserwuje się rosnące zainteresowanie zastosowaniem chlorofiliny zarówno jako naturalnego barwnika jak i dodatku stabilizującego dietę. Prezentowana praca dotyczy oddziaływań pomiędzy CHLA i fericytochromem *c* (feriCY) w roztworach wodnych w warunkach fizjologicznych, badanych przy zastosowaniu metod spektralnych: absorpcji i fluorescencji w zakresie widzialnym oraz dichroizmu kołowego w zakresie

ultrafioletu (UV-CD). W roztworze 0,1 M buforu fosforanowego (pH 7) CHLA podlega w ciemności transformacji od formy absorbującej światło czerwone 645 nm do formy absorbującej światło dalekiej czerwieni 735 nm, z półokresem transformacji wynoszącym $16 \cdot 10^3$ s. Reakcja transformacji jest aktywowana światłem lasera rubinowego. FeriCY w roztworze z CHLA podlega redukcji i tworzy kompleks z dodatkowym pasmem absorpcji 694 nm. Wykazano, że w kompleksie feriCY-CHLA występuje jedno miejsce wiązania w stosunku stechiometrycznym 1:1 obu składników. Stała asocjacji kompleksu wynosi $K_A = 1{,}1 \cdot 10^6$ M^{-1}, a energia swobodna $\Delta G = -33{,}8$ kJ·mol^{-1}. Widma UV-CD ujawniają spadek zwartości helikalnej struktury proteiny w kompleksie od 22,4% (występującej w cytochromie natywnym) do 16,05%. Prezentowane badania dowodzą, że CHLA wiążąc się z białkiem jest efektywnym reduktantem (antyutleniaczem).

Słowa kluczowe: chlorofilina *a*, fericytochrom *c*, struktura helikalna α