The Physiological Response of Green Algae (*Chlorella vulgaris*) to pH-Dependent Inhibitory Activity of Some Zinc(II) Compounds: Carboxylato- and Halogenocarboxylatozinc(II) Complexes

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Received 13 October 2003

The inhibition of chlorophyll content in statically cultivated green algae *Chlorella vulgaris* caused by $ZnSO_4 \cdot H_2O$ and nine carboxylatozinc(II) complexes was observed at pH = 5.8 and 7.2, respectively. At pH = 5.8 comparable inhibitory activity with that of $ZnSO_4 \cdot H_2O$ was shown only by $Zn(ClCH_2COO)_2 \cdot 2H_2O$ (IC_{50} = 0.111 mmol dm^{-3} and 0.104 mmol dm^{-3}, respectively). The inhibitory activity of all other compounds was lower (the corresponding IC_{50} values varied in the range of 0.204—0.571 mmol dm^{-3}) and it showed a decrease in the following order: $Zn(ICH_2COO)_2$, $Zn(BrCH_2COO)_2 \cdot 2H_2O$, $Zn(CH_3COO)_2 \cdot 2.5H_2O$, $Zn(ClCH_2CH_2COO)_2$, $Zn(CH_3CH_2CH_2COO)_2$ or $Zn(CH_3CH_2COO)_2$, $Zn(CH_3CH(Cl)COO)_2$, $Zn((CH_3)_2CHCOO)_2$. At pH = 7.2 the inhibitory activity of all tested halogenocarboxylatozinc(II) complexes was higher than that of $ZnSO_4 \cdot H_2O$ (0.300 mmol dm^{-3}). The inhibition of chlorophyll *a* by $ZnSO_4 \cdot H_2O$ and by the studied zinc(II) complexes was more strongly affected than that of chlorophyll *b*. The insertion of halogen atom into ligand led to the increase of biological activity whereas the branching of the alkyl chain was connected with activity decrease. In aqueous solutions in the investigated zinc(II) complexes zinc atom is coordinated with carboxylato ligands as well as with H_2O molecules. We assume that its toxic effect can be manifested after exchange of these ligands (mainly H_2O) with potential *O*- and *N*-donor ligands occurring in the algal cells. The higher the complex stability, *i.e.* the lower is the complex ability to exchange its ligands, the lower is its biological activity. Organic ligands could also affect the transport of metal ions through the lipophilic regions of cell membranes.

Zinc as an essential trace element is structural and catalytic component of some proteins and enzymes. However, when applied in excess it is extremely toxic also to algal cells, which react by defensive mechanism such as organometal complexation in the cell wall, metal accumulation in vacuoles, and synthesis of phytochelatins [1]. The metabolic functions of zinc are based on its strong tendency to form tetrahedral complexes especially with *N*-, *O*- and particularly *S*-ligands and it thereby plays both a functional (catalytic) and structural role in enzyme reactions [2].

Chlorella sp. are very resistant to changes in environmental conditions and they are distributed very widely in almost all areas of the world where conditions are such as to permit growth of green plants. In freshwater ecosystems the total zinc concentration is usually higher than in ocean water [3]. Experimental evidences indicate that growth and toxicity are mostly a function of the activity of the free metal cation [4]. Zinc appears to be complexed to a much lesser extent than *e.g.* copper [5]. The ratio of amount of Zn^{2+} to total dissolved zinc was about 0.02 to 0.1 in the fresh water [6].

The higher concentrations of Zn^{2+} decrease total chlorophyll (Chl) content and Chl *a*/*b* ratio in higher plants [7] as well as in *Chlorella vulgaris* [8] and in *Euglena gracilis* [9]. The reduction of Chl may be due to sensitivity of the enzymes of Chl biosynthesis towards heavy metal ions [10]. *Rai* and *Kumar* [8] reported that the toxicity of zinc to *Ch. vulgaris* decreased at alkaline pH whereas increased toxicity at pH 4—5 was recorded. It was found that zinc has a preference to chelate with ligands containing *S*-, *O*-, and *N*-donors [2]. Accumulation of Zn in *Ch. vulgaris* was estimated to be predominantly mediated by metabolic processes and it was confirmed that metallothionein-like protein was inductively biosynthesized in *Ch. vulgaris* by exposure to Zn [11]. Photosystem II as the primary site

Table 1. Concentrations of $ZnSO_4 \cdot H_2O$ and Zn(II) Complexes Causing 50 % Decrease of Chlorophyll Content in Statically Cultivated Green Alga *Chlorella vulgaris* (IC_{50}) at pH = 5.8

Compound	$\frac{IC_{50} \pm C.L._{0.05}}{mmol\ dm^{-3}}$		
	Chl *total*	Chl *a*	Chl *b*
$ZnSO_4 \cdot H_2O$	0.111 (0.099—0.123)	0.097 (0.086—0.108)	0.151 (0.127—0.173)
$Zn(CH_3COO)_2 \cdot 2.5H_2O$	0.204 (0.174—0.236)	0.170 (0.129—0.210)	0.255 (0.236—0.276)
$Zn(CH_3CH_2COO)_2$	0.329 (0.261—0.405)	0.284 (0.207—0.368)	0.420 (0.366—0.483)
$Zn(CH_3CH_2CH_2COO)_2$	0.324 (0.256—0.387)	0.310 (0.265—0.351)	0.333 (0.252—0.410)
$Zn((CH_3)_2CHCOO)_2$	0.571 (0.487—0.639)	0.550 (0.403—0.601)	0.593 (0.498—0.677)
$Zn(CH_3CH(Cl)COO)_2$	0.421 (0.375—0.483)	0.416 (0.357—0.502)	0.434 (0.365—0.544)
$Zn(ClCH_2CH_2COO)_2$	0.284 (0.262—0.308)	0.293 (0.271—0.317)	0.262 (0.221—0.300)
$Zn(ClCH_2COO)_2 \cdot 2H_2O$	0.104 (0.073—0.133)	0.112 (0.080—0.141)	0.172 (0.092—0.248)
$Zn(BrCH_2COO)_2 \cdot 2H_2O$	0.178 (0.152—0.205)	0.133 (0.110—0.154)	0.223 (0.209—0.239)
$Zn(ICH_2COO)_2$	0.166 (0.135—0.196)	0.142 (0.106—0.173)	0.198 (0.158—0.245)

of action of Zn^{2+} ions in *Chlorella vulgaris* has been reported by *Rai et al.* [12].

The aim of this paper was to investigate the effects of some carboxylatozinc(II) and halogenocarboxylatozinc(II) complexes on chlorophyll content in freshwater alga *Chlorella vulgaris* statically cultivated at two different pH values.

EXPERIMENTAL

The studied zinc(II) complexes $Zn(CH_3COO)_2 \cdot 2.5H_2O$, $Zn(CH_3CH_2COO)_2$, $Zn(CH_3CH_2CH_2COO)_2$, $Zn((CH_3)_2CHCOO)_2$, $Zn(CH_3CH(Cl)COO)_2$, $Zn(ClCH_2CH_2COO)_2$, $Zn(ClCH_2COO)_2 \cdot 2H_2O$, $Zn(BrCH_2COO)_2 \cdot 2H_2O$, and $Zn(ICH_2COO)_2$ were synthesized from respective carboxylic acid and zinc(II) carbonate according to procedures described elsewhere [13, 14]. $ZnSO_4 \cdot H_2O$ of anal. grade was purchased from Lachema (Brno, Czech Republic). Anal. grade chemicals were employed for the preparation of all solutions. Freshly distilled water was used in all experiments.

The algae *Chlorella vulgaris* were statically cultivated (photoperiod 16 h light/8 h dark; irradiation: 90 μmol m^{-2} s^{-1} PAR) at mean air temperature ((23 ± 1) °C) according to *Kráľová et al.* [15] at pH 5.8 and 7.2, respectively. After 7 days of cultivation the effect of the tested compounds on algal Chl content was determined spectrophotometrically after extraction into methanol according to *Wellburn* [16]. The Chl content in the suspensions at the beginning of cultivation was 0.5 mg dm^{-3}. All tested concentrations were triplicated. The IC_{50} values and their 95 % confidence limits ($C.L._{0.05}$) were calculated using the least-squares regression. For the calculation nominal concentrations of Zn(II) compounds were used. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

For *Ch. vulgaris* the inhibitory effect of $ZnSO_4 \cdot H_2O$ and nine Zn(II) complexes on total chlorophyll, chlorophyll *a*, and chlorophyll *b* content was observed. The IC_{50} values are summarized in Tables 1 and 2.

Two possible structure types are known for the compounds $Zn(carboxylate)_2 \cdot nH_2O$ in the solid state. The structure of zinc acetate dihydrate is molecular, with octahedrally coordinated zinc(II) atoms by two bidentate carboxylate oxygens and two water molecules [17]. The same structure is proposed for $Zn(ClCH_2COO)_2 \cdot 2H_2O$ and $Zn(BrCH_2COO)_2 \cdot 2H_2O$ on the basis of spectroscopic data. Structures of anhydrous complexes are polymeric [18] with tetrahedrally coordinated zinc(II) atoms connected by *syn-anti* carboxylate bridges to polymeric sheets.

At pH = 5.8 (Table 1) the comparable inhibitory activity with that of $ZnSO_4 \cdot H_2O$ was shown only by $Zn(ClCH_2COO)_2 \cdot 2H_2O$ (IC_{50} values determined for the total Chl content were 0.111 mmol dm^{-3}

Table 2. Concentrations of $ZnSO_4 \cdot H_2O$ and Zn(II) Complexes Causing 50 % Decrease of Chlorophyll Content in Statically Cultivated Green Alga *Chlorella vulgaris* (IC_{50}) at pH = 7.2

Compound	$\frac{IC_{50} \pm C.L._{\cdot 0.05}}{mmol\ dm^{-3}}$		
	Chl *total*	Chl *a*	Chl *b*
$ZnSO_4 \cdot H_2O$	0.300 (0.264—0.334)	0.277 (0.243—0.310)	0.346 (0.309—0.384)
$Zn(CH_3COO)_2 \cdot 2.5\ H_2O$	0.483 (0.413—0.598)	0.418 (0.356—0.509)	0.578 (0.444—0.777)
$Zn(CH_3CH_2COO)_2$	0.582 (0.530—0.643)	0.573 (0.516—0.620)	0.592 (0.539—0.658)
$Zn(CH_3CH_2CH_2COO)_2$	0.383 (0.316—0.486)	0.366 (0.285—0.463)	0.441 (0.379—0.606)
$Zn((CH_3)_2CHCOO)_2$	0.531 (0.477—0.601)	0.513 (0.458—0.577)	0.567 (0.508—0.652)
$Zn(CH_3CH(Cl)COO)_2$	0.565 (0.502—0.628)	0.540 (0.486—0.605)	0.622 (0.541—0.690)
$Zn(ClCH_2CH_2COO)_2$	0.225 (0.216—0.247)	0.231 (0.218—0.252)	0.219 (0.204—0.238)
$Zn(ClCH_2COO)_2 \cdot 2H_2O$	0.117 (0.088—0.141)	0.096 (0.065—0.121)	0.161 (0.134—0.188)
$Zn(BrCH_2COO)_2 \cdot 2H_2O$	0.278 (0.221—0.327)	0.258 (0.206—0.302)	0.318 (0.244—0.285)
$Zn(ICH_2COO)_2$	0.258 (0.239—0.282)	0.250 (0.227—0.278)	0.271 (0.259—0.285)

and 0.104 mmol dm^{-3}, respectively). The inhibitory activity of all other compounds was lower and the corresponding IC_{50} values varied in the range of 0.204—0.571 mmol dm^{-3}. The inhibitory effectiveness of these Zn(II) complexes decreased in the following order: $Zn(ICH_2COO)_2$, $Zn(BrCH_2COO)_2 \cdot 2H_2O$, $Zn(CH_3COO)_2 \cdot 2.5H_2O$, $Zn(ClCH_2CH_2COO)_2$, $Zn(CH_3CH_2CH_2COO)_2$ or $Zn(CH_3CH_2COO)_2$, $Zn(CH_3CH(Cl)COO)_2$, $Zn((CH_3)_2CHCOO)_2$.

The IC_{50} values obtained for the studied Zn(II) compounds at pH = 7.2 (Table 2) were higher than those obtained at pH = 5.8. At pH = 7.2 the most active compound was also $Zn(ClCH_2COO)_2 \cdot 2H_2O$ (IC_{50} = 0.117 mmol dm^{-3}). The inhibitory effectiveness of the further studied Zn(II) compounds decreased in the following order: $Zn(ClCH_2CH_2COO)_2$, $Zn(ICH_2COO)_2$, $Zn(BrCH_2COO)_2 \cdot 2H_2O$, $ZnSO_4 \cdot H_2O$, $Zn(CH_3CH_2CH_2COO)_2$, $Zn(CH_3COO)_2 \cdot 2.5H_2O$, $Zn((CH_3)_2CHCOO)_2$, $Zn(CH_3CH(Cl)COO)_2$, $Zn(CH_3CH_2COO)_2$.

The content of chlorophyll *a* in the algal suspension was more strongly affected by $ZnSO_4 \cdot H_2O$ and also by the studied carboxylatozinc(II) complexes than that of chlorophyll *b*. This was reflected in the calculated IC_{50} values for investigated compounds. In general, this parameter calculated for Chl *a* was lower than for Chl *b*. Therefore IC_{50} values for Chl *total* were determined between the above-mentioned values (Tables 1 and 2). These results fit with the findings of *DeFilippis* and *Pallaghy* [7] and *DeFilippis et al.* [9].

The studied zinc(II) complexes are water-soluble compounds and so it could be assumed that also in solution the $RCOO^-$ ligands remain coordinated to Zn^{2+} similarly as in solid state and Zn^{2+} will coordinate also H_2O molecule(s). From Table 1 it is evident that the inhibitory activity of the studied compounds depends on the chemical structure of $RCOO^-$ ligands. At pH = 5.8 for compounds with R = methyl, ethyl, propyl, and isopropyl the increase in the lipophilicity of R substituent led to decreased inhibitory activity. We assume that this activity decrease could be connected with the reduced aqueous solubility of more lipophilic compounds. Even the unfavourable steric effect of the branching of the R substituent caused a decrease of the inhibitory activity of the compound. This was reflected in lower inhibitory activity of $Zn((CH_3)_2CHCOO)_2$ with respect to that of $Zn(CH_3CH_2CH_2COO)_2$. Comparable inhibitory effectiveness to that of $ZnSO_4 \cdot H_2O$ was exhibited only by $Zn(ClCH_2COO)_2 \cdot 2H_2O$. The inhibitory activity of all other compounds was lower. Halogenocarboxylato Zn(II) compounds $Zn(ClCH_2COO)_2 \cdot 2H_2O$, $Zn(BrCH_2COO)_2 \cdot 2H_2O$, and $Zn(ICH_2COO)_2$ exhibited higher inhibitory activity than the corresponding carboxylatozinc(II) complex $Zn(CH_3COO)_2 \cdot 2.5H_2O$ indicating that the electron-withdrawing substituent contributed to the increase of inhibitory effectiveness.

Also, compound $Zn(ClCH_2CH_2COO)_2$ with halogen substituent bonded to the β-carbon was somewhat more toxic than $Zn(CH_3CH(Cl)COO)_2$. The obtained results are supported by our previous findings concerning the effects of the studied Zn(II) compounds on the inhibition of photosynthetic electron transport in suspensions of *Chlorella vulgaris* [19].

With the exception of $Zn(CH_3CH_2CH_2COO)_2$ also at pH = 7.2 the increase in the lipophilicity of R substituent in $RCOO^-$ led to decreased toxicity of the compounds. The further results fit those obtained with the studied Zn(II) compounds at pH = 5.8. Whereas at pH < 7.0 zinc is present predominantly as Zn^{2+}, in alkaline environment $[Zn(OH)]^+$, $[Zn(OH)_2]$, $[Zn(OH)_3]^-$, and $[Zn(OH)_4]^{2-}$ species are in equilibrium.

From the above-discussed results it is evident that with the increase of pH value from 5.8 to 7.2 a decrease in bioavailability of Zn(II) complexes for alga occurred, which was reflected in higher IC_{50} values obtained at pH = 7.2 (Tables 1 and 2). Our results support the findings of *Rai* and *Kumar* [8] who observed decreased toxicity of zinc to *Ch. vulgaris* at alkaline pH related to its toxicity in the concentration range of pH 4—5. It was reported that very high abundances of soluble zinc are present under well oxidization conditions and at pH from 5 to 6.5, whereas low abundances of soluble zinc are present at pH = 8 under all redox conditions and at pH from 5 to 6.5 under moderately and strongly reducing conditions [20]. *Chuan et al.* [21] found that lead, cadmium, and zinc in soil were sparingly soluble under alkaline conditions (pH = 8.0) and their solubility increased already under slightly acidic conditions (pH = 5.0).

In general, it could be concluded that the insertion of halogen atom into the ligand led to the increase of inhibitory activity of the compound, whereas the branching of the alkyl chain was connected with activity decrease. Increase of photosynthesis-inhibiting activity by introduction of halogen substituent(s) into the compound was found previously for some organic pollutants [22] and the activity-decreasing effect of the branching of the alkyl chain substituent has been observed, too [23].

In aqueous solutions of the investigated zinc(II) complexes, zinc(II) is coordinated with carboxylate ligands as well as with H_2O molecules. We assume that its toxic effect can be manifested after exchange of these ligands (mainly H_2O) with potential *S*-, *O*-, and *N*-donor ligands occurring in the algal cells. Therefore the inhibitory effectiveness of zinc(II) compounds depends on the stability of zinc(II) complexes. The lower ability of the complex to exchange its ligands is reflected in its lower biological activity. In general, organic ligands could also affect the transport of metal ions through the lipophilic regions of cell membranes. However, it is also possible that some metal complexes are not able to reach their site of action in the algal cell in sufficient concentration due to their decreased aqueous solubility. The obtained results support our earlier findings concerning the study of effects of some Cu(II) complexes on the inhibition of photosynthetic electron transport in spinach chloroplasts [24, 25] and suspensions of *Chlorella vulgaris* [15] as well as inhibition of chlorophyll content in the statically cultivated green alga *Ch. vulgaris* [15, 25].

Acknowledgements. The research was supported by the Grant Agency VEGA, Grant No. 1/0089/03.

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