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SUPEROXIDE DISMUTASE MIMETIC ACTIVITY OF CYTOKININ-COPPER(II) COMPLEXES

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SUMMARY: Dissociation constants of cytokinins, derivatives of purine which form complexes with cupric ion, were determined by spectrophotometry and the stability constants of their copper complexes by pH titration. The values found for kinetin were 3.76, 9.96, 7.8, and 15.3 for pK_1, pK_2, $\log k_1$, and $\log \beta_2$, respectively, and those for 6-benzylaminopurine were, in the same order, 3.90, 9.84, 8.3, and 15.9. The copper(II) complexes with kinetin and 6-benzylaminopurine had superoxide dismutase mimetic activity, and the reaction rate constants with superoxide, which were determined by polarography, were $2.3 \times 10^7\ M^{-1}s^{-1}$ for kinetin and $1.5 \times 10^7\ M^{-1}s^{-1}$ for 6-benzylaminopurine at pH 9.8 and 25°C.

Senescence is a multifactorial syndrome, and free radicals, particularly oxy free radicals, seem to play an important role in aging processes (1). In higher plants, free radical-associated phenomena include postharvest fruit spoilage, leaf senescence, and accelerated wilt of cut flowers (2-6), and senescence-associated parameters can be effectively retarded by cytokinins (CK), a group of plant growth regulators. Thus, the interaction of cytokinin with free radicals needs to be elucidated. Leshem et al. demonstrated that CK interaction with free radicals could occur in two ways: (i) CK may act as a direct free radical scavenger; (ii) CK may serve as an incipient preventative of free radical formation by inhibiting oxidation of plant purine compounds by enzyme such as xanthine oxidase (7-9). Frimer et

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Abbreviations; SOD, superoxide dismutase; CK, cytokinin; 3,5-DIPS, 3,5-diisopropylsalicylic acid; 6-BAP, 6-benzylaminopurine.

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al. (8) demonstrated that superoxide reacts with (arylmethyl)-amines by extraction of the hydrogen of the α-carbon atom in the amine bond in an aprotic solvent, but it is not clear whether superoxide reacts with CK so efficiently in an aqueous solution. Now we investigated the possibility that CK might form a complex with copper and effectively remove superoxide by SOD mimetic activity. Since the chloroplast is abundant in copper, if a CK-copper complex is formed and has SOD-like activity, it may participate in senescence retardation by quenching of superoxide. This communication describes the stability constants and SOD mimetic activities of CK-copper complexes.

Materials and Methods

Materials and apparatus: Kinetin was obtained from Tokyo Chemical Industry Co., Ltd., 6-benzylaminopurine (6-BAP) from Wako Pure Chemical Industries, Ltd., and copper(II) sulfate (99.6 %) from Kanto Chemical Co., Inc. UV and visible spectra were obtained with a Hitachi 557 double-beam spectrometer. The pH was determined with a pH meter, Model PT-30 (Tokyo Kagaku Sangyo Co., Ltd.).

Determination of dissociation constants: The dissociation constants of kinetin and 6-BAP were determined by spectrophotometric method. Solutions containing 0.1 mM of kinetin or 6-BAP and various concentrations of HCl or NaOH were prepared. The ionic strength of the solution was maintained at 0.1 by adding NaCl. The UV spectra and pH were measured at 25 °C.

Determination of stability constants: The stability constants of CK-copper complexes were determined by Bierrum's method (10,11). Solutions containing 0.02 mM $CuSO_4$ and 0.2 mM kinetin or 6-BAP were prepared and titrated with standard NaOH (0.01 N) at 25 °C. The ionic strengths of the solutions were maintained at 0.1 by adding NaCl.

Determination of SOD mimetic activity: SOD mimetic activities of copper complexes were polarographically determined (12,13) using a Yanaco voltammetric analyzer Model P-1000. A copper complex was prepared in solution by mixing the ligand and $CuSO_4$. A copper complex concentration of between 10^{-8} and 10^{-6} M was achieved by subsequent addition of the copper complex to a buffered solution of 0.1 M sodium borate containing 0.5 mM triphenylphosphine oxide. After each addition, the height of the polarographic wave was recorded. The rate constant of dismutation was calculated by measuring the increment of the polarographic wave as a function of the copper complex concentration, according to the equation of Koutecky et al. (14).

$$((7.42 I_1/I_d) - 7.42)/(2.25 - 1.25(I_1/I_d)) = k[C]t_g$$

I_1 and I_d are the mean limiting currents either in the presence or absence of the copper complex, respectively, t_g the drop time of mercury electrode, [C] the concentration of the copper complex and k the rate constant of the reaction.

Results and Discussion

The formation of complexes between CK and Cu^{2+} in ethanol was observed by spectrophotometric measurements. The compound formed in ethanol solution by reaction of 6-BAP and Cu^{2+} was identified by the method of continuous variation (15,16). The results indicated the existence of a compound having the composition $Cu(6\text{-}BAP)_2$ (Fig. 1). In aqueous solution, the formation of a complex by 6-BAP and Cu^{2+} was also observed by spectrophotometric measurement, but the absorption was too small to determine the composition by the continuous variation method. However, as the release of proton from 6-BAP with the formation of the copper complex was maximum when the ratio of 6-BAP and Cu^{2+} was 2:1, the composition was expected to be $Cu(6\text{-}BAP)_2$.

The dissociation constants (pK_1 and pK_2) of kinetin and 6-BAP were determined by spectrophotometric method (Table I). The dissociation constants and the pH titration results for solutions containing Cu^{2+} and CK were used to draw the formation curves by the method of Bjerrum (Fig. 2), and the stability constants were

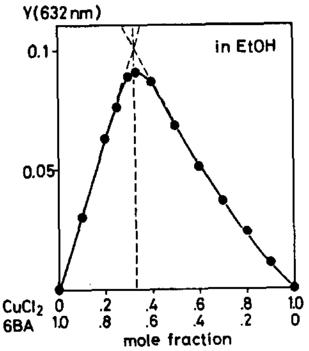

Fig. 1. Continuous variation plot for copper(II)-6-benzylaminopurine complex in ethanol.

Total concentration of $CuCl_2$ and 6-BAP was 1.0 mM.

Table I. Dissociation constants of cytokinins and stability constants of their copper complexes (25°C, ion strength 0.1)

	pK_1	pK_2	$\log k_1$	$\log \beta_2$
Kinetin	3.76 ± 0.02	9.96 ± 0.04	7.8	15.3
6-Benzylaminopurine	3.90 ± 0.10	9.84 ± 0.19	8.3	15.9

calculated (10,11) (Table I). The constants of the following equilibria 1 and 2 are expressed by k_1 and k_2, respectively, and β_2 is the overall stability constant (i.e., $\beta_2 = k_1k_2$).

$$Cu^{2+} + CK^- \xrightleftharpoons{k_1} [Cu(CK)]^+ \quad (1)$$

$$[Cu(CK)]^+ + CK^- \xrightleftharpoons{k_2} Cu(CK)_2 \quad (2)$$

As the reduction of nitrobluetetrazolium by KO_2 was inhibited by $Cu(6\text{-}BAP)_2$, the SOD mimetic activity of the complex was expected (data not shown). The rate constants of the reaction between superoxide and the copper complexes were determined by polarographic procedure (12,13) (Table II). The SOD mimetic

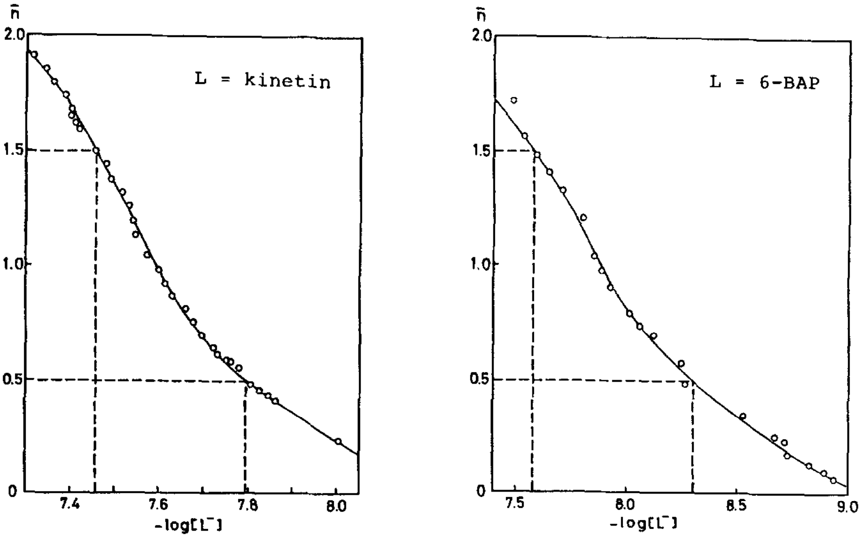

Fig. 2. Formation curves of copper(II)-cytokinin complexes at 25°C and ionic strength 0.1.

Table II. Rate constants for reaction of superoxide with copper compounds

	pH	k, $M^{-1}s^{-1}$	
SOD	9.8	$(1.8 \pm 0.2) \times 10^9$	this work
	7-9	2×10^9	ref. 22
$CuSO_4$	9.8	$(1.7 \pm 0.1) \times 10^7$	this work
	6	5.0×10^8	ref. 23
$Cu(kinetin)_2$	9.8	$(2.3 \pm 0.1) \times 10^7$	this work
$Cu(6\text{-benzylaminopurine})_2$	9.8	$(1.5 \pm 0.2) \times 10^7$	this work
$Cu(salicylic\ acid)_2$	9.8	$(2.0 \pm 0.2) \times 10^7$	this work

activity of complexes formed between CK and Cu^{2+} were equivalent at pH 9.8 to that of Cu(salicylic acid)$_2$, which is known to have SOD-like activity ($k = (1.64 \pm 0.15) \times 10^9\ M^{-1}s^{-1}$ at pH 7.5) (17). The rate constant for spontaneous second-order decay of superoxide is about $10^3\ M^{-1}s^{-1}$ at pH 9.8 and $10^5 M^{-1}s^{-1}$ at pH 7 (18). The pH level does not affect SOD because of the participation of lysine residues (19), but it did affect the activities of the copper complexes. The CK-copper complex may be more reactive with superoxide at pH 7 than at pH 9.8.

Superoxide is more stable and reactive in an aprotic solvent than in a protic solvent. The protonation is considered to control the reactivity and longevity of superoxide, therefore, in living systems, superoxide may be more stable and reactive at a hydrophobic site, e.g., in a membrane (20) or in a hydrophobic core of protein, than in a cytosol. SOD catalyzes disproportionation of superoxide and depresses oxygen toxicity. Native SOD is a water-soluble enzyme which has a high molecular weight and as a result is thought to be less effective against superoxide present at a hydrophobic site.

Recently, low molecular weight, lipophilic copper complexes with SOD-mimetic action, such as Cu(3,5-DIPS)$_2$ have been attracting attention. Sorenson (21) reported that copper complexes including copper anthranilate, aspirinate and 3,5-DIPS have anti-inflammatory activity stronger than the ligands. As we found that CK forms a complex with copper, which has SOD mimetic activity and is lipophilic, the complex may possess activity like $Cu(3,5\text{-}DIPS)_2$ and be able to participation in senescence retardation.

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