

# The 1,10-phenanthroline micelles–copper(I) complex catalyzes protein degradation

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**Abstract** The 1,10-phenanthroline micelles–copper(I) coordinated complex, not the mono-dispersed bis 1,10-phenanthroline–copper(I) one, rapidly degraded proteins in the presence of  $\beta$ -mercaptoethanol in the neutral-to-mild acidic region under aerobic conditions. The degradation products derived from  $\alpha$ -casein were peptides and amino acids.

**Key words:** Artificial protease; 1, 10-Phenanthroline; Copper(I)

## 1. Introduction

There are various types of protease in nature [1]. However, a powerful protease, natural or artificial, that completely degrades a protein to amino acids is not known. When we examined the effects of chemical compounds on the cysteine proteases from rat endoplasmic reticulum [2–4] using a commercial protein-disulfide isomerase (PDI) as the substrate, PDI was rapidly degraded on the addition of 1,10-phenanthroline (OP) to the reaction mixture. However, PDI further purified by hydroxyapatite column chromatography was resistant to the degradation. Then, a very small amount of copper ions was found to have contaminated the commercial PDI preparation. On the basis of these findings, we succeeded in showing that OP micelles complexed with copper(I) degrade  $\alpha$ -casein to peptides and amino acids in the presence of  $\beta$ -mercaptoethanol. However, mono-dispersed bis OP–copper(I) showed no activity. Such a reaction, which should facilitate the hydrolysis of polypeptides to amino acids with a single catalyst, has not previously been reported.

## 2. Experimental

### 2.1. Materials

OP, 2,9-dimethyl 1,10-phenanthroline, and  $\alpha$ -casein were purchased from Sigma. Bovine liver PDI was from Takara Shuzo Co., Kyoto. All other chemicals were of reagent grade.

### 2.2. Assay of the protease reaction

The protease reaction was carried out in 10 mM bis(2-hydroxyethyl) iminotris (hydroxymethyl) methane (bis-Tris)-HCl (bis-Tris buffer), pH 6.3, containing 1.5  $\mu$ g  $\alpha$ -casein or PDI, 10  $\mu$ M cupric sulfate, 3 mM OP and 100 mM  $\beta$ -mercaptoethanol. The final volume was 10  $\mu$ l. After a 1-h incubation at 37°C, the reaction was terminated by the addition of

4  $\times$  SDS sample buffer (4  $\mu$ l) [5], followed by analysis by SDS-PAGE [5].

### 2.3. Analysis of peptides among the reaction products

The reaction (1 mg  $\alpha$ -casein in 30 ml) was carried out under the above assay conditions except that 10 mM acetate buffer, pH 6.0, was used instead of bis-Tris buffer. After a 3-h incubation, the reaction mixture was lyophilized and the residue dissolved in 0.5 ml of 0.1% trifluoroacetic acid. 50  $\mu$ l of the sample solution was applied to a  $\mu$ Bondasphere C<sub>18</sub> high performance liquid chromatography (HPLC) column (0.39  $\times$  15 cm). Amino acids and peptides were eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid at 30°C, at the flow rate of 1 ml/min. The elution was monitored as to the absorbance at 214 nm. Fractions were collected and analyzed with a Protein Sequencer, Model 477A (Applied Biosystems Inc.).

### 2.4. Analysis of amino acids among the reaction products

The reaction was carried out as described in section 2.3. After the reaction, norleucine was added to the reaction mixture as an internal standard. In order to remove OP, the reaction mixture was applied to a Dowex 50W x8 (H<sup>+</sup>) column and eluted with 1 N ammonium in water. The effluent was dried in a rotary evaporator. The residue was dissolved in 0.2 M sodium citrate buffer, pH 2.2, for amino acid analysis. An aliquot of the sample was analyzed with an Amino Acid Analyzer, Model 835 (Hitachi Ltd., Tokyo).

## 3. Results and discussion

Almost all of the  $\alpha$ -casein was degraded under the assay conditions given in section 2 (Fig. 1, lanes 1, 2 and 6). The reaction required OP, Cu<sup>2+</sup> and  $\beta$ -mercaptoethanol. With the omission of any one of the components,  $\alpha$ -casein was not degraded at all (Fig. 1, lanes 3–5). PDI was also degraded (Fig. 1, lanes 7 and 8). Bovine serum albumin and soy glycinine were similarly degraded (data not shown).  $\alpha$ -Casein was mostly degraded within 1 h under the assay conditions used (Fig. 2A). The reaction proceeded in the mild acidic region but not in the mild basic region (Fig. 2B). This degradation depended on the temperature (Fig. 2C). OP could not be replaced by ethylenediaminetetraacetic acid, 2,2'-bipyridine or 2,9-dimethyl 1,10-phenanthroline (data not shown). Fe<sup>3+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup> and Ni<sup>2+</sup> could not be substituted for Cu<sup>2+</sup> (data not shown). This suggests an unusual reactivity of the OP–copper(II) coordinated complex.

As for the degradation products derived from  $\alpha$ -casein, polypeptide fragments were hardly detected on the SDS gel (Fig. 1, lanes 2 and 6), suggesting that polypeptides were further degraded. In order to identify the reaction products, the reaction mixture was analyzed by reverse-phase HPLC and with an Amino Acid Analyzer. Individual major fractions obtained on HPLC (fraction numbers 4–8) were composed of two or more peptide fragments (Fig. 3A). When  $\alpha$ -casein was assumed to be completely hydrolyzed to amino acids, the yield of glycine was 14.6% (Fig. 3B). This value is much higher than those for the other amino acids. Thus, the OP–copper(II) complex may pref-

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**Abbreviations:** PDI, protein-disulfide isomerase; OP, 1,10-phenanthroline; bis-Tris buffer, bis(2-hydroxyethyl) iminotris (hydroxymethyl) methane-HCl; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

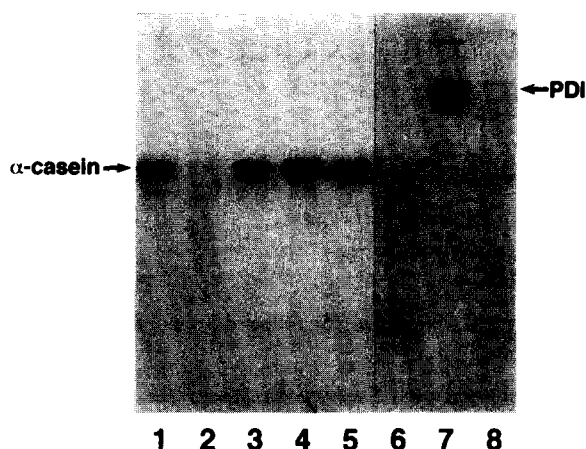
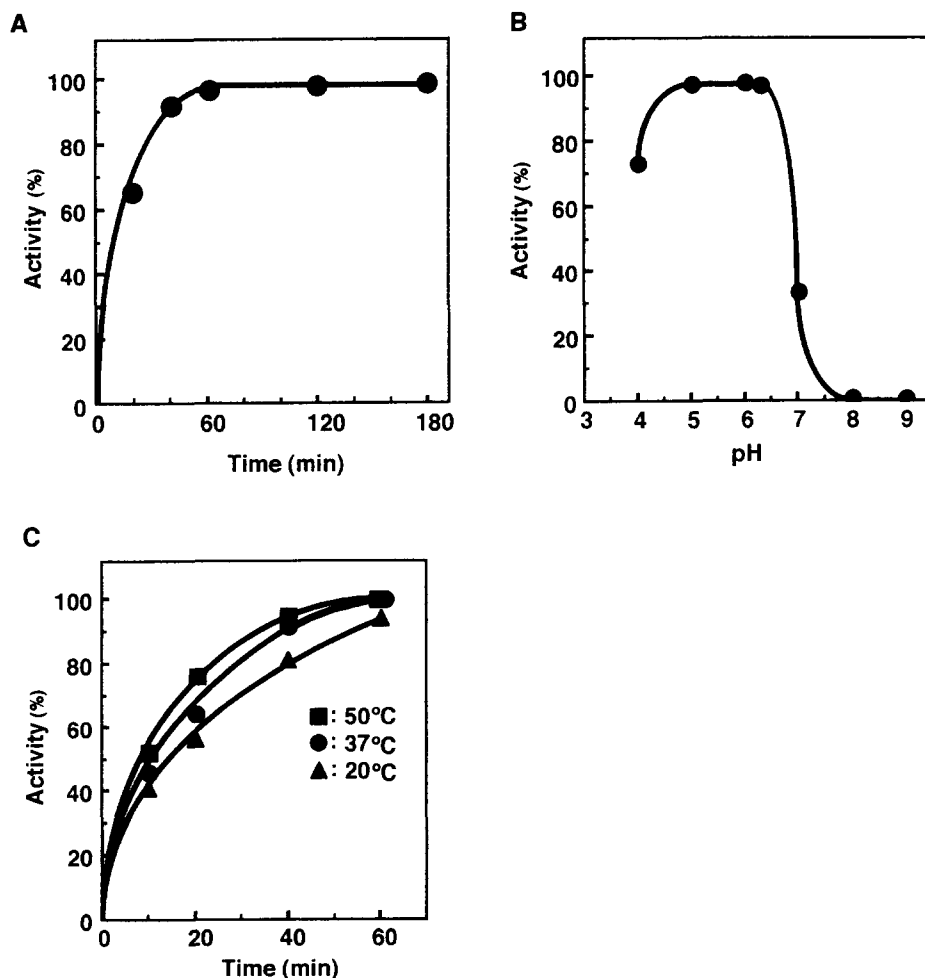


Fig. 1. Degradation of proteins by the OP-copper ion complex.  $\alpha$ -Casein (lanes 1–6) or PDI (lanes 7 and 8) were incubated under the assay conditions given section 2 except that PDI was incubated for 3 h: lanes 1 and 7, without incubation; lane 3, without 3 mM OP; lane 4, without 10  $\mu$ M cupric sulfate; lane 5, without 100 mM  $\beta$ -mercaptoethanol; lane 6, 10 mM acetate buffer, pH 6.0, instead of bis-Tris buffer. After the incubation, the samples were subjected to SDS-PAGE on 15% gels.

entially cleave some specific peptide linkages containing especially glycine. These results suggest that the OP-copper(II) complex behaves like an endoprotease rather than an exoprotease. However, this complex may also have peptidase-like activity, since free amino acids were produced.

It appears that OP, at ten times the concentration, will sequester all  $\text{Cu}^{2+}$  as a 2:1 complex of OP-copper(II) to bis OP-copper(II). However, under such conditions (100  $\mu$ M OP and 10  $\mu$ M cupric ions), the 2:1 complex failed to degrade  $\alpha$ -casein (Fig. 4A). The OP-copper(II) complex exhibited protease activity at OP concentrations above 650  $\mu$ M (Fig. 4A). Thus, extremely high OP concentrations were required for the reaction. This suggests that OP assembles to form certain aggregates, since it has an amphipathic structure. Then, its colloidal behavior was determined in 10 mM bis-Tris buffer, pH 6.3, at 37°C (Fig. 4B). As expected, OP was found to form micelles, with a critical micellar concentration of 650  $\mu$ M (Fig. 4B). Therefore, it seems likely that the OP micelles-copper(II) complex behaves like a chemical enzyme, catalyzing protein degradation.

2,9-Dimethyl 1,10-phenanthroline, a cuprous-specific chelating agent [6], inhibited the degradation of  $\alpha$ -casein (data not shown). This may indicate that the OP micelles-copper(I) complex rather than the OP micelles-copper(II) one plays an essential role in the hydrolysis of peptide linkages.  $\beta$ -Mercaptoetha-



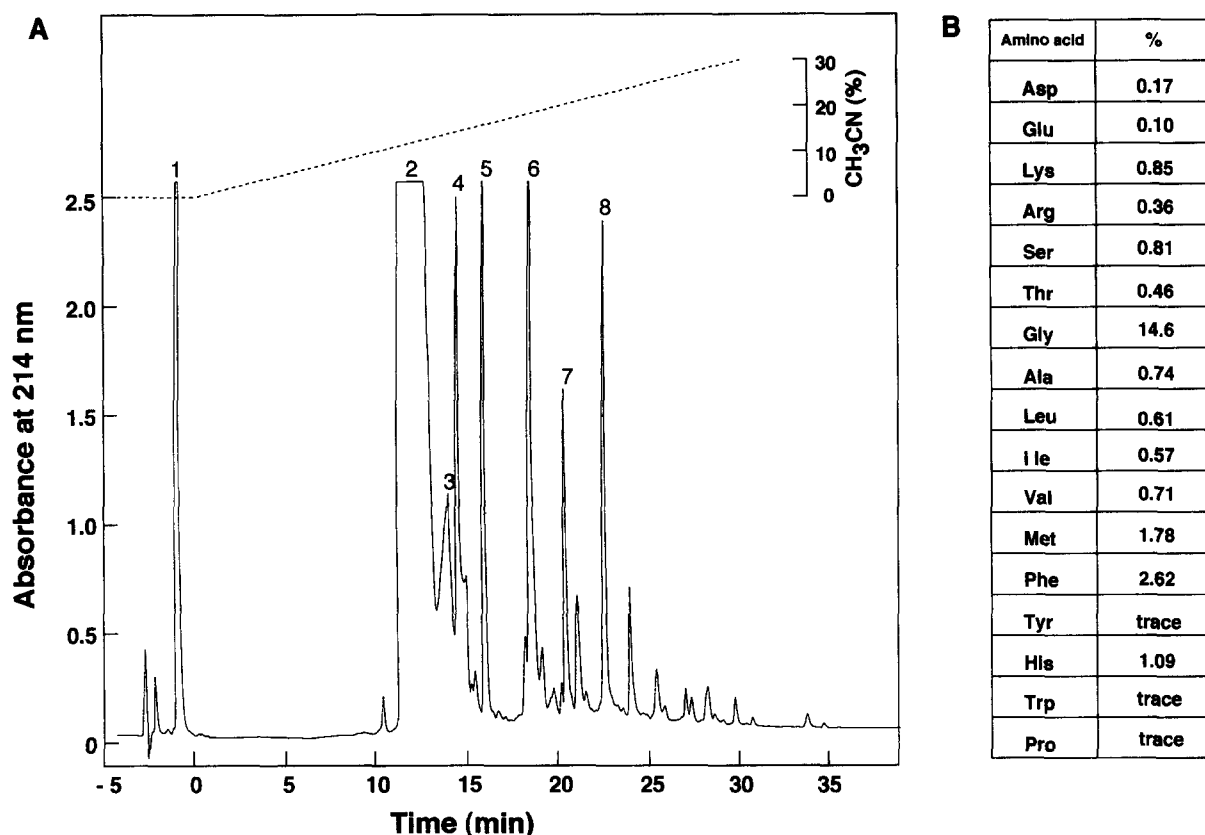


Fig. 3. Reaction products derived from  $\alpha$ -casein. The reaction products derived from  $\alpha$ -casein were analyzed as described in section 2. (A) The elution profile on reverse-phase HPLC. 1 and 3, amino acids; 2, OP; 4–8, two or more peptides in each fraction. (B) Production of amino acids. The yields (%) of amino acids were calculated, the amount of each amino acid obtained on hydrolysis of  $\alpha$ -casein in 6 N HCl being taken as 100%.

nol may generate the OP micelles–copper(I) complex by reducing the OP micelles–copper(II) one. On the other hand, the degradation reaction proceeded under aerobic conditions but not under anaerobic conditions (Fig. 5, lanes 2 and 3). However, the reaction proceeded on the addition of H<sub>2</sub>O<sub>2</sub> to the reaction mixture under anaerobic conditions (Fig. 5, lane 4), whereas this effect of H<sub>2</sub>O<sub>2</sub> was not seen in the absence of Cu<sup>2+</sup> (lane 5). From the several lines of evidence described above, it is assumed that oxygen serves as a precursor of H<sub>2</sub>O<sub>2</sub> via superoxide radical formation [7–10]. This reaction may be coupled to the in situ oxidation of  $\beta$ -mercaptoethanol catalyzed by either the bis OP–copper(II) or OP micelles–copper(II) complex, of which copper(II) will be reduced to copper(I). Then, a hydroxy free radical ( $\cdot$ OH) may be formed from H<sub>2</sub>O<sub>2</sub> [10,11] by the complexed copper(I). However, mannitol or ethanol, hydroxy free radical scavengers, did not inhibit the reaction (data not shown). This suggests that reactive diffusible species do not participate in the reaction. However, we cannot exclude the case of a hypothetical intermediate, 'crypto  $\cdot$ OH', bound to

the copper(II), which may be resistant to hydroxy radical scavengers.

The bis OP–copper(I) complex failed to act as a catalyst for the protein hydrolysis (Fig. 4B), although this complex may produce  $\cdot$ OH [9,11] and cleave the phosphoester of DNA [8]. It was found that the OP micelles–copper(I) complex may serve as a chemical protease. Probably, OP micelles provide a structure necessary for their association with proteins. Contrary to in the case of this complex, it seems difficult for the mono-dispersed bis OP–copper(I) one to bind properly to proteins. Thus, an important feature of the degradation reaction may be the binding of the OP micelles–copper(I) complex to a protein. Thus, it is speculated that a 'crypto  $\cdot$ OH' of the OP micelles–copper(I) complex may effectively attack the target peptide linkages.

Although the exact mechanism of action remains to be elucidated, our findings strongly suggest an important role for the micelle formation of inactive mono-dispersed components in protease activity.

Fig. 2. Time-course, optimal pH and temperature-dependent manner of the degradation of  $\alpha$ -casein. The reaction was carried out under the assay conditions given in section 2 except that the incubation time was changed (A), pH was changed (B), and the temperature was changed (C), as shown in the figure. Then, the samples were subjected to SDS-PAGE on 15% gels.  $\alpha$ -Casein fractions stained with Coomassie brilliant blue R-250 were determined at 550 nm with a densitometer. The activity was calculated according to the equation:  $\alpha$ -casein [at 0 time–after the reaction]/ $\alpha$ -casein at 0 time  $\times$  100 (%). The mean values for duplicate experiments are plotted.

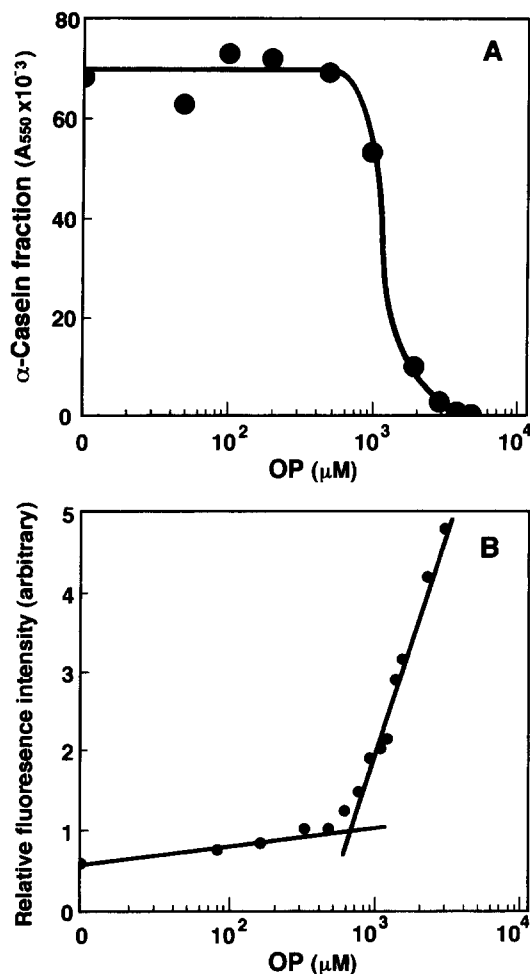


Fig. 4. Relation between protease activity and micelle formation of OP. (A) The degradation of  $\alpha$ -casein was carried out under the assay conditions given in section 2 except that the OP concentration was changed. Then, samples were subjected to SDS-PAGE on 15% gels.  $\alpha$ -Casein fractions stained with Coomassie brilliant blue R-250 were determined at 550 nm with a densitometer. The mean values for duplicate experiments are plotted. (B) The critical micellar concentration of OP was determined according to Chattopadhyay and London [12] in 10 mM bis-Tris buffer, pH 6.3, with various concentrations of OP. Fluorescence was measured at an excitation wavelength of 358 nm and an emission wavelength of 430 nm. The critical micellar concentration was read at the intercept of the lines through the data points. The data are means for duplicate experiments.

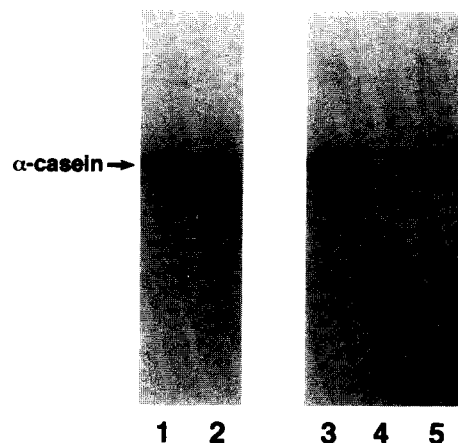


Fig. 5. The degradation of  $\alpha$ -casein under aerobic or anaerobic conditions. The reactions were carried out under the assay conditions given in section 2 (control), under aerobic or anaerobic conditions, the solutions being de-aerated by bubbling with  $O_2$ -free  $N_2$  gas. 1, 0-time control; 2, control under aerobic conditions; 3–5, under anaerobic conditions; 3, control; 4, +  $H_2O_2$ ; 5, +  $H_2O_2$  without  $Cu^{2+}$ .

## References

- [1] Webb, E.C. (1992) Enzyme Nomenclature, pp. 371–421, Academic, San Diego.
- [2] Urade, R., Nasu, M., Moriyama, T., Wada, K. and Kito, M. (1992) J. Biol. Chem. 267, 15152–15159.
- [3] Urade, R., and Kito, M. (1992) FEBS Lett. 312, 83–86.
- [4] Urade, R., Takenaka, Y. and Kito, M. (1993) J. Biol. Chem. 268, 22004–22009.
- [5] Laemmli, U.K. (1970) Nature 227, 680–685.
- [6] Hall, J.R., Marchant, N.K. and Plowman, R.A. (1962) Aust. J. Chem. 15, 480–485.
- [7] Sigman, D.S., Graham, D.R., D'Aurora, V. and Stern, A.M. (1979) J. Biol. Chem. 254, 12269–12272.
- [8] Marshall, L.E., Graham, D.R., Reich, K.A. and Sigman, D.S. (1981) Biochemistry 20, 244–250.
- [9] Goldstein, S. and Czapski, G. (1983) J. Am. Chem. Soc. 105, 7276–7280.
- [10] Misra, H.P. (1974) J. Biol. Chem. 249, 2151–2155.
- [11] Johnson, G.R.A. and Nazhat, N.B. (1987) J. Am. Chem. Soc. 109, 1990–1994.
- [12] Chattopadhyay, A. and London, E. (1984) Anal. Biochem. 139, 408–412.