Substrate Specificities of Deuterolysin from *Aspergillus oryzae* and Electron Paramagnetic Resonance Measurement of Cobalt-substituted Deuterolysin

Yuko DOI,1 Byung Rho LEE,2 Masamichi IKEGUCHI,1,2 Yasunori OHBA,3 Tadaaki IKOMA,3
Shozo TERO-KUBOTA,3 Seigo YAMAUCHI,3 Koji TAKAHASHI,4 and Eiji ICHISHIMA1,2,†

1Department of Bioengineering, Graduate School of Engineering, Soka University, Hachioji, Tokyo 192-8577, Japan
2Department of Bioengineering, Faculty of Engineering, Soka University, Hachioji, Tokyo 192-8577, Japan
3Institute of Multidisciplinary Research for Advanced Materials (IMRAM), Tohoku University, Katahira 2-1-1, Sendai 980-8577, Japan
4Department of Applied Biological Science, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-0054, Japan

Received June 25, 2002; Accepted October 9, 2002

The substrate specificities of deuterolysin, a 19-kDa zinc-protease (EC 3.4.24.39) from *Aspergillus oryzae*, were investigated at pH 9.0 with various fluorogenic acyl-peptide-4-methylcoumaryl-7-amides (peptide-MCAs). N-Butoxycarbonyl-Arg-Val-Arg-Arg-MCA was the best substrate for deuterolysin. We therefore measured its kinetic parameters. Deuterolysin had high activity toward the peptide bonds next to pairs of basic residues in calf thymus histone H4. The specificity of cobalt-substituted deuterolysin (Co-deuterolysin) for peptide-MCAs was similar to that of native deuterolysin. The CD spectrum of Co-deuterolysin was similar to that of the native deuterolysin. The metal coordination sphere of Co-deuterolysin was analyzed by Q-band (33.9570 GHz) electron paramagnetic resonance (EPR) spectroscopy. Using computer simulation of EPR, we found the $g$ principal values to be $g_x = 5.20$, $g_y = 4.75$, and $g_z = 2.24$; the metal center was a divalent cobalt ion in a high spin state.

Key words: cobalt-substituted; deuterolysin; electron paramagnetic resonance; substrate specificity; zinc-protease

Deuterolysin (EC 3.4.24.39) from *Aspergillus oryzae* contains one mole of zinc per mole of enzyme, and has a single chain of 177 amino acid residues, three disulfide bonds, and a molecular mass of 19,018 Da.1,2 The enzyme has a hitherto-unknown zinc-binding motif, aspzin, defined as the “HEX-XH+D” motif with an aspartic acid residue as the third ligand.3 Deuterolysin from *Aspergillus sojae* is extremely stable at 100°C, but unstable near 75°C,4 possibly because of self-digestion. The thermal stability at 100°C of deuterolysin from *A. oryzae* has been reported and discussed.5 Deuterolysin from *A. sojae* is highly active toward basic nuclear proteins, but has low activity toward substrates routinely used in the laboratory.5) Deuterolysin from *A. sojae* seems not to act on small synthetic peptides.6) Activity of deuterolysin from *A. oryzae* have been measured using a fluorescent substrate N-butoxycarbonyl-Val-Leu-Lys-4-methylcoumaryl-7-amide (Boc-Val-Leu-Lys-MCA).1)

Recently, McAuley et al.7) described the application of a new direct-method program, ACORN, to identify the structure of a zinc metalloproteinase, the deuterolysin from *A. oryzae*, for which atomic resolution data could be measured but no similar model was available. This paper reports the first identification of the structure of an unknown protein with ACORN. The C-terminal sequence of 156 amino acids of the pro-region of deuterolysin has Arg-Arg.1) It suggests that catalytic cleavage of the peptide bond at Arg→Thr1 at the junction between the pro-region and the mature region may occur during self-digestion of deuterolysin. Site-directed mutagenesis experiments showed that three essential amino acid residues, Glu129, Asp143, and Tyr106, are catalytically crucial in the deuterolysin from *A. oryzae*.3) In a previous paper, we found that three site-directed mutants, Y106F, E129Q, and D143N, have complete loss of the converting activity of proenzyme to mature form.7) The results meant that the catalytic cleavage of this peptide bond at Arg→Thr1 did occur through self-digestion of deuterolysin. We
hypothesized that the dibasic amino acids in the peptide-MCAs are useful substrates of deuterolysin. We describe here the cleavages of pairs of basic residues by deuterolysin from *A. oryzae* with fluorogenic peptide-MCAs. We also report the specificity toward the dibasic residues for calf thymus histone H4.

The value of EPR spectroscopy in establishing electronic structures and their dependence on metal coordination-sphere composition and geometry has been illustrated by its application to several aspects of cobalt bioinorganic chemistry. From *A. oryzae* has a zinc-binding motif, but, the zinc is not detected EPR. Removal of the zinc ion from *A. oryzae* deuterolysin yields the inactive apoenzyme, which can also be reactivated by stoichiometric amounts of zinc ions. Moreover, the addition of cobalt ions to the apoenzyme results in an enzymatically active species. In this study, we purified Co-deuterolysin to examine cobalt coordination and geometry by EPR. We are interested in the structural basis for the observed EPR spectral perturbation.

**Materials and Methods**

**Materials.** Protease M from *Aspergillus oryzae* was the gift of Amano Enzyme, Co. (Nagoya, Japan). N-Butoxycarbonyl-L-arginyl-L-valyl-L-arginyl-L-arginyl-4-methylcoumaryl-7-amide (Boc-Arg-Val-Arg-Arg-MCA) and other fluorogenic peptide-MCAs were purchased from the Peptide Institute, Inc. (Mino, Osaka, Japan). Histone H4 from calf thymus was purchased from Boehringer (Mannheim, Germany).

**Purification of deuterolysin.** Fifty grams of commercially available Protease M was dissolved in 10 volumes of distilled water. The supernatant of the crude enzyme solution was treated at 70°C for 15 min and then placed on ice for 10 min. The solution was centrifuged at 6,000 × g for 20 min. Ammonium sulfate was added to the supernatant to a concentration of 80% saturation. The solution was left for 3 h at 4°C and centrifuged at 6,000 × g for 20 min. The precipitate was suspended in 50 ml of Milli Q water. The solution was dialyzed overnight against 10 mM sodium acetate buffer, pH 5.0, at 4°C. The mixture was centrifuged at 10,000 × g for 20 min, and the supernatant was put on a Super Q column (2.5 × 20 cm) equilibrated with 10 mM sodium acetate buffer, pH 5.0. Elution was done with a linear gradient of 5–11% NaCl in 10 mM sodium acetate buffer, pH 5.0. The active fractions were collected and dialyzed overnight against 10 mM sodium acetate buffer, pH 5.0. Purity was confirmed by SDS-PAGE with a 12% (w/v) gel, which was stained with Coomassie brilliant blue R250. On SDS-PAGE, a protein with a molecular mass of 15.3 kDa was seen. The specific activity of deuterolysin was 0.13 mkat per kg of protein with Boc-Arg-Val-Arg-Arg-MCA as the substrate. The yield of deuterolysin was about 2%.

**Purification of Co-deuterolysin.** The purified deuterolysin was incubated in 10 mM sodium acetate buffer, pH 5.0, in the presence of 0.5 mM EDTA at 30°C for 20 min. The solution was put on a Sephadex TM G-50 Fine column (2.5 × 100 cm) and eluted with 10 mM sodium acetate buffer, pH 5.0, containing 0.5 mM EDTA. The apo-deuterolysin was collected and dialyzed against Milli Q water at 4°C overnight. The apo-deuterolysin was incubated in 1 mM cobalt sulfate at 0°C for 30 min. The solution was put on a Sephadex TM G-50 Fine column (1 × 30 cm) and eluted with Milli Q water. Co-deuterolysin was dialyzed against Milli Q water at 4°C overnight and concentrated by ultrafiltration, Resource Q chromatography, or both. The specific activity of Co-deuterolysin was 0.09 mkat per kg of protein. The yield of Co-deuterolysin was about 1%.

**Assay of enzyme activity.** Standard conditions for the assay of enzyme activity was Boc-Arg-Val-Arg-Arg-MCA as the substrate, in 50 mM Tris-HCl buffer, pH 9.0, with the assay at 30°C. The purified enzyme (42 nmol) was first incubated in 895 μl of 50 mM Tris-HCl buffer, pH 9.0, at 30°C for 10 min to which 5 μl of 10 mM peptide-MCA substrate was added. The initial rate of increase in the 7-amino-4-methylcoumarine (AMC) in enzymatic hydrolysates by deuterolysin was monitored fluorometrically at excitation λex at 360 and emission λem at 440 nm, respectively, with a Shimadzu RF-5000 spectrophotometer; 1 kat of deuterolysin activity was defined as the amount of enzyme needed to release 1 mol of AMC from Boc-Arg-Val-Arg-Arg-MCA at 30°C and pH 9.0.

**Heat stability experiment.** Deuterolysin or Co-deuterolysin at the concentration of 42 nmol (0.8 mg/ml) in Tris-HCl buffer, pH 9.0, was incubated at different temperatures for 10 min in a temperature-controlled bath. After incubation, the enzyme solution was cooled immediately on ice for 10 min and the remaining activity was measured with Boc-Arg-Val-Arg-Arg-MCA as the substrate at pH 9.0.

**Location of cleavage site of histone.** Ten nanomole of histone H4 was incubated with 20 nmol of deuterolysin at 30°C in 0.1 ml of 50 mM phosphate buffer, pH 7.0. After 60 min, the enzymatic reaction was stopped by the addition of 10 μl of 100% trifluoroacetic acid (TFA) and the mixture was centrifuged at 12,000 × g for 20 min. Small peptides generated in 20 μl of supernatant were separated by high-pressure liquid chromatography with a Shimadzu LC6AD system and ODS column with a
linear gradient of acetonitrile from 10 to 60% containing 0.05% TFA in 30 ml. The flow rate was 1 ml min\(^{-1}\) and detection was done at 220 nm. N-Terminal amino acid sequences of the small peptides obtained were analyzed with an Applied Biosystems 743A protein sequencer.

**Protein measurement.** Protein concentrations were measured by the Bradford method,\(^{10}\) with a bovine serum albumin as the standard.

**CD measurement.** Deuterolysin and Co-deuterolysin were dialyzed in 50 mM Boric buffer, pH 9.0, and diluted in the same buffer at the concentration of 21 mM (0.4 mg/ml). The far-UV CD spectra of the two samples were measured with a Jasco J-720 spectrophotometer (Tokyo, Japan) at 25°C in a cuvette with a 1 mm path length.\(^{11,12}\) The proportions of \(\alpha\)-helix, \(\beta\)-structure and other kinds of secondary structure of the enzyme were calculated with the CONTIN circular dichroism diskette (CPC Program Library, Queen’s University of Belfast, Ireland).\(^{13,15,16}\)

**Atomic absorption spectrophotometry.** Cobalt analysis of the Co-deuterolysin was done by atomic absorption spectrophotometry with a Shimazu model AA-660 (P/N206-1000-02) apparatus (Kyoto, Japan).

**Optical absorption spectra.** An optical absorption spectrum of Co-deuterolysin at the concentration of 0.57 mM was measured on a Shimadzu UV-240 spectrophotometer (Tokyo, Japan) at 25°C in a cuvette.

**EPR spectra.** Q-bands (33.9570 GHz) EPR spectra were measured on an ESP300 EPR spectrometer (Bruker, Karlsruhe, Germany). The temperature of the sample was kept at 5 to 10 K with a helium flow cryostat (ESP300 equipped with an Oxford CF935, Oxford, UK). The magnetic fields and microwave frequencies were measured with a FEM2000 NMR gauss meter (Echo Electronic, Shiki, Japan) and a Takedariken TR5204 frequency counter (Advantest, Tokyo, Japan), respectively. Computer-simulated EPR spectra of Co-deuterolysin were obtained with our own program for an effective spin 1/2 system,\(^{13,14}\) taking into account the anisotropic \(g\) factor and \(g\)-strain linewidth,\(^{13,15,16}\)

### Results

**Specificity of deuterolysin**


The kinetic parameters for hydrolysis by deuterolysin of some dibasic-MCA derivatives are in Table 1. The lowest \(K_m\) was 26.4 \(\mu\)M with Boc-Arg-Val-Arg-Arg-MCA, and the \(k_{cat}\) was highest at 2.2 \(\times\) 10\(^{-2}\) s\(^{-1}\). The \(k_{cat}/K_m\) at 833 s\(^{-1}\) M\(^{-1}\), for Boc-Arg-Val-Arg-MCA therefore was the highest of the peptide-MCAs tested. The action of deuterolysin on calf thymus histone H4 is summarized in Fig. 1. Deuterolysin hydrolyzed histone H4 to yield a variety of peptides with various amino acid residues at the P\(_1\) position including basic arginine and lysine, acidic aspartic and glutamic acids, and proline. Deuterolysin had high activity toward peptides next to a pair of basic residues (Lys-Arg, Arg-His, Arg-Lys, and Arg-Arg) in the calf thymus histone H4. Deuterolysin hydrolyzed bonds between Arg\(^{13}\)-His\(^{18}\), His\(^{18}\)-Arg\(^{19}\), Arg\(^{21}\)-Asp\(^{24}\), and Arg\(^{36}\)-Leu\(^{37}\). On the other hand, deuterolysin also hydrolyzed Pro\(^{32}\)-Arg\(^{35}\), Glu\(^{62}\), Asn\(^{64}\), Asp\(^{68}\)-Ala\(^{69}\), and Met\(^{64}\)-Asp\(^{65}\). The specificity of deuterolysin was different from the specificity of

---

**Table 1. Kinetic Parameters of Deuterolysin and Co-Deuterolysin toward Fluorogenic MCA Substrates**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Deuterolysin</th>
<th>Co-deuterolysin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) ((\mu)M)</td>
<td>(k_{cat}) (s(^{-1}))</td>
</tr>
<tr>
<td>P(_1)</td>
<td>26.4 ± 4.0</td>
<td>2.2 (\times) 10(^{-2})</td>
</tr>
<tr>
<td>P(_2)</td>
<td>23.7 ± 7.4</td>
<td>2.2 (\times) 10(^{-3})</td>
</tr>
<tr>
<td>P(_3)</td>
<td>146.0 ± 21.0</td>
<td>9.0 (\times) 10(^{-3})</td>
</tr>
<tr>
<td>P(_4)</td>
<td>70.0 ± 3.5</td>
<td>4.5 (\times) 10(^{-3})</td>
</tr>
<tr>
<td>Z-Arg-Arg-MCA</td>
<td>43.7 ± 10.0</td>
<td>9.0 (\times) 10(^{-4})</td>
</tr>
</tbody>
</table>

\* The rate of hydrolysis of Boc-Arg-Val-Arg-Arg-MCA by deuterolysin is taken to be 100, and other such values are relative to that. Results are shown as means ± S.D. of three determinations.
Fig. 1. Summary of Cleavage Specificity of Deuterolysin from A. oryzae toward Calf Thymus Histone H4.

The arrows (|) indicate cleavage sites. The underlined sequences (ß) of amino acid residues correspond exactly to the sequences found by analysis of the deuterolysin hydrolysates. The numbers are identical to corresponding residues of calf thymus histone H4.

Cleavage sites following pairs of basic residues

GAKR17 | H18RKVLRDNIQGITKPA
AKRH18 | R19KVLRDNIQGITKPA
AIRR36 | L37ARRGKV

Other cleavage sites

KVLR23 | D24NIQGITKQ
ITKP32 | A33IRRLA
VFLE63 | N64VIRD
VIRD68 | A69VTYTHEAKRKTVMV
VTAM84 | D85VYY

Fig. 2. Thermal Stability of Deuterolysin and Co-Deuterolysin. Deuterolysin or Co-deuterolysin at the concentration of 42 mM in Tris-HCl buffer, pH 9.0, were incubated for 10 min in a temperature-controlled bath, separat. After incubation, the enzyme solution was immediately cooled in an ice for 10 min and the remaining activity was measured with Boc-Arg-Val-Arg-Arg-MCA at pH 9.0. Error bars are shown in perpendicular lines. Data are expressed as the means ± S.D. of 3 independent experiments. ○, Deuterolysin. ●, Co-deuterolysin.

Fig. 3. CD Spectra of Deuterolysin and Co-Deuterolysin.

Optical absorption and EPR spectra of Co-deuterolysin

The observed and calculated optical absorption spectra of Co-deuterolysin are shown in Fig. 4. To measure peak wavelength and amplitude, we used a Lorentzian lineshape as shown in Fig. 4B. Two d-d transitions were observed, at 18,450 cm⁻¹ (ε = 245 M⁻¹ cm⁻¹) and 20,100 cm⁻¹ (ε = 200 M⁻¹ cm⁻¹); they were quite strong.

In Fig. 5 we show the Q-band EPR spectra of Co-deuterolysin measured at 10 to 5 K. Preliminary observations of the X- and S-band EPR (9 and 3 GHz, respectively) showed an axial feature but line-broadening prevented us from identifying the g principal values from the spectrum. Resolution improvement with respect to g values is one advantage of use of a high microwave frequency. From the spectra at
Fig. 4. Optical Absorption Spectra of Co-Deuterolysin.
Electronic absorption spectra of 0.57 mM Co-deuterolysin in distilled water. Two fairly strong d-d transitions are observed at 18,450 cm⁻¹ (ε = 245 M⁻¹ cm⁻¹) and 20,100 cm⁻¹ (ε = 200 M⁻¹ cm⁻¹). A: ———, Spectrum calculated. ……, Spectrum observed. B: Spectrum of component lines used in reconstruction of the observed absorption spectrum.

Fig. 5. Q-Band EPR Spectra of Co-Deuterolysin.
A: Spectrum observed. B: Simulated spectrum made with the g and g-strain tensor principal values described in the text.

33.9570 GHz, g principal values could be unambiguously measured.

The dominant line-broadening mechanism of Co-deuterolysin was unresolved hyperfine splitting of cobalt nucleus and g-strain. The line width from a g-strain mechanism increases with increasing microwave frequency, but the contribution from hyperfine splitting is independent of the microwave frequency. On the basis of the Q-band spectrum giving better resolution than that of the X- and S-bands, we concluded that the dominant line-broadening mechanism at Q-band EPR was g-strain and that there was broadening in the X- and S-band spectra. From spectra simulation of the Q-band spectrum, we obtained the g principal values of $g_{xx} = 5.20$, $g_{yy} = 4.75$, and $g_{zz} = 2.24$, and the g-strain tensor principal values $dg_{xx} = 1.11$ (0.2), $dg_{yy} = 1.11$ (0.2), and $dg_{zz} = 0.19$ (0.01).

Discussion
Specificity of deuterolysin
Despite an earlier report that deuterolysin does not hydrolyze synthetic small peptides,6) we examined this enzyme for hydrolytic amidase activity toward fluorogenic peptide-MCAs. Here, we found Boc-Arg-Val-Arg-Arg-MCA to be the best substrate for deuterolysin of the substrates tried. The substrates with dibasic motifs were readily cleaved by deuterolysin from A. oryzae. The lack of activity toward Boc-Gly-Arg-Arg-MCA, Boc-Gln-Arg-Arg-MCA, and Boc-Gln-Lys-Lys-MCA was largely due to the effect of the P₁ position of the substrates. It appears that the P₁ position of the MCA-substrates may be important for the S₁-P₁ interaction of the enzyme-substrate complex formation. The specificity of deuterolysin was different from the specificities of ther-
temperature-dependence of the EPR spectrum of Co-deuterolysin is less sensitive than that of Co-carboxypeptidase A; that is electron spin relaxation is less effective in Co-deuterolysin. The long spin relaxation of Co-deuterolysin can be explained by a larger energy splitting between the ground and excited states in which a spin-orbit coupling interaction operates. Therefore, the number of coordinating ligands or coordination bonds in Co-deuterolysin is larger than that of Co-carboxypeptidase A. Thus the EPR results suggest that Co-deuterolysin may be four- or five-coordination.

These absorption bands of a high spin divalent cobalt complex are assigned to $^4T_1 (F) \rightarrow ^4T_1 (P)$ electronic transitions. Empirical observations for Co$^{3+}$ complexes with well-defined coordination spheres suggest that the energy and the extinction coefficient of the ligand field band can be used as an indicator to the coordination number: in a four-coordinate site, $\sim 16,000$ cm$^{-1}$, $\epsilon > 300$ M$^{-1}$ cm$^{-1}$, as observed in carbonic anhydrase; in a five-coordinate site, $16,000 \sim 19,000$ cm$^{-1}$, $500 > \epsilon > 250$ M$^{-1}$ cm$^{-1}$, as observed in methionyl aminopeptidase; and in a six-coordinate site, $19,000$ cm$^{-1}$, $\epsilon < 50$ M$^{-1}$ cm$^{-1}$, as observed in glyoxalase I. Moreover, a characteristic absorption (16,000 cm$^{-1}$) is observed in a five-coordinate site. The absorption spectrum of Co-deuterolysin indicates that the number of coordinating ligands can be four or five, that is, tetrahedral or trigonal-bipyramidal, respectively. As a four-coordinate site, the absorption energy of Co-deuterolysin indicates that the number of coordinating ligands or coordination bonds in Co-deuterolysin is less sensitive than that of Co-carboxypeptidase A. Thus the EPR results suggest that Co-deuterolysin may be four- or five-coordination.

It can be concluded that a distorted tetrahedral or a two-triangular pyramidal environment has been assigned to the Co-deuterolysin from the correlation of the g principal values, $g_{xx} = 5.20$, $g_{yy} = 4.75$, and $g_{zz} = 2.24$.

Acknowledgments

We thank K. Hirano of Amano Enzyme Co. for the gift of the enzyme source. We also thank M. Furukawa and Y. Yamada for technical assistance in purification of deuterolysin and CD measurement, respectively.

References


