Deuterolysin (EC 3.4.24.39; formerly designated as neutral proteinase II) from Aspergillus oryzae, which contains 1 g atom of zinc/mol of enzyme, is a single chain of 177 amino acid residues, includes three disulfide bonds, and has a molecular mass of 19,018 Da. Active-site determination of the recombinant enzyme expressed in Escherichia coli was performed by site-directed mutagenesis. Substitution of His128 and His132 with Arg of Glu129 with Gln or Asp, of Asp143 with Asn or Glu, of Tyr106 with Phe in complete almost loss of the catalytic function. It can be concluded that His128, His132, and Asp143 provide the Zn2+ ligands of the enzyme according to a 65Zn binding assay. Based on site-directed mutagenesis experiments, it was demonstrated that the three essential amino acid residues Glu129, Asp143, and Tyr106 are catalytically crucial residues in the enzyme. Glu129 may be implicated in a central role in the catalytic function. We conclude that deuterolysin is a member of a family of Zn2+ metalloendopeptidases with a new zinc-binding motif, aspzin, defined by the "HEXXH + D" motif and an aspartic acid as the third zinc ligand.

Metalloendopeptidases are physiologically important proteases for processing proteins in eukaryotes and prokaryotes and are one of the best known classes of proteolytic enzymes (1, 2). From recent data, Hooper (3) attempted to present a scheme based on the zinc-binding site, and this has been extended to classify zinc metalloproteases into distinct families. There are glu zincins, met zincins, inver zincins, the carboxypeptidase family, and the D-D-carboxypeptidase family. Rawlings and Barrett (4) divided the families of metalloendopeptidases into five groups based on the zinc-binding motif: "HEXXH + E", "HEXXH + H", "HEXXH + ?", heterogeneous other than HEXXH, and unknown.

The molds Aspergillus oryzae and Aspergillus sojae are of great practical importance in Japanese fermentation industries and enzyme technologies (5). In the fermented vegetable protein, soy sauce, the cooked soybeans are mixed with equal amounts of roasted wheat and then inoculated with a pure cultured starter of A. sojae or A. oryzae, which is called "koji starter" or "seed mold" (6). Sekine et al. (7) previously reported that both neutral proteinases I and II were found to have an effect equal to that of alkaline serine proteinase (8, 9) on the hydrolysis and liquefaction of soybean protein. Neutral proteinase II is now designated as deuterolysin (EC 3.4.24.39) according to the Enzyme Nomenclature Commission (10). The metalloproteinases (neutral proteinases I and II) from A. sojae were characterized by Sekine (11–15).

Neutral proteinase I contains 1 g atom of zinc and 2 g atoms of calcium per mol of enzyme, and the zinc is essential for activity (13). Neutral proteinase I with a molecular mass of 41,700 Da has enzymatic properties similar to those of the neutral proteinase from Bacillus thermoproteolyticus, thermolysin (EC 3.4.24.27) (15). In the digestion of the oxidized insulin B-chain with neutral proteinase I, the cleavage sites produced by the enzyme are very similar to those of the other neutral proteinases.

Deuterolysin from A. sojae possesses a preference for basic proteins as substrates, showing high activities on the basic nuclear proteins histone, protamine, and salmine, but very low activities on milk casein, hemoglobin, albumin, and gelatin (12, 14). Deuterolysin is extremely stable at 100 °C, but relatively unstable around 75 °C (16). Elucidation of the thermal stability at 100 °C of the deuterolysin from A. oryzae has also been reported and discussed. It contains 1 g atom of zinc and 2 g atoms of calcium per mol with a molecular mass of 19,800 Da (13) and includes three disulfide bonds (16). The cloning and expression in yeast cells of a cDNA clone (S53810) encoding deuterolysin from A. oryzae were reported by Tatsumi et al. (17).

The elucidation of substrate specificity with an oxidized insulin B-chain (18) and bioactive oligopeptides (19) led to the discovery of penicilloylase as an enzyme thought to be a new 18-kDa metalloendopeptidase from Penicillium citrinum, having a distinct mode of action and a specificity unique from those of other metalloendopeptidases (2, 3, 20–22). Penicilloylase also possesses a preference for basic proteins as substrates, showing high activities on basic nuclear proteins such as histone, protamine, and salmine, but very low activities on milk casein, hemoglobin, albumin, and gelatin (19); however, the enzyme has no heat stability above 60 °C. Penicilloylase contains 1 g atom of zinc/mol of enzyme and three disulfide bonds. The enzyme is a single-chain protein of 177 amino acid residues with a molecular mass of 18,529 Da and pl 9.6. Cloning of a cDNA clone (D25535) encoding penicilloylase from P. citrinum was carried out by Matsumoto et al. (23). Penicilloylase shows 68% sequence identity to deuterolysin from A. oryzae. We previously assumed that His128, His132, and Glu143 of penicilloylase (23) corresponded to zinc ligands in thermolysin (24) and the neutral proteinases (2, 3).
In this paper, the possibility of a zinc-binding role for Glu⁶⁵ in deuterolysin was ruled out because the mutant E65Q still had catalytic activity. The predicted amino acid sequence, including aspartic acid and glutamic acid, of deuterolysin (17) has strong similarity to other members of the deuterolysin family: MEP20 from Aspergillus flavus (25) and Aspergillus fumigatus (26), metalloendopeptidases from Grifola frondosa and Pleurotus ostreatus (27), and penicilloysis (23). This finding may indicate that enzymes in the deuterolysin family are coded for by evolutionarily related genes at the enzymatic level. We hypothesized that Asp¹⁴ or Asp¹⁶ in the highly conserved region of the C terminus may be the third ligand of deuterolysin.

We describe here site-directed mutagenesis studies of deuterolysin from A. oryzae for active-site determination. We found a new zinc-binding motif, aspzm, defined as the “HEXX + D” motif with aspartic acid as the third zinc ligand.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases, T4 DNA polymerase, alkaline phosphatase, and Taq DNA polymerase were purchased from Takara Shuzo (Kyoto, Japan). T4 DNA ligase and poly(A)⁻/reverse transcriptase were Life Technologies, Inc. T4 polynucleotide kinase was from Nippon Gene (Tokyo, Japan). Protamine sulfate was from Sigma. Antifoam DB110N was purchased from Nacalai Tesque (Kyoto), was as isoamyl-β-D-thiogalactopyranoside.

**Strains, Plasmids, and Media**—A. oryzae IFO 4251 was used as a source of native deuterolysin and mRNA because we could not obtain the strain (A. oryzae ATCC 20386) used in previous work (17). Escherichia coli DH5α (supE44 lacIqlacZΔM15 lacY1 endA1 gyr96 thi-1 relA1) was used for plasmid isolation and cloning. E. coli TG1 (supE44 thi Δlac-proAB/F' [traD36 proAB lacIq lacY1]) was used for propagation bacteriophage M13 vectors. E. coli BL21(DE3) (F'ompT lacIqθ (φ80 dlac proAB lacY1 proDE80 lacIq lacY1 lacZΔM15)) was used to propagate bacteriophage M13 vectors. Plasmids pUC19, M13mp18, and pET32a were purchased from Takara Shuzo. E. coli cells were grown in LB medium (1% Bacto-Trypton, 1% Bacto yeast extract, and 1% NaCl), and 50 μg/ml ampicillin was added when necessary.

**Isolation of mRNA from A. oryzae**—A. oryzae IFO 4251 was cultured in 50 ml of wheat bran medium (1.5% defatted soya bean peptone, 1% potassium phosphate, and 0.02% zinc chloride dissolved in wheat bran extract (6 g/liter at 37°C for 80 h), and the culture was centrifuged at 15,000 g to obtain 2 g of wet cells. Total RNA was purified and the purified by the method of Chomczynski and Sacchi (28). Poly(A)⁻/RNA was isolated using an Oligotex-dT30 (Takara Shuzo).

**cDNA Synthesis**—A. oryzae single-stranded cDNA (dlnO) was synthesized from poly(A)⁻/RNA using oligo(dT)₁₂₋₁₈ primer and SuperScript RNase H reverse transcriptase (Life Technologies, Inc.), followed by cDNA synthesis from poly(A)⁻/RNA according to the manufacturer’s instructions. The cDNA was amplified by polymerase chain reaction (29) using the following primer sets designed for adapting the BamHI site (5'-TTTGGATCCGGCCAGATGGGTTGTCATTACCTACT-3' (sense) and 5'-CAGAGATCCCAACAGCCGTTAGCCTTGCATTTAGCGCTTGGCTGCTGAC-3' (antisense), based on the DNA sequence of deuterolysin from A. oryzae reported by Tatsumi et al. (17). The underlined letters in the sequences are the 

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed by the method of Kunkel et al. (30). The EcoRI-BamHI fragment containing deuterolysin cDNA from pUCDLN(EB) was subcloned into M13mp18 to serve as a template for mutagenesis. The following mutagenic primers were used: E42Q, 5'-GGTCCTGAGATGTGCGCAGCTGCTGAC-3' (sense), 5'-GCGGGAGGACTTTAGCTATCCACG-3' (antisense); E65Q, 5'-GGTCCTGAGATGTGCGCAGCTGCTGAC-3' (sense), 5'-GCGGGAGGACTTTAGCTATCCACG-3' (antisense); and E70Q, 5'-GGTCCTGAGATGTGCGCAGCTGCTGAC-3' (sense), 5'-GCGGGAGGACTTTAGCTATCCACG-3' (antisense). All the mutagenic primers were designed to be antisense. Mismatches with the original sequence were indicated in lowercase letters. The mutation sites with the original sequence of dlnO are indicated in lowercase letters. The mutation was verified by DNA sequencing before subcloning the gene into the expression vector pETDLN.

**Construction of Expression Plasmids in E. coli Cells**—The expression plasmid for pETDLN, pETDLN, was constructed by inserting the deuterolysin cDNA fragment into the T7 promoter/operator and T7 terminator and thioredoxin fusion gene (31) into pET32a (Novagen). The cDNA was modified by polymerase chain reaction to remove the following signal sequence and to introduce a start codon at the N terminus of deuterolysin. The sense primer 5'-TTGAGATCCCAACAGCCGTTAGCCTTGCATTTAGCGCTTGGCTGCTGAC-3' (sense), 5'-GCGGGAGGACTTTAGCTATCCACG-3' (antisense), 5'-GAGGGAGGACTTTAGCTATCCACG-3' (antisense), and 5'-GAGGGAGGACTTTAGCTATCCACG-3' (antisense) were designed to be antisense. Mismatches with the original sequence of dlnO are indicated in lowercase letters. A Met start codon was created at the Ndel site at the N terminus of deuterolysin. The amplified 1.0-kilobase pair cDNA fragment was digested with EcoRI and BamHI and cloned into pUC19 to generate pUCDLN(EB). The amplified cDNA was confirmed to lack the undesired mutation by sequencing. The Ndel-BamHI fragment from the pUCDLN(EB) was subcloned into the pET32a vector to generate pETDLN (Fig. 1).

**Expression of Prodeuterolysin in E. coli**—E. coli BL21(DE3) cells transformed with pETDLN were cultured at 37°C in a 5-liter jar fermentor containing 2 liters of 4-fold concentrated LB medium containing 50 mg/ml ampicillin, 10 mM calcium chloride, and 0.25% (v/v) antifoam DB110N emulsion until A₆₀₀ nm reached 7; then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and incubation was continued for 3 h (aeration and agitation rate was 3 liters/min and 300 rpm, respectively). The cells were harvested by centrifugation at 15,000 × g for 20 min, resuspended in 200 ml of 10 mM Tris-HCl buffer, pH 8.0, and frozen at −80°C.

**Purification of Recombinant Prodeuterolysin by Fast Protein Liquid Chromatography**—Part of the frozen cells were melted and ruptured by
sonication. The cell homogenate was centrifuged at 20,000 × g for 10 min. The supernatant was re-centrifuged, and the resulting supernatant was used as crude proteodeuterolysin preparation. The crude proteoenzyme solution was loaded on a RESOURCE Q column (0.64 × 3 cm, Amersham Pharmacia Biotech) and eluted with 5 mM sodium phosphate, pH 7.0. The fractions containing proteodeuterolysin were pooled. The pooled fraction was loaded on a HiLoad Superdex 75 column (1.6 × 60 cm, Amersham Pharmacia Biotech) and eluted with 5 mM sodium phosphate buffer, pH 7.0, containing 0.3 mM NaCl. The fractions containing proteodeuterolysin were dialyzed against 5 mM sodium phosphate buffer, pH 7.0, and used as a purified proteodeuterolysin preparation material.

Conversion of Proteodeuterolysin to the Mature Form—The purified proteoenzyme fraction was incubated at 37 °C for 30 min with an equal amount of trypsin (mol/mol, Merck). After the addition of 1-choro-3-(4-tosylamido)-7-aminoo-2-heptanone hydrochloride at 100 μM as a trypsin inhibitor, the reaction mixture was loaded on a HiLoad 16/60 Superdex 75 column (1.6 × 60 cm, Amersham Pharmacia Biotech) and eluted with 5 mM sodium phosphate buffer, pH 7.0, containing 0.3 mM NaCl. The fractions containing deuterolysin were pooled. After dialysis against 5 mM sodium phosphate buffer, pH 7.0, the pooled fraction was loaded on two HiTrap Q anion-exchange columns (0.77 × 10 cm, Amersham Pharmacia Biotech) and eluted with 5 mM sodium phosphate, pH 7.0, with a linear gradient of 0–0.5 mM NaCl. The fractions containing mature enzyme were pooled. Maturation from prodeuterolysin to the active deuterolysin was also observed with the addition of 5 mM ZnCl2.

Proteolytic Activity Assay—Proteolytic activities with salmon protamine sulfate (salmine) were assayed at pH 7.0 and 30 °C. Fifty μl of sample and 400 μl of 100 mM sodium phosphate buffer, pH 7.0, were mixed and preincubated for 5 min, and then 150 μl of 2% (w/v) salmon protamine sulfate (previously denatured at 100 °C for 30 min in 100 mM sodium phosphate buffer, pH 7.0) was added and incubated for various periods. Six-hundred μl of 12.5% (w/v) trichloroacetic acid with 20% (w/v) NaCl was added to stop the reaction, and the mixture was then filtered with Advantec No. 2 filter paper. Two-hundred μl of the filtrate and 3.0 μl of 0.5 M sodium citrate buffer, pH 5.0, were added to the reaction mixture; 1 μl of freshly prepared ninhydrin reagent (19) was also added. The linearity of the assay was checked, and the amount of amino acid produced in the reaction mixture was determined. One katal is defined as the amount of enzyme yielding the color equivalent of 1 mol of tyrosine/s with ninhydrin reagent using protein substrates at pH 7.0 and 30 °C according to previous work (19).

Protein Concentration—Protein concentration was determined using the BCA protein assay reagent (Fierce) following the manufacturer’s instructions. Known concentration of protein standards was prepared by diluting the bovine serum albumin standard solution purchased with this kit. Ten μl of each standard or unknown protein sample was pipetted into the tube, and then 200 μl of working reagent was added to each tube and mixed well. All tubes were incubated at 60 °C for 30 min and cooled to room temperature, and the absorbance at 520 nm was measured.

Native Amino Acid Sequence Analyses—Protein samples were loaded onto a RESOURCE RPC column (0.64 × 3 cm, Amersham Pharmacia Biotech) and eluted with 0.1% (w/v) trifluoroacetic acid with a linear gradient of 0–80% (v/v) acetonitrile. The peak fractions were concentrated with a TAITEC VA-500F Speed Vac and then subjected to N-terminal sequence determination on an Applied Biosystems 473A protein sequencer with a 610A data analysis system.

SDS-PAGE—SDS-PAGE supernatants (0.5 ml) of the E. coli transformants with wild-type and mutant dinO genes were treated with trichloroacetic acid and centrifuged. The pellets were dissolved in 100 mM sodium phosphate buffer, pH 8.0, containing 0.01% SDS, heated, and denatured. The proteins were separated by SDS-PAGE as described by Laemmli (32) and then stained with Coomassie Brilliant Blue R-250 dissolved in 50% methanol and 9.5% acetic acid and destained in 5% methanol and 9.5% acetic acid.

CD Measurements—Samples were dialyzed in 5 mM phosphate buffer, pH 7.0, and then diluted in the same buffer to 0.2 mg of protein/ml by adjusting A280 nm at 0.2. The CD measurements were done as described by Yamaguchi et al. (19) using a Jasco J-710 spectrophotometer. Measurements of α-helix and β-structure of the enzyme were calculated by the SSE-338 program described by Yang et al. (33). The molecular mass value of 19,800 Da was used.

Zinc Blotting for the 65Zn Binding Ability Assay—The zinc blotting technique, after which the filter was washed in metal-binding buffer (1 mM NaOH, pH 7.0, 0.3 M NaCl) for 3 h. The filter was probed for 1 h with 5 μCi of 65ZnCl2 (2.71 Ci/mg; 1 Ci = 37 GBq; NEN Life Science Products) in 15–20 ml of metal-binding buffer. It was then washed in Saran Wrap and exposed to an imaging plate (Fuji Photo Film Co., Tokyo) for 12 h. After exposure, the imaging plate was analyzed by a BioImage BAS-2000 Analyzer System (Fuji Photo Film Co.). Proteins immobilized on the polyvinylidene difluoride membrane were detected by staining with Coomassie Brilliant Blue R-250 (0.05%) in 50% methanol and 10% acetic acid; the protein staining was done in duplicate.

Atomic Absorption Spectrophotometric Analyses—Zinc analysis in the enzyme was also done by atomic absorption spectrophotometry using a Perkin-Elmer Model 3100 apparatus.

Molecular Mass Determination on Superdex 75 HR 10/30—Molecular mass determination of the recombinant wild-type deuterolysins activated with ZnCl2 and trypsin was done by gel filtration with a Superdex 75 HR 10/30 column. The elution time of proteins through the column was plotted against logarithms of a molecular mass of standard proteins. Bovine serum albumin (67 kDa), ovalbumin (43 kDa), and myoglobin (17.4 kDa) were used as molecular mass standards.

RESULTS

Recombinant wild-type proteodeuterolysin was expressed in E. coli and purified as described under “Experimental Procedures.” The purified proteodeuterolysin could be activated by 5 mM ZnCl2 at 4 °C for 24 h. A high yield of the mature deuterolysin was obtained by the activation method with ZnCl2, however, the method required a lengthy incubation time. A more effective method of activating the purified proteoenzyme fractions was by incubation at 37 °C for 30 min with an equal amount of trypsin (mol/mol), although the yield of the activation method with trypsin was lower than that of the method with ZnCl2. We obtained homogeneous preparations on SDS-PAGE of deuterolysin activated with ZnCl2 and trypsin, as shown in Fig. 2. The molecular mass of the recombinant deuterolysin activated by ZnCl2 was determined as 19,800 Da by gel filtration on Superdex 75 HR 10/30 (data not shown), which was similar to that of the native enzyme (19,000 Da) reported by Tasumi et al. (17). The specific activity of deuterolysin activated by trypsin treatment was 0.144 katal/kg of protein with salmine as a substrate, and this was also the same specific activity as that of the native enzyme purified from A. oryzae. Activity resistance to 100 °C treatment with Zn2+ and inhibition with EDTA showed that the recombinant deuterolysin expressed in E. coli was similar to native deuterolysin.

The N-terminal sequence of recombinant wild-type deuterolysin activated with an equal amount (mol/mol) of trypsin was found to be Thr-Glu-Val-Asp, which is the N-terminal sequence of the recombinant wild-type enzyme described by Laemmli (32). The N-terminal sequence of deuterolysin from A. oryzae (17), as shown in Table I. The result suggests that autoproteolytic cleavage of the peptide bond between Arg1-Thr1 in proteodeuterolysin by trypsin may occur. The N-terminal sequence of the recombinant wild-type enzyme activated with 5 mM ZnCl2 for 24 h at 4 °C, in contrast, was found to be Glu-Val-Thr-Asp, which was one amino acid residue shorter than that of native deuterolysin from A. oryzae. These results suggest that autoproteolytic cleavage of the propeptide may occur ahead of threonine and that the product is trimmed by aminopeptidase(s) in bacterial culture broth. The ultraviolet CD spectrum of recombinant wild-type deuterolysin activated by trypsin predicted a conformation of 70% α-helix, 16% β-structure, and 14% random structure, which was almost identical to that of the native enzyme (71% α-helix and 29% β-structure).

Several specific mutations were introduced by site-directed mutagenesis into the predicted active sites and substrate-bind-
ing regions of deuterolysin. The mutations were selected on the basis of sequence comparison of the enzyme (17) with other zinc metalloendopeptidases (2–4). Mutations were focused at Glu 42, Arg 58, Glu 65, Asp 86, Glu 86, Asp 104, Tyr 106, His 118, Asp 121, His 126, Glu 129, His 132, Glu 142, Asp 143, and Asp 164. These amino acid residues were highly conserved in deuterolysin (17), 23-kDa metalloproteinases from A. flavus MEP20 (25) and A. fumigatus MEP20 (26), penicillolysin from P. citrinum (23), and metalloendopeptidases from G. frondosa and P. ostreatus (27), as shown in Fig. 3.

The ultraviolet CD spectra of the five site-directed mutants H128R, E129Q, H132R, D143N, and D164N, were almost identical to that of wild-type deuterolysin. The contents of mutant enzymes H128R and D164N for salmine hydrolysis at pH 7.0. However, the magnitude of the specific activity of mutant D143N was determined as 0.90% (Table I). Furthermore, in these site-directed mutagenesis experiments, we demonstrated that two carboxylic acid residues, Glu 129 and Asp 143, were catalytically crucial residues of the enzyme. Although the mutants E129Q and D143N have the ability to incorporate 65Zn2+, as do the native and wild-type enzymes (Fig. 6B and Table III). It was concluded that His 126, His 132, and Asp 164 provide the three Zn2+ ligands in the active site of deuterolysin; it was also demonstrated that the two mutants E129Q and D143N have zinc-binding capacity. Atomic absorption spectrophotometric analysis confirmed that E129Q and D143N had 1 g atom of zinc/mol of enzyme (data not shown). Substitutions of Tyr 106 with Phe and of Asp 164 with Asn caused drastic decreases in proenzyme maturation and proteolytic activity for salmine hydrolysis at pH 7.0. However, the magnitude of the specific activity of mutant D143N was larger than that of the Zn2+ ligand mutants H128R, H132R, and D164N. The relative specific activity of mutant D143N was determined as 0.90% (Table III).

The two histidine residues in the HEXXH + D motif in which an aspartic acid is the third ligand.

**DISCUSSION**

Earlier comparison (23) of the sequences of deuterolysin and penicillolysin with the metalloendopeptidases thermolysin and carboxypeptidase A showed that the primary structures of deuterolysin and penicillolysin have only a low degree of sequence identity to other metalloproteases. We previously assumed that His 126, His 132, and Glu 165 of penicillolysin corresponded to the zinc ligands in the homologous thermolysin and neutral proteinases (23). The two histidine residues in the HEXXH motif of deuterolysin from A. oryzae were confirmed to be zinc ligands of the enzyme by site-directed mutagenesis in the case of the mutants H128R and H132R. The possibility that Glu 165 is a zinc ligand in deuterolysin was ruled out, however, because mutant E65Q had showed proenzyme maturation and proteolytic activity for salmine hydrolysis at pH 7.0. The discovery of the third zinc ligand of deuterolysin was the major purpose of this work.

It is easy to identify the crucial residue of Glu 129 in the HEXXH motif of deuterolysin. The present results from site-directed mutagenesis confirmed that Glu 129 of deuterolysin is a catalytically crucial residue of this enzyme from A. oryzae. Furthermore, in these site-directed mutagenesis experiments, we demonstrated that two other amino acid residues, Asp 143 and Tyr 106, were also catalytically crucial residues of the enzyme. Although the mutants E129Q and D143N disrupted the catalytic function, the 65Zn-binding abilities of the mutants were maintained. These experiments showed that Glu 129 and Asp 143 were not the zinc ligands of the enzyme. It was therefore concluded that the three residues Glu 129, Asp 143, and Tyr 106 are crucial for the catalysis of deuterolysin from A. oryzae.
Comparison of amino acid sequences of deuterolysin from *A. oryzae*, penicillolysin (23), *A. flavus* MEP20 (25), *A. fumigatus* MEP20 (26), *G. frondosa* metalloendopeptidase (27), and *P. ostreatus* metalloendopeptidase (27).

Deuterolysin from *A. oryzae*

| Enzyme | N-terminal sequence | Secondary structure |
|--------|---------------------|---------------------|
| Native | TEVTD               | α-Helix: 71 β-Structure: 0 β-Turn: 29 Random coil: 0 |
| Wild-type (Zn<sup>2+</sup>) | EVTVD | α-Helix: 70 β-Structure: 0 β-Turn: 17 Random coil: 13 |
| Wild-type (trypsin) | TEVTD | α-Helix: 70 β-Structure: 0 β-Turn: 16 Random coil: 14 |
| H128R | TEVTD | α-Helix: 63 β-Structure: 0 β-Turn: 17 Random coil: 20 |
| E129Q | TEVTD | α-Helix: 71 β-Structure: 0 β-Turn: 17 Random coil: 12 |
| H132R | TEVTD | α-Helix: 70 β-Structure: 0 β-Turn: 27 Random coil: 3 |
| D143N | TEVTD | α-Helix: 68 β-Structure: 0 β-Turn: 19 Random coil: 13 |
| D164N | TEVTD | α-Helix: 75 β-Structure: 0 β-Turn: 22 Random coil: 3 |

**TABLE I**

Comparison of N-terminal sequences and estimated secondary structures by CD determination of native, wild-type, and site-directed mutant deuterolysins expressed in *E. coli*

| Enzyme | N-terminal sequence | α-Helix | β-Structure | β-Turn | Random coil |
|--------|---------------------|---------|-------------|--------|-------------|
| Native | TEVTD               | 71      | 0           | 29     | 0           |
| Wild-type (Zn<sup>2+</sup>) | EVTVD | 70      | 0           | 17     | 13          |
| Wild-type (trypsin) | TEVTD | 70      | 0           | 16     | 14          |
| H128R | TEVTD | 63      | 0           | 17     | 20          |
| E129Q | TEVTD | 71      | 0           | 17     | 12          |
| H132R | TEVTD | 70      | 0           | 27     | 3           |
| D143N | TEVTD | 68      | 0           | 19     | 13          |
| D164N | TEVTD | 75      | 0           | 22     | 3           |

Fig. 3. Comparison of amino acid sequences of deuterolysin from *A. oryzae*, penicillolysin (23), *A. flavus* MEP20 (25), *A. fumigatus* MEP20 (26), *G. frondosa* metalloendopeptidase (27), and *P. ostreatus* metalloendopeptidase (27). The closed inverted triangles show His<sup>128</sup> (deuterolysin numbering), His<sup>132</sup>, and Asp<sup>164</sup> identified as a new zinc ligand motif in deuterolysin. The open circles show Tyr<sup>106</sup>, Glu<sup>129</sup>, and Asp<sup>143</sup> identified as the catalytically crucial sites of deuterolysin.

**Fig. 4.** SDS-PAGE of soluble proteins of the site-directed mutants of prodeuterolysin expressed in *E. coli* with Zn<sup>2+</sup>. Conversions of prodeuterolysins to mature deuterolysins with 5 mM ZnCl<sub>2</sub> for 24 h at 4 °C were observed. No conversion of prodeuterolysin to the mature form with Zn<sup>2+</sup> was observed in the site-directed mutants H128R, E129Q, H132R, D143N, and D164N. The gel was stained with Coomassie Brilliant Blue R-250.

**MW (kDa)**

- Native: 66.4
- Wild-type: 55.6
- R128Q: 42.7
- Y106F: 36.5
- E129Q: 26.6
- D143N: 20.0
- Mature: 20.0

Trx-proDLN
**New Zn$^{2+}$-binding Sites of Deuterolysin from A. oryzae**

**TABLE II**

| Mutant     | Conversion of proenzyme to mature form with Zn$^{2+}$ | Proteolytic activity$^b$ |
|------------|------------------------------------------------------|-------------------------|
| Wild-type  | +                                                   | ++                     |
| E42Q       | +                                                   | +                      |
| E58Q       | +                                                   | +                      |
| E65Q       | +                                                   | +                      |
| D89N       | +                                                   | +                      |
| E86Q       | +                                                   | +                      |
| D104N      | +                                                   | ++                     |
| H118A      | +                                                   | ++                     |
| D121N      | +                                                   | ++                     |
| E142Q      | +                                                   | ++                     |

$^a$ + represents the proenzyme is able to convert to the mature enzyme.
$^b$ ++++, +++, +, and + represent 70–100, 30–70, and 5–30% activity of the wild-type enzyme, respectively.

**TABLE III**

| Enzyme     | Specific activity | $^{65}$Zn-binding ability$^a$ |
|------------|------------------|-------------------------------|
| Wild-type  | 0.144            | +                             |
| H128R      | 0.000183         | –                             |
| E129Q      | 0.000334         | +                             |
| H132R      | ND$^b$           | –                             |
| D143N      | 0.00129          | +                             |
| D164N      | 0.000416         | –                             |

$^a$ +, $^{65}$Zn incorporated; −, $^{65}$Zn not incorporated.
$^b$ Not detected.

From the present findings, Glu$^{129}$ and Asp$^{143}$ of deuterolysin are probably in the ionized COO$^-$ form, and Tyr$^{106}$ of the enzyme is probably the binding site of substrate.

Amino acid sequences of metalloendopeptidases for acyllysine bonds from *G. frondosa* and *P. ostreatus* fruiting bodies were reported by Nonaka et al. (27). They suggested that these proteases, *G. frondosa* and *P. ostreatus* metalloendopeptidases, do not have conserved third and/or fourth liganding amino acid residues seen in the metzincin or thermolysin superfamily of proteases, but belong instead to a novel zinc metalloendopeptidase superfamily. A zinc atom in thermolysin has been demonstrated to be bound to His$^{142}$, His$^{146}$, and Glu$^{166}$ by extensive studies of the tertiary structure (24). Nonaka et al. also indicated the presence of the homologous regions GXXDXXYG and AXNNXD among the sequences of six fungal metalloproteinases (deuterolysin from *A. oryzae* (17), MEP20 from *A. flavus* (25) and *A. fumigatus* (26), penicillolysin (23), and metalloendopeptidases from *G. frondosa* and *P. ostreatus* (27)) of the deuterolysin (EC 3.4.24.39) family, in which the potential zinc ligand residues Asp, Asn, and Tyr are conserved. They suggested that Asp, Asn, and/or Tyr in these regions is likely to be the third and/or fourth zinc ligand in these metalloproteinases and that the six fungal metalloproteinases might be classified into a novel subfamily of zinc metalloproteinases (27).

From the present results of site-directed mutagenesis and the $^{65}$Zn-binding assay, His$^{128}$ and His$^{132}$ of deuterolysin from *A. oryzae* were shown to correspond to the zinc-binding sequence in thermolysin (24), whereas the third aspartate zinc ligand, Asp$^{164}$, of deuterolysin was replaced by Glu$^{166}$ in thermolysin. Site-directed mutagenesis experiments showed that mutant D164N had no detectable catalytic activity for maturation from the proenzyme to the mature form or proteolytic activity for salmine hydrolysis (Fig. 4).

The Zn$^{2+}$ atom is bound to both thermolysin and carboxypeptidase A by interaction with the imidazole side chains of two His residues and with the carboxyl side chain of a Glu residue (1). A further coordination position of each Zn$^{2+}$ atom is occupied by a water molecule, which plays a crucial role in the catalytic activity of each enzyme (1). It is concluded that the two particular residues His$^{128}$ and His$^{132}$ are involved in binding zinc. However, the present finding showed that a unique amino acid residue, Asp$^{164}$, is a third Zn$^{2+}$ ligand of deuterolysin. In deuterolysin, the spacer between His$^{128}$ and His$^{132}$ is replaced by a water molecule, which plays a crucial role in the catalytic activity of each enzyme (1). It is concluded that the two particular residues His$^{128}$ and His$^{132}$ are involved in binding zinc. However, the present finding showed that a unique amino acid residue, Asp$^{164}$, is a third Zn$^{2+}$ ligand of deuterolysin. Asp$^{164}$, defined by the HEXXXH + D motif and aspartic acid as the third zinc ligand, is the proposed family name for the zinc metalloendopeptidase deuterolysin.

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