Amino Acid Sequence and Characterization of a Nuclease (Nuclease Le3) from *Lentinus edodes*

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The fruit body of shiitake (*Lentinus edodes*) produces two acid nucleases, nuclease Le1 and nuclease Le3, both of which are thought to be candidates for the enzyme that produces a flavorful substance, 5′-GMP, and the primary structure of one of the nucleases, nuclease Le1, has been analyzed by both protein chemistry and gene cloning [Biosci. Biotechnol. Biochem. 64, 948–957 (2000)].

In this study the amino acid sequence of nuclease Le3 was analyzed by protein chemistry and gene cloning. Nuclease Le3 is a glycoprotein that contains 280 amino acid residues, and the molecular mass of the protein moiety of nuclease Le3 is 31,045. The nucleotide sequence of the cDNA and genomic DNA encoding nuclease Le3 revealed the presence of an 18-residue putative signal peptide.

Nuclease Le3 contains 170, 108, and 98 amino acid residues that are identical to residues of nuclease Le1, nuclease P1, and nuclease S, respectively. The amino acid residues involved in coordination with Zn$^{2+}$ atoms in nuclease P1 are all conserved in nuclease Le3. Nuclease Le3 contains 9 half-cystine residues, and 7 of them are located in the same positions as in nuclease Le1.

Key words: cDNA; *Lentinus edodes*; nuclease; 3′-nucleotidase; primary structure

Single-strand-specific nucleases are widely distributed in many organisms, from microbes to higher animals. Some of them are widely used as tools in molecular biology and gene manipulation as well as for the production of artificial flavors.1,2) Knowledge of the structure-function relationships of these nucleases, however, is very limited. The primary structures of only four nucleases have been reported to date,3–7) and the three- dimensional structure of only one enzyme, nuclease P1 from *Penicillium citrinum*, has been reported.8)

The nucleases of *Lentinus edodes* have been thought to be related to the production of a flavorful substance, 5′-GMP, and many attempts have been made to identify these nucleases.9–11) During the course of research to characterize the RNA-degrading enzymes of *L. edodes*,12,13) we isolated two acid nucleases, nuclease Le1 and nuclease Le3, from fresh fruit bodies and reported their N-terminal sequences.14,15) In order to establish the role of these nucleases in production of the flavorful substance and discover the structure-function relationship of single-strand-specific nucleases, in this study, we analyzed the amino acid sequence of one of the nucleases, nuclease Le3, by protein and gene technology. In addition, the sequence and some of the enzymatic characteristics of nuclease Le3 were compared with those of nuclease Le1.

Materials and Methods

**Enzyme purification.** Since the nuclease activity of semi-dried shiitake is higher than that of fresh fruit bodies, nuclease Le3 was prepared from semi-dried shiitake by a modified procedure reported previously.1,3)

**Step 1.** Extraction. Powdered dried shiitake (6.0 kg) were suspended in 10 l of 0.1 M sodium acetate buffer (pH 6.0) and vigorously stirred for 30 sec at 0°C, five times, at 60-sec intervals. The suspension was centrifuged at 10,000 rpm for 5 min, and the supernatant (the crude extract) was used for further purification.

**Step 2.** Ammonium sulfate fractionation. The crude extract was fractionated with ammonium sulfate, and the precipitate between 0.4–0.9 saturation was collected. The precipitate was dissolved in 10 mM sodium acetate buffer (pH 6.0) containing 0.2 mM...
Nuclease Le3 was further purified in steps 3–10 below, as described in Chart 1 and Table 1. As shown in Fig. 1, in step 4, nuclease Le3 was separated from nuclease Le1 and ribonuclease Le2 (RNase Le2). The subsequent steps were effective in completely separating it from other nucleases (nuclease Le1 and RNase Le2), and step 8 effectively separated the nucleases from other proteins. As shown in Fig. 2, the nuclease Le3 thus purified appeared as a single band on SDS-PAGE and showed the characteristic pH optimum as seen in Fig. 9.

**Enzyme assay.** Enzyme activity was measured as described in the previous report. A small volume of enzyme solution was added to 1 ml of sodium acetate buffer (50 mM, pH 5.0) containing yeast RNA (2.5 mg/ml), and the reaction mixture was incubated at 37°C. The reaction was stopped by the addition of MacFadyen reagent (0.5 ml). The supernatant (150 µl) obtained by centrifugation of the reaction mixture at 3,000 rpm for 5 min was diluted with 1 ml of deionized water, and the absorbance of the diluted solution was measured at 260 nm. One unit of enzyme activity was defined as an increase in absorbancy of 1.0 at 260 nm over 5 min under the experimental conditions described above.

**Sodium dodecylsulfate-polyacrylamide gel electrophoresis.** (SDS-PAGE) SDS-PAGE was done in a 12% polyacrylamide gel by the method of Laemmli. The gel was silver stained [Daiichi Kagaku (Tokyo)], and molecular mass was measured by SDS-PAGE with marker proteins (Oriental Yeast Co, Tokyo).

**Protein concentrations.** The protein concentration of the final enzyme preparation was calculated based on the estimated absorbancy of 2.30 of a 0.1% protein solution at 280 nm. This value was estimated on
Table 1. Purification of Nuclease Le3 from Semi-dried Shiitake
The starting material for purification was 6 kg of semi-dried fruit bodies of Lentinus edodes (Shiitake).

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Activity (units)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>24,200</td>
<td>103,000</td>
<td>0.23</td>
<td>100</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄ (0.4s–0.9s)</td>
<td>23,400</td>
<td>38,000</td>
<td>0.62</td>
<td>96.7</td>
</tr>
<tr>
<td>3. Sephadex G-50 (pH 6.0)</td>
<td>21,000</td>
<td>27,000</td>
<td>0.77</td>
<td>86.8</td>
</tr>
<tr>
<td>4. DEAE-Toyopearl (pH 6.0)</td>
<td>7,000</td>
<td>8,700</td>
<td>0.80</td>
<td>28.9</td>
</tr>
<tr>
<td>5. Sephadex G-50 (pH 6.0)</td>
<td>5,850</td>
<td>3,200</td>
<td>1.83</td>
<td>24.2</td>
</tr>
<tr>
<td>6. Ultrogel AcA 54 (pH 6.0)</td>
<td>5,400</td>
<td>320</td>
<td>16.8</td>
<td>22.3</td>
</tr>
<tr>
<td>7. DEAE-Toyopearl (pH 7.0)</td>
<td>2,630</td>
<td>33</td>
<td>80.7</td>
<td>10.9</td>
</tr>
<tr>
<td>8. SP-Sephadex (pH 5.0)</td>
<td>690</td>
<td>1.6</td>
<td>431</td>
<td>2.9</td>
</tr>
<tr>
<td>9. Heparin Sepharose CL-6B (pH 6.0)</td>
<td>720</td>
<td>1.5</td>
<td>480</td>
<td>3.0</td>
</tr>
<tr>
<td>10. Shodex Protein KW-802.5</td>
<td>614</td>
<td>0.96</td>
<td>640</td>
<td>2.5</td>
</tr>
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</table>

Amino acid sequence of Nuclease Le3 from L. edodes on the basis of the amino acid composition of the enzyme.

Amino acid analysis. Amino acid analysis of the protein and peptides was done by the method of Bidlingmeyer et al.18) by using a Pico Tag amino acid analysis system (Millipore Japan, Tokyo). Tryptophan was measured by the method of Pajot.19)

Hexosamine content. The hexosamine content was measured by the method of Bidlingmeyer et al.18) with a Pico Tag amino acid analysis system after hydrolysis of glycoproteins with 2 N HCl at 100°C for 12 h, as described in our previous paper.13)

Carbohydrate content. The carbohydrate content was measured by the method of Mikami and Ishida20) after hydrolysis with 4 N trifluoroacetic acid at 100°C for 4 h. The neutral sugars were separated with a cation exchanger (Shimpack-ISA07/S2594) and then measured fluorometrically by reaction with arginine.

Reduction and S-carboxymethylation of nuclease Le3. Nuclease Le3 was reduced and S-carboxymethylated by the method of Crestfield et al.21) The reduced and S-carboxymethylated nuclease Le3 (RCM nuclease Le3) was separated from the excess reagents by dialysis against deionized water.

Protease digestion. RCM nuclease Le3 was digested at 37°C for 2 h with staphylococcal V8 protease in 0.1 M TMA buffer (pH 7.9) at an enzyme/substrate ratio of 1/100.

Separation of the protease digest by reversed-phase HPLC and of the peptides formed by reduction of nuclease Le3 by SDS-PAGE. (a) Staphylococcal V8 protease digestion products were separated with a column of Shisiedo Capcell pak C-18 (10 x 250 mm) equilibrated with 40 mM TMA buffer (pH 8.9) and an acetonitrile gradient up to 80%. (b) Reduction of nuclease Le3 yielded two peptides, peptide M1 (21 kDa) and peptide M2 (13 kDa), detected by SDS-PAGE. These peptides were transferred to a PVDF membrane and used for sequence analysis.

Amino acid sequencing. The amino acid sequences of the protein and peptides were identified by Edman degradation with an Applied Biosystems Procise 492 protein sequencer.22)

mRNA extraction and cDNA synthesis. mRNAs were extracted from the 2nd mycelia of L. edodes stored at -80°C by using the acid guanidinium-phenol-chloroform method with Trizol Reagent [Gibco BRL, Life Technologies, Inc.(Rockville, MD)]. The cDNA was synthesized by using 1 μg of the purified total RNA and M-MuLV reverse-transcriptase [New England Biolab(Beverly, MA.)], with HIK-18VN(oligo dT) as the primer.23)

Primers and adaptors. The primers and adapters used were synthesized by Amersham Pharmacia Biotechnol. K. K. (Tokyo) and were as follows: Le3-1F, TGGGGNATGAARGGNCAYGARGCN-GT; Le3-25F, TCATTCGTCGAAACCTCTCTGAGT; Le3-35F, CAGTACCATTCTTCTCTTGAGCTTGCC; Le3-65R, GTNGGNGGYTGRTCYTC-NGCRTCNACRAARTG; Le3-155R, GTTTATATTACCATCGTCCCACACAGA; Le3-165R, AGCCGTCGATTTTATCATCGAGTAACTT; Le3-270R, AAAACGAAAAACTGGTACGGGATTGG; Hik-18VN, ATGGTAGCCTGCGATTAGACTCTCA(T)18VN; HIK-ad1, ATGGTAGCCTGCGATTAGACTCTCA; AUAP, GGCCACGCGTCGACTAGTAC; Le3-+34F, ATTCTATCACCACCATT-CTTCTTTC. Abridged Anchor Primer and Abridged Universal Amplification Primer (AUAP) were included in the 5'-RACE system for rapid Amplification of cDNA Ends, Ver. 2.0 (Gibco BRL, Life Technologies, Inc., Rockville, MD).

Nucleotide sequencing. cDNA encoding nuclease
Le3 was amplified from cDNAs by PCR with mixed primers Le3-1F and Le3-65R (see latter section), primer and adapters which are synthesized from known protein sequences of nuclease Le3. Sequencing of the amplified cDNA indicated that it was the cDNA of nuclease Le3. The cDNA was cloned with a pGEM-T Easy Vector system [Promega Co. (Madison, WI)]. To analyze the unknown sequence downstream of the cDNA analyzed as above, the cDNAs were cloned by PCR with Le3-25F and HIK-ad1 adapter primer (3'-RACE method). Analysis of the nucleotide sequence of the cDNA encoding the N-terminal region, signal peptide, and 5'-flanking region of nuclease Le3 was done with the 5'-RACE system for rapid amplification of cDNA ends Version 2.0 [Gibco BRL].

mRNAs were reverse-transcribed with Le3-270R as an antisense primer. Deoxynucleotidyltransferase (TdT) was used to add an oligo C sequence to the 5'-end of the cDNA amplified and the cDNA was then amplified by PCR with Le3-155R and an anchor primer having a sequence complementary to the oligo C, Abridged Anchor Primer. The cDNA was further cloned by PCR with AUAP and Le3-155R as primers. Both strands of DNA were sequenced by the dyeoxy method using a Thermo Sequence Cycle Sequencing kit (Amersham Pharmacia Biotech. Cleveland, OH) with a DNA sequencer, DNA analyzer, and GENE REDIR4299 (Li-COR. Inc., NE).

## Results

### Amino acid sequence of RCM nuclease Le3 found by protein chemistry

Nuclease Le3 was reduced and S-carboxymethylated (RCM nuclease Le3), and the RCM nuclease Le3 was used to analyze the amino acid sequence. RCM nuclease Le3 was digested with staphylococcal V8 protease, and the protease digest was fractionated by reversed-phase HPLC (Fig. 3). The digest was separated into 7 peptides (V1-V7), and the amino acid sequences of the peptides were analyzed by Edman degradation. The results as well as the N-terminal

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**Fig. 3.** Fractionation of the Staphylococcus V8 Protease Digest of RCM Nuclease Le3 by Reversed-phase HPLC.

The staphylococcal V8 protease digest of RCM Nuclease Le3 was fractionated on a Capcell pak C-18 column (10 × 250 mm) equilibrated with 40 mM TMA buffer (pH 8.0). The digest was separated into 7 peptides (V1-V7), and the amino acid sequences of the peptides were analyzed by Edman degradation. The results as well as the N-terminal

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**Fig. 4.** Partial Amino Acid Sequence of RCM Nuclease Le3 Analyzed by Protein Chemistry.

The arrows designate the amino acid sequences analyzed by Edman degradation. "V1-V7" is RCM nuclease Le3 peptide (Fig. 3). B1 and B2 are peptides obtained by reduction of nuclease Le3 (Fig. 2). The locations of the peptides were estimated from the similarity with the sequence of nuclease Le1.
sequence previously reported are shown in Fig. 4. Nuclease Le3, which yielded a single band on SDS-PAGE (Fig. 2), yielded two bands on SDS-PAGE in the presence of a reducing agent (Fig. 2), indicating that the reduced nuclease Le3 consists of two peptides connected by disulfide bridge(s). The amino acid sequences of these two peptides, peptide B1 (21 kDa) and peptide B2 (13 kDa), were analyzed after blotting on a PVDF membrane, and the sequences are also shown in Fig. 4.

The N-terminal sequence previously reported\(^1\) and V1 were included in the B1 sequence. The sequences of V2 and V3 are very similar to the 35th-71st amino acids of nuclease L1. The sequences of V4 and V5 are similar to the 110th to the 169th amino acids of nuclease L1. The amino acid sequences of B2, V6, and V7 are also very similar to that of the 222nd to the 258th amino acids of nuclease L1. We therefore analyzed approximately 60% of the sequence of nuclease Le3 based on the similar molecular masses of the two nuclease according to the results of SDS-PAGE (Fig. 2).

**Nuclease sequence analysis of nuclease Le3 from cDNA cloning**

Since we were unable to establish the complete amino acid sequence of nuclease Le3 by protein chemistry, we tried to deduce the amino acid structure from nucleotide structures of the cDNA and genomic DNA encoding nuclease Le3. The strategy of cDNA sequencing of nuclease Le3 is summarized in Fig. 5. The mRNAs isolated from the 2nd mycelia of *L. edodes* were reverse-transcribed by using reverse-transcriptase with HIK-18VN (oligo dT) as primer.\(^{25}\) cDNA encoding nuclease Le3 was cloned from cDNA by RT-PCR with mixed primers, Le3-1F and Le3-65R, synthesized according to the known protein sequences of nuclease Le3.
By analyzing both strands of the cloned cDNA, we confirmed that the clone containing the nucleotide sequence to encode the N-terminal amino acid sequence of nuclease Le3. To analyze the unknown sequence downstream of the cDNA analyzed as above, the cDNA of the 3'-side was analyzed by PCR of the sequence between Le3-25F and HIK-ad1 as primers. The DNA sequence of the amplified DNA analyzed from both directions is shown in Fig. 5. Analysis of the N-terminal region of nuclease Le3 was done by using the 5'-RACE system for rapid amplification of cDNA ends Ver 2.0 with AUAP and Le3-165R as an antisense primer.

The oligo C sequence was added to the cDNA with deoxynucleotidyltransferase (TdT), and it was then amplified by PCR with Le3-165R and an anchor primer having a sequence complementary to that of oligo C. The cDNA was cloned further, and analysis of the amplified cDNA yielded the nucleotide sequence of the 5'-terminal part of nuclease Le3, including an 18-amino acid putative signal peptide. These experiments allowed analysis of the nucleotide sequence coding nuclease Le3 and 46- and 123-nucleotide sequences upstream and downstream, respectively, of mature nuclease Le3 (Fig. 5).

The results of the sequence analysis of the cDNA encoding nuclease Le3 indicated that the protein portion of nuclease Le3 is a single polypeptide consisting of 280 amino acid residues.

**Nucleotide sequence of genomic DNA encoding nuclease Le3**

The genomic DNA of *L. edodes* was prepared to check the nucleotide sequence of cDNA encoding nuclease Le3. The strategy for sequencing the genomic DNA encoding nuclease Le3 is shown in Fig. 6. The DNA was digested with *Sau*3Al, and the fragments were inserted into λDASHII and transfected into *E. coli*. Plaque hybridization with cDNA obtained by PCR of cDNA with Le3-35F and 270R as primers was used to screen for genomic DNAs encoding nuclease Le3. The genomic DNA encoding nuclease Le3 from the positive clone was further purified and used for sequencing.

The purified DNA was digested with *Bam*HI, and further digestion of the *Bam*HI fragment (5.4 kbp) with *Sal*I yielded four fragments of 1.9 kbp, 2.0 kbp, and 0.75–0.8 kbp. The nucleotide sequence of the 1.9 kbp was analyzed from both directions up to 800 bp. The *Bam*HI fragment (5.4 kbp) was digested with *Sal*I and *Eco*RV, yielding a 0.75-kbp fragment, and its sequence was analyzed from both directions. The genomic DNA was amplified by PCR with +34F and 270R as primers, and the sequence of the DNA was analyzed. The sequence of the DNA was completely identical to the 3'-part of the 1.9-kbp *Sal*I fragment and the 5'-part of the 0.75-kbp fragment of the *Sal*I/*Eco*RV fragment, indicating a definite connection between these two fragments. These experiments made it possible to establish the nucleotide sequence of a 2.65-kbp length of the genomic DNA fragment including the region of the encoding nuclease Le3. The results showed that the genomic DNA encoding nuclease Le3 consists of 13 exons and 12 introns and confirmed the sequence of the cDNA described previously (Fig. 7).

**Comparison of the amino acid sequence of nuclease Le3 with those of the other four nucleases**

The amino acid sequence of nuclease Le3 was compared with those of the other four nucleases studied previously (Fig. 8). Since nuclease Le3 contains 170, 108, 98, and 59 amino acid residues that are identical to residues of nuclease Le1,7) nuclease P1,3,4) nuclease S,5) and a nuclease from *L. donovani* (tentatively named nuclease Ld),6) respectively, these five
Fig. 7. Nucleotide Sequence of the Genomic DNA Encoding Nuclease Le3.

The amino acid sequence deduced from the nucleotide sequence is shown in bold capital letters; the 5'- and 3'-flanking regions are shown in capital letters; and the intron is shown in lower case letters. The putative signal sequence is shown in italic capital letters. The asterisk indicates the stop codon. The sites of cleavage by the restriction enzymes are boxed. Accession number: AB075026

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Fig. 8. Amino Acid Sequence of Nuclease Le3 from L. edodes nucleases are similar enzymes. As shown in Fig. 8, nuclease Le3 contains an extra sequence consisting of 9 amino acid residues between Asp196 and Asn197 (nuclease P1 numbering), and it is 14 residues shorter than the extra sequence of nuclease Le1. There is a short deletion, from the 221st to the 224th residue (nuclease P1 numbering) in nuclease Le3 and nuclease Le1, compared to sequences of nuclease P1 and nuclease S. The amino acid composition of nuclease Le3 shown in Table 2 agreed well with the amino acid composition calculated from the nucleotide sequence.

Some characteristics of nuclease Le3 As shown in Fig. 9a, the pH optimum of nuclease...
Le3 was about 6.5, approximately 1 pH unit higher than that of nuclease Le1. As shown in Fig. 9b, the pH-stability curve showed that nuclease Le3 is active at a wider range of pHs than nuclease Le1. Heat stability testing of nuclease Le3 at pH 5.5 and 65°C for 5 min showed 70% residual activity, slightly higher than for nuclease Le1 (35%). These experiments suggested that nuclease Le3 is active in a wider range of environments than nuclease Le1.

Discussion

Possible modification of nuclease Le3 by proteases

The results of the nucleotide sequence analysis indicated that nuclease Le3 consists of 280 amino acid residues, and SDS PAGE electrophoresis in the presence of a reducing agent (Fig. 2) indicated that it consists of two peptides having molecular masses of about 21 kDa and 10 kDa. These results suggested that mature nuclease Le3 is modified by proteases between the 208th and 209th residue, in vivo or during purification, without separating from each other, probably connecting by disulfide bridges. Since a similar modification was observed in nuclease Le1, the sequences of nuclease Le1 and Le3 around that point may be more susceptible to proteolysis.

Disulfide bridges

As mentioned previously, nuclease Le3 contains 9 half-cystine residues (Cys73, Cys81, Cys86, Cys142, Cys186, Cys203, Cys214, Cys227, and Cys240 in the nuclease Le3 numbering), and since 4 of them are superimposable with the Cys72, Cys80, Cys85, and Cys217 of nuclease P1, they may form disulfide bridges: Cys80-Cys85 and Cys72-Cys217 (nuclease P1 numbering). Since nuclease Le1 contains no free SH groups, it has 4 more half-cystine residues, Cys141, Cys195, Cys227, and Cys253 (nuclease Le1 numbering). Because nuclease Le3 is highly similar to nuclease Le1, we suspected that the three-dimensional structure of nuclease Le1 is very similar to that of nuclease P1. If they are similar, then two disulfide
Table 2. Amino Acid Composition of Nuclease Le3

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Experimental (residue/mol)</th>
<th>Calculated from the sequence (residue/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>38.8</td>
<td>39</td>
</tr>
<tr>
<td>Glu</td>
<td>25.6</td>
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<td>CM-Cys</td>
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<td>Ser</td>
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<tr>
<td>Mannose</td>
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</table>

![Fig. 9](image)

(a) pH Optima of Nucleases Le1 and Le3, with RNA as Substrate, and (b) pH Stability of Both Enzymes at Given pH for 5 min at 37°C.

●, nuclease Le3; ○, nuclease Le1

bridges, Cys141-Cys253 and Cys195-Cys227, probably form between the two closely located half-cystine residues. Among the other 5 half-cystine residues, disulfide bridges between Cys142-Cys240 can be assumed to form based on the analogy with nuclease Le1. However, we were unable to specify the counterpart of the Cys214 residue, because we do not know which of the other three half-cystine residues (Cys214, Cys203, and Cys186) is in the free form. Thus, more precise studies are necessary to obtain exact or probable information concerning the S-S bridge.

Site of carbohydrate attachment

Since nuclease Le3 contains approximately 2 residues of N-acetylglucosamine and 5 residues of mannose, it is a glycoprotein, like nuclease Le1. Nuclease Le3 has a potential carbohydrate attachment site at the same position as nuclease Le1, and thus nuclease Le3 may be an N-glycosylated protein like nuclease Le1. Instead of a lower molecular mass of the protein moiety of nuclease Le3, compared to nuclease Le1, the mobility of nuclease Le3 on SDS-PAGE is roughly the same as that of nuclease Le1. This phenomenon also suggests that nuclease Le3 is a glycoprotein.

Active site of nuclease Le3

The amino acid residues concerned with the active site of nuclease P1 estimated from the X-ray analysis of nuclease P1-nucleotide-complex8) were traced on the amino acid sequence of nuclease Le3, and the amino acid residues in nuclease P1 thought to be concerned with the coordination to Zn$^{2+}$, His6, His61, His117, His127, His150, Asp46, Asp121, and Trp1 (nuclease Le3 numbering), were all found to be conserved in nuclease Le3. Thus, the Zn$^{2+}$ binding site of nuclease Le3 seems to be very similar to that of nuclease P1. Nuclease P1 contains two nucleotide binding sites, a phosphate binding site at Arg48 and Asp45 and base binding site at Phe61 and Asp63 (nuclease P1 numbering). In nuclease Le3 and nuclease Le1, all of these residues are conserved. However, the 2nd nucleotide binding site of nuclease P1 consists of Tyr144 and Tyr155, both of which seemed to stack with the adenine base of the nucleotide, and they were replaced by Lys and Gly in nuclease Le3 and Glu and Gly in nuclease Le1. Of both amino acid residues making the base recognition site 2 of nuclease P1, Asp146 is replaced by Asn in nuclease Le3 and by Thr in nuclease Le1. Thus, the 2nd nucleotide binding site does not seem to be present in nucleases Le1 and Le3.

Comparison of the locations of the exons and introns in nucleases Le1 and Le3
As shown in Fig. 10, the genomic DNA of nuclease Le3 consists of 13 exons and 12 introns, and their locations and sizes are very similar to those of the exons and introns in the gene encoding nuclease Le1, especially the sizes of each exon (Fig. 10). These results seem to suggest that nuclease Le1 and nuclease Le3 genes were duplicated and then evolved at different rates after duplication, retaining a similar exon structure as shown in the phylogenetic tree constructed by the most-likelihood method developed by Adachi and Hasegawa (Fig. 11).26)

References

19) Pajot, P., Fluorescence of proteins in 6 m guanidine hydrochloride: A method for the quantitative


