The Gen and Deduced Protein Sequences of the Zymogen of *Aspergillus niger* Acid Proteinase A*

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Proteinase A obtained from the culture medium of *Aspergillus niger* var. *macrosporus* is a unique acid endopeptidase that is insensitive (or less sensitive) to specific inhibitors of ordinary acid or aspartic proteinases, such as pepstatin, diazoacetyl-DL-norleucine methyl ester, and 1,2-epoxy-3-(p-nitrophenoxyl)-propene. In the preceding paper (Takahashi, K., Inoue, H., Sakai, K., Kohama, T., Kitahara, S., Takishima, K., Tanji, M., Atanda, S. B. P., Takahashi, T., Akahuma, H., Mamiya, G., Yamazaki, M. J. Biol. Chem. 266, 19480–19483), we reported the complete primary structure of the mature enzyme determined at the protein level. The enzyme has a unique two-chain structure with a 39-residue light (L) chain and a 173-residue heavy (H) chain linked noncovalently. As an extension of this study, we isolated genomic and cDNA clones encoding this proteinase and determined their nucleotide sequences. To isolate a genomic clone, the genomic DNA was selectively amplified by polymerase chain reaction using mixed oligonucleotide primers designed from the amino acid sequence of the H chain, and a specific probe thus generated was used for screening a λgt10 genomic library. A cDNA for the enzyme was also selectively amplified by polymerase chain reaction using primers synthesized based on the sequence of the genomic DNA. Sequencing of the cloned genomic DNA and cDNA revealed the nucleotide sequence of the structural gene for the enzyme of 846 base pairs without introns. It encodes the precursor form of proteinase A, including an NH2-terminal preprosequence of 59 residues, the L chain of 39 residues, an intervening sequence of 11 residues, and the H chain of 173 residues linked in that order. Thus, proteinase A is thought to be synthesized as a single peptide chain preproenzyme of 282 residues, which is processed to generate the mature two-chain form.

Two types of acid endopeptidases are known to be present in the culture medium of *Aspergillus niger* var. *macrosporus*, i.e. acid proteinases A and B, which are commercially named Proctase A and B, respectively (1, 2). Proteinase B is a typical aspartic proteinase, like mammalian and fungal peptidases, which is inhibited by pepstatin, diazoacetyl-DL-norleucine methyl ester (DAN) in the presence of cupric ions, and 1,2-epoxy-3-(p-nitrophenoxyl)propene (EPNP) (4, 5). On the other hand, proteinase A is a different type of acid proteinase (1, 6). Its substrate specificity and responses to inhibitors are quite different from those of the ordinary aspartic proteinases (1–5). It is not inhibited by pepstatin and EPNP and only partially inhibited by DAN in the presence of cupric ions under conditions in which proteinase B is completely inhibited (4).

As described in the preceding paper (6), we have determined the complete amino acid sequence of proteinase A by conventional methods of protein chemistry, which revealed that the enzyme has a two-chain structure consisting of a 39-residue light (L) chain and a 173-residue heavy (H) chain bound noncovalently, and has none of the consensus active-site sequences of ordinary aspartic proteinases (6).

Non-pepsin type acid proteinases like proteinase A have also been found in some species of molds, mushrooms, bacteria, and archaeabacteria (7–15). Among them only the sequences of the acid proteinase B from *Scytalidium lignicolium* (15) and thermopsin from *Sulfolobus acidocaldarius* (16) have been reported. The former is insensitive to pepstatin and DAN, but sensitive to EPNP. On the other hand, thermopsin is inhibited by pepstatin, but only slowly and nonspecifically with DAN and EPNP. The amino acid sequence of proteinase A shows no homology with thermopsin, but approximately 50% homology with *Scytalidium* proteinase B. We could not find any other proteins homologous to proteinase A by computer comparisons with 10,856 proteins in the GenBank data base. Proteinase A and *Scytalidium* proteinase B thus seem to belong to the same subclass of non-pepsin type acid proteinases. However, there are three major structural differences. First, the former is a two-chain enzyme, whereas the latter is a single-chain enzyme. Second, the former has two disulfide bonds, whereas the latter has three, and only one of them is common to both enzymes. Third, the former has no counterparts to Glu and Asp, the proposed active-site residues of the latter (17, 18). It therefore remains to be seen which residues really participate in the catalytic function of proteinase A and how the mechanism operates. It will also be interesting to elucidate the mechanism of processing of the precursor form to the mature enzyme.

In the present study, we isolated the gene and cDNA for this enzyme and sequenced them as a further step in this direction. Thus, we have deduced the amino acid sequence, as well as the gene structure of the single-chain 282-residue precursor form of the enzyme, which is composed of a 59-residue prepropeptide, the 39-residue L chain, an 11-residue

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M68871.

*The abbreviations used are: DAN, diazoacetyl-DL-norleucine methyl ester; EPNP, 1,2-epoxy-3-(p-nitrophenoxyl)propene; L chain, light chain; H chain, heavy chain; bp, base pair(s); kb, kilo base(s).
DNA Blotting Analysis and Cloning of the Gene for Proteinase A—When the amplified radiolabeled DNA prepared by using oligonucleotide primers (Fig. 1) was hybridized to a blot of A. niger DNA that had been digested with EcoRI, BamHI, or both, a single major band was found to hybridize with the probe in each digest, and their lengths were approximately 9 kb in the EcoRI digest and approximately 2.8 kb in the BamHI and EcoRI/BamHI digests (Fig. 2). These results suggested that the region from residues 41 to 154 of the H chain was encoded in the 2.8-kb BamHI fragment. In the screening of 40 × 10^6 individual plaques of the λgt10 genomic library, 10 plaques were positive. After single-plaque purification, one of the positive clones was chosen for further study.

Nucleotide Sequence and the Deduced Amino Acid Sequence of the Zymogen—The cloned DNA was shown to be 2,714 bp long by sequence determination (Figs. 3 and 4). Assuming the absence of an intron, the clone would have an open reading frame of 846 bp encoding a protein of 282 amino acid residues.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Intervening peptide, and the 173-residue H chain connected in that order.

**A. niger Acid Proteinase A Zymogen Sequences**

![Image with nucleotide sequences and amino acid sequences]
DNASIS (Hitachi SK) at a span setting of Doolittle profile of preproproteinase A bond. The NHz-terminal glutamine residue is thought to have been converted to a pyroglutamic acid in the latter. The NHz-terminal glutamine residue is thought to have been converted to a pyroglutamic acid residue after cleavage at the Arg'Og-Gln"o bond in the oxidized insulin B chain (28-30, Tyr8-Gly9 bond, since it was reported to cleave the Tyr-Thr bond in the oxidized insulin B chain (5) and the Tyr-Gly bond in [Tyr]-substance P (35). Another question of interest is whether the putative intermediate enzyme whose L and H chains are linked with the intervening sequence is active or

As described in the preceding paper (6), the mature form of proteinase A is a 212-residue protein composed of a 59-residue L chain and a 173-residue H chain bound noncovalently. On the other hand, by nucleotide sequencing of the cDNA and genomic DNA for the enzyme performed in the present study, the precursor protein of proteinase A was deduced to be a single polypeptide of 282 amino acid residues containing both H and L chains, as shown in Figs. 3 and 6a. Thus, this precursor protein is 70 residues larger than the mature enzyme (6), containing a 59-residue preprosequence (residues 1–59) at the NHz terminus and an 11-residue sequence (residues 99–109) intervening between the L and H chains. So far, neither the cleavage nor the intervening sequence has been found in the mature enzyme isolated from the culture filtrate. The amino acid sequences deduced for the H and L chains by DNA sequencing agree completely with those obtained by protein sequencing (6), except that the NHZ-terminal amino acid residue of the H chain is glutamine in the former and pyroglutamic acid in the latter. The NHZ-terminal glutamine residue is thought to have been converted to a pyroglutamic acid residue after cleavage at the Arg109-Gln110 bond.

The sequence of uncharged amino acid residues with a high content of hydrophobic amino acids from residues 3 to 22 in the NHZ-terminal region is characteristic of a signal sequence. This area is the most hydrophobic region in the protein, as can be seen from the hydropathy profile (Fig. 6b), according to Kyte and Doolittle (22). Further, a lysine residue at position 2 is frequently found in the signal sequences. The most probable site of signal sequence cleavage was predicted by the method of von Heijne (23, 24) to be between residues Ala18 and Ala19 (Fig. 7). The location of alanine residues at positions -1 and -3 relative to the putative cleavage site satisfies the (-3, -1) rule (25), and the amino acid residue at position -2 is leucine, which is most frequently found in known signal sequences (23). The next probable site of signal sequence cleavage is between Ala16 and Leu17. In either case, the length of the signal sequence is roughly the same as those of pepsinogens (26). Since proteinase A is excreted into the culture medium, either of these sequences is thought to function as a signal sequence.

In contrast to the abundance of acidic residues in the H and L chains, basic residues are abundant in both the prosequence and the intervening sequence (Fig. 8). Basic residues are particularly prevalent in the regions from residues 24 to 45 (in the prosequence) and from residues 106 to 109 (in the intervening sequence). These basic residue-rich sequences may stabilize the conformation of the proenzyme by interacting electrostatically with acidic residues of the H or L chain, or may be necessary for proper folding of the proenzyme molecule. The precursors of aspartic proteinases such as pepsinogen also contain a prosequence that resembles that of proteinase A in the abundance of basic amino acid residues. In pepsinogen, indeed, the basic residues of the prosequence are thought to interact with acidic residues of the pepsin moiety to stabilize thezymogen molecule at neutral pH inside cells (27). The prosequence of proteinase A may play the same role as that of pepsinogen.

Fig. 9 shows a comparison of the putative prosequence of proteinase A with the prosequences of some typical pepsinogens (28–32) and procathepsin D (33). Interestingly, there appears to be some homology between the prosequence of the proteinase A and those of pepsinogens and procathepsin D, especially in the NHZ-terminal half of the prosequences. Since organic acids such as citric acid excreted by A. niger make the environment acidic, the proenzyme may be activated just after secretion like pepsinogen. However, the possibility also remains that activation takes place before secretion.

The mature form of proteinase A composed of the H and L chains is considered to be generated by removal of the NHZ-terminal prosequence and the intervening sequence from the one-chain proenzyme. Interestingly, these two sequences are the most hydrophilic ones in the protein, as judged from the hydropathy profile shown in Fig. 6b. The three sites, Ala19, Glu50, Tyr94-Gly98, and Arg109-Gln110, therefore, should be cleaved in the activation process of the proenzyme. They may be located at the surface of the enzyme and easily attacked by proteinases. The secondary structure prediction of the proenzyme according to Chou and Fasman (34) suggests that these sites are all in turn structures (Fig. 10). It remains to be elucidated what kind of proteinase(s) participates in processing. Proteinase A or proproteinase A itself may process the proenzyme intra- or intermolecularly like pepsinogen, or another proteinase(s) such as A. niger acid proteinase B may be involved. It may be possible that proteinase A cleaves the Tyr94-Gly98 bond, since it was reported to cleave the Tyr-Thr bond in the oxidized insulin B chain (5) and the Tyr-Gly bond in [Tyr]-substance P (35). Another question of interest is whether the putative intermediate enzyme whose L and H chains are linked with the intervening sequence is active or...
not. So far, however, the proenzyme containing the pro part and/or the intervening sequence has not been isolated from the culture medium. Studies are in progress to investigate the processing and activation of proteinase A by preparing the proenzyme by expressing the cDNA in Escherichia coli and yeast.

The fact that the amino acid sequence of proteinase A is approximately 50% identical with that of Scytalidium acid proteinase B seems to suggest that the two enzymes share the same active sites. However, neither Glu nor Asp, the proposed active site residues of the Scytalidium enzyme (17, 18), are conserved in proteinase A. Both amino acid and cDNA sequencing showed that the corresponding residues in proteinase A are Gln and Lys, respectively (6). Neither of the consensus sequences of the active sites of ordinary aspartic proteinases, Asp-Thr-Gly- and Asp-Ser-Gly-, are present in proteinase A. It remains to be seen which residues in proteinase A are the catalytic residues. Studies are under way to elucidate the active site residues of this enzyme by several methods, including protein engineering and site-directed mutagenesis.

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EXPERIMENTAL PROCEDURES

Materials

Reagents used were purchased as follows: DNA cloning system (Takara, Japan), STRT oligonucleotides (Toyobo, Osaka, Japan), T4 DNA ligase (Toyobo), Taq DNA polymerase (Perkin-Elmer-Cetus), and 

Culture of Aspergillus niger var. macrourus and extraction of total DNA and RNA

Cells were grown in 1% maltose, 1% glucose, 1% peptone, 0.1% MgCl₂, and 0.1% NaH₂PO₄ medium for 30 h, collagenase was added to the medium to a final concentration of 5% (w/v). After additional cultivation for 24 h, the cells were collected and frozen in liquid nitrogen. Total DNA and RNA were prepared as described (12,37).

Amplification of the DNA probe specific for the proteinate A by polymerase chain reaction

Polymerase chain reaction (PCR) was used to generate a probe specific for uncoiled proteinate A gene (38,39). The degenerate nucleotide sequences of the primers used for PCR were designed based on the amino acid sequences of the heavy (H) chain of the proteinate A (6) as shown in Fig. 1. A mixture of 17-mers oligonucleotides was prepared as the sense primers (8), which contained all the degenerate nucleotide sequences encoding the amino acid sequence from residues 11 to 40 of the H chain (Fig. 1). The antisense primers were prepared based on the amino acid sequence from residues 101 to 144 of the H chain. All the possible twenty-four nucleotide sequences (17-mers) were synthesized in a model 380A DNA synthesizer (Applied Biosystems, Inc.), and purified by electrophoresis on 10% polyacrylamide gel containing 8 M urea. The reaction mixture for PCR contained 10 ng of EcoRI-digested genomic DNA as a template, 1 mol each of sense and antisense primers, and 2.5 units of Thermus aquaticus Taq DNA polymerase (Perkin-Elmer-Cetus) in 10 mM Tris-HCl buffer (pH 8.3) containing 1.5 mM MgCl₂, 0.1 mM dNTPs, and 0.01% (v/v) gelatin to a total volume of 50 μl. Samples were heated for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 2 min, and extension at 72°C for 2 min. The amplified probe was 5% agarose gel electrophoresis revealed a major band of approximately 348 base pairs (bp), corresponding to the length of 341 bp expected in the absence of intron. Much more bands could be amplified by using primer C-1 or C-2 as the antisense primer. The band was cut out, and the DNA extracted, purified by phenol extraction, and precipitated with ethanol, and labeled by the multiprimer DNA labeling system (Amersham) with [α-32P]dCTP (40).

DNA blot hybridization

Southern blotting was carried out as described (11). Chromosomal DNA from A. niger var. macrourus was digested with EcoRI and hybridized with the sense and antisense probes (EcoRI-digested chromosomal DNA was fractionated by sucrose density gradient centrifugation, the fractions containing fragments of 2 to 4 kilo base pairs were pooled, and the DNA was precipitated by ethanol). The DNA was immobilized on nylon membrane filters followed by digestion with EcoRI and hybridization. The Southern blot was washed at 65°C in 0.1× SSC containing 0.1% sodium dodecyl sulfate (SDS).

Cloning and sequencing

The EcoRI-digested chromosomal DNA was fractionated by sucrose density gradient centrifugation, the fractions containing fragments of 2 to 4 kilo base pairs were pooled, and the DNA was precipitated by ethanol. The DNA was digested with 17-mers oligonucleotides prepared as the sense primers (8) and hybridized with the antisense primer (8). The DNA was then digested with EcoRI, followed by cloning into the EcoRI site of the M13 mp18 vector and sequenced with the Sequenase sequencing system (US Biochemical Corp.). The resulting sequencing strategy is illustrated in Fig. 4.

Preparation of cDNA

Synthesis of the first strands of cDNA was performed according to the standard method (40). PCR was carried out for selective amplification of cDNA for proteinate A using the first cDNA strands as a template and specific primers. Preparative PAGE for 3% and 3% and restriction of the positive bands from the preparative PAGE with a combination of EcoRI and HindIII were performed. The cDNA was ligated with EcoRI to be cloned into the EcoRI site of M13 mp18 vector, and served for sequencing.

Fig. 1. Oligonucleotide primer sequences used for amplification of the specific probes for the gene of the proteinate A. Primer S corresponds to the amino acid sequence of H chain (36). Primer A-Tyr-Phe-His-Arg (8). Primer G-Tyr-Pro-Phe (8).

Fig. 2. Southern blot analysis of the genomic DNA of A. niger var. macrourus with specific probes for the proteinate A. DNA (10 μg) was digested with HindIII, followed by agarose gel electrophoresis. After the DNA fragments were transferred to a nitrocellulose filter, the 32P-labeled probe amplified by PCR was added to carry out the hybridization. Lambda DNA digested with HindIII was used as a size marker (lane Wi).

Fig. 3. Restriction enzyme map and nucleotide sequencing strategy of the proteinate A gene. The restriction sites are shown on the top. The sequence strategy is summarized on the bottom. The arrows represent the sequencing directions. The bold lines represent the proteinate A gene.

Fig. 4. Determination of exon size and nucleotide sequencing strategy of the proteinate A gene. The restriction sites are shown on the top. The sequence strategy is summarized on the bottom. The arrows represent the sequencing directions. The bold lines represent the proteinate A gene.

Fig. 5. Oligonucleotide primer sequences used for selective amplification of cDNA. Sequences derived from the proteinate A gene are underlined. The recognition sites of EcoRI are dotted.
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ACIDIC RESIDUES

BASIC RESIDUES