Cloning and Heterologous Expression of the Antibiotic Peptide (ABP) Genes from *Rhizopus oligosporus* NBRC 8631

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We carried out protein sequencing of purified Antibiotic Peptide (ABP), and cloned two genes encoding this peptide as *abp1* and *abp2*, from *Rhizopus oligosporus* NBRC 8631. Both genes contain an almost identical 231-bp segment, with only 3 nucleotide substitutions, encoding a 77 amino acid peptide. The *abp* gene product comprises a 28 amino acid signal sequence and a 49 amino acid mature peptide. Northern blot analysis showed that at least one of the *abp* genes is transcribed in *R. oligosporus* NBRC 8631. A truncated form of *abp1* encoding only the mature peptide was fused with the α-factor signal peptide and engineered for expression in *Pichia pastoris* SMD1168H. Culture broth of the recombinant *Pichia* displayed ABP activity against *Bacillus subtilis* NBRC 3335 after induction of heterologous gene expression. This result indicates that mature ABP formed the active structure without the aid of other factors from *R. oligosporus*, and was secreted.

**Key words:** *Rhizopus oligosporus; Bacillus subtilis; Antibiotic Peptide*

*Rhizopus oligosporus* is one of the common filamentous fungi used for fermented food production in Asia. For example tempeh, a traditional food in Indonesia, is prepared by fermenting soybeans with this fungus, and is now popular throughout the world, especially in the U.S.A., as a low fat, low calorie health food. Other *Rhizopus* species are also used for making many kinds of alcoholic beverages in China, such as shao hsing chiu.

In 1969, Wang et al. reported the partial purification of a bacteriostatic agent during production of tempeh using *R. oligosporus*.1 In 1992, we reported the purification of an antibiotic peptide (ABP) from the submerged cultivation broth of *R. oligosporus* NBRC 8631.2 The ABP is a simple cysteine-rich (20%) peptide with a molecular mass of approximately 5,500 Da. It is thought that the high cysteine content of ABP might contribute to its pH and thermal stability. Interestingly, the antibiotic spectrum of ABP was relatively narrow, but it was very active against some of the *Bacillus* species, especially against *Bacillus subtilis* (*natto*), a major contaminant of tempeh production.

Given the proven safety record of *R. oligosporus* in tempeh production, together with the evident antibiotic specificity and stability, ABP might be a useful antibiotic agent for food processing. For such applications, functional analysis of ABP is necessary. In this paper, we describe the protein sequencing of purified ABP, the cloning of this gene from *R. oligosporus* NBRC 8631, and its heterologous expression in *Pichia*. This represents the first step towards a structural analysis of ABP and a full characterization of its unique properties.

**Materials and Methods**

**Strains, plasmids, and media.** *Rhizopus oligosporus* NBRC 8631 was used as the gene source and *Bacillus subtilis* NBRC 3335 was used as an indicator strain for the antibiotic assays. *Escherichia coli* strain DH5α, and *Pichia pastoris* SMD1168H were used as hosts, and pCR®2.1-TOPO, pCR®-Blunt, and pPICZαA (Invitrogen, Tokyo) were used as vectors for cloning and expression of the ABP genes. *R. oligosporus* was grown in 5% casamino acid (Difco) medium. *B. subtilis* was cultivated in CM-medium consisting of 4% glucose, 1% polypeptone, 0.5% yeast extract, 0.5% KH₂PO₄, and 0.2% MgSO₄·7H₂O with or without 2% agar. *E. coli* was grown in LB medium with or without 2% agar supplemented with the appropriate antibiotics. BMGY and BMMY medium consisting of 1% yeast extract, 2% polypeptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% Yeast Nitrogen Base (Difco), 4 x 10⁻⁵% biotin, and 1% glycerol or 0.5% methanol were used for *Pichia* ABP gene expression studies.

**Protein sequencing of purified ABP.** The N-terminal amino acid sequence of ABP was determined after alkylation with iodine acetate followed by Edman degradation using a 471A protein sequencer (Applied Biosystems, Tokyo). The internal amino acid sequence of ABP was obtained by treatment with endoproteases to

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generate peptides for N-terminal sequencing. V8 Protease (Wako, Osaka) and clostripain (Sigma, St. Louis, MO)-digested peptides of the ABP were purified by HPLC using an Inertisol-ODS column (4.6 mm × 15 cm, GL Science, Tokyo). The C-terminal amino acid of the ABP was obtained after treatment with carboxypeptidase Y (Takara, Otsu, Japan) and determined using a Hitachi 853 amino acid analyzer (Hitachi, Tokyo).

Cloning and sequencing of the ABP gene. Genomic DNA was extracted from R. oligosporus NBRC 8631 mycelia cultivated with 5% casamino acid medium for 3 d at 30°C according to Lee et al. A degenerate pair of PCR primers designed from the protein sequence data (ABP-N, 5'-GCNTGYGGNGCNGARGGNSWTGY-CA-3'; ABP-C, 5'-TARTACNCRAANGNGCNGCY-TGYTT-3'; N, A, T, G or C; Y, T or C; R, A or G; S, G or C; W, A or T) were used for partial ABP gene fragment amplification using genomic DNA as a template. Primers ABP-F (5'-ACAGATGCAAAACGATGCTCTGCGCCCTAC-3') and ABP-R (5'-GTAAGGCGCAGGACATGCTTTGGCATGCT-3') were used to clone DNA flanking the ABP gene from a library constructed with the Universal Genome Walker kit (BD Biosciences Clontech, Tokyo). The ABP gene that contains 5' and 3' UTR was amplified with primers LABP-F (5'-TGGTCTGAGTGACCTGAGCGTCTATG-3') and LABP-R (5'-GCCTTTAGAAAGATTGAAGGCCA-3') from R. oligosporus genomic DNA, subcloned into pCR®-Blunt to generate pCR-LABP1, 2, 3, and 4, and sequenced. Nucleotide sequences were determined by the dideoxyxynucleotide chain termination method using a DNA sequencer ABI 310 (Applied Biosystems, Tokyo).

Southern blot analysis of the ABP gene. R. oligosporus NBRC 8631 genomic DNA was digested with several restriction enzymes, separated by electrophoresis and blotted on a nylon membrane (Hybond-N+, Amersham, Tokyo). A probe was amplified using a PCR DIG probe synthesis kit (Roche Diagnostics, Tokyo) with R. oligosporus genomic DNA as a template and the oligonucleotide primers ABP-ORF1 (5'-GAAGCCCTATGCTAGTTCTTTCTCAATGA-3') and ABP-ORF2 (5'-GAAGCCCTTTAGCTGTAATAGCAC-3'). Hybridization was carried out according to the manufacturer’s instructions.

Northern blot analysis of the ABP gene. R. oligosporus NBRC 8631 was cultivated with 5% casamino acid medium at 30°C and total RNA was extracted using Isogen (Nippongene, Tokyo). Northern blot analysis was carried out as described by Akao et al. The same probe was used for Southern blot analysis.

Heterologous expression of the ABP gene. The gene encoding ABP was amplified from plasmid pCR-LABP1 using primers α-ABP (5'-CTCGAGAAGAGACATG-5') and ABP-ORF2. The PCR product was subcloned directly into pCR®-Blunt and sequenced. The gene fragment was digested with XhoI and NotI, and inserted into pPICZαA to construct the Pichia expression vector, pPIC-αABP2. The plasmid was then digested with PmeI and introduced into P. pastoris SMD1168H using the EasySelect Pichia Expression Kit (Invitrogen, Tokyo). Transformants were selected on the basis of resistance to Zeocin and pre-cultivated with BMGY for 2 d at 30°C before being transferred to BMMY for several hours. An aliquot (5 μl) of culture broth was spotted directly onto an ABP activity test plate prepared as described by Kobayashi et al. Briefly, S. subtilis NBRC 3335 was incubated overnight in CM-medium at 30°C. An aliquot (200 μl) of 25-times diluted culture was added to 20 ml of molten agar (1.5% agar containing CM-medium) cooled to 50°C. The medium was then poured into a Petri dish and hardened to make an ABP activity test plate. Then the plates were incubated for 16 h at 30°C.

Results

Protein sequencing of purified ABP
Previously we reported the purification of ABP from the submerged cultivation broth of R. oligosporus NBRC 8631. We carried out protein sequencing of purified ABP and determined 43 residues as ACGAEG-SCHFGGGELCNRDKCSGPTGKYKRAGCCGT-LKQA from the N-terminal (Fig. 1B). We also carried out C-terminal amino acid analysis and obtained it as YS. Then three fragments generated by V8 Protease and clostripain treatments were obtained and analyzed. Two fragments from V8 Protease treatment were thought to correspond to the N-terminal region of ABP. From the fragment generated by clostripain, the sequence GKYK-RGACCGLKQAAPCGYYS was obtained, but the signals of the C-terminal region, especially the part APCGYYS, were relatively weak. Given these results, though the predicted cysteine content (16%) was lower than that determined from the amino acid analysis (20%) of purified ABP, we predicted the complete amino acid sequence of ABP, as shown in Fig. 1B.

Cloning of the ABP gene
From the protein sequencing data, a pair of primers, ABP-N and ABP-C, were designed, and a partial ABP gene fragment was amplified from R. oligosporus genomic DNA and sequenced. Then primers ABP-F and ABP-R were used to clone the upstream and downstream regions of the partial gene fragment. Unexpectedly, we found two distinct fragments amplified by both primers, suggesting the existence of a pair of abp genes in the R. oligosporus genome. To obtain these genomic regions, primers LABP-F and LABP-R were designed and used for PCR amplification. Sequencing analysis of four individual clones resulted in two groups, entirely identical pCR-LABP1 and pCR-
LABP2, and entirely identical pCR-LABP3 and pCR-LABP4. As shown in Fig. 1A, both gene fragments were predicted to include an almost identical 231-bp segment encoding 77 residues, with only 3 nucleotide differences, so we designated them \textit{abp1} and \textit{abp2}. Using the PSORT II program (http://psort.ims.u-tokyo.ac.jp/), a 28 amino acid signal sequence was predicted for both peptides, followed by a 49 amino acid length of mature peptide. One amino acid substitution was identified in the signal peptide region (ABP1: 15 Phe and ABP2: 15 Leu) between these products, but the mature portions of the peptides were identical. These predicted translation products based on both gene sequences were also in good agreement with that of protein sequencing results from 29 Ala to 71 Ala, but one deletion and two substitutions were found in the C-terminal region (Fig. 1B). To clarify whether another gene(s) corresponding to ABP exist in the \textit{R. oligosporus} genome, Southern blot analyses were carried out under standard and low stringency conditions. Using a probe amplified from the mature ABP region of pCR-LABP1 and restriction enzymes that do not cut the ABP coding region, only two signals corresponding to \textit{abp1} and \textit{abp2} were detected under the standard condition (Fig. 2A), and no additional signal was obtained in low stringency hybridization (Fig. 2B). Given these results and the technical difficulties of protein sequencing, we conclude that the sequence predicted from gene analysis is correct. This conclusion is supported by the calculated molecular mass data for the mature ABP1 and ABP2 (5,050 Da) which is almost equal with that of purified ABP (5,000 Da) estimated by SDS-PAGE. Furthermore the predicted cysteine content (20.4%) was consistent with that of the amino acid analysis (20.0%) of purified ABP. Southern analysis results also suggest that \textit{abp1} and \textit{abp2} are allelic genes, as in the case of \textit{chi1} and \textit{chi2} or \textit{chs1} and \textit{chs2}.

Comparison of the 5' and 3' regions of the \textit{abp1} and \textit{abp2} genes indicated several nucleotide deletions, substitutions, and insertions. Several putative TATA sequences and a poly adenylation signal (5' -AATAAA- 3') were found in both the \textit{abp1} and the \textit{abp2} gene, but no typical CCAAT sequence was found. To confirm expression of the ABP gene, Northern blot analysis was
Heterologous expression of the ABP gene

To clarify whether the \(\text{abp1}\) and/or \(\text{abp2}\) gene(s) encode active ABP, we constructed a heterologous expression plasmid, pPICZ-\(\alpha\)-ABP2, which contains the mature part of ABP from \(\text{abp1}\) for expression in \(P.\) pastoris (Fig. 4A). In this construct, ABP gene expression is under the control of the methanol-inducible \(\text{AOX1}\) gene promoter. We also arranged that the mature peptide of ABP be secreted using the \(\text{C11}\)-factor signal sequence and cleavage by the \(\text{KEX2}\) protein (Fig. 4A).

\(P.\) pastoris SMD1168H was transformed with pPICZ-\(\alpha\)-ABP2 or pPICZ as a negative control. Several transformants were tested for the production of ABP activity. Only cells harboring pPICZ-\(\alpha\)-ABP2, after 3 h induction in the methanol-containing BMMY medium, displayed ABP activity against \(B.\) subtilis NBRC 3335 (Fig. 4B). This result clearly indicates that at least one of the ABP genes, \(\text{abp1}\), encodes active ABP that are expressed in \(P.\) pastoris under the control of the \(\text{AOX1}\) gene promoter. Furthermore, this result indicates that mature ABP formed the active structure without the aid of additional factors.

Discussion

We carried out protein sequencing of purified ABP and cloned two genes, \(\text{abp1}\) and \(\text{abp2}\), encoding this peptide from \(R.\) oligosporus NBRC 8631. The 5’ and 3’ regions of \(\text{abp1}\) and \(\text{abp2}\) showed some differences in nucleotide sequence (deletion, substitution, and insertion), but both genes were predicted to include an almost identical 231-bp segment encoding a 77 amino acid peptide. Yanai \textit{et al.}\textsuperscript{5} reported the cloning of allelic chitinase genes, \(\text{chi1}\) and \(\text{chi2}\), and Motoyama \textit{et al.}\textsuperscript{6} also cloned two pairs of allelic chitin synthase genes, \(\text{chs1}\) and \(\text{chs2}\), from \(R.\) oligosporus. Although the ploidy of \(R.\) oligosporus is unknown, we conclude that \(\text{abp1}\) and \(\text{abp2}\) also represent a pair of allelic genes.

Both \(\text{abp}\) genes were predicted to encode a 77 amino acid peptide, comprising a 28 amino acid signal sequence and a 49 amino acid mature peptide. The N-terminal sequence of the predicted mature peptide is identical to the sequence obtained from purified ABP. Furthermore, both the predicted molecular mass of the mature peptide and the predicted cysteine content were in good agreement with the experimentally determined values. The disparity between the amino acid sequencing data at the C-terminal region of ABP and the DNA analysis is probably due to technical difficulties associated with protein sequencing.

The 10 cysteine residues within the mature 49 amino acid peptide perhaps contribute to the pH and thermal stability of ABP. Disulfide bonds are thought to be essential for biological activity because treatment with 2-mercaptoethanol (1% \(v/v\)) drastically reduces the bacteriostatic properties of ABP (data not shown). Furthermore, SDS–PAGE electrophoresis of ABP in the absence of 2-mercaptoethanol gave an apparent
molecular mass of 15 kDa, indicating that native ABP might be a homo-trimer (data not shown). These data suggest that disulfide bonds form between ABP molecules to give the homo-trimer and thereby contribute to ABP activation and stability. The X-ray crystal structure of ABP ought to clarify the tertiary structure and might help to elucidate the mechanism of antibiotic activity.

We fused the mature part of ABP with an α-factor signal sequence for heterologous expression in P. pastoris SMD1168H. Results from the recombinant expression studies indicate that \( \text{abp1} \) encodes active ABP, and that the mature form of ABP is secreted from \( P. \) pastoris. Though further analysis is needed, it was thought that the correct tertiary structure of ABP was formed without the aid of other factors from \( R. \) oligosporus. Furthermore, ABP does not appear to require post-translational modification for activity, in contrast with the lantibiotics, which include the rare nonprotein amino acid lanthionine. Lantibiotics are known to be converted into biologically active peptide by a series of post-translational modifications of specific amino acids.\(^7\)

The physiological function of ABP is not clear. Interestingly, the antibiotic spectrum of ABP is relatively narrow, but ABP is very active against several \( \text{Bacillus} \) species, especially \( B. \) subtilis (natto) a major contaminant of tempeh production. We found that a related strain of \( R. \) chinensis B12, identified from Chinese fermented food, produces a very similar antibiotic peptide to ABP (data not shown). These data suggest that these peptides might have an ecological role that increases the competitive fitness of the producing organisms.

In this paper, we describe the protein sequencing of purified ABP, cloning of two \( \text{abp} \) genes from \( R. \) oligosporus NBRC8631, and the heterologous expression of one of the genes in a \( Pichia \) system. This represents the first step toward a structural analysis of ABP and a better understanding of its unique characteristics. We intend to study the mechanism of antibiotic activity and elucidate the physiological role of ABP. In addition, ABP may prove to be of considerable practical value as an antibiotic in food processing.

References

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