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ORIGINAL PAPER

A highly thermostable antimicrobial peptide from *Aspergillus clavatus* ES1: biochemical and molecular characterization

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Abstract Antimicrobial peptides (AMPs) are extremely attractive candidates as therapeutic agents due to their wide spectrum of antimicrobial activity and mechanism of action, which differs from that of small-molecule antibiotics. In this study, a 6.0-kDa antimicrobial peptide from *Aspergillus clavatus* ES1, designated as AcAMP, was isolated by a one-step heat treatment. AcAMP was sensitive to proteolytic enzymes, stable between pH 5.0 and 10.0, and heat resistant (15 min at 100°C). The *acamp* gene encoding AcAMP peptide was isolated by reverse-transcriptase polymerase chain reaction (RT-PCR) and cloned in pCR®II-TOPO vector. Sequence analysis of the complementary DNA (cDNA) *acamp* gene revealed an open reading frame of 282 bp encoding a peptide of 94 amino acid residues consisting of a 21-aa signal peptide, a 22-aa pro-peptide, and a 51-aa mature peptide. The deduced amino acid sequence showed high identity with other ascomycete antifungal peptides. AcAMP belongs to the group of small, cysteine-rich, basic proteins with antimicrobial activity. In addition to its antifungal activity, AcAMP is the first fungal peptide exhibiting antibacterial activity against several Gram-positive and Gram-negative bacteria. Based on all these features, AcAMP can be considered as a promising new member of the restraint family of ascomycete antimicrobial peptides that might be used in biological control of plant diseases and also for potential applications in food preservation.

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Introduction

During recent decades, bacteria resistance to most clinically available antimicrobial agents has emerged at an alarming rate. Consequently, discovery of new antimicrobials has become increasingly crucial [29]. Most research has focused on investigation of natural products as sources of novel bioactive molecules. Antimicrobial peptides (AMPs), widely expressed by a diverse range of organisms, represent a new family of antibiotics that have stimulated research and clinical interest as new therapeutic options for infections caused by multidrug-resistant bacteria and fungi [29]. It has been shown that AMPs possess antibacterial, antiviral, antifungal, antitumor, and immunomodulatory activities [11]. These peptides are generally short, with a broad spectrum of antimicrobial activity, strong thermal stability, and low immunogenicity [31]. AMPs are generally defined as peptides of fewer than 100 amino acid residues with an overall positive charge imparted by the presence of multiple lysine and arginine residues and a substantial portion (≥30% or more) of hydrophobic residues. To date, more than 800 AMPs with several different sequence motifs have been isolated from a wide range of organisms including bacteria, fungi, plants, and vertebrates [10, 13]. A common feature shared among the cationic AMPs is their ability to fold into amphipathic conformations, often induced by interaction with membranes or membrane mimics [11]. Alongside their direct antimicrobial activities against Gram-positive and Gram-negative bacteria, these peptides play additional roles as antibiotics against fungi [23] and protozoa [1].

Filamentous fungi are valuable sources of small antifungal peptides. A limited number of peptides have so far been isolated from this restraint family. The first filamentous fungus described as a potent producer of an antifungal peptide, called AFP, was *Aspergillus giganteus* [16, 19]. An antifungal peptide, named PAF, was then isolated from *Penicillium chrysogenum* [20], and later another antifungal peptide, termed AnAFP, was identified from *Aspergillus niger* [18]. In addition, NAF peptide was isolated from *Penicillium nalviogense* and found to be identical to PAF [10]. In fact, AFP showed 35.6% and 47.1% homology to AnAFP and PAF/NAF, respectively. Recently, AcAFP antifungal peptide was isolated from *A. clavatus* VR1 strain [27]. All these reports dealt with the antifungal activity of isolated peptides, whereas no antibacterial activity was described for these strains, especially for *A. clavatus* species.

In this paper, we report for the first time the isolation of a potent antibacterial peptide from *A. clavatus* ES1. We also investigate its biochemical properties. The gene encoding the antibacterial peptide was cloned and sequenced.

Materials and methods

Strains, plasmids, and growth conditions

A. clavatus ES1 was isolated from wastewater (Sfax, Tunisia). It was identified on the basis of 860 bp of the 18S ribosomal RNA (rRNA) analysis (EF151929) [12]. The strain was propagated on potato dextrose agar (PDA) plates at 30°C, and conidia were prepared from 7-day-old colonies by flooding with 10 ml sterile distilled water and scraping the agar plates.

Escherichia coli TOP10 (F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) was used as a host for plasmid propagation. The strain was purchased from Invitrogen Life Technologies. Culture of different *E. coli* clones was done in Luria–Bertani (LB) medium composed of (g l^{-1}): peptone, 10; yeast extract, 5; and NaCl, 5.

The plasmid pCR®II-TOPO® (Invitrogen Corporation, USA) was used for gene cloning. Wizard® *Plus* SV Minipreps DNA Purification System kit (Promega, USA) was used for plasmid preparation.

Production of AcAMP peptide

Production of AcAMP was carried out in medium containing (g l^{-1}): yeast extract, 10; peptone, 5; NaCl, 5; glucose, 20; pH 7.0. Culture media were inoculated with 10^7 spores ml^{-1} and incubated on a rotatory shaker (150 rev min^{-1}) for 96 h at 30°C in 300-ml Erlenmeyer flasks with working volume of 50 ml. The culture supernatant was obtained by filtration and centrifugation at 8,000 rpm during 15 min. The resultant supernatant was used as crude peptide preparation.

One-step purification of AcAMP and N-terminal amino acid sequence

The thermostable character of AcAMP was exploited in the purification procedure. The supernatant was incubated at 100°C for 10 min, and then thermolabile and insoluble materials were removed by centrifugation at 13,000 rpm for 15 min. The purity of the AcAMP was examined by 15% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing conditions as described by Laemmli [17]. The molecular weight of the purified AcAMP was estimated using a low-molecular-mass calibration kit as markers consisting of bovine serum albumin (66 kDa), egg white ovalbumin (45 kDa), glyceraldehyde-3-P dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine a-lactalbumin (14.2 kDa). The peptide concentration was measured by the method of Bradford [5] using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

The region containing the AcAMP band was excised and electrophoretically transferred from an SDS–PAGE to a polyvinylidene difluoride (PVDF) membrane. Then, the N-terminal amino acid sequence was determined by the Edman degradation method using an ABI Procise 494 protein sequencer (Applied Biosystems).

Antimicrobial assay

Antibacterial activities of purified AcAMP were tested against seven bacterial strains: *Staphylococcus aureus* (ATCC 25923), *Micrococcus luteus* (ATCC 4698), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 13883), *Bacillus cereus* (ATCC 11778), and *Enterococcus faecalis* (ATCC 29212). Antifungal activities were tested using *Aspergillus niger*, *Fusarium solani*, and *F. oxysporum*. The strains were kindly provided from the Center of Biotechnology, Sfax, Tunisia.

The antibacterial and antifungal activities were assessed according to the method described by Berghe and Vlietinck [4]. Briefly, 200 µl of tested microorganisms [10^6 colony-forming units (cfu) ml^{-1} of bacteria cells and 10^8 spores ml^{-1} of fungal strains] was spread on Luria–Bertani (LB) agar and PDA medium, respectively. Then, wells (3 mm depth, 4 mm diameter) were made using a sterile borer and were loaded with 50 µl peptide solution at desired concentrations as estimated by Bradford assay. Supernatant from

uninoculated medium was used as negative control. The Petri dishes were kept, firstly for 1 h at 4°C, and then were incubated for 24 h at 37°C for bacteria and 72 h at 30°C for fungal strains. Antimicrobial activity was evaluated by measuring the growth inhibition zone diameter in millimeters (including well diameter of 4 mm). Measurements of inhibition zones were carried out for three sample replications, and values are the average of three replicates.

Effects of enzymes, heat, and pH on AcAMP activity

In order to evaluate the sensitivity of AcAMP to proteolytic enzymes, a solution of the isolated peptide (200 μg ml^{-1}) was incubated at 37°C for 1 h with 2 mg ml^{-1} final concentration of the following enzymes: trypsin, proteinase K, Alcalase, and chymotrypsin in 100 mM Tris–HCl buffer (pH 8.0) and pepsin in 100 mM sodium acetate buffer (pH 4.0). The sensitivity of AcAMP to EndoH, Thermamyl®, and lipase was also investigated. Samples were then boiled for 2 min to inactivate the enzyme. The antimicrobial activities were then measured by the agar well diffusion method against *Bacillus cereus* (ATCC 11778) as bacterial indicator strain. Trypsin, proteinase K, chymotrypsin, pepsin, EndoH, and lipase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Thermamyl® and Alcalase were purchased from Novozymes A/S, Bagsvaerd-Denmark.

To investigate thermal stability, purified AcAMP peptide was incubated at 50°C, 70°C, 90°C, and 100°C for 5, 10, and 15 min. After cooling the treated samples on ice for 10 min, antimicrobial activity against *B. cereus* was measured as mentioned above.

pH stability was determined by adjusting samples of purified AcAMP peptide with 5 M NaOH or 5 M HCl to different pH values ranging from 3.0 to 11.0, followed by incubation for 1 h at room temperature. Antimicrobial activity against *B. cereus* was then assayed as mentioned above.

Determination of minimum inhibitory concentration (MIC)

MIC values were determined by microwell dilution method [8]. The purified AcAMP peptide was subjected to dilution series in a 96-well microtiter plate. The antibiotic gentamycin was used as positive standard and AcAMP free solution was used as negative control. Each well of the microplates included 40 μl growth medium, 10 μl inoculum (10^6 cfu ml^{-1}), and 50 μl diluted AcAMP peptide. Then, the microplates were incubated overnight at 37°C. As an indicator of microorganisms growth, 40 μl *p*-iodonitrotetrazolium violet (INT) dissolved in water was added to the wells and incubated at 37°C for 30 min. The colorless tetrazolium salt acts as an electron acceptor and is reduced to a red-colored formazan product by biologically active organisms [8]. Where microbial growth was inhibited, the solution in the well remained clear after incubation with INT. Evaluation of MIC values was carried out in triplicate.

RNA isolation and reverse transcription

A. clavatus ES1 was grown in the medium described above. After 96 h, mycelia from a 50-ml culture were harvested by filtration through no. 1 Whatman filter, washed with sterile distilled water, quickly frozen in liquid nitrogen, and disrupted by grinding; total RNA was extracted with Trizol reagent (Invitrogen). The RNA pellet was collected by centrifugation (14,000*g*, 15 min) and washed twice with 75% ethanol (v/v). The RNA preparation was resuspended in Diethylenepyrocarbonate (DEPC)-treated water, and the purity of the preparation was estimated by calculating $A_{260\,nm}/A_{280\,nm}$ ratios. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using the SuperScript™ III Reverse Transcription System (Invitrogen). Total RNA (1.0 μg), random primers (250 ng) in nuclease-free water, 10 mM dNTP mix (1.0 μl), and sterile distilled water to 13 μl were preheated for 5 min at 70°C and then chilled in ice-water for 5 min. The following reagents were then added: ×5 First-Strand buffer (250 mM Tris HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) (4.0 μl), 0.1 M 1,4-Dithiothreitol (DTT) (1.0 μl), RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen) (1.0 μl), and SuperScript™ III Reverse Transcriptase (200 units μl^{-1}) (1.0 μl). The reaction was incubated at 50°C for 50 min, and the enzyme was then inactivated at 70°C for 15 min. The resultant cDNA was used as template.

Cloning and cDNA sequencing of *acamp* gene

The primers used for isolation, sequencing, and cloning of the open reading frame (ORF) encoding AcAMP peptide were designed according to *A. clavatus* NRRL1 gene (accession number: XM_001272037). PCR was performed with forward primer AcAMPF: 5′-GCACAACCCCCGCC GCAGTTT-3′ and reverse primer AcAMPR: 5′-ACTC CGCCCTTTCGCCAGCTCC-3′. PCR was carried out in a Thermo Hybaid PCR Express thermocycler. The amplification reaction mixtures (50 μl) contained 20 ng cDNA as template with 1.0 unit *Go Taq* DNA polymerase, 10 mM dNTP, ×5 *Go Taq* buffer, and 20 μM of each primer. Amplification of DNA was carried out under the following conditions: denaturation (1 min at 94°C), annealing (45 s at 53°C), and extension (1 min at 72°C) for 35 cycles, followed by 10 min at 72°C. Amplified PCR products were analyzed on a standard 1% (w/v) agarose gel containing ethidium bromide and purified by PureLink™ PCR purification kit (Invitrogen).

The purified PCR product was cloned in pCR®II-TOPO plasmid vector according to the manufacturer's protocol

(Invitrogen Corporation). Competent cells of *E. coli* TOP10 were transformed with the ligation mixture. The recombinant plasmids were then purified with Wizard® *Plus* SV Minipreps DNA Purification System kit (Promega, USA). The presence of the appropriate insert was checked by PCR.

The nucleotide sequence of *acamp* gene was determined by dideoxynucleotide chain termination method using an automated DNA sequencer ABI Prism_3100-Avant Genetic Analyzer (Applied Biosystems) using M13 primers from both strands. In order to confirm the fidelity of the sequence, the gene encoding *acamp* of *A. clavatus* ES1 was sequenced three times using recombinant plasmids from three different clones as templates, in each case, to exclude potential sequencing errors that can occur during PCR reaction.

Database searches were performed online with the programs blastx, blastp, and blastn provided by the BLAST E-mail server [2]. CLUSTALW was used to align the amino acid sequences. All parameters were set at their default values.

Nucleotide sequence accession number

The nucleotide sequence of *acamp* gene has been submitted to the GenBank database and assigned the accession number GU390689.

Statistical analysis

Values are expressed as mean ± standard deviation. Analysis of variance (ANOVA) was conducted, and differences between variables were tested for significance by one-way ANOVA using the Statistical Package for the Social Sciences (SPSS, version 11) program. Differences at $P \leq 0.05$ were considered statistically significant. Correlation and regression analysis was carried out using Excel software (Microsoft Corporation, USA).

Results

Purification and N-terminal sequence of AcAMP peptide

An antimicrobial peptide from *A. clavatus* ES1, named AcAMP, was purified in one step. The thermostable character of the AcAMP peptide was exploited after having confirmed this feature in culture supernatant. Heat treatment for 10 min at 100°C effectively removed a large amount of inactive and thermosensitive proteins from the crude supernatant. After removing the precipitated proteins, the resulting supernatant contained only one small protein with molecular mass of about 6.0 kDa as estimated by SDS–PAGE (Fig. 1). A 50-µl sample of the obtained fraction, at 200 µg ml^{-1}, was used to test antimicrobial activity against *B. cereus* as indicator strain. A large inhibition zone (diameter about 22 mm) was detected, showing that the 6.0-kDa peptide had antimicrobial activity. It is interesting to note that AcAMP peptide was produced only when cultures were incubated at 30°C. No antimicrobial activity was detected in the supernatant from culture incubated at 37°C. This observation suggests that the *acamp* gene could be regulated by a thermosensitive activator.

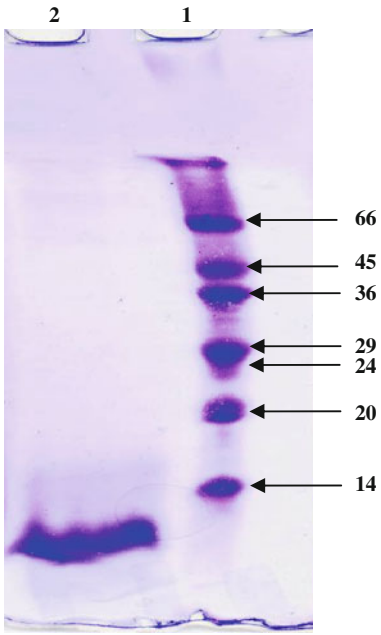

Fig. 1 Sodium dodecyl sulfate–polyacrylamide gel 15% (w/v) electrophoresis of the purified AcAMP peptide: *lane 1* molecular mass marker (Amersham Biosciences); *lane 2* purified AcAMP peptide obtained after heat treatment for 15 min at 100°C

The N-terminal sequence of the first 19 amino acid residues of the purified AcAMP peptide was found to be ATYDGKCYKKDNICKYKAQ (Table 1). Findings showed that the N-terminal sequence of the mature AcAMP peptide is identical to that of antifungal peptide (AcAFP) from *A. clavatus* VR1 [27]. A single change at the fourth residue was detected compared with AFP from *A. giganteus*: Asp in AcAMP peptide is replaced by Asn in AFP [24]. The N-terminal sequence of AcAMP peptide exhibits a lower degree of homology with PAF peptide from *P. chrysogenum* at 58% identity. Furthermore, AnAFP peptide from *A. niger* has no similarity with AcAMP peptide from *A. clavatus* ES1. No antibacterial activity was reported for these peptides from described strains.

Antimicrobial spectrum of AcAMP peptide

AcAMP peptide from *A. clavatus* ES1 was tested against various pathogenic strains by agar well diffusion method. The purified AcAMP peptide exhibited inhibitory activities

Table 1 N-terminal sequence comparison of AcAMP with AcAFP, AFP, PAF, and AnAFP antifungal peptides

Peptide	Microorganism	N-terminal sequence	Reference
AcAMP	*A. clavatus* ES1	ATYDGKCYKKDNICKYKAQ	Present report
AcAFP	*A. clavatus* VR1	ATYDGKCYKKDNICKYKAQ	[27]
AFP	*A. giganteus*	ATYNGKCYKKDNICKYKAQ	[25]
PAF	*P. chrysogenum*	AKYTGKCTKSKNECKYKND	[20]
AnAFP	*A. niger*	LSKYGGECSLEHNTCTYRK	[18]

Table 2 Spectrum of antimicrobial activity of AcAMP peptide from *A. clavatus* ES1

Microorganism	Inhibition zone (mm)			MIC (μg ml^{-1})	
	50 μg ml^{-1}	100 μg ml^{-1}	200 μg ml^{-1}	AcAMP peptide	Gentamycin
S. aureus	11.0 ± 1.0	16.0 ± 0.5	19.0 ± 1.0	20.0	08.0
B. cereus	15.0 ± 2.0	18.0 ± 1.0	22.0 ± 1.0	10.0	08.0
M. luteus	12.0 ± 1.0	14.0 ± 0.5	18.0 ± 1.0	10.0	12.0
E. faecalis	10.0 ± 1.5	12.0 ± 1.0	13.0 ± 1.0	50.0	10.0
E. coli	12.0 ± 1.0	13.0 ± 2.0	16.0 ± 1.0	30.0	20.0
P. aeruginosa	8.0 ± 1.0	11.0 ± 1.0	12.5 ± 1.0	50.0	10.0
K. pneumonia	0	0	0	>200.0	16.0
A. niger	7.0 ± 1.0	8.0 ± 1.0	11.0 ± 0.5	NT	NT
F. solani	10.5 ± 2.0	12.0 ± 1.0	15.0 ± 1.0	NT	NT
F. oxysporum	10.0 ± 1.0	10.0 ± 1.0	12.0 ± 1.0	NT	NT

NT not tested

towards Gram-positive bacteria including *S. aureus*, *B. cereus*, *M. luteus*, and *E. faecalis*. Purified AcAMP peptide was also active against some Gram-negative bacteria such as *P. aeruginosa* and *E. coli*, but was not active towards *K. pneumonia* (Table 2). MIC was also assessed by liquid growth inhibition assay. Antibacterial activity was greater against Gram-positive bacteria, especially *B. cereus* and *M. luteus* strains with MIC value of 10 μg ml^{-1}, which was similar to gentamycin as positive control. *E. coli* and *P. aeruginosa* were also sensitive toward purified AcAMP peptide, with MIC values of 30 and 50 μg ml^{-1}, respectively. The highest MIC value occurred with *K. pneumonia* (>200 μg ml^{-1}) (Table 2).

The antifungal activity of purified AcAMP peptide was also evaluated against *A. niger*, *F. solani*, and *F. oxysporum*. The results showed inhibitory effects on the growth of all studied fungi (Table 2). The inhibition diameter zones were 11, 15, and 12 mm, using 50 μl at 200 μg ml^{-1}, respectively. *F. solani* was found to be the most sensitive fungal strain to AcAMP peptide. As can be seen in Table 2, the inhibitory effects of AcAMP peptide increased with increasing concentration.

Effects of enzymes, heat treatment, and pH on antibacterial activity of AcAMP peptide

After purified AcAMP peptide was treated with several enzymes, heat, and pH, its antimicrobial activity was assayed against *B. cereus*. As reported in Table 3, the antimicrobial activity of AcAMP was totally lost only when treated with the proteolytic enzymes Alcalase, trypsin, chymotrypsin, pepsin, and proteinase K, indicating that the antimicrobial substance was proteinaceous. However, antimicrobial activity was unaffected by Thermamyl and EndoH, suggesting that the peptide was not glycosylated or its activity was independent of glycosylation (Table 3).

Table 3 Effects of enzymes and pH on the activity of purified AcAMP peptide from *A. clavatus* ES1 toward the indicator strain *B. cereus* (ATCC 11778) at concentration of 200 μg ml^{-1}

	Inhibition diameter zone (mm)
Control	22.0 ± 1.0
Enzymes	
Alcalase	0
Trypsin	0
Chymotrypsin	0
Pepsin	0
Proteinase K	0
Thermamyl®	22.0 ± 1.0
Lipase	22.0 ± 1.0
EndoH	22.0 ± 1.0
pH	
3.0–5.0	15.0 ± 1.0
6.0–10.0	20.0 ± 1.0
11.0	13.0 ± 1.0

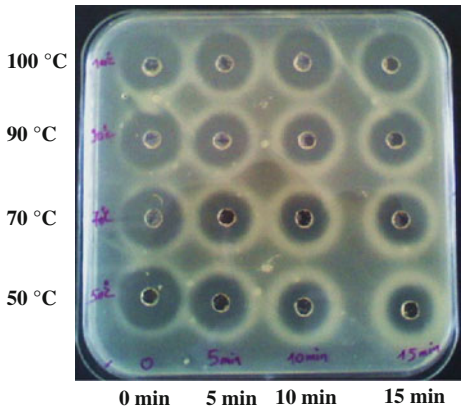

Fig. 2 Effect of heat treatment on purified AcAMP peptide activity. The same amount of purified AcAMP peptide was heated for 5, 10, and 15 min at 50°C, 70°C, 90°C, and 100°C. A sample without heat treatment was used as positive control

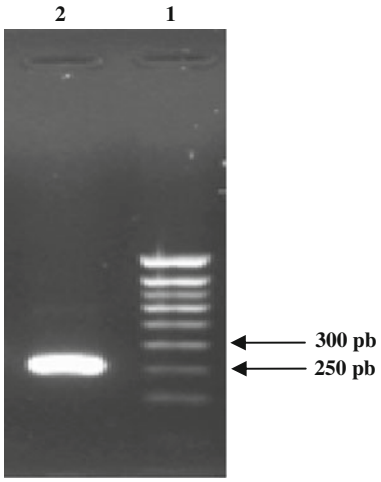

Fig. 3 Amplification of a DNA fragment encoding the pre-pro-AcAMP peptide of *A. clavatus* ES1: *lane 1* DNA marker (low DNA molecular marker kit, Invitrogen); *lane 2* PCR product from cDNA

AcAMP peptide exhibited high thermal stability (Fig. 2). Antimicrobial activity against *B. cereus* remained stable after 15-min incubation at 100°C. Heat stability is potentially a useful characteristic during food preservation because many food-processing procedures involve a heating step.

AcAMP remained stable after incubation for 1 h at pH values from 6.0 to 10.0, yielding a similar inhibition zone of about 20 mm using 50 µl at 200 µg ml^{-1}. However, its activity was slightly reduced at pH 3.0, 4.0, 5.0, and 11.0, with an inhibition zone of 15 mm at acidic conditions and 13 mm at alkalophilic conditions (Table 3).

Cloning of *acamp* gene and sequence analysis

Using the *acafp* gene sequence [28] and the codon usage database of *A. clavatus* strains at www.kazusa.org.jp/codon, two oligonucleotide primers called AcAMPF and AcAMPR were designed and used to amplify the gene encoding AcAMP peptide. Amplification from cDNA gave a fragment of about 300 bp (Fig. 3). Subsequently, the PCR product was cloned in pCR®II-TOPO plasmid vector and transferred into *E. coli* TOP10. The nucleotide sequence of the cDNA *acamp* and the deduced primary structure of the protein encoded by this gene are shown in Fig. 4. Analysis of the nucleotide sequence of the cDNA *acamp* gene revealed the presence of an ORF of 285 bp which encodes a pre-pro-AcAMP with an ATG start codon and a TAG stop codon. The ORF encodes a pre-pro-peptide of 94 amino acid residues with calculated molecular mass of 9874.4 Da and was predicted to belong to the antifungal peptide family (Fig. 4). This ORF was confirmed as the gene encoding AcAMP peptide, since the 19 residues of the N-terminal amino acid sequence of the purified AcAMP reported in Table 1 perfectly matched the deduced amino acid sequence.

SignalP version 3.0 predicted a signal peptide of 21 aa (M1-A21) and a pro-peptide of 22 aa (S22-Q43). The cleavage sites of the pre- and the pro-peptide were conserved among other antimicrobial peptides. Based on amino acid analysis by Protparam software in Expasy (http://ca.expasy.org/cgi-bin/protparam), the mature peptide, consisting of 51 aa (A44-C94), has predicted molecular weight of 5777.7 Da and an isoelectric point estimated at 9.06. Under physiological conditions, therefore, the AcAMP peptide was positively charged. The most significant feature of the amino acid composition of the mature AcAMP peptide is its high Lys and Cys content. The mature AcAMP contains 11 Lys residues and 8 Cys residues, corresponding to 21% and 15%, respectively.

Sequence alignment revealed 34–97% identity between the amino acid sequence of the deduced AcAMP pre-pro-peptide and other ascomycete antifungal peptides (Fig. 5). A more advanced search revealed a high degree of identity at the amino acid level with AcAFP from *A. clavatus* VR1. Both contained a high amount of hydrophobic and cationic residues, and eight Cys residues. Nevertheless, there were two and nine differences between the AcAMP peptide from *A. clavatus* ES1 and AcAFP and AFP peptides from *A. clavatus* and *A. giganteus*, respectively. In the case of AcAFP, the two differences are in the pre-sequence (F3V and V20A). However, in the case of AFP, three modifications were located in the pre-sequence (F3V, A19V, and V20A), four in the pro-sequence (T22S, E25D, T30A, and L43Q), and two in the mature peptide (N47D and K75V). Furthermore, the PAF peptide from *P. chrysogenum* and

Fig. 4 Nucleotide sequence of cDNA *acamp* gene of *A. clavatus* ES1 and its deduced amino acid sequence. *Numbers* on the *left side* of the amino acid and nucleotide sequences denote amino acid and nucleotide positions, respectively. Nucleotide sequence numbered from the first base initiation codon. The first amino acid Met of the enzyme is counted as +1. The putative starting residues of the pre-peptide (pre), pro-peptide (pro), and mature peptide (mature) are indicated. *Asterisk* indicates a stop codon

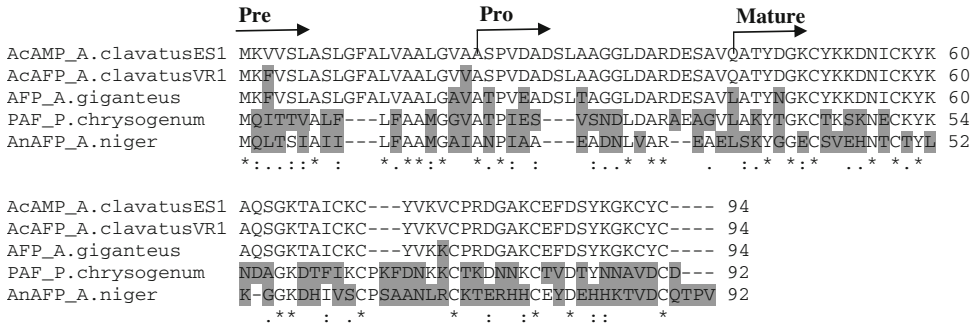

Fig. 5 Amino acid sequence alignment of pre-pro-peptide AcAMP with antifungal peptides from *A. clavatus* [27], *A. giganteus* [19], *P. chrysogenum* [10], and *A. niger* [18]. *Asterisk* indicates that the residues in that column are identical in all sequences in the alignment. *Colon* indicates that conserved substitutions have been observed. *Dot* indicates that semiconserved substitutions are observed. *X* indicates amino acid changes between AcAMP and other fungal peptides

AnAFP peptide from *A. niger* contained only six Cys residues, with Cys69 and Cys92 being missing (Fig. 5).

Discussion

A highly thermostable antibacterial and antifungal peptide from *A. clavatus* ES1, named AcAMP, was purified by one-step heat treatment and was characterized biochemically. The purification process differed from those previously described for other ascomycete antifungal peptides that employed ultrafiltration and cation exchange chromatography [19, 27]. A single band of about 6.0 kDa was observed on SDS–PAGE analysis. This molecular mass was close to the AcAFP peptide from *A. clavatus* VR1 [27] and the AFP peptide from *A. giganteus* [16], determined to be 5.77 and 5.8 kDa, respectively. AnAFP peptide from *A. niger* and PAF peptide from *P. chrysogenum* have molecular mass of 6.6 and 6.3 kDa, respectively [21]. All peptides reported from ascomycetes had only antifungal activity. To our knowledge, this is the first description of an antibacterial activity from an *A. clavatus* strain.

The purified AcAMP peptide from *A. clavatus* ES1 had pronounced antibacterial activity against Gram-positive bacteria including *S. aureus*, *B. cereus*, and *E. faecalis*. The AcAMP peptide was also active against some Gram-negative bacteria such as *P. aeruginosa* and *E. coli* but was not active toward *K. pneumonia*. These findings differed from those related to the inhibition data for bifidocin B, a bacteriocin produced by *Bifidobacterium bifidum* [30]. Indeed, bifidocin B was unable to inhibit *S. aureus*, *Clostridium butyricum*, *Streptococcus thermophilus*, and Gram-negative bacteria. On the other hand, the AcAMP peptide had some similar inhibition activities to those of bifidin [3] and bifilong [15], two bacteriocin types able to inhibit some Gram-positive and Gram-negative bacteria.

The effects of various enzymes on purified AcAMP peptide were evaluated. Complete inactivation of AcAMP inhibition activity was observed after treatment with proteolytic enzymes. These results differed from those of Cheikhyoussef et al. [6, 7], who reported partial inactivation of a 3.0-kDa bacteriocin of *B. infantis* BCRC 14602 after treatment with proteolytic enzymes. Enzyme treatment with Thermamyl, lipase, and EndoH did not affect inhibition activity of AcAMP peptide, suggesting that carbohydrate

and lipid moieties are not required for its activity. Similar results of enzyme treatments with proteases, lipases, and amylases were reported for other antimicrobial peptides, including bacteriocin produced by *Pediococcus pentosace* [14], *Pseudomonas* sp. strain 4B [9], and *Bacillus* sp. [24].

Purified AcAMP peptide was highly heat-stable in the range from 50°C to 100°C (Fig. 2). AcAMP peptide exhibited similar heat stability to AcAFP from *A. clavatus* VR1 [27]. The pH stability profile of AcAMP peptide is in agreement with the findings by Meghrous et al. [22]. Bacteriocins from 13 strains of *Bifidobacteria* were active at pH values ranging from 2.0 to 10.0. AcAMP peptide exhibited a wider pH stability range compared with the partially purified bifidin from *B. bifidum* 1452, which had optimum pH of 4.8 and only a maximum inhibition activity in the pH range of 4.8 to 5.5 [3]. AcAMP was relatively stable at pH values below 6.0 and above 10.0, while bifidocin B showed maximum pH stability in the range from 2.0 to 12.0 [30]. The heat and pH stability and the wide spectrum of activity are very useful characteristics for the potential application of AcAMP peptide as a food preservative [18].

The cDNA *acamp* gene that encoded AcAMP peptide was isolated by RT-PCR from total RNA. The PCR product was cloned in pCR®II-TOPO vector, and the entire nucleotide sequence of the open reading frame was determined. Alignment and comparison of the protein sequence deduced from cDNA gene with sequences from the protein databases using BLAST program showed high similarity to other ascomycete antifungal peptides. The *acamp* ORF encodes a 94-amino-acid precursor protein (pre-pro-AcAMP). The first 21 amino acids correspond to a predicted signal sequence. The pre-sequence of AcAMP from ES1 differed from AcAFP produced by *A. clavatus* VR1 by two amino acids: Val and Ala at positions 3 and 20 in AcAMP peptide were modified to Phe and Val in AcAFP, respectively [28]. The 22 amino acids from residues 22 to 43 constitute a pro-sequence that would be removed before or during release of mature AcAMP [20]. Pro-sequence plays an important role by preventing protein activity before secretion. The mature protein would adopt its active conformation after the pro-sequence is cleaved. Antimicrobial peptides produced as pre-pro-proteins from ascomycetes include AFP, AcAFP, and PgAFP from *A. giganteus*, *A. clavatus* VR1, and *P. chrysogenum*, respectively [25, 26, 28].

AcAMP peptide had an overall positive charge (+7) imparted by the presence of 12 lysine and arginine residues and a substantial portion of hydrophobic residues (about 40). Furthermore, the predicted tertiary structure of the mature AcAMP peptide using AcAFP as template had five antiparallel β-strands, defining a small and compact β-barrel which was stabilized by four internal disulfide bridges (data not shown). This property could explain the high thermostability of the AcAMP peptide. Similar three-dimensional (3D) structures were obtained with antifungal peptides from ascomycetes such as AcAFP [28], PcAFP [26], and AFP [25]. The common feature shared among the cationic antimicrobial peptides is their ability to fold into amphipathic conformations in the presence of hydrophobic amino acid residues. AcAMP peptide amphipathic character could be due to the presence of Y29, V30, Y45, Y50, and a cationic K9 and K10 residues [28]. These conformations could induce interaction with membranes or membrane mimics [11]. In addition to their direct antimicrobial activities against Gram-positive and Gram-negative bacteria, these peptides play additional roles as antibiotics against fungi [23].

In conclusion, this work attempted to show evidence for the high thermostability and strong antibacterial activity of a 6.0-kDa peptide from *A. clavatus* ES1. AcAMP peptide showed characteristics common to the group of small, basic, cysteine-rich antifungal proteins from molds. An ORF of 282 bp encoding a 94-amino-acid protein was characterized. The protein sequence deduced from *acamp* gene exhibited high identity with peptides from other fungi strains. Considering the heat and pH stability and the wide spectrum of action against fungi and bacteria, AcAMP peptide can be an excellent candidate for application as a food preservative and for biological control of plant diseases.

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