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cDNA cloning, functional expression and antifungal activities of a dimeric plant defensin SPE10 from *Pachyrrhizus erosus* seeds★

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Abstract

SPE10 is an antifungal protein isolated from the seeds of *Pachyrrhizus erosus*. cDNA encoding a 47 amino acid peptide was cloned by RT-PCR and the gene sequence proved SPE10 to be a new member of plant defensin family. The synthetic cDNA with codons preferred in yeast was cloned into the pPIC9 plasmid directly in-frame with the secretion signal α-mating factor, and highly expressed in methylotrophic *Pichia pastoris*. Activity assays showed the recombinant SPE10 inhibited specifically the growth of several pathogenic fungi as native SPE10. Circular dichroism and fluorescence spectroscopy analysis indicated that the native and recombinant protein should have same folding, though there are eight cystein residues in the sequence. Several evidence suggested SPE10 should be the first dimeric plant defensin reported so far.

Introduction

Plant defensins are basic, cystein-rich peptides that can inhibit a broad range of fungi and/or bacteria growth. They are mainly identified in peripheral cell layer and can be induced by pathogen attacks. Before isolated from plant, defensins had been identified in insects and mammalians. They are considered to play important roles in the innate immunity response among plant, invertebrate and vertebrate. Recently, the critical role of human defensins in adaptive immunity has also been more and more recognized (Raj *et al*., 2002; Yang *et al*., 2002).

The main activity of defensin is antimicrobial, with plant defensins usually against fungi, while insect and mammalian defensins are mainly active against bacteria. The activities of defensins from different species are normally suffered from increasing cationic strength in solution (Lehrer *et al*., 1988; Terras *et al*., 1992; Cociancich *et al*., 1993). However, unlike human and insect defensins, which are proposed to act by direct interaction with membrane negative phospholiphid, plant defensins seem to bind specific membrane receptor at the initial step (Thevissen *et al*., 1996, 1997). Though great efforts have been made to elucidate the mechanism of plant defensins' activities, most details are still unknown.

Plant defensin family shows very limited sequence conservation. Except the eight cystein and two glycine as well as one glutamate acid residues, there are few consensus residues among this family (Figure 1). Though most plant defensins inhibit only fungal growth, some exceptions display antibacterial ability (Moreno *et al*., 1994; Segura *et al*., 1998) and some others display no antimicrobial activity at all (Bloch and Richardson, 1991). Even for the antifungal subfamilies, they usually exhibit distinct microbial spectrum and make different influences on the morphology

★Nucleotide sequence data reported in this paper are available in the DDBJ/EMBL/GenBank database under accession number AY679170

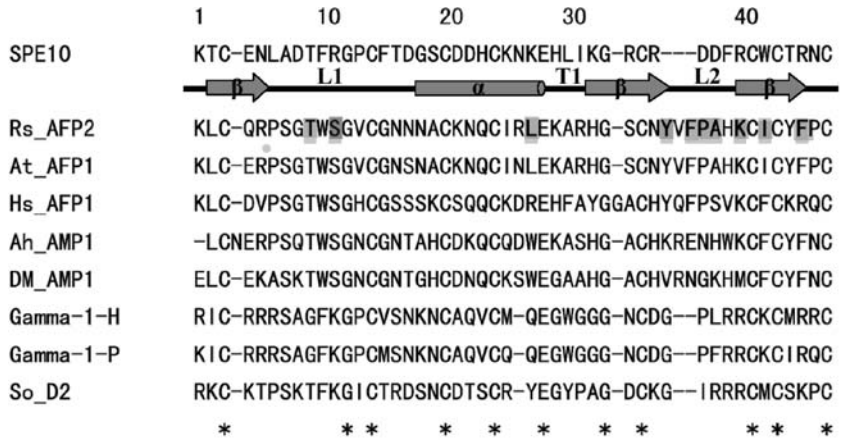


Figure 1. Multiple sequence alignment of SPE10 with several typical plant defensins. Rs_AFP2 from radish seeds (Terras *et al*., 1992), At-AFP1 from *Arabidopsis thaliana* seeds (Terras *et al*., 1995) and Hs-AFP1 from *Heuchera sanguinea* seeds (Osborn *et al*., 1995) can cause germ tubes and hyphae to swell and form mutiple hyphal buds. Ah-AMP1 from *Aesculus hippocastanum* and Dm-AMP1 from *Dahlia merckii* (Osborn *et al*., 1995) also inhibit the growth of fungi but do not cause morphologic change of hypha. Gamma-1-H and Gamma-1-P from barley and wheat endosperm exhibit no detectable antifungal or antibacterial activity (Mendez *et al*., 1990). So_D2 from spinach is active against some bacterial pathogens, as well as against fungi. The residues possibly involved in binding receptor in Rs_AFP2 are marked by shadow (De Samblanx *et al*., 1997) and the secondary structure of Rs_AFP2 based on that of Rs_AFP1 (Fant *et al*., 1998) are presented just above the sequence. Residues conserved across them are indicated by asterisks (*). This alignment was performed with CLUSTAL X software (Thompson *et al*., 1994).

of the target fungal hypha (Terras *et al*., 1992, 1995; Osborn *et al*., 1995).

We recently reported the purification and preliminary crystallographic analysis of SPE10, a plant defensin from *Pachyrrhizus erosus* seeds (Song *et al*., 2004). Here we described its cDNA cloning, heterologous functional expression and the antifungal activities. It is worthy to mention that SPE10 should be the first plant defensin existing as a dimer, which would provide a suitable model to study the relationship between the quaternary structure and plant defensin activity mechanism.

Materials and methods

Total RNA extraction and RT-PCR

Protein purification and N-terminal amino acid sequencing of SPE10 has been described previously (De Samblanx *et al*., 1997). After the seeds of *P. erosus* were germinated at 28 °C for 6 days, the germules were collected and homogenated in liquid nitrogen. Total RNA was isolated with Trizol reagent (BioBasic, Canada). The first strand of cDNA was synthesized with oligo(dT) primer and M-MLV reverse transcriptase. Briefly, total RNA and oligo(dT) primer was firstly incubated at 70 °C for 5 min and immediately put on ice. Other reagent including reaction buffer, dNTP, MMLV RT, RNase inhibitor were then added and the reaction was performed at 37 °C for 1 h. Subsequent PCR was carried out with two degenerate oligo-nucleotide primers using ordinary cycle condition. The primers were designed based on the N-terminal and C-terminal amino acid sequences determined by atomic resolution crystal structure of SPE10 (will be published elsewhere) and the cDNA sequences of five plant defensins most homologous with SPE10.

The sense primer: 5′-GGAATTCCATATGAA (A/G)AC(T/A)TG(T/C)GAGAA(T/C)-(T/C)T(G /A)GC-3′. The antisense primer: 5′-GCGCTCG AGTTAACA(G/A)TTTCT(G/A)GT(G/A)CACC A(A/G)C-3′. *Nde*I and *Xho*I restriction sites (underlined) were added for in-frame cloning into the PET22b vector for sequencing.

Construction of the plasmid for P. pastoris *expression*

The expression of SPE10 in *E. coli* AD494 (a thioredoxin reductase mutant strain) with PET22b plasmid failed to produce detectable protein. The methylotrophic yeast *Pichia pastoris* was then used for expression. For efficient expression of the gene,

we synthesized the full-length gene according to the codon usage preference in *S. cerevisiae* (Bennetzen and Hall, 1981). It has been reported that the N-terminal residues of plant defensin are possibly related to its activity (Almeida *et al.*, 2001). So *Xho*I enzyme site of pPIC9 vector was selected for no additional amino acid added to the N-termini of the recombinant protein. SPE10 gene was cloned into the pPIC9 expression vector directly in-frame with the secretion signal α-mating factor, downstream of the alcohol oxidase I promoter. A sequence of KEX2 sites comprising 12 nucleotides was placed ahead of the 141 bp SPE10 cDNA for cleaving the signal peptide. While the sequence encoding the STE13 cleavage sites (Glu-Ala-Glu-Ala) in pPIC9 was deleted since there were some cases where STE13 protease cleavage of Glu-Ala repeats was not efficient and Glu-Ala repeats are not necessary for cleavage by KEX2. A stop codon and an *Eco*RI restriction site were added at the 3′-end. This synthesized fragment was isolated from pGEM-T plasmid by digestion with *Xho*I and *Eco*RI enzymes and ligated into the *Eco*RI/*Xho*I-digested pPIC9. After transformed into *E. coli* JM109 host strain, the plasmid pPIC9/SPE10 was isolated and linearized with *Sal*I to favor integration at the *his*4 locus of *P. pastoris* genome, and transformed into yeast by electroporation (according to the manual of the Pichia expression kit v.E, Invitrogen).

P. pastoris *expression and purification of recombinant SPE10*

Selection of His^+ transformants was done on minimal selective MD medium (1.34% YNB, 4×10^{-5} % biotin, 1% dextrose, and 1.5% agar). Then 30 clones were selected and inoculated on MM (1.34% YNB, 4×10^{-5} % biotin, 0.5% methanol, and 1.5% agar) and MD medium respectively to screen the mut^+ clone. These thirty clones were all proved to be mut^+ type. After PCR screening, 17 positive clones were selected and analyzed for the amount of secreted rSPE10 (recombinant SPE10) through small-scale expression trials in 5-ml cultures. 100 μl supernatant was taken out and concentrated into about 10 μl for SDS-PAGE analysis. After optimization of expression conditions, finally the highest SPE10-expressing *P. pastoris* colony was grown in 200 ml BMG (100 mM potassium phosphate, pH 6.0, 1.34 %YNB, 4×10^{-5} biotin, 1% glycerol) medium at 30°C with constant shaking until OD600 reached 2.0–3.0(~36 h). The pellet was collected, re-suspended in 1 l BMM (replace the glycerol in BMG with 0.5%methanol) and induced for 96 h at 30 °C with acute shaking. During this course, growing in BMM was supplemented daily with 0.5% methanol.

The cell mass was centrifuged with 2500 *g* at room temperature. The supernatant was precipitated by saturated ammonium sulfate and the pellet was resolved in 20 mM Tris–HCl (pH 7.5) buffer. After desalted and concentrated by ultrafiltration, the sample was applied to Superdex 75 column pre-equilibrated with buffer of 50 mM Tris–HCl (pH 7.5) and 0.15 M NaCl on ÄKTA FPLC system (Amersham-Pharmacia). Fractions containing rSPE10 were collected and prepared for subsequent studies. The purified rSPE10 was spotted onto a PVDF membrane and sequenced in University of Science & Technology of China. The purification of wtSPE10 was almost the same as described before (Song *et al.*, 2004) except another superdex 75 separation step was used for measuring the molecular weight of SPE10 in solution. Pure protein was further vacuum-dried for mass spectrometric analysis. Mass spectrometric analysis was carried out with a BRUKER BIFLEX III mass spectrometer equipped with a nitrogen laser of 337 nm, with the protein resolved in pure water.

Light-scattering experiments

Protein samples (1 mg/ml determined by the Bio-Rad protein assay with BSA as the standard marker, in 20 mM Tris–HCl pH 7.5, 20 mM NaCl,) were used for light-scattering studies. DLS experiments were performed at 25 °C on a DynaPro-MS800 instrument (Protein Solutions Inc., Charlottesville, VA) operating at a wavelength of 824.2 nm. Molecular masses were determined using the software Dynamic V6.2.

Circular dichroism and fluorescence analysis

Circular dichroism (CD) measurements were obtained on a Jasco J-810 spectropolarimeter at 20 °C. Intrinsic fluorescence intensities were measured using AMINCO Bowman series 2 Luminescence Spectrometer (Thermo Spectronic, USA) at 25 °C. Fluorescence of the sample was excited at

290 nm and emission spectra were recorded between 300 and 400 nm. The excitation spectra were recorded between 250 and 330 nm with emission measured at 340 nm. The concentration of native SPE10 and rSPE10 in the assay was diluted to 0.12 and 0.085 mg/ml respectively, in 10 mM Tris–HCl buffer at pH 7.5.

Antifungal activity assay

Inhibitory activities of native SPE10 and rSPE10 on the growth of fungi were determined by microspectrophotometry as described before (Cammue *et al.*, 1992) with the following fungi used: *Fusarium oxysporum f.* sp. *vasinfectum, Verticillium dahliae, Aspergillus flavus, Penicillium* spp., *Colletotrichum gloeosporides, Botrytis cinerea, Bipolaris maydis, Aspergillus niger, Fusarium oxysporum f.* sp. *lycopersici, Rhizopus stolonifer*.

Briefly, 10 μl of the filter-sterilized protein diluted with different concentrations was pipetted into wells of a 96-well titer plate containing 90 μl of the test fungal spore suspension ($\sim 4 \times 10^4$ spores/ml) in potato dextrose (PD) medium, which was placed in an incubator at 28 °C. Fungal spore germination and growth were observed under a microscope and measured with a microplate reader at a wavelength of 595 nm after inoculation for 30 min and 48 h. Controls were tested identically except that the protein samples were omitted. All experiments were done in triplicate. Values of growth inhibition lower than 10% were not considered as significant. (Growth inhibition is defined as the ratio of the corrected absorbance at 595 nm of the control minus the corrected absorbance of the test sample, divided by the corrected absorbance of the control. The corrected absorbance is defined as the absorbance at 48 h minus that at 30 min. IC50 is defined as the protein concentration when 50 inhibition was reached)

Results

cDNA Cloning and yeast expression of SPE10

The result of RT-PCR was submitted to Genbank. Gene sequence alignment of peptides most homologous to SPE10 leaded to the identification of several conserved codons, reducing greatly the degeneracy of the primers. A 141-bp cDNA amplified from germless total mRNA encodes a 47 amino acid protein with the theoretical molecular weight of 5.5 kDa.

Purification of rSPE10

Thanks to the very low levels of native protein secreted by *P. pastoris*, only two steps were needed for purification of rSPE10. After saturated ammonium sulfate precipitation and superdex 75 size exclusion chromatography, 7 mg pure protein could be finally obtained from 1 l culture. The N-terminal sequencing of rSPE10 and Mass spectroscopy experiments confirmed that the recombinant SPE10 was expressed correctly.

Molecular weight determination

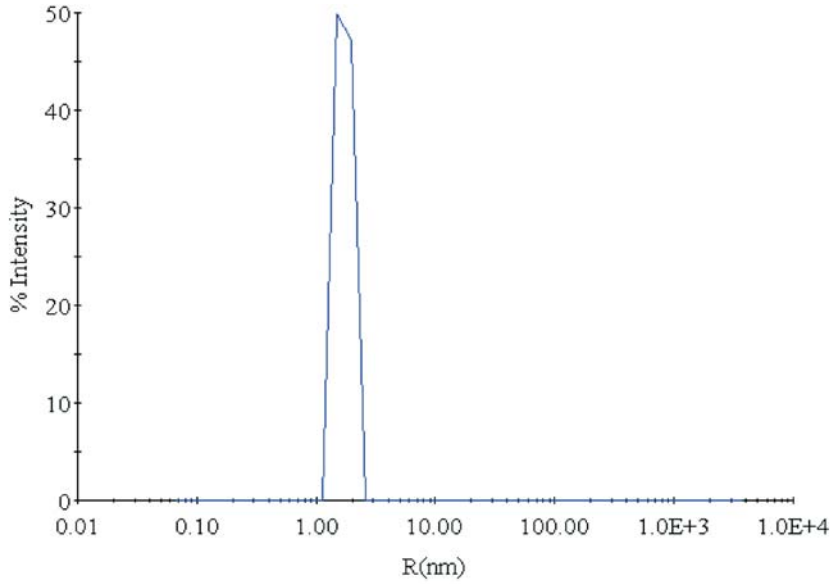
The molecular mass calculated from amino acid sequences of SPE10 is 5.5 kDa. The size exclusion chromatography of superdex 75 showed SPE10 appeared as a single peak, corresponding to a molecular weight of 13 kDa, consistent with the fact that Dynamic Light Scattering experiment also measured the molecular weight to be 12 KDa with no discernible polydispersity (Figure 2). These data suggest that SPE10 forms a homogenous dimer in solution. In addition, mass spectra of both native SPE10 and recombinant SPE10 showed $[2M + H]^+$ and $[M + H]^+$ peaks, indicating the dimer is quite stable and couldn't be fully dissociated during mass spectrometric analysis.

Spectroscopy analysis of the native SPE10 and recombined SPE10

Since there is a tryptophan residue (Trp42) in the sequence, SPE10 has intrinsic fluorescence. As Figure 3A shows, the fluorescence spectrum features of rSPE10 are almost identical to that of the native SPE10 with the maximum excitation peak at 340 nm. CD analysis also displays that rSPE10 has the similar curve features with the native SPE10 (Figure 3B). These results indicate that rSPE10 has a similar fold with the native one.

Antifugal activity

It was observed that the antifungal activity of SPE10 is strongly dependent on the target fungus.



Item	R(nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	1.7	13.9	12	100.0	100.0

Figure 2. Light scattering analysis of SPE10. The measured molecular weight is 12 kDa corresponding to a dimmer.

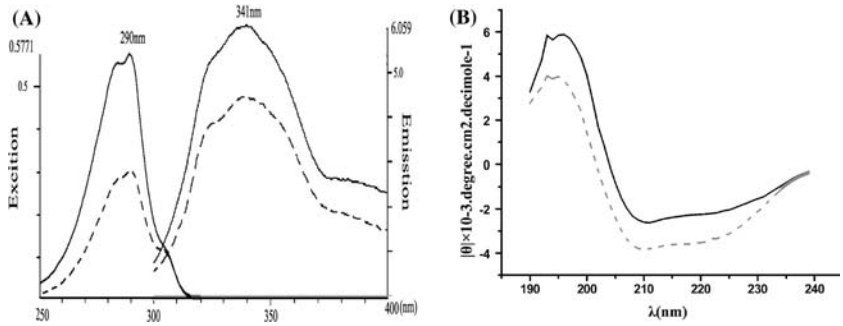


Figure 3. Spectroscopic properties of recombinant SPE10 and native SPE10. The fluorescence (A) and circular dichroic (B) spectra of rSPE10 (real line) and the native SPE10 (dashed line). The maximum peaks of excitation and emission fluorescence spectra are indicated.

The results are listed in Table 1. The IC50 values of Rs-AFP2 were also listed for comparison (Terras *et al*., 1992). It should be noted that the value of IC50 is also affected by assay conditions such as the used medium, the initial concentration of fungal spore, the incubation time, etc. So the IC50 values of Rs-AFP2 in Table 1 are only for a qualitative comparison. Among the tested fungi, *Bipolaris maydis* appeared to be most sensitive to SPE10 with an IC50 of 15 μg/ml. However, when 10 mM $CaCl_2$ was added, the activity of SPE10 was completely inhibited even the protein concentration was increased up to 100 μg/ml.

The antifungal potency of rSPE10 was assessed on the three fungi, *Aspergillus flavus, Bipolaris maydis and Aspergillus nige.,* which have been proved to be sensitive to the native SPE10. The recombinant peptide rSPE10 presented almost the same antifungal potency for the tested fungi. Besides, the antifungal activity of rSPE10 is also greatly reduced when 10 mM $CaCl_2$ was added to the reaction buffer.

Table 1. Antifungal activities of SPE10.

Fungi	IC50[a] (μg/ml) of SPE10	IC50 (μg/ml) of Rs-AFP2
Aspergillus flavus	30	ND
Aspergillus niger	45	ND
Bipolaris maydis	15	ND
Botrytis cinerea	>100	2
Colletotrichum gloeosporides	>100	ND
Fusarium oxysporum f.sp. lycopersic	>100	2
Fusarium oxysporum f.sp. vasinfectum	>100	ND
Penicillium spp.	>100	ND
Rhizopus stolonifer	>100	>100
Verticillium dahliae	>100	1.5

[a]Protein concentrations required for 50% growth inhibition (IC50) after 48 h of incubation were determined from the dose-response curves (percent growth inhibition vs. protein concentration).

Discussion

As for an alarming increase of resistance of microorganisms to classical antibiotics, the introduction and expression of antimicrobial peptides like plant defensin in crops is emerging as an intriguing biotechnological application for enhancing disease resistance (Punja, 2001; Osusky *et al.*, 2000). In this work, a new plant defensin, SPE10, was characterized and expressed successfully. SPE10 contains eight cysteine residues forming four disulfide bonds. It is known that disulfide bridge formation of eukaryotic proteins expressed in prokaryotes is often erratic, which prompted us to use the *P. pastoris* eukaryotic expression system and synthesize the full cDNA sequence of SPE10 according to the yeast codon usage bias. Good expression level of SPE10 is achieved in *P. pastoris*. Multiple evidences indicate that the recombined SPE10 has the same three-dimensional folding with the native one.

The antifungal activities of SPE10 on several fungi greatly decreased when 10 mM $CaCl_2$ was added. This phenomenon was also observed in other types of defensins. For insect and mammalian defensins, high cationic strength in solution will disturb the positively charged defensin to interact with the negative microbial membrane surface (Cociancich *et al.*, 1993; Wimley *et al.*, 1994). However, since plant defensins are considered to exert their function through binding specific receptor, the influence of ion strength is not likely to result from the same way as their insect and mammalian counterparts. One possibility is that the specific binding between plant defensins and receptor also includes some key electrostatic interaction, which would be disturbed by the high ion strength in solution. However, there is little evidence for such proposal by now and the information about residues involved in such interaction is also lack. On the other hand, with the mutation studies of Rs-AFP2, two patches were identified to be putative receptor binding sites. One patch is composed of residues Tyr38, Phe40, Pro41, Ala42, Lys44, and Ile46, most of which are hydrophobic. The other patch contains Thr10, Ser12, Leu28, and Phe49 (De Samblanx *et al.*, 1997). As shown in Figure 1, SPE10 shows very low similarity with Rs-AFP2 and most of the above residues important for Rs-AFP2 activity are not present in SPE10 at all. SPE10 also exhibits different inhibitory spectrum compared with Rs-AFP2 (Table 1), suggesting that SPE10 possibly should have different working mode or different receptor. The structural and functional research of SPE10 will be expected to provide answers to those important questions and contribute to the establishment of the possible diverse working mechanism for plant defensin family (Figure 4).

For mammalian defensins, it is assumed that the affinity to form a dimer is related to their antibacterial activity (Schibli *et al.*, 2002). A popular hypothesis of pore formation theory proposes that mammalian defensin can form multimeric ion-permeable pores through interaction of dimer, leading release of cellular contents (Schibli *et al.*, 2002; Kagan, 1990). Structures of some mammalian defensins associated in dimers or multimers provide evidence for such a theory (Hill *et al.*, 1991; Hoover *et al.*, 2000). However, little has

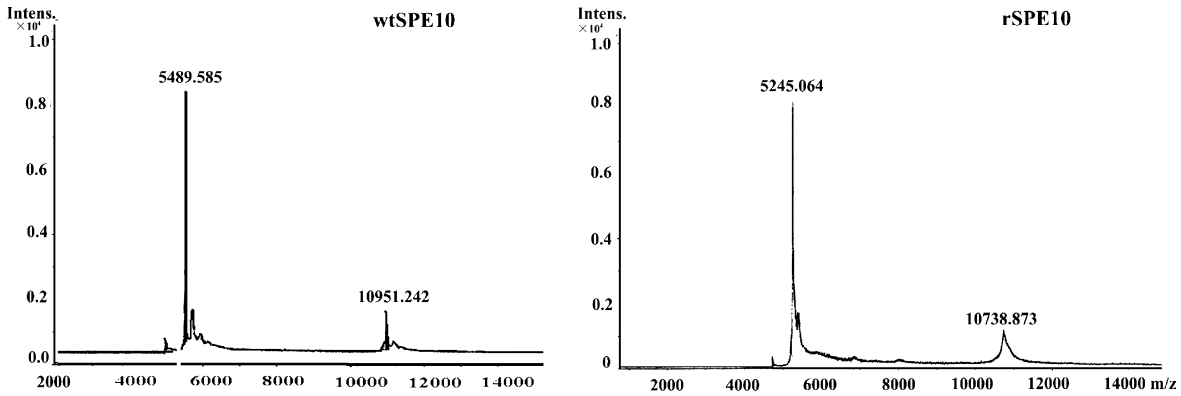


Figure 4. Mass spectra of native SPE10 (wtSPE10) and recombinant SPE10 (rSPE10). There is a peak of $[2M + H]^+$ in both spectra, indicating a dimeric SPE10.

been known about the mechanism of plant defensins. If any quaternary structures are involved in their functions remains unknown. To our knowledge, SPE10 is the first dimeric plant defensin reported so far. This protein would be a good candidate to address this question. An interesting problem is how SPE10 forms a dimer. We tend to believe such a dimer is mainly mediated by hydrophobic interactions as most cases of dimer or multimer in nature. The structure of Rs_AFP1 (Fant *et al*., 1998) has a hydrophobic head composed of loop 1 and loop 2 as Figure 5 shows. The amino acid sequence of SPE10 corresponding to loop 1 and loop 2 regions of Rs_AFP2 contains several hydrophobic residues such as Ala7, Phe10, Pro13, Phe15 and Phe39. Considering that the loop2 of SPE10 is shorter, which could make these hydrophobic residues located closer, providing the driving force to form a dimer.

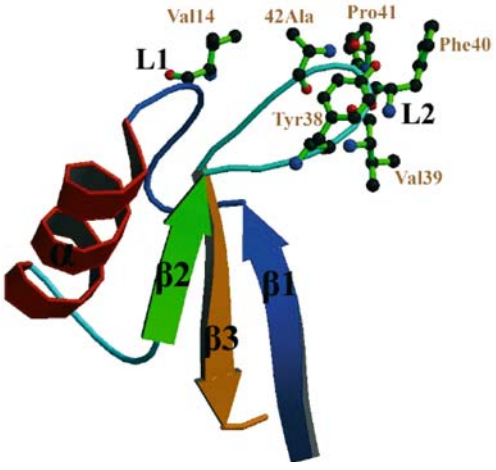


Figure 5. The NMR structure of Rs_AFP1 (Fan *et al*., 1998). The structure displayed that the molecule has a hydrophobic head. This Figure was generated by MOLSCRIPT (Kraulis, 1991) and rendered by Raster3D (Merritt and Bacon, 1997).

The characterization and cDNA cloning of SPE10 provides a key step for the further research on this novel plant defensin. In addition, the large amount of functional rSPE10 expressed in the *P. pastoris* system will allow a more detailed structural and functional analysis.

Acknowledgement

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